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Title: CENP-A, a histone H3 variant with key roles in centromere architecture in healthy and diseased states

Authors: Daniel Jeffery, Marina Lochhead, Geneviève Almouzni[#]

Author information: Institut Curie, PSL Research University, CNRS, Sorbonne Université, Nuclear Dynamics Unit, UMR3664, Equipe Labellisée Ligue contre le Cancer, Paris, France

[#]Corresponding author: genevieve.almouzni@curie.fr

Abstract:

Centromeres are key architectural components of chromosomes. Here, we examine their construction, maintenance, and functionality. Focusing on the mammalian centromere-specific histone H3 variant, CENP-A, we highlight its co-evolution with both centromeric DNA and its chaperone, HJURP. We then consider CENP-A *de novo* deposition and the importance of centromeric DNA recently uncovered with the added value from new ultra-long-read sequencing. We next review how to ensure the maintenance of CENP-A at the centromere throughout the cell cycle. Finally, we discuss the impact of disrupting CENP-A regulation on cancer and cell fate.

Keywords:

Centromere, CENP-A, alpha-satellite, HJURP, histone variant, genome architecture, cell cycle, cancer

Abbreviations:

CCAN: Constitutive Centromere Associated Network

CDE: Cell cycle-Dependent Element

CDK: Cyclin Dependent Kinase

CENP-A: Centromere Protein A

CHR: Cell cycle genes Homology Region

CIN: Chromosomal Instability

DREAM: Dimerization partner, Rb-like, E2F, And MuvB

EMT: Epithelial-to-Mesenchymal Transition

ESC: Embryonic Stem Cell
FISH: Fluorescence In Situ Hybridization
GIC: Glioblastoma Initiating Cell
HAC: Human Artificial Chromosome
HAT: Histone Acetyltransferase
HJURP: Holliday Junction Recognition Protein
HOR: Higher-Order Repeat
KO: Knockout
MEF: Mouse Embryonic Fibroblast
ncRNA: non-coding RNA
PLK1: Polo-Like Kinase 1
siRNA: small interfering RNA
T2T: Telomere-to-Telomere
TCGA: The Cancer Genome Atlas

1. Introduction:

Centromeres are a key architectural component of eukaryotic chromosomes essential for faithful chromosome segregation during cell division. First described as the primary constriction between sister chromatids (Flemming 1882), centromeres provide the foundation for kinetochore assembly that is the sites where spindle microtubules attach to chromosomes in mitosis and meiosis (Darlington 1936). In 1985, using the sera of scleroderma patients with autoantibodies targeting human centromeres (Moroi et al. 1980), William Earnshaw and Naomi Rothfield identified the first CENtromere/kinetochore Proteins: CENP-A, -B, and -C (Earnshaw and Rothfield 1985). Among these centromeric proteins, CENP-A proved to be a variant of histone H3 (Palmer et al. 1987, 1991; Sullivan et al. 1994), one of four major classes of core histones (including H3, H4, H2A, H2B), the proteins contributing to the core particle of the nucleosome, the basic unit of chromatin. The presence of CENP-A as a specific mark of centromeres raised the concept of its key role as an epigenetic mark defining centromere location (reviewed in (Probst et al. 2009)). With less than 50% sequence identity compared with the other H3 variants in humans, CENP-A is the most divergent H3 variant (reviewed in (Ray-Gallet and Almouzni 2021)). In turn, CENP-A-containing nucleosomes show distinct features and wrap only 121 bp of DNA, compared to the 147 bp in nucleosomes containing

H3.1, H3.2, or H3.3 (Tachiwana et al. 2012; Hasson et al. 2013; Lacoste et al. 2014). CENP-A is incorporated into chromatin specifically at centromeres via a dedicated chaperone protein called Holliday Junction Recognition Protein (HJURP) (Dunleavy et al. 2009; Foltz et al. 2009), initially identified for its ability to bind Holliday junction cruciform DNA (Kato et al. 2007). Importantly, this CENP-A deposition determines where on DNA active centromeres can form (Barnhart et al. 2011; Fachinetti et al. 2013). Directly binding to CENP-A nucleosomes (Carroll et al. 2010; Kato et al. 2013), CENP-C is a key centromeric protein (Fukagawa et al. 1999), considered the organizing scaffold of the inner kinetochore (Klare et al. 2015). The inner kinetochore complex, called the constitutive centromere associated network (CCAN), includes, along with CENP-C, several sub-complexes (CENP-LN, CENP-HIKM, CENP-OPQUR, and CENP-TWSX) (**Figure 1**). These components form a hierarchy of interactions that promote the stability of the CCAN at the centromere throughout the cell cycle and enable the recruitment of the outer kinetochore components in mitosis and meiosis (reviewed in (Navarro and Cheeseman 2021)). The multi-protein kinetochore complexes couple the centromeres to the spindle microtubules, enabling the orchestrated movement and alignment of the chromosomes to the metaphase plate and ensuring faithful segregation of the genetic material to the two daughter cells as cells divide (reviewed in (Musacchio and Desai 2017)). Thus, CENP-A-containing nucleosomes are the foundation for centromere formation enabling kinetochore assembly (**Figure 1**).

This chapter is about understanding how to build and maintain functional centromeres. Following a summary concerning how CENP-A, centromeric DNA, and HJURP have co-evolved across diverse species, we then focus on the human centromere. We highlight the genetic characteristics of human centromere DNA that favor the establishment of CENP-A chromatin. We then discuss how to maintain CENP-A at the centromere throughout the cell cycle. Finally, we discuss the regulation and misregulation of CENP-A in healthy and diseased states.

2. Co-evolution of centromere DNA, CENP-A and HJURP

2.1. Diversity of centromere DNA and CENP-A

There is an incredible diversity in the organization of centromeres, both in nature and size across eukaryotes (reviewed in (Talbert and Henikoff 2020)). Three major categories of centromeres can be distinguished: point centromeres exemplified in *S. cerevisiae*; regional

centromeres, observed in most organisms studied to date; and holocentromeres spanning over an entire chromosome, found in diverse groups of animals and plants. Centromere sizes range from the smallest ~125 bp in *S. cerevisiae* to a variety of sizes depending on the nature of the centromere, the organism, and even the chromosome. Regional centromeres range from short (~1–5 kb) to very long (up to several megabases), as is the case in humans. In short regional centromeres, the centromeric DNA usually consists of unique sequences flanked by pericentric repeats. Larger regional centromeres are often strongly enriched for transposons or highly structured repetitive sequences, flanked by less structured but still highly repetitive DNA. Within this complex repetitive structure, frequent DNA breaks at centromeres can arise when challenged during replication (reviewed in (Barra and Fachinetti 2018)) and by mitotic spindle defects (Guerrero et al. 2010). They are thus prone to an increased rate of mutations. Indeed, a common feature of centromeres across different categories and sizes is that centromere DNA sequences are rapidly evolving (Melters et al. 2013)(reviewed in (Thakur et al. 2021)). Most centromeric DNA also tends to be AT-rich, gene-free and very lowly transcribed, suggesting a conserved role of these characteristics in centromere function (reviewed in (Talbert and Henikoff 2020)). Importantly, point centromeres (Carbon and Clarke 1984; Kobayashi et al. 2015), and potentially some short regional centromeres (e.g., red alga (Maruyama et al. 2007), diatoms (Diner et al. 2017)), are determined genetically by their centric DNA sequences. However, in most centromeres, the centromeric DNA is neither sufficient nor necessary for centromere function, suggesting an epigenetic mechanism of centromere determination (reviewed in (Das et al. 2017)). Since in most eukaryotic organisms studied to date, orthologs of CENP-A are essential centromere components, the presence of the centromeric histone variant has been considered as a key epigenetic parameter in defining centromere sites (reviewed in (Balzano and Giunta 2020)). Notably, CENP-A, like centromere DNA, is also highly divergent across even closely related species (Malik and Henikoff 2001; Cooper and Henikoff 2004; Schueler et al. 2010; Kursel and Malik 2017). Thus, both centromere DNA and the centromeric histone variant show high levels of evolutionary change. This observation led to studies exploring the functional co-evolution of the two components.

2.2. Centromere drive

To explain the apparent paradox of the diversity and rapid evolution of centromere components with the centromere's essential conserved function, researchers proposed the

“centromere drive” model (Henikoff et al. 2001). In this model, centromere proteins and centromere DNA are under constant co-evolutionary forces in species where one sex has asymmetric meiosis, and the other has symmetric meiosis, as is the case for many plants and animals. Female gametogenesis in mammals is an example of asymmetric meiosis, as only one of the four daughter cells forms a gamete, while the other cells cannot pass on their DNA. This creates an environment for the positive selection of mutations in centromeric DNA that increase the likelihood that a chromosome will migrate to the gamete. Indeed, changes to centromere DNA sequences that enhance CENP-A recruitment (Iwata-Otsubo et al. 2017), enrichment of microtubule-destabilizing activity (Akeru et al. 2019) and increase spindle microtubule binding (Chmátal et al. 2014) show increased retention in the gamete of female mouse models. However, when gametogenesis is symmetric, this imbalance in centromere strength between homologous chromosomes can be deleterious for the species, resulting in skewed sex ratios or male sterility (Fishman and Saunders 2008). This suggests that positive selection also works on mutations that suppress centromere strength (Henikoff et al. 2001), such as alterations to centromere proteins that change how efficiently kinetochores are recruited to centromeres (Rosin and Mellone 2016; Hori et al. 2020; Kumon et al. 2021). As predicted by the centromere drive model, clades with only symmetric meiosis (such as Ferns, *Saccharomyces*, and *Plasmodium*) show much less adaptive evolution of CENP-A than clades with asymmetric meiosis (including *Brassicaceae*, *Drosophila*, and Primates) (Zedek and Bureš 2016). Thus, centromere drive depicts a tug-of-war between changes in centromere sequences that increase centromere strength in asymmetric meiosis while mutations in CENP-A, its chaperone, or other DNA-interacting centromere proteins counteract the DNA-dependent advantages.

2.3. Co-evolution of HJURP and CENP-A

Importantly, but perhaps not surprisingly, the histone chaperone responsible for depositing CENP-A into centric chromatin (HJURP in humans) is also highly divergent across species (Sanchez-Pulido et al. 2009; Phansalkar et al. 2012). Indeed, the *Drosophila* chaperone (called CAL1) does not share common ancestry with either the vertebrate HJURP chaperone or the related yeast ortholog (Scm3), and so far a chaperone specific for the centromere- H3 variant has not been identified in plants or protists (reviewed in (Drinnenberg et al. 2016; Zasadzińska and Foltz 2017)). This variability in the chaperone likely occurred through co-evolution to

maintain its interaction with the divergent centromeric H3 variant (Phansalkar et al. 2012; Rosin and Mellone 2016). Notably, in mice, *Hjurp* was among the genes with the highest level of copy number variation, exhibiting population-specific expansions indicative of involvement in local adaptations (Pezer et al. 2015), with common lab strains C57BL/6 and BALB/cJ both showing multiple duplication events encompassing the *Hjurp* locus (Filipescu et al. 2017). Interestingly, a recent study by Hori et al. identified a lethal mutation in chicken CENP-A that inhibits its interaction with HJURP in chicken cells, while the equivalent mutation in human cells does not prevent CENP-A-HJURP interaction and is non-lethal (Hori et al. 2020). With a humanized HJURP mutant, the researchers could rescue the chicken CENP-A-HJURP interaction and cell survival. Therefore, this study provides an interesting example of how HJURP mutation can compensate for a mutation in CENP-A to maintain their critical interaction. Overall, the interlinked co-evolution of centromere DNA, CENP-A, and HJURP highlights the evolutionary pressures on each of these centromere components. Notably, this indicates that while CENP-A-containing nucleosomes contribute epigenetically to defining the centromere, the importance of centromeric DNA should not be underappreciated (reviewed in (Dumont and Fachinetti 2017)), as we will discuss below. Excitingly, with the advent of high fidelity long-read sequencing technologies, the complex highly repetitive sequences of large regional centromeres, like those in humans, are now finally accessible for detailed analysis (reviewed in (Suzuki and Morishita 2021)). In the next section, we discuss the structure and organization of centromere DNA in humans and its impact on centromere function.

3. Building the centromere foundation on DNA

3.1. Characterizing the human centromere *before* long-read sequencing

The original characterization of human centromeric sequences began in the mid-1970s, long before long-read sequencing could be used to accurately sequence and annotate centromeres (Miga et al. 2020; Logsdon et al. 2021). The restriction enzyme digestion of purified human heterochromatic DNA enabled the identification of prominent ~170 bp monomer and ~340 bp dimer satellite DNA repeats (Manuelidis 1976). In situ hybridization revealed that these satellite repeats localized to centromeres (Manuelidis 1978). Sanger sequencing identified the sequence of the 171 bp monomer (Manuelidis and Wu 1978), now known as alpha satellite DNA, the core repeating unit of human centromeric sequences. Later work revealed that each human centromere contains a distinct organization of alpha satellite DNA (Willard 1985). The

alpha satellites are organized into sets ranging from two to 34 monomers, called higher-order repeat (HOR) units, with 50-70% sequence identity between alpha satellite monomers within each HOR unit (Mitchell et al. 1985; Jørgensen et al. 1987; Tyler-Smith and Brown 1987; Waye and Willard 1987). These HOR units—generally chromosome-specific—are arranged in thousands of tandem-repeated sequences within the centromere, forming long stretches of HOR arrays of up to five million base pairs in length. The repeating HOR units are 97-99% identical within a HOR array (reviewed in (Hartley and O’Neill 2019)). In contrast, total HOR array length varies substantially between individuals for every chromosome (Wevrick and Willard 1989) and even chromosome-specific HOR units can display significant variation in monomer number between homologous chromosomes, despite otherwise maintaining 99.8% sequence identity (Roizès 2006). Importantly, kinetochores attach to only a discrete domain within the HOR arrays, namely the functional core of the centromere. Interestingly, evolutionarily younger HOR arrays (more homogenous/less mutated) locate close to the functional core of the centromere, flanked by layers of increasingly divergent ancestral alpha satellites (Schueler et al. 2005; Rudd et al. 2006; Shepelev et al. 2009). Finally, the regions flanking the HOR arrays, called the pericentromeres, devoid of HOR units, contain arrays of alpha satellite monomers interspersed with LINEs, SINEs, and various other repeat elements (Schueler et al. 2001; Nusbaum et al. 2006; Rudd et al. 2006). These regions are marked by characteristic constitutive heterochromatin, namely Heterochromatin Protein 1 (HP1) binding, enrichment of H3K9me2/3, H3K27me3 and H4K20me3, hypoacetylation of histones H3 and H4, and transcriptional silencing (reviewed in (Fioriniello et al. 2020)). In contrast, the functional centromeres display unique features, where CENP-A nucleosomes are interspersed with both H3.1 and H3.3 nucleosomes (Dunleavy et al. 2011). The latter present an enrichment for H3K4me2 and H3K36me2 marks and hypoacetylation of histones H3 and H4, as shown using immunofluorescence of stretched chromatin fibers (Sullivan and Karpen 2004; Bergmann et al. 2011, 2012). Further work based on immunoprecipitation and mass spectrometry of CENP-A-associated chromatin also found enrichment for dual H3K9me2-K27me3 and H4K20me1 (Bailey et al. 2016). Thus, the functional centromere presents marks distinct from both heterochromatin and euchromatin. Overall, a first detailed framework of the centromeres emerged with their repeated sequence properties and the associated chromatin features.

3.2 New insights from telomere-to-telomere sequencing

The Human Genome Project announced the completion of the human genome sequence in 2001 (Lander et al. 2001). However, due to the limitations of short-read DNA sequencing technology at the time, up to 20% of the genome annotations remained largely unassembled, corresponding mainly to regions of heterochromatin and repetitive DNA, including all centromeres. Recently, in 2020 and 2021, the Telomere-to-Telomere (T2T) consortium used long-read sequencing technologies to fully sequence human chromosomes from telomere to telomere without gaps for the first time (chromosome X (Miga et al. 2020) and 8 (Logsdon et al. 2021, p. 8); see also **Figure 2**), using CHM13hTERT cells, a human cell line derived from a complete hydatidiform mole, where a single sperm undergoes post-meiotic chromosomal duplication resulting in uniformly homozygous alleles across the genome. Shortly after, they revealed the first complete sequence of an entire human genome (preprint, Nurk et al., 2021), accompanied by a sister preprint focused on the centromeres (Altemose et al. 2021). The sequencing and assembly of the entire human genome open up many possibilities, in particular, to illuminate variability at centromeres. On average, centromeres show more than double the structural variation between human individuals compared to the rest of the genome (45.05 events/Mb vs. 21.16 events/Mb), according to long-read sequencing data analysis (Suzuki and Morishita 2021). This approach confirmed the previously established framework of centromere DNA structure at base-pair resolution for all human chromosomes, including the characterization of all alpha satellite monomers, HOR units and arrays, and all pericentromeric repeats and satellite arrays (Altemose et al. 2021). They resolved the minute differences in centromeres with near-identical HOR arrays (including chromosomes 13/21, 14/22, and 1/5/19), identified previously hidden HOR array inversions and interruptions, and confirmed the high degree of centromeric genetic variation between individuals. Based on the unique and region-specific markers of the centromeres, they provide a method for repeat-sensitive mapping of short-read sequencing results to the completed centromere sequences. Applying this method to CENP-A ChIP-Seq and CUT&RUN, they confirm across all chromosomes the layered expansion of centromere arrays with CENP-A enrichment mapping to the evolutionarily youngest HOR arrays. Notably, alpha satellite DNA contains methylated CpG repeats that are important in human health (reviewed in (Scelfo and Fachinetti 2019)). However, precise sequencing of DNA methylation of centromeres was previously limited to non-repetitive neocentromeres, which revealed DNA hypermethylation of the domain with

notable pockets of active transcription and hypomethylation (Wong et al. 2006). Importantly, the current long-read sequencing methods simultaneously delineate DNA methylation, making it possible to accurately assess DNA methylation in endogenous centromeres for the first time. Strikingly, they revealed that while centromeres are indeed hypermethylated, CENP-A enrichment corresponds to a clear domain of reduced CpG methylation (Miga et al. 2020; Logsdon et al. 2021). Interestingly, CENP-A enrichment extends outside of this reduced CpG hypermethylation domain by several hundred kilobases for every chromosome (Altemose et al. 2021), including some chromosomes with smaller regions of CENP-A enrichment overlapping a secondary methylation dip or no dip at all. Interestingly, the drop in methylation also correlates with extreme inaccessibility, linked to the enrichment of CENP-A binding (Gershman et al. 2021). Overall, this work marks the first exploration of the fully assembled centromere sequences, solidifying and expanding the findings of previous studies on centromere structure and providing a detailed reference in specific cultured human cells. Future studies will need to undertake analyses in other normal cells, tissues, and organisms to comprehend centromere diversity and function fully.

3.3 Impact of centromere DNA on centromere function

CENP-A nucleosomes wrap discrete blocks of centromere DNA within a single HOR array on each chromosome, but CENP-A binding is not sequence-specific, and the subdomains of CENP-A localization are not limited to particular sequence motifs (Blower et al. 2002; Sullivan et al. 2011; Hayden et al. 2013; Ross et al. 2016; Aldrup-MacDonald et al. 2016). In fact, neocentromeres, whether naturally occurring in the human population (Tyler-Smith et al. 1999; Amor et al. 2004), in disease (Marshall et al. 2008), or artificially-induced (Murillo-Pineda et al. 2021), can support stable, fully functional centromeres in the absence of HOR arrays or pericentromeric repeats. Similarly, evolutionarily new centromeres in humans typically lack HORs (reviewed in (Rocchi et al. 2012)). This is also observed in many other species (Rothfels and Mason 1975; Suja et al. 1986; Ventura et al. 2001; Carbone et al. 2006; Giulotto et al. 2017), with the most striking examples in equids that have been studied in detail (Giulotto et al., 2017). Finally, even in the absence of any detectable alpha satellite DNA, the establishment of stable human artificial chromosomes (HACs) can be efficiently achieved (Logsdon et al. 2019). Thus, the centromere is indeed a prime example of an epigenetically determined chromosomal domain. Why then do all endogenous human centromeres occupy

these complex repetitive DNA regions? What are the evolutionary advantages offered by this DNA? The fact is that neocentromeres or centromeres devoid of these sequences do not harbor the same properties as those that do have these sequences, in particular when assessing genome stability. Indeed, both HOR arrays (Hartley and O'Neill 2019; Dumont et al. 2020) and pericentromeres (Janssen et al. 2018) provide significant advantages to maintaining genome stability. Thus, below we discuss several mechanisms for how these complex repetitive DNA elements contribute to the establishment, maintenance, and function of human centromeres.

3.3.1. CENP-B binding

One of the most explicit advantages of centromeric HOR arrays is their capacity to recruit one of the centromeric proteins, CENP-B, in a DNA sequence-specific manner. CENP-B binds specifically to the alpha satellite DNA at a specific 17 bp sequence, called the CENP-B box (Masumoto et al. 1989). This sequence is present in the HOR arrays of all human centromeres, except for the Y chromosome (Earnshaw et al. 1987). Since CENP-B proved dispensable for cell survival (Hudson et al. 1998; Perez-Castro et al. 1998; Kapoor et al. 1998), it was initially discarded as a critical component in defining centromeres. However, uterine and reproductive defects are associated with CENP-B deficiency (Fowler et al. 2000, 2004). Furthermore, to produce *de novo* functional HACs carrying centromeric DNA, CENP-B was necessary (Ohzeki et al. 2002; Okada et al. 2007), except when bypassed with CENP-A seeding (Logsdon et al. 2019). Of note, CENP-A itself can be bypassed by artificially targeting CENP-C or CENP-T, which is sufficient for kinetochore assembly and microtubule binding (Gascoigne et al. 2011; Hori et al. 2013). So, CENP-B is considered more as a stabilizer of kinetochores through its interaction with CENP-A nucleosomes and CENP-C (Suzuki et al. 2004; Fachinetti et al. 2013, 2015; Fujita et al. 2015). These properties of CENP-B culminate to enhance centromere fidelity, particularly upon CENP-A perturbation (Fachinetti et al. 2015). Notably, while kinetochores do not assemble in the absence of CENP-A, CENP-B can sustain kinetochore binding and efficient chromosome segregation if CENP-A is degraded after kinetochore assembly, including removal of all detectable CENP-A from centromere chromatin (Hoffmann et al. 2016). Furthermore, centromeres with higher concentrations of CENP-B boxes (and CENP-B) show increased chromosome segregation fidelity when CENP-A is degraded (Dumont et al. 2020). Interestingly, upon loss of CENP-A from the centromere, CENP-B can also act as a memory

signal for CENP-A localization, as observed naturally in the immune system. Indeed, quiescent human lymphocytes, CD4⁺ T cells, start to show detectable CENP-A only once activated and re-assemble CENP-A at centromeres as they re-enter the cell cycle, but only if CENP-B is present (Hoffmann et al. 2020). CENP-B also helps in recruiting histone chaperones and chromatin modifiers required for the unique chromatin characteristics that are important for efficient CENP-A assembly (Otake et al. 2020). Thus, CENP-B binding represents an important aspect of HOR array functionality through direct CENP-A-kinetochore stabilization, as a mark for centromere memory and as a multi-functional recruitment hub for the maintenance of centromeric chromatin.

3.3.2 Centromere transcription

As recently reviewed (Corless et al. 2020; Leclerc and Kitagawa 2021), all human HOR arrays produce low levels of array-specific non-coding RNAs (ncRNAs) transcribed by RNA polymerase II (Chan et al. 2012; McNulty et al. 2017; Bury et al. 2020). Specific depletion of ncRNAs transcribed from a given (CENP-A bound) HOR array, in turn, reduces CENP-A localization and prevents *de novo* recruitment of CENP-A to the corresponding targeted array, without affecting CENP-A localization at other centromeres (McNulty et al. 2017). Thus, centromere transcription is important for CENP-A recruitment *in cis*. Notably, recent single-molecule RNA FISH studies revealed that centromere ncRNAs did not stably remain at centromeres; thus, the *cis* function of centromeric ncRNAs could arise because of the act of nascent transcription (Bury et al. 2020). However, since the two studies above targeted different centromere ncRNAs, one cannot exclude distinct dynamics for the respective centromeres considered. How these ncRNAs, or the act of transcription, precisely aid in CENP-A recruitment is not yet clear. Another role for nascent transcription at centromeres could operate through a positive feedback loop involving cohesin and SGO1 proteins (Zhang and Liu 2020) to maintain centromere cohesion (Chen et al. 2021). Finally, low levels of centromere transcription may also help in maintaining the delicate balance of euchromatin and heterochromatin marks at functional centromeres. Several engineering studies modifying different aspects of chromatin marks in HAC centromeres showed that centromeres are remarkably permissive to alterations to histone marks. However, both too much and too little transcription can interfere with the recruitment and maintenance of CENP-A and ultimately results in loss of centromere function (Nakano et al. 2008; Bergmann et al. 2011, 2012; Molina

et al. 2016). On the one hand, H3K4me2 is particularly important for centromere transcription to prevent the spread of H3K9me3 from pericentromeric heterochromatin, which otherwise blocks CENP-A assembly (Molina et al. 2016). This aspect of maintenance echoes work in *Drosophila* cell lines (Bobkov et al. 2018), where centromere transcription may be required to ensure a stable presence of the *Drosophila* centromeric H3 variant. On the other hand, too much transcription actively displaces pre-established CENP-A and may interfere with CENP-A loading by HJURP (Bergmann et al. 2012). Thus, how precisely low levels of centromere transcription is tuned in the setting of this unusual chromatin with mixed features is an important question. One regulatory factor, the zinc finger protein ZFAT promotes transcription of centromeric ncRNAs by binding an 8 bp motif present in all centromere sequences of human chromosomes (Ishikura et al. 2020). By recruiting the histone acetylase KAT2B, H4K8ac levels increase and, in turn, recruits the bromodomain protein BRD4, which brings RNA polymerase II and thereby increases centromere transcription. Conversely, repression of centromere transcription may depend on proximity to nucleoli, as alpha satellite transcription showed a strong negative correlation with nucleolus proximity across a panel of cell lines, and disruption of the nucleoli increased alpha satellite transcription (Bury et al. 2020). Further investigation into the specific factors and mechanisms that control centromere transcription is still needed. Overall, HOR arrays produce low levels of ncRNAs that are important for maintaining the specialized chromatin environment that enables stable CENP-A nucleosome assembly on centromere DNA.

3.3.3 Nucleosome phasing and cruciform formation

Another potential advantage of the centromeric DNA derives from the physical properties of the repeating alpha satellite sequences. Nucleosome phasing is the phenomenon where underlying DNA sequences influence the positioning and spacing of an array of nucleosomes (Blank and Becker 1996). This occurs in centromeres as the 10 bp periodicity of AA dinucleotides, observed in the satellite monomers of many species, reduces the bending energy required for wrapping DNA around core histones and favors nucleosome formation (Struhl and Segal 2013). Furthermore, alpha satellite sequences can affect nucleosome positioning by enriching CENP-A nucleosome occupancy within the 5' end of the monomer, just 3' of the CENP-B box (Hasson et al. 2013). Notably, this phasing also occurs in HOR units devoid of CENP-B boxes, though to a lesser extent. Thus, HOR arrays may favor a regular

spacing of CENP-A nucleosomes where they all interact with the same “face” of the DNA double helix to possibly stabilize the centromere-kinetochore interaction during the tension of anaphase. This property could provide a selective advantage for these sequences (reviewed in (Talbert and Henikoff 2020)). HOR arrays are also highly amenable to the formation of non-B form DNA, since AT-rich sequences are considered as more open (Dekker 2007) and CENP-B binding bends DNA (Tanaka et al. 2001), both of which could promote negative supercoiling (reviewed in (Talbert and Henikoff 2020)). Negative supercoiling enables cruciform formation (Palecek 1991; van Holde and Zlatanova 1994; Krasilnikov et al. 1999). Interestingly, centromeres that lack CENP-B boxes, including the Y chromosome and neocentromeres, often present short dyad symmetries that, like CENP-B binding, could potentially promote negative supercoiling (Kasinathan and Henikoff 2018). Furthermore, permanganate sequencing shows that cruciform structures correlate with CENP-A occupancy (reviewed in (Kasinathan and Henikoff 2018; Talbert and Henikoff 2020)). Considering that HJURP was originally identified as a Holliday Junction recognizing protein (Kato et al. 2007), the formation of cruciform DNA in HOR arrays is interesting. These secondary DNA structures could facilitate HJURP binding and consequently CENP-A recruitment for nucleosome formation. Thus, the physical properties of HOR alpha satellite DNA may promote advantageous CENP-A nucleosome phasing and facilitate HJURP binding.

3.3.4 Pericentromere heterochromatin

Pericentromeric DNA is important for centromere function as a hub for constitutive heterochromatin. Heterochromatin is required for sister chromatid cohesion, promoted by HP1 α , which assembles the SGO1 cohesion complex and chromosomal passenger complex INCENP (reviewed in (Janssen et al. 2018)). Pericentric heterochromatin also recruits cohesin through H4K20me3, which is required for developing tension between sister chromatids and ensuring the bi-orientation of sister chromatids during mitosis and meiosis (reviewed in (Smurova and De Wulf 2018)). Furthermore, pericentric heterochromatin prevents internal recombination events between centromere sequences, including meiotic crossing over, which can result in acentric or dicentric chromosomes and subsequent mitotic catastrophe (reviewed in (Nambiar and Smith 2016)). In mouse cells, disrupting the integrity of pericentric heterochromatin by inhibition of histone or DNA methylation resulted in increased CENP-A levels in the centric domains, suggesting an interplay between centric and pericentric

chromatin (Boyarchuk et al. 2014). The pericentromeric heterochromatin may also play an important role in regulating centromere clustering in interphase (Jagannathan et al. 2018). Common features between the pericentromere and the nucleolus, such as DNA looping, condensin and cohesin binding, recombination control, and phase separation (reviewed in (Lawrimore and Bloom 2019)) suggest interesting parallels that remain to be explored between these two major architectural components of the nucleus. Overall, the constitutive silencing of pericentromeres ensures the fidelity of the centromere during cell division and protects against genome instability. Further studies are required to characterize how precisely the pericentromeric DNA performs these functions.

4. Deposition and maintenance of CENP-A at the centromere

4.1. Licensing centromere chromatin during kinetochore assembly

Once a functional centromere is established, it should be maintained throughout the cell cycle (reviewed in (Muller and Almouzni 2017; Navarro and Cheeseman 2021)). New CENP-A must be loaded specifically to the CENP-A-bound centromere domains each cell cycle to ensure the continued function of the centromere through multiple cell divisions (**Figure 3**). In humans, new CENP-A loading occurs exclusively in late telophase/early G1 (Jansen et al. 2007). The discovery of HJURP by biochemically purifying the complex of proteins associated with CENP-A was key to understanding how CENP-A could be deposited specifically at centromeres (Dunleavy et al. 2009; Foltz et al. 2009). This specificity was linked to the CENP-A targeting domain (CATD) in the HJURP N-terminal region that is key for binding soluble CENP-A-H4 (Shuaib et al. 2010; Bassett et al. 2012). HJURP (loaded with a CENP-A-H4 dimer) is only stably targeted to centromeres in late telophase/early G1 through a process of centromere licensing (**Figure 3a-b**), whereby CENP-C recruits the Mis18 complex (Moree et al. 2011; Dambacher et al. 2012; McKinley and Cheeseman 2014), which in turn recruits HJURP (Barnhart et al. 2011). This licensing occurs simultaneously with the assembly of the kinetochore and chromosome segregation (summarized in **Figure 1**; reviewed in (Navarro and Cheeseman 2021)), and is tightly regulated according to the cell cycle by Cyclin Dependent Kinases (CDKs) (reviewed in (Hochegger et al. 2008; Ding et al. 2020)). In late G2, as CDK1/2 activity increases, there is an increase in the localization of several CCAN components at the centromere, including CENP-C, -TW, and -HI (Prendergast et al. 2011; Gascoigne and Cheeseman 2013). During the M phase, CENP-C is phosphorylated by CDK1, which stabilizes its interaction with CENP-A

nucleosomes (Watanabe et al. 2019). CENP-C then recruits the Mis18 complex subunit M18BP1 (also known as Knl2) to centromeres (Moree et al. 2011; Dambacher et al. 2012; McKinley and Cheeseman 2014). However, CDK1/2 phosphorylation of the Mis18 complex components (M18BP1, MIS18 α , and MIS18 β) prevents the recruitment of MIS18 α/β to the centromere until anaphase onset, when reduced CDK1/2 activity allows their site-specific dephosphorylation (Silva et al. 2012; McKinley and Cheeseman 2014). This dephosphorylation enables the binding of MIS18 α/β to M18BP1, forming a hetero-octameric complex at a 4:2:2 ratio (MIS18 α : β :M18BP1) (Pan et al. 2017; Spiller et al. 2017). Reduced CDK1/2 activity at anaphase also allows dephosphorylation of HJURP (loaded with CENP-A-H4), which promotes its interaction with the Mis18 complex (Wang et al. 2014; Stankovic et al. 2017) and targeting to the centromere (Muller et al. 2014). In this way, dephosphorylated HJURP brings soluble CENP-A-H4 dimers to the centromere for loading through interaction with the Mis18 complex only after anaphase onset. At the level of chromatin, increased centromere histone acetylation also affects licensing of CENP-A loading. The histone binding proteins RBAP46 and RBAP48 are recruited to centromeres with the MIS18 complex and are similarly required for CENP-A recruitment (Hayashi et al., 2004). RBAP46 and RBAP48 are subunits of many chromatin-remodeling complexes, chromatin-assembly complexes, and histone-modifying enzymes, including histone acetyltransferases (HATs). Work exploring the impact of chromatin modifications in HACs demonstrated that tethering a HAT to alpha satellite DNA could promote HAC establishment (Bergmann et al. 2012) and even bypass the requirement for MIS18 α (Ohzeki et al. 2012). Furthermore, the acetyltransferase KAT7/HBO1/MYST2 interacted directly with M18BP1, and tethering KAT7 to ectopic alpha satellite DNA promoted CENP-A deposition (Ohzeki et al. 2016). Thus, centromeric histone acetylation may play an important function in counteracting heterochromatinization at the centromere to enable new CENP-A loading. Altogether, as kinetochores assemble and chromosomes divide, centromere chromatin is primed for CENP-A reloading. In the late M phase, CENP-C recruits the Mis18 complex, which in turn recruits HJURP bound to new CENP-A-H4, which is timed in the cell cycle through CDK phosphorylation.

4.2 Loading of new CENP-A in early G1

As cells complete mitosis, Polo-like kinase 1 (PLK1) site-specifically phosphorylates the Mis18 complex to maintain its localization at the centromere (**Figure 3c**) (McKinley and Cheeseman

2014). Artificial inhibition of PLK1 or alanine mutations of key PLK1 consensus sites in M18BP1 (which cannot be phosphorylated), prevent the centromeric localization of the Mis18 complex in G1, while not affecting its recruitment to centromeres in the M phase. These perturbations also prevent centromeric localization of HJURP in G1 and thereby prevent the incorporation of *de novo* CENP-A. Thus, while CDK phosphorylation of M18BP1 in the late G2/M phase negatively regulates centromere licensing by preventing the formation of the Mis18 complex, PLK1 phosphorylation in late M/early G1 positively regulates centromere licensing by maintaining M18BP1 localization. In complex with Mis18, HJURP brings together two dimers of CENP-A-H4 for deposition into chromatin (Zasadzinska et al. 2013; Nardi et al. 2016). As such, new CENP-A is loaded onto chromatin in early G1 (Jansen et al. 2007). This is achieved by exploiting the histone chaperone HJURP in promoting CENP-A-H4 deposition onto DNA (Dunleavy et al. 2009; Foltz et al. 2009). Recent work by Pan and colleagues revealed that a single HJURP binds to the 4:2:2 Mis18 hetero-octamer complex through the R1 and/or R2 regions in the C-terminal half of HJURP and suggests that for HJURP to tetramerize CENP-A-H4 dimers for nucleosome assembly, two Mis18-HJURP complexes must simultaneously or consecutively deposit CENP-A-H4 (Pan et al. 2019). Previous work also indicated the involvement of remodeling factors in the stable integration of CENP-A nucleosomes in the latter half of G1, as the kinetochore disassembles (**Figure 3d**). First, for example, there is the Remodeling and Spacing Factor (RSF) complex, which can reconstitute and space CENP-A nucleosomes *in vitro*, and induced loss of centromeric CENP-A when depleted (Perpelescu et al. 2009). Second, another complex enabled the stable incorporation of new CENP-A at centromeres, consisting of a Rho family GTPase activating protein (MgcRacGAP) that interacts with M18BP1, as well as Ect2, a Rho family guanine nucleotide exchange factor, and GTPases Cdc42 and Rac (Lagana et al. 2010). Various CENP-A post-translational modifications also play important and, in many cases, disputed roles in CENP-A recruitment and/or assembly (reviewed in (Mahlke and Nechemia-Arbely 2020)), with a recent article confirming previous findings (and contradicting others) that CENP-A-K124Ub is non-essential (Salinas-Luybaert et al. 2021) and a current preprint re-affirming the disputed essentiality of CENP-A-S68 phosphorylation for centromere function in mice (Liu et al. 2021). These conflicting findings highlight the potential impacts of different model systems and molecular perturbations. Altogether, maintenance of CENP-A at the centromeres is a dynamic, cell cycle-regulated process that is essential for the faithful segregation of chromosomes during cell division.

4.3 Recycling CENP-A at the replicating centromere

Every time the DNA replicates (**Figure 3e**), it dilutes the number of CENP-A nucleosomes at the centromere by half (Jansen et al. 2007). Previously established (parental) nucleosomes containing H3.1/2, H3.3 and CENP-A are disrupted by the replication fork and need to be recycled to the new daughter strands (Dunleavy et al. 2011). This recycling can possibly take advantage of the histone chaperone activity of the subunit of the DNA helicase MCM2 together with the general histone chaperone ASF1 (Richet et al. 2015; Clement and Almouzni 2015; Huang et al. 2015). Interestingly, the chaperone needed for CENP-A *de novo* deposition, HJURP, is also important for CENP-A recycling at centromeres during replication (Zasadzińska et al. 2018). Indeed, HJURP transiently localizes to centromeres and binds parental CENP-A during the S phase, co-purifies with MCM2 and CENP-A simultaneously *in vitro*, and its rapid degradation in the S phase dramatically reduces parental CENP-A retention (Zasadzińska et al. 2018). Thus, in the centromeres, each daughter strand receives an equal distribution of CENP-A nucleosomes through CENP-A recycling, which is usually constrained to the original CENP-A chromatin domain (Ross et al. 2016; Nechemia-Arbely et al. 2019). However, outside of the centromeres, replication evicts ectopic CENP-A without recycling them (Nechemia-Arbely et al. 2019), acting as an intrinsic cellular mechanism to protect genome integrity by preventing the accumulation of CENP-A in chromosome arms. Interestingly, similar to the effect of degradation of HJURP (Zasadzińska et al. 2018), degradation of CENP-C in the S phase significantly diminishes retention of centromeric parental CENP-A at centromeres (Nechemia-Arbely et al. 2019), suggesting that CENP-C is also an important player in ensuring CENP-A retention during centromere replication. Importantly, since loading of new CENP-A by HJURP is restricted to the end of telophase/early G1, cells go through mitosis with only half the full complement of CENP-A. In contrast, new H3.1/2 incorporation is coupled to replication via CAF-1, and new H3.3 is incorporated throughout the cell cycle via HIRA and DAXX/ATRX (reviewed in (Mendiratta et al. 2019)). Notably, while both H3.1 and H3.3 are incorporated into centromeric chromatin during the S phase, only H3.3 is lost from centromeres at the time of CENP-A loading (Dunleavy et al. 2011), supporting the hypothesis that H3.3 acts as a placeholder for CENP-A. Furthermore, HIRA fills nucleosome gaps using H3.3 when CAF-1 depletion limits H3.1/2 incorporation behind the replication fork (Ray-Gallet et al. 2011), suggesting that a similar mechanism could be employed at centromeres when new CENP-A is

not available. Future work investigating the dynamics of H3.1, H3.3, and CENP-A at the replicating centromere will be important to understand how CENP-A is retained through the cell cycle, and how centromeric H3.3 influences CENP-A loading and ultimately centromere function.

5. CENP-A regulation from healthy to diseased states

5.1. Transcriptional regulation of CENP-A

Beyond positional and post-translational modification, CENP-A is also tightly regulated at the transcriptional level (reviewed in (Mendiratta et al. 2019)). CENP-A in mammals is encoded by a single gene that undergoes conventional processing, splicing, and poly-adenylation (Sullivan et al. 1994; Régnier et al. 2003), located in the p-arm of chromosome 2 in humans. Unlike its loading to centromeres in early G1 (Jansen et al. 2007), transcription of *CENPA* peaks in G2, which is important for its later targeting to the centromere (Shelby et al. 1997). This expression is coordinated with the expression of *HJURP* as well as a major network of other kinetochore and centromere-related factors, including *AURKB*, *PLK1*, and *CENPB*, by direct binding of the FOXM1 master transcriptional regulator (Wang et al. 2005; Chen et al. 2013). These FOXM1 target genes, including *CENPA* and *HJURP*, all contain a Cell cycle-Dependent Element (CDE) and a Cell cycle genes Homology Region (CHR) in their promoters (Müller et al. 2014), which are coordinately repressed in G1 phase by the binding of the Dimerization partner, Rb-like, E2F, and MuvB (DREAM) complex, and then subsequently activated in late S/G2 phase by the binding of FOXM1 to MuvB (reviewed in (Sadasivam and DeCaprio 2013)). Notably, activation of the tumor suppressor p53 inhibits transcription of *CENPA* and *HJURP* through the CDE/CHR elements, in a manner dependent on p21 (Filipescu et al. 2017), likely by promoting repressive DREAM binding (Quaas et al. 2012; Fischer et al. 2016). The p53 gene *TP53* is the most commonly mutated gene in cancer. It acts as a master regulator of stress response through activation or repression of a variety of target genes controlling key cellular processes, including cell cycle, apoptosis, senescence, cell metabolism, DNA repair, and stem cell differentiation, amongst others (reviewed in (Levine 2020)). Interestingly, recent work has revealed that CENP-A transcription is also regulated by a CDK5 regulatory subunit (*CDK5RAP2*), involved in spindle formation and spindle checkpoint signaling (Zhang et al. 2009; Barrera et al. 2010), and is a direct transcriptional activator of *CENPA* in human cells (Wang et al. 2021). Notably, depletion of *CDK5RAP2* globally reduced CENP-A levels and caused mitotic defects

that were partially rescued by exogenous expression of CENP-A. How or if p53 or Cdk5rap2 impacts the cell cycle timing of CENP-A transcription remains to be explored. Overall, the tight regulation of CENP-A transcription involving the DREAM complex, FOXM1, p53/p21, and CDK5RAP2 is essential for faithful chromosome segregation and cell division in healthy cells.

5.2 Potential role for CENP-A in stem cell differentiation

As a regulator of stem cell differentiation, the negative control of p53 in CENP-A expression is intriguing as high CENP-A levels are linked to stemness. Human pluripotent stem cells maintain p53 in an inactive state through post-translational regulation (Jain and Barton 2018) and have been shown to naturally overexpress CENP-A mRNA (Ambartsumyan et al. 2010; Milagre et al. 2020). Cultured human cardiac progenitor cells showed high CENP-A protein levels that reduced as cells aged. Similarly, in the mouse heart, CENP-A levels were highest in the embryo and lowered in the adult (McGregor et al. 2014). Furthermore, a meta-analysis of microarray data from various human cell types revealed increased levels of CENP-A, along with various other CENPs, upregulated predominantly in undifferentiated cell types, including embryonic stem cells (ESCs), neural stem cells, and glioblastoma initiating cells (GICs), relative to more differentiated cells. As well, siRNA knockdown of CENP-A in GICs led to reduced stemness, as measured by sphere-forming ability (reduced size and frequency) and immunofluorescence of the stemness marker SOX2 (Behnan et al. 2016). In *Drosophila*, intestinal stem cells preferentially retain pre-existing CENP-A during asymmetric divisions, when one daughter cell differentiates and the other daughter cell maintains stemness (García del Arco et al. 2018), suggesting that CENP-A nucleosomes may epigenetically mark stem cell identity in this system. Indeed, asymmetric mitosis depends on the asymmetric loading of CENP-A loading on sister chromatids in both male (Ranjan et al. 2019) and female (Dattoli et al. 2020; Carty et al. 2021) *Drosophila* germline (reviewed in (Kochendoerfer et al. 2021)). This asymmetry in CENP-A is also associated with asymmetry in CENP-C, CAL1 (HJURP-like chaperone for *Drosophila* CENP-A), and NDC80. Remarkably, overexpression of CENP-A (Dattoli et al. 2020) but not CENP-C or CAL1 (Carty et al. 2021) promotes stem cell self-renewal in the female germ line. Thus, how CENP-A overexpression contributes to stem cell renewal and pluripotency in human cells, as well as its connection to p53 regulation, will be an important avenue for future research.

5.3 CENP-A misregulation in cancer

CENP-A is commonly overexpressed in many cancer types (Sun et al. 2016; Saha et al. 2020) and correlates with tumor aggressiveness in patients, including associations with increased tumor stage/size (Ma et al. 2003; Li et al. 2011; Qiu et al. 2013; Gu et al. 2014), increased invasiveness/metastasis (Ma et al. 2003; McGovern et al. 2012; Gu et al. 2014; Sun et al. 2016), increased rates of recurrence (McGovern et al. 2012; Sun et al. 2016) and poor patient prognosis (McGovern et al. 2012; Wu et al. 2012; Qiu et al. 2013; Gu et al. 2014; Sun et al. 2016). Similarly, HJURP overexpression is also associated with poor patient survival (Kato et al. 2007, p. 200; Hu et al. 2010; de Tayrac et al. 2013; Valente et al. 2013; Montes de Oca et al. 2015) and increased tumor grade (Hu et al. 2010; de Tayrac et al. 2013). Indeed, CENP-A and HJURP, along with other centromere, kinetochore and cell cycle-regulated genes, are coordinately overexpressed in tumors (Thiru et al. 2014; Zhang et al. 2016; Saha et al. 2020), which may be a result of deregulated FOXM1 (Thiru et al. 2014). In human cell lines, overexpression of CENP-A leads to its mislocalization to chromosome arms, in addition to increased localization at centromeres (Van Hooser et al. 2001; Lacoste et al. 2014; Nechemia-Arbely et al. 2017). In particular, mislocalized CENP-A is enriched at areas of high histone turnover, corresponding to promoters, enhancers, and CTCF binding sites, amongst others, which overlap with H3.3-enriched DNA (Lacoste et al. 2014). Remarkably, this mislocalization in HeLa cells can be attributed to the H3.3-dedicated chaperone DAXX, as siRNA depletion of DAXX eliminated the bulk of CENP-A mislocalization, presenting a model whereby DAXX promiscuously picks up excess CENP-A-H4 and loads it onto chromatin in place of H3.3-H4 (Lacoste et al. 2014). Mislocalization of CENP-A by ATRX and HIRA has also been proposed in colorectal cancer cell lines with naturally high levels of CENP-A (Athwal et al. 2015; Nye et al. 2018). Importantly, induced CENP-A overexpression results in chromosomal instability (CIN) as evidenced by various mitotic defects, including increased rates of both congression and segregation errors, associated with decompaction of endogenous centromeres and reduced recruitment of CENP-T and components of the Ndc80 complex (Shrestha et al. 2017, 2021). Interestingly, depletion of DAXX rescued these phenotypes, suggesting that mislocalization of CENP-A, rather than CENP-A overexpression itself, may cause the mitotic errors. Notably, CIN, defined as an increased rate of chromosome segregation errors that results in numerical and/or structural chromosomal abnormalities, occurs in up to ~80% of cancers (Carter et al. 2012), contributes to tumor progression, aggressiveness, and therapeutic resistance, and correlates with tumor stage, relapse, and metastasis (reviewed in (Bakhoun and Cantley

2018)). Furthermore, centromere breaks, neocentromeres and dicentric chromosomes are all linked to CIN in disease (reviewed in (Barra and Fachinetti 2018)). Thus, CENP-A overexpression and mislocalization could be a driver of cancer aggressiveness through the promotion of CIN. On the other hand, excessive CIN can also be deleterious to cancer cells by creating unsustainable levels of genotoxicity (reviewed in (Potapova et al. 2013)). Accordingly, while CENP-A overexpression is generally associated with worse cancer patient prognosis, as discussed above, some lung and breast cancer cohorts also showed a link between high CENP-A levels and increased sensitivity to genotoxic agents (McGovern et al. 2012; Sun et al. 2016; Zhang et al. 2016). Overall, both CENP-A and HJURP overexpression are common in cancer and correlate with tumor aggressiveness. However, what drives their overexpression and how it impacts cancer biology is still under investigation.

5.4 Impact of p53 status on CENP-A overexpression in cancer

In line with its negative regulation of CENP-A and HJURP transcription, p53 loss-of-function mutations correlate with high CENP-A and HJURP levels across cancers (Filipescu et al. 2017). Importantly, ectopic CENP-A or HJURP overexpression in a classic two-hit tumorigenesis model combining the knockout of p53 with the expression of a putative oncogene in mouse embryonic fibroblasts (MEFs) was not sufficient alone to drive oncogenesis in this system. However, p53 knockout in MEFs increased HJURP and CENP-A levels, which increased further upon oncogenic transformation, and tumorigenic cells proved addicted to this overexpression (Filipescu et al. 2017). Importantly, inducible HJURP knockout (KO) by CRISPR-Cas9 in MEFs, human cancer cell lines, and MEF *in vivo* grafts resulted in distinct phenotypes depending on p53 status. In p53-WT cells, HJURP KO led primarily to cell cycle arrest, but in p53-null cells, it led to severe aneuploidy and apoptosis, halting tumor growth *in vivo* (Filipescu et al. 2017). Thus, excess HJURP becomes essential for maintaining tolerable genome integrity and survival in cancer cells, particularly with loss of p53 and cell cycle checkpoints. Interestingly, while ectopic overexpression of CENP-A in mouse cells showed mislocalization to chromosome arms, similar overexpression levels upon p53 knockout and oncogenic transformation did not (Filipescu et al. 2017). This suggests that CENP-A and HJURP levels need to be co-regulated. When unbalanced, this could lead to a deleterious situation for cells and represent an Achilles heel in tumor cells. Studies comparing the impact of different CENP-A and HJURP dosages on cell survival and how it translates to prognosis in human cancers are needed.

The impact of p53 status on cell fate upon CENP-A overexpression is also significant. Notably, CENP-A levels vary widely between tumors and even different cell types in healthy tissue (**Figure 4**). Induced CENP-A overexpression in isogenic human cell lines with or without defective p53 revealed dramatically distinct effects on both cell state and cell identity (Jeffery et al. 2021). In terms of cell state, CENP-A overexpression in p53-WT cells led to mitotic defects, prolonged cell cycle arrest, and ultimately senescence, showing reduced proliferation, increased sensitivity to X-irradiation, and decreased colony-forming ability. Meanwhile, the p53-defective cells escaped cell cycle arrest, evaded senescence, and showed tolerability for X-irradiation. A striking change in cell identity also occurred that depended on p53 status, specifically in the non-tumoral breast epithelial cell line MCF10-2A (**Figure 5**). While the p53-WT cells maintained their epithelial identity long-term, prolonged CENP-A overexpression promoted epithelial-to-mesenchymal transition (EMT) in the p53-defective cells. Since EMT is associated with increased stemness and cell plasticity, this provides another link between CENP-A and its role in stem cell identity, discussed above. EMT is also strongly linked to invasiveness and metastasis (reviewed in (Shibue and Weinberg 2017; Pastushenko and Blanpain 2019)). Thus, these findings support a mechanism by which CENP-A overexpression could promote these aggressive phenotypes in p53-defective cancers. In agreement, a recent study by Shrestha et al. found that induced CENP-A overexpression in p53-defective cancer cells (DLD1) increases cell invasion *in vitro* (Shrestha et al. 2021). Overall, these studies demonstrate that while CENP-A or HJURP overexpression may not necessarily be direct drivers of oncogenesis, they are not passive passengers either. Notably, the very concept of driver mutations is evolving, as even mutations of well-established tumor suppressors (*TP53*) and oncogenes (*KRAS*) cause a continuum of phenotypic effects, rather than categorizing them into discrete categories (Ursu et al. 2022). In any event, depending on p53 status, misregulation of CENP-A and HJURP can result in distinct cellular consequences with implications for cancer prognosis and therapy.

5.5. Centromere nuclear positioning in healthy tissues and cancer

Centromere clustering in the nucleus influences 3D genome interactions that regulate diverse cellular processes (reviewed in (Muller et al. 2019)). From yeast to humans, centromeres from different chromosomes cluster near the nuclear periphery or the nucleolus throughout

interphase. This contrasts with the spatial organization of chromosomal territories, which favor intra-chromosomal rather than inter-chromosomal interactions. In humans, studies assessing centromere positioning in solid tissues and malignancies are rare, but FISH and/or immunofluorescence microscopy in hematopoietic progenitor cells, mature blood cells (B and T lymphocytes, granulocytes, monocytes) (Salníková et al. 2000; Alcobia et al. 2003; Ollion et al. 2015), primary fibroblasts (Bartholdi 1991), and ESCs (Wiblin et al. 2005) have shown consistent centromere clustering. Noncycling lymphocytes and terminally differentiated monocytes showed robust centromere clustering to the nuclear periphery, with only rare single foci associated with the nucleolus (Weimer et al. 1992; Weierich et al. 2003; Solovei et al. 2004). Centromere clustering in cycling cultured cells is more dynamic. During G1, centromere foci only briefly associated with the nuclear periphery, with a greater proportion adjacent to the nucleolus in comparison to the non-cycling cells (Solovei et al. 2004; Ollion et al. 2015). In late G1/early S, centromere foci partially disperse in non-random patterns that often resemble rings or lines, followed by doubling upon replication and further dispersal as cells enter G2. Finally, in prometaphase, with condensation of chromosomes and chromosome alignment, the centromere foci separate entirely for entry into mitosis (Bartholdi 1991; Solovei et al. 2004). Notably, dynamic centromere clustering is also linked to cell differentiation. Human embryonic stem cells showed a smaller proportion of centromere foci at the nuclear periphery than differentiated lymphoblastoid cells or primary fibroblasts (Wiblin et al. 2005). Meanwhile, different centromeres clustered together according to cell type and at different stages of hematopoietic differentiation (Alcobia et al. 2000, 2003). More recently, chromatin capture experiments (e.g., 4C, Hi-C) confirmed the strong interaction of centromeres and pericentromeres in sub-compartments across many species, including *S. cerevisiae*, *S. pombe*, *C. albicans*, *D. melanogaster*, *A. thaliana*, and vertebrates, though to a weaker extent in mouse and human cells (reviewed in (Muller et al. 2019)). Chromatin capture experiments also demonstrated that centromeres act as a topological barrier to intra-chromosomal contacts between p and q arms for inactive regions in human cells (Kalhor et al. 2011). Centromere clustering may also play a key role in genome integrity as its disruption led to nuclear budding, formation of micronuclei, DNA damage and cell death in both *Drosophila* and mouse cells (Jagannathan et al. 2018). Interestingly, phase separation has been proposed for centromere clustering considering pericentric heterochromatin foci bound by HP1 in *Drosophila* and mouse cells (Strom et al. 2017). However, which factors are involved in

regulating centromere clustering during the cell cycle and as cells differentiate remains to be determined. Furthermore, the impact of centromere clustering on gene expression and the functional consequences of this clustering in human cells are still open questions.

Another major question is how centromere positioning is altered in human cancers. Recent work, using a variety of fixed normal and malignant tissue sections from patients, assessed centromere positioning by CENP-A immunohistochemistry (Verrelle et al. 2021). Remarkably, all healthy and benign tissues tested, independently of proliferation, consistently showed regularly spaced CENP-A clustering close to the nuclear periphery, including tissues from breast, head and neck, stomach, kidney, pancreas, liver, lymph node, gall bladder, colon, and placenta. In striking contrast, all neoplastic tumors showed loss of regular centromere spacing, reduced localization to the nuclear periphery, diversification of the number and size of CENP-A foci, and greater heterogeneity in CENP-A patterning and intensity across nuclei (**Figure 6a**). Thus, disruption of centromere positioning, likely accompanied by changes in genome architecture, can provide a hallmark of malignancy. Furthermore, CENP-A nuclear patterning for a cohort of patients with head and neck cancer treated with chemoradiation predicted curability with 96% accuracy and a clear overall survival advantage (**Figure 6b and c**), independent of proliferation (as measured by Ki67 immunohistochemistry). The distinct CENP-A pattern, termed “Pattern-C”, proved to be a prognostic/predictive marker that outperformed all other tumor characteristics, including HPV status and tumor stage. Given the conservation of CENP-A patterning across tissues, the ability of Pattern-C to predict sensitivity may extend to other cancers treated with genotoxic agents. Future work should focus on validating the current findings in other cohorts and identifying the functional mechanisms behind this striking correlation. So far, our understanding of the relationship between 3D genome architecture and tumor heterogeneity remains limited, but CENP-A staining by immunohistochemistry represents a simple and efficient method to explore this relationship further. Until recently, the use of genome-wide chromatin capture technologies in primary human cancers has been limited to acute lymphoblastic leukemia (Kloetgen et al. 2020) and gastric cancer (Ooi et al. 2020) due to high input requirements. However, low input methods are also emerging (Díaz et al. 2018; Animesh et al. 2021). These are avenues opening up the possibility of characterizing alterations to 3D genome architecture across cancers. Finally, the complete sequencing of the human genome (Nurk et al. 2021; Altomose et al. 2021) and the

accompanying methods for mapping short-read sequencing results to centromeres enables the precise characterization of centromere chromatin contacts genome-wide in healthy and diseased states.

Overall, the tight cell cycle regulation of CENP-A expression is essential for the faithful inheritance of DNA in healthy cells, and CENP-A levels are held in check by the tumor suppressor p53. But CENP-A is commonly overexpressed in cancer, with links to CIN and tumor aggressiveness. However, it may also play an important role in stemness and cell identity, depending on p53 status. Finally, CENP-A staining as a readout of centromere positioning represents a novel marker of tissue malignancy and may help predict treatment outcomes.

6. Conclusions:

Centromeres are a striking example illustrating how genome architecture can change in healthy and diseased cells across species. Essential for proper chromosome segregation as cells divide, the rapid co-evolution of centromeric DNA sequences, the centromere-specific histone H3 variant, and its dedicated chaperone underline how evolutionary forces on each of these centromere components can align with a functional role. While CENP-A represents a prime example of a bona fide transgenerational epigenetic mark for defining centromeres, the complex genetic repetitive structure of human centromeric DNA provides advantages for enhancing centromere fidelity. Importantly, centromere establishment and maintenance across cell divisions depends on coordinated transcriptional and post-translational control, operating on a key network of centromere, kinetochore, and cell cycle factors across the cell cycle. Finally, perturbation of CENP-A and centromere architecture commonly occurs in cancer. Thus, evaluation of centromere function represents an important area to further explore with implications for normal development, cell fate, tumor aggressiveness, and patient treatment.

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Figure Legends

Figure 1. *Schematic diagram of human centromere and kinetochore components in M phase.*

Recently reviewed in (Kixmoeller et al. 2020; Navarro and Cheeseman 2021), the centromere is the site of kinetochore assembly and microtubule attachment in mitosis and meiosis, visible as a chromosomal constriction in condensed chromosomes (represented on the left). Centromere chromatin contains CENP-A nucleosomes interspersed with H3.1 and H3.3 nucleosomes (not shown). CENP-B binds a specific 17 bp motif (CENP-B box) within centromeric alpha satellite DNA and stabilizes kinetochores through direct interaction with CENP-A and CENP-C. CENP-C also interacts directly with CENP-A nucleosomes, forming the scaffold for the CCAN sub-complexes CENP-NL and CENP-HIKM, and the Mis12 complex of the outer kinetochore. In addition to CENP-C, CENP-NL interacts directly with CENP-A, CENP-T, and CENP-HIKM, which binds CENP-OPQUR. CENP-T binds DNA non-specifically and interacts directly with CENP-HIKM, as well as Knl1 and Mis12. While all 16 CCAN components remain localized to the centromere throughout the cell cycle, the different CCAN sub-complexes show dynamics in their intra-CCAN interactions and relative abundance. The outer kinetochore assembles in late G2/M. The Mis12 complex is phosphorylated by Aurora B, which allows its binding to CENP-C. The Knl1 complex then binds Mis12. Mitotic CDK1 phosphorylates CENP-T in early M phase, enabling CENP-T to recruit Mis12 as well. After nuclear envelope breakdown, the Ndc80 complex is recruited to Mis12 and/or phosphorylated CENP-T. Ska1 and Astrin/SKAP bind Ndc80 to promote its binding to spindle microtubules. In the absence of microtubule interaction, the spindle assembly checkpoint proteins and fibrous corona are also recruited (not shown). Once microtubule interaction is established and kinetochores bi-oriented, the spindle assembly checkpoint proteins are released, and segregation occurs. Simultaneously with outer kinetochore assembly, CENP-C recruits the Mis18 complex (M18BP1, MIS18 α , MIS18 β), which recruits HJURP loaded with new CENP-A-H4 dimers so they can be incorporated into centromere chromatin in early G1. Complex names are indicated in bold.

Figure 2. *Schematic diagram of chromosome 8 with zoom on centromere showing DNA structure and organization, DNA methylation, and CENP-A enrichment, adapted from (Logsdon et al. 2021).*

Schematic diagram of G-banded chromosome 8 with approximate centromere highlighted in pink. Approximate “pericentric” and “centric” domains are indicated within the centromere, with the “functional domain” defined by CENP-A localization indicated at the bottom. The diagram shows the precise structure, organization, DNA methylation, and CENP-A localization for the centromere of chromosome 8, as characterized by (Logsdon et al. 2021). Repeat elements in top rectangle colored according to classification type: α -satellites, grey; β -satellites, blue; γ -satellites, purple; LINEs, pink; LTRs, orange; SINEs, turquoise. The α -satellite structure colored by HOR unit length: monomeric/divergent, black; 4-monomer, blue; 7-monomer, red; 8-monomer, green; 11-monomer, purple.

Figure 3. *Dynamics of centromere components during the cell cycle, adapted from (Muller and Almouzni 2017).*

A) Increased enrichment of various CCAN components begins in late S/G2/M, including CENP-C (Gascoigne and Cheeseman 2013) and CENP-T–CENP-W, recruited *de novo* in late S phase–G2 phase (Prendergast et al. 2011) via the chaperone FACT (Prendergast et al. 2016). Kinetochore assembly begins.

B) Centromeres are licensed in the late G2 phase in a process involving the Mis18 complex (M18BP1, MIS18 α , MIS18 β). M18BP1 is recruited to CENP-C (Moree et al. 2011; Dambacher et al. 2012; McKinley and Cheeseman 2014). The Mis18 complex (Silva et al. 2012; Pan et al. 2017; Spiller et al. 2017) and HJURP (Wang et al. 2014; Muller et al. 2014) are site-specifically dephosphorylated and sequentially recruited after anaphase onset upon reduced cyclin-dependent kinase 1 (CDK1) and CDK2 (CDK1/2) activity (Stankovic et al. 2017).

C) Mis18 complex is phosphorylated site-specifically by Polo-like kinase 1 (PLK1) and maintained at centromeres (McKinley and Cheeseman 2014). HJURP dimerizes in complex with Mis18 (Zasadzinska et al. 2013; Pan et al. 2019), interacts with DNA (Muller et al. 2014), and deposits CENP-A-H4 in early G1 (Jansen et al. 2007; Dunleavy et al. 2009; Foltz et al. 2009). It also interacts directly with CENP-C and possibly helps to reposition CENP-C at centromeric chromatin (Tachiwana et al. 2015). H3.3, as a placeholder for CENP-A, is possibly evicted in the process (Dunleavy et al. 2011).

D) In G1, kinetochores are disassembled, and the Mis18 complex and HJURP leave the centromere.

E) During late S phase, centromeric chromatin is replicated. H3.1, H3.3, and CENP-A nucleosomes are disrupted as the replication fork progresses, and old histones are recycled in a process involving MCM2 and ASF1 (Richet et al. 2015; Clement and Almouzni 2015; Huang et al. 2015). Recycling of old CENP-A depends on HJURP (Zasadzińska et al. 2018) and CENP-C (Nechemia-Arbely et al. 2019). New H3.1 and H3.3 are deposited through mechanisms involving the dedicated chaperone complexes indicated. CENP-A is diluted onto both daughter strands, and H3.1 and/or H3.3 may be deposited to fill the gaps (Dunleavy et al. 2011). The H2A–H2B pool is handled by the FACT complex (Formosa 2013). PTM, post-translational modification.

Figure 4. *Variation in CENP-A expression levels in cancer, normal tissues, and single cells.*

A) Box and dot plots indicating *CENPA* mRNA expression levels in patient tumors relative to normal samples (z-score, log₂ RNA SeqV2 RSEM) from cBioPortal (TCGA PanCancer Atlas studies) (Cerami et al. 2012; Gao et al. 2013). The plot was generated using the cBioPortal web interface. X-axis indicates Cancer Type: Bladder Urothelial Carcinoma (Blad.), Cholangiocarcinoma (Chol.), Colorectal Adenocarcinoma (Col.), Endometrial Carcinoma (Endo.), Esophageal Squamous Cell Carcinoma (ESCC), Esophagogastric Adenocarcinoma (Eso.), Fibrolamellar Carcinoma (FLC), Head and Neck Squamous Cell Carcinoma (HNSCC), Hepatocellular Carcinoma (Liver), Invasive Breast Carcinoma (Breast), Non-Small Cell Lung Cancer (Lung), Prostate Adenocarcinoma (Pros.), Renal Clear Cell Carcinoma (RCCC), Renal Non-Clear Cell Carcinoma (RNCCC), Undifferentiated Stomach Adenocarcinoma (Stom.), Well-Differentiated Thyroid Carcinoma (Thy.). TCGA: The Cancer Genome Atlas.

B) RNA expression consensus data of normal tissues obtained from the Human Protein Atlas (Uhlén et al. 2015) showing normalized Transcripts Per Million (nTPM) expression values from the Human Protein Atlas RNA-seq data and RNA-seq data from the Genotype-Tissue Expression (GTEx) project. X-axis indicates tissue type. Bar plot colored according to tissue groups, as indicated in the legend on the right. Plot source: <http://www.proteinatlas.org/ENSG00000115163-CENPA/tissue>

C) Single-cell RNA sequencing data from the Human Protein Atlas meta-analysis of 10X Genomics Chromium single-cell gene expression databases containing healthy human tissues (Karlsson et al. 2021), showing normalized Transcripts Per Million (nTPM) expression values. The X-axis indicates cell type. Bar plot colored according to cell type groups, as indicated in

the legend on the right. Plot source: <https://www.proteinatlas.org/ENSG00000115163-CENPA/single+cell+type>

Figure 5. *CENP-A overexpression promotes distinct cell fates depending on p53 status, adapted from (Jeffery et al. 2021).*

A) High CENP-A promotes EMT in p53-defective cells: Immunofluorescence images of breast epithelial cells (MCF10-2A) with wild-type p53 (p53-WT) or dominant-negative p53 (p53-DN), with or without prolonged CENP-A overexpression (34 days). Scale bars = 40µm. DAPI in cyan. E-cadherin (epithelial marker) in yellow. Vimentin (mesenchymal marker) in magenta.

B) CENP-A overexpression reprograms cell fate with distinct effects on cell state and cell identity depending on p53 status. Perturbation (a) by CENP-A overexpression (in blue) induces mitotic defects in both wild-type p53 (p53-WT, top panel blue) and p53-defective cells (p53-DN, dominant-negative, bottom panel green). These defects provoke distinct cell fate decisions according to p53 status, impacting cell state (b) or identity (c). When p53 is functional, the cell state shifts towards acute cell cycle arrest and senescence, reducing self-renewal capacity. Additional stress, like DNA damage from X-irradiation, amplifies this response, resulting in radiosensitivity. Furthermore, functional p53 ensures the preservation of epithelial identity. In contrast, when p53 is defective, the cells evade arrest and continue cycling, allowing CENP-A overexpression to promote epithelial-mesenchymal transition (EMT).

* symbol: reprogramming stimulated by CENP-A overexpression.

Figure 6. *CENP-A staining patterns distinguish normal from malignant tissues and predict prognosis for chemoradiation-treated head and neck cancers, adapted from (Verrelle et al. 2021).*

A) Example images of CENP-A staining by immunohistochemistry in breast normal and breast tumor tissues. Samples were fixed with AFA and paraffin-embedded. Normal tissues across all tissue types tested show a similar CENP-A staining pattern, characterized by the presence of CENP-A loci at the nuclear periphery, disrupted in all malignant tissues tested. The scale bar is 10 µm. Zoom in the inset shows an example nucleus.

B) Example images, as in (A), but comparing different CENP-A staining patterns between malignant head and neck tumors. Pattern-C is characterized by homogeneity in the number, size, shape, localization and intensity of CENP-A foci across all the nuclei in the tissue section,

with intra-nuclear rather than peripheral localization of centromeres, medium to strong intensity of CENP-A immunostaining, and mild anisokaryosis (i.e., mild heterogeneity in size and shape of nuclei). Any other patterns were designated Pattern Non-C.

C) Kaplan–Meier survival curve of patients treated by concurrent chemoradiation therapy with CENP-A pattern-C (black) and CENP-A pattern non-C (red).

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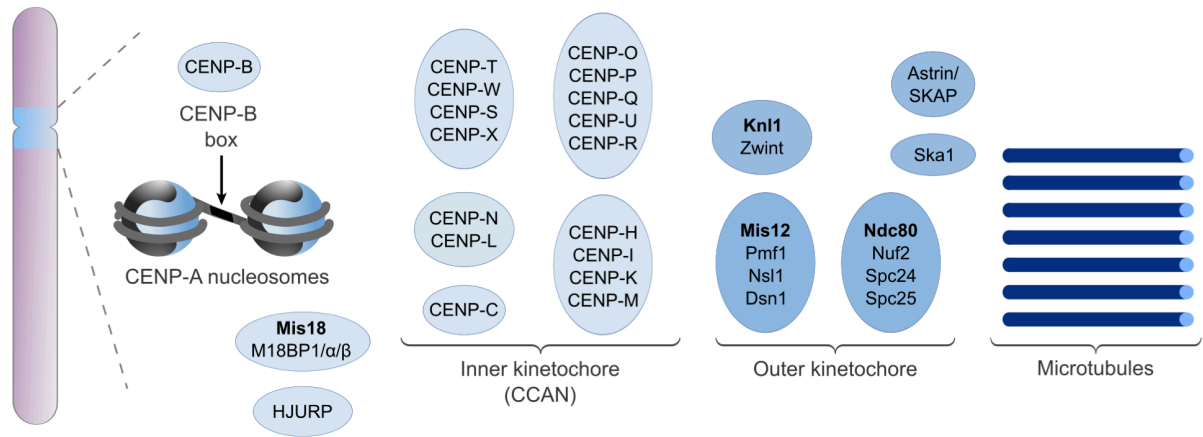


Figure 1. Schematic diagram of centromere and kinetochore components in M phase.

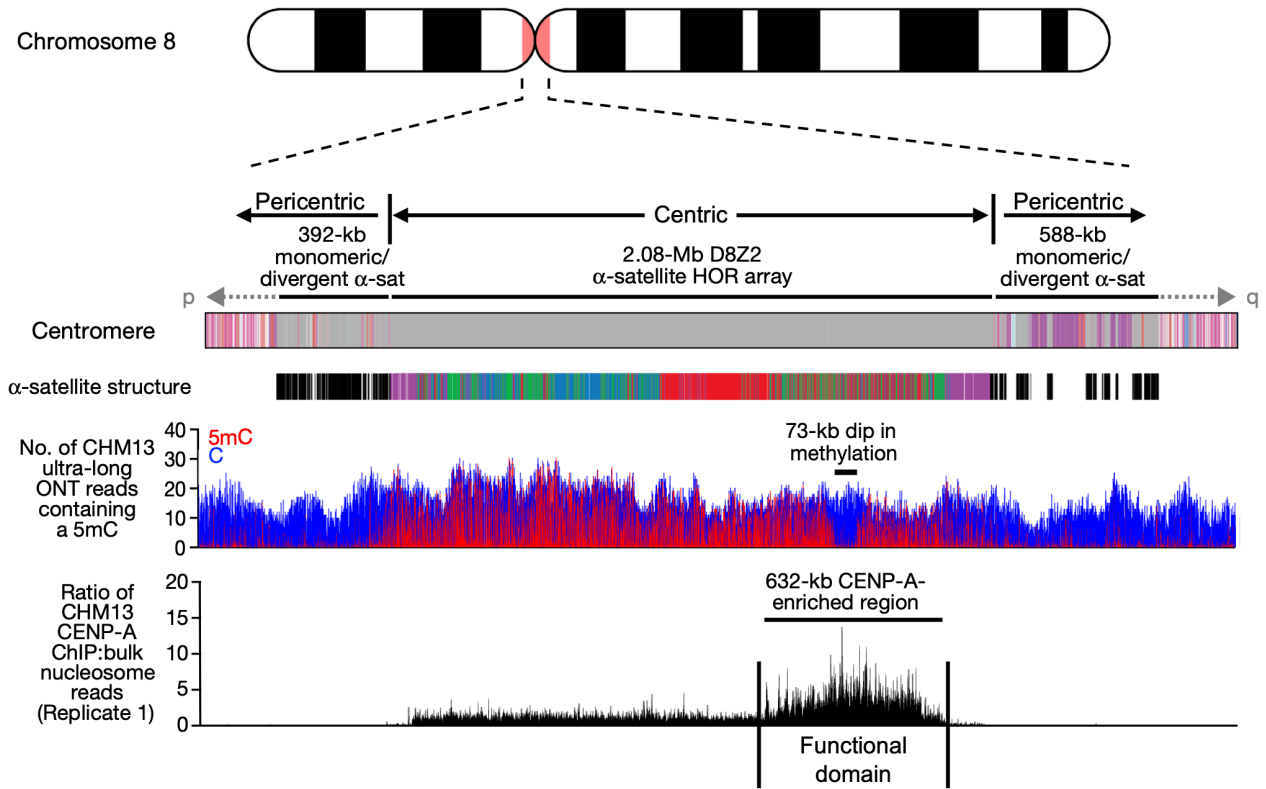


Figure 2. Schematic diagram of chromosome 8 with zoom on centromere showing DNA structure and organization, DNA methylation, and CENP-A enrichment, adapted from (Logsdon et al., 2021).

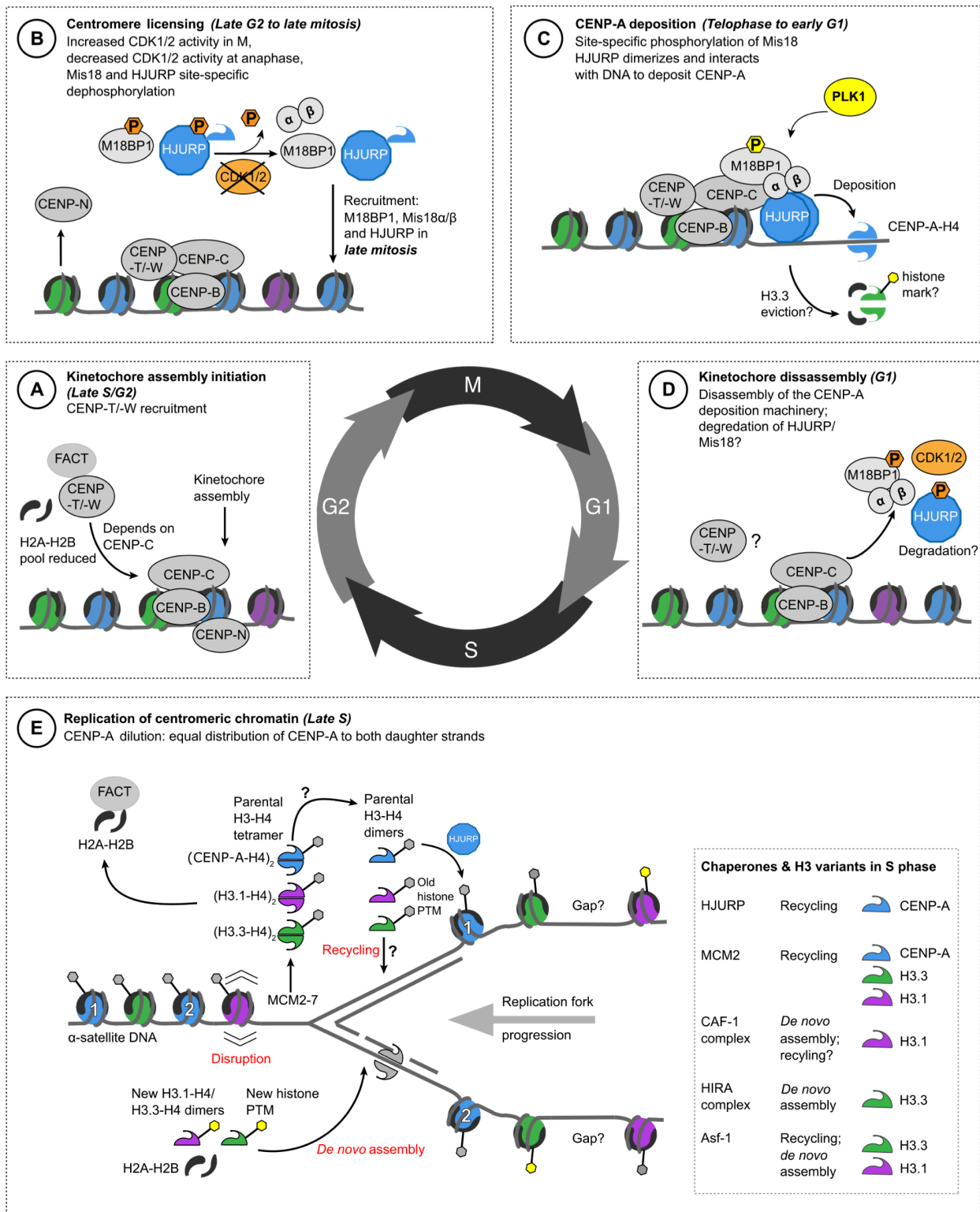


Figure 3. Dynamics of centromere components during the cell cycle, adapted from (Muller and Almouzni, 2017).

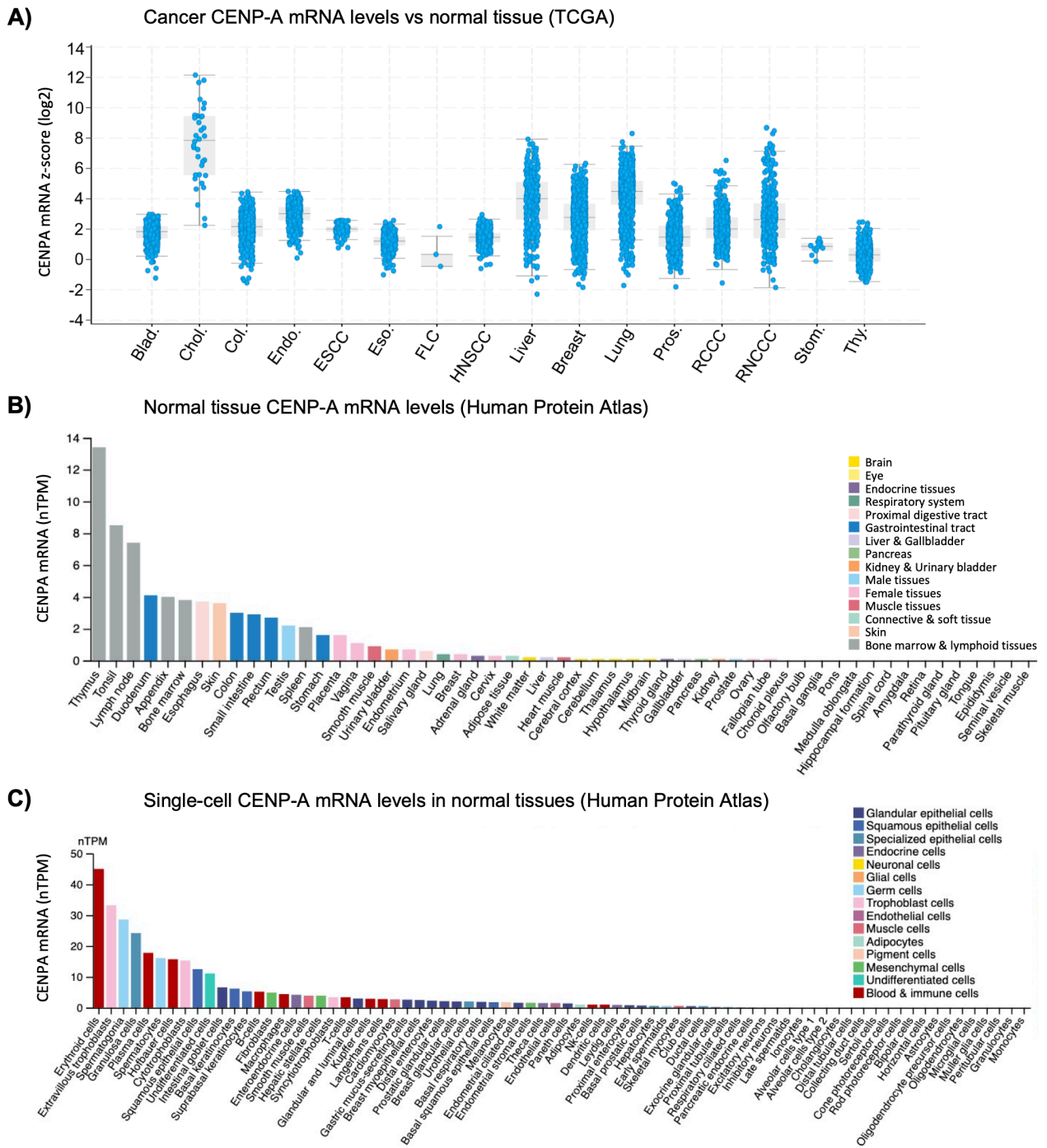


Figure 4. Variation in CENP-A expression levels in cancer, normal tissues, and single cells.

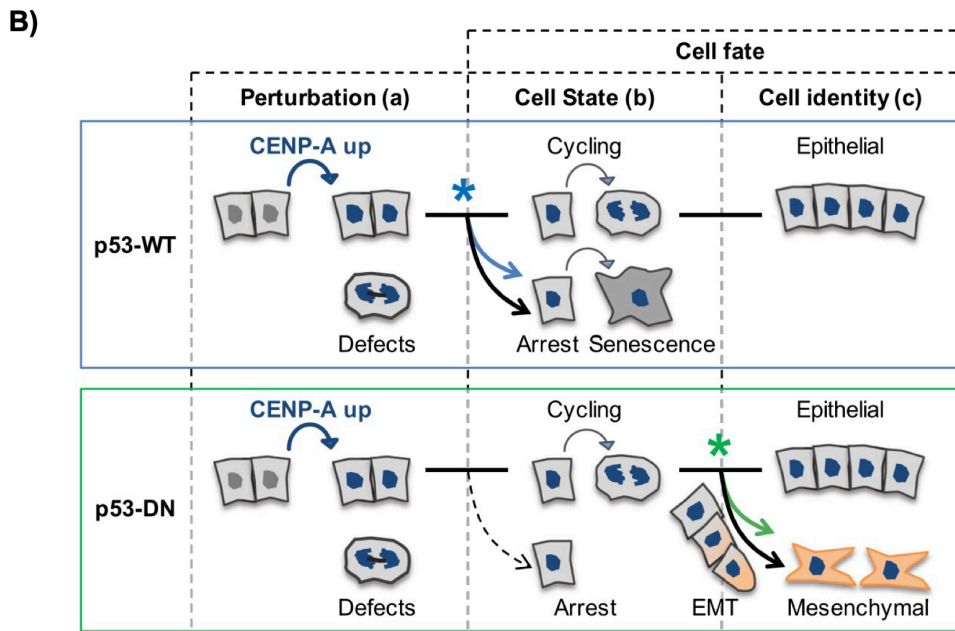
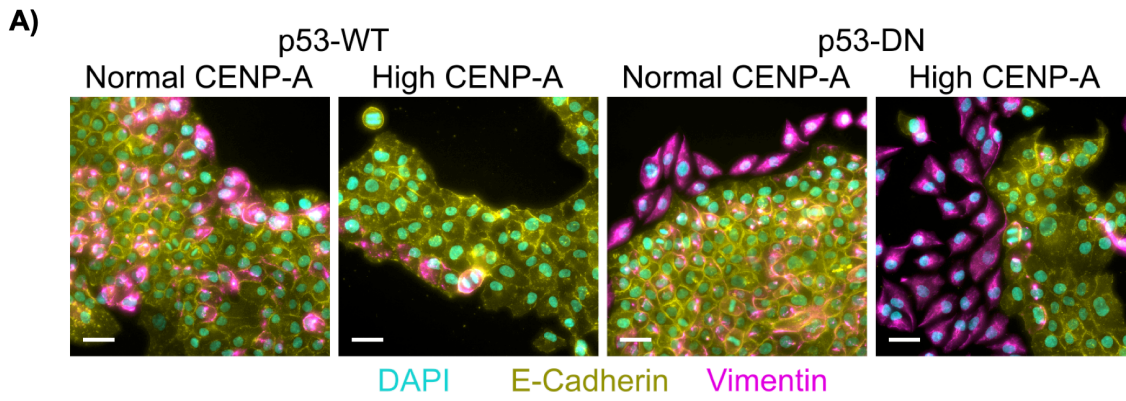


Figure 5. CENP-A overexpression promotes distinct cell fates depending on p53 status, adapted from (Jeffery et al., 2021).

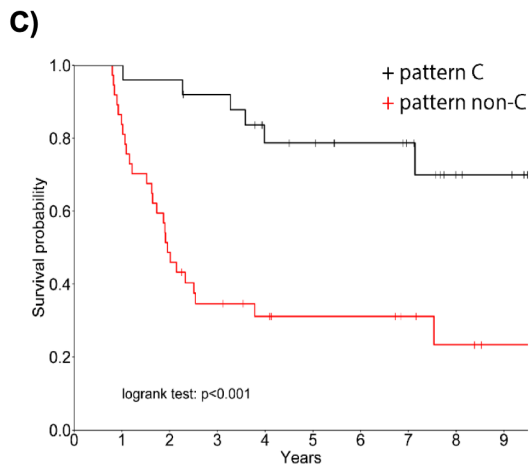
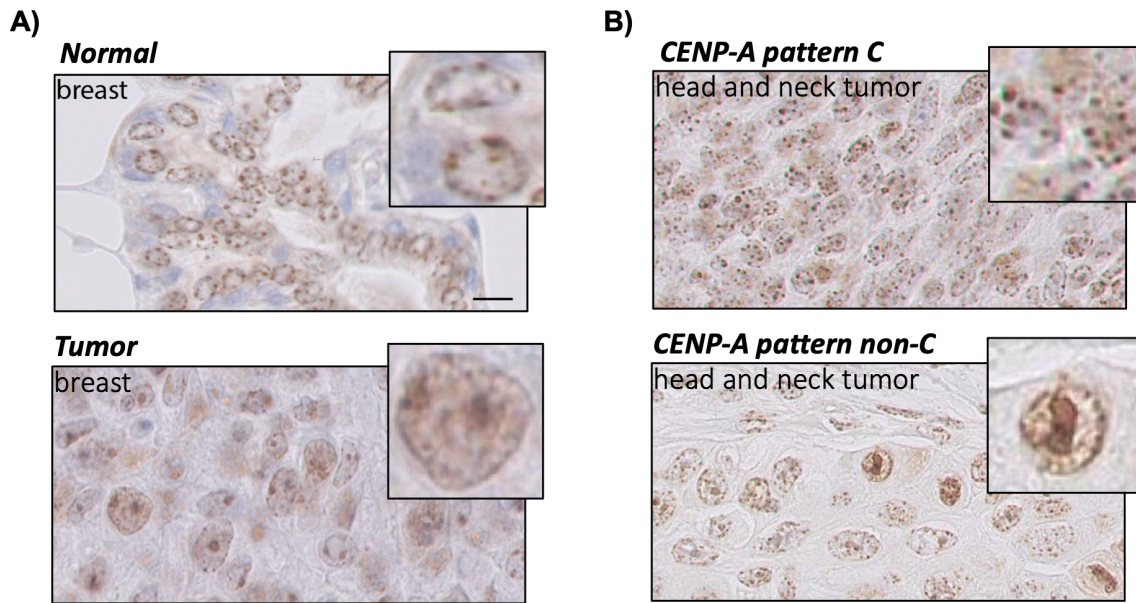


Figure 6. CENP-A staining patterns distinguish normal from malignant tissues and predict prognosis for chemoradiation-treated head and neck cancers, adapted from (Verrelle et al., 2021).