

# Using human artificial chromosomes to study centromere assembly and function



Chromosoma

# USING HUMAN ARTIFICIAL CHROMOSOMES TO STUDY CENTROMERE ASSEMBLY AND FUNCTION

--Manuscript Draft--

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| **Abstract:** | Centromeres are the site of assembly of the kinetochore, which directs chromosome segregation during cell division. Active centromeres are characterized by the presence of nucleosomes containing CENP-A and a specific chromatin environment that resembles that of active genes. Recent work using Human Artificial Chromosomes (HAC) sheds light on the fine balance of different histone post-translational modifications and transcription that exists at centromeres for kinetochore assembly and maintenance. Here, we review the use of HAC technology to understand centromere assembly and function. We put particular emphasis on studies using the alphoidtetO HAC, whose centromere can be specifically modified for epigenetic engineering studies. |
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| **Author Comments:** | We suggest Professor Erich Nigg as an Editor (not found in the "Request Editor" list), who invited us to write this review. |
| **Response to Reviewers:** | Comments for the Author:<br><br>Reviewer #1: Since there has been significant progress over the last few years with regards to HAC technology, particularly with alphoidtetO HACs, a review on this topic would be welcomed in the field, especially by these authors, who are world experts on |

this topic. The review is mostly satisfying, but I would suggest two areas where the authors would be well served to make additions/changes to avoid disappointing readers hoping for more focus/organization/clarity. First, since the goal of this review is to "review the use of HAC technology to understand centromere assembly and function", it would be especially helpful if the authors summarized the evolution of HAC technology and the findings that came from targeting different tetR fusion proteins to the alphoidtetO HACs (probably most effectively in a table or schematic).

We thank the reviewer for the thoughtful review of our MS. Following the reviewer´s advice, a table has been added explaining the findings that came from the alphoidtetO HAC studies in a timeline manner (Table 1).

Second, more discussion on the pitfalls of current HACs would be desirable (currently only a couple sentences is mentioned in the future directions).
From how it is written they sound so great that a reader would probably wonder why they are not used more widely in the research lab and/or clinic. There are problems with them, and it would be more clear and interesting, in my opinion, if more of the problems were laid out/challenges addressed.

A description of limitations of the HAC technology have been added in the text, first limitations for HAC clinical use (p. 8-9) and additional limitations that should be overcome in the final remarks (p. 23). Some other changes that I'd suggest:

1. It's confusing to refer to the same DNA as "alpha satellite", "α-satellite", and "alpha-satellite" throughout the review (most notably in the first two lines of the second paragraph). Why not just refer to it as "α-satellite" DNA?

We changed the term to α-satellite DNA throughout the text to maintain consistency.

2. The authors mention a result by Ikeno et al, 1998 that states that "introduction of YAC vectors with this α-satellite DNA into HT1080 cells efficiently formed HACs in 11 out of 13 clones analyzed". Especially after the authors go through the details of the faults of the Willard approach from 1997, stating it like they did for the Ikeno paper is misleading. In 7 out of those 11 clones, only a very minor proportion of cells actually had a HAC in them, the others were usually insertions into chromosomes…only 4 out of the 11 positive clones had a HAC in >50% of cells (as shown in Table 1 of Ikeno et al). Combined with using descriptive words like 'efficiently' to describe HAC formation, I think this part of the review doesn't come across as accurate as it should be.

We added information about the frequency of HAC-bearing cells in the clones isolated by Ikeno et al., 1998 (p. 7).

3. One pg 19, the authors state that an "open euchromatin signature and a flanking heterochromatin domain is necessary for proper chromosome segregation", following a long discussion of their HAC experiments. But there isn't really any data from their HAC experiments that would support this statement. At the end of the review, it seems this is precisely what the authors are now setting out to do. So the mis-statement on what can be concluded from the published HAC experiments should be removed on pg 19. The data mentioned in the text demonstrates that heterochromatin generated within a centromere can destabilize a kinetochore, but evidence of flanking heterochromatin being required for proper chromosome segregation is not directly shown or mentioned.

We agree with the referee that it is soon for such a definitive statement, as we are working further on this at the present time with the development of new HACs. However, strong evidence obtained using the HAC and other studies in yeast and Drosophila (cited in the text) suggest this as a very possible situation. Therefore, we changed this sentence for “data strongly suggest that an open euchromatin signature and a flanking heterochromatin domain is necessary for chromosome segregation”. (p. 19)

Typos:
4. Pg 3: Remove the comma between "primates" and "are".
5. Pg 3: Should be "more than 90% identity" instead of "more 90% identity".

6. Pg 10: Repeated the word "the" on 2nd to last line.
7. Pg18: Second full paragraph says ". . .tetracycline repressor fusion chimeras made it a suitable system", but shouldn't it be "make" since we are discussing the advantages and future directions of this system in the review?
8. Pg 19: "such H3K4me2" should be "such as H3K4me2"
9. Pg 20: HJURP is misspelled as "HURP"
10. Pg 22: "Identity" is misspelled as "identify"

All typos have been corrected.

Reviewer #2: Authors of this review had been studying on centromere/kinetochore assembly for long time, especially using human artificial chromosomes (HACs). They focus on HACs in this review and introduced studies using them from history to recent topics. This MS was written quite well, and I believe that this review would be widely read. As I gave some minor comments, it would be great if authors incorporate my points.

We thank the reviewer's for these helpful comments.

1. On page4, line 33-34, authors mentioned about di-centromere. If two centromeres are very close, it would be possible that there are two active centromeres. Sullivan and Willard published about this chromosome (Nature Genet., 1998: PMID: 9806536).

A sentence and corresponding citation was added (p. 4).

2. On page4, line 47, authors cited Fukagawa and Earnshaw, Dev Cell, 2014. This is OK, but Fukagawa and Earnshaw also wrote another review on neocentromeres in Curr. Biol. 2014. In addition to Dev Cell, please add Review in Curr. Biol. (PMID: 25291631).

Reference was added in the text (p. 4)

3. On page 5, line 2-3, author describe about ectopic CENP-A. Hori et al ( Dev Cell, 2014: PMID: 24960696) clearly showed ectopic CENP-A cloud based on ChIP-seq. Please cite this citation here.

Reference was added in the text (p. 5)

4. On page 5, line 28-30, authors mentioned that CENP-A containing nucleosomes occupy in HOR that ranges 200 and 2000 kb. I am not sure whether CENP-A containing nucleosomes occupy 2000 kb region. Although Sullivan et al. (2011) did such a conclusion, this may not ne consensus idea.

We recognize that as Karen Miga has yet to publish her maps of centromeric sequence contigs, mapping is difficult and there may still be some discussion of the exact size of the alphoid DNA array occupied by CENP-A nucleosomes. Although 2000 kb may be regarded as a possibly unusual upper limit, we respect the work of Beth Sullivan and would prefer to give this as an upper limit. We have changed the wording in the text to read "up to a limit of 2000 kb".

5. On page 8 line 7, authors mentioned that "…since these first studies…". I agree that Willard and Masumoto works are pioneer work, but "top-down" approach were taken before Willard and Masumoto works.

We agree with the reviewer that "top-down" approaches were done before the "bottom-up" ones and we did not comment enough about this. We clarified this in the text where we talk about the different approaches for HAC formation. Also, we specified in the "historic view" section that we are focusing on de novo HAC formation (i. e. bottom-up approach).

P. 6 – We added "de novo artificial chromosome construction…"
in p. 8 – "They have been constructed by either a "top-down" approach, by which the first mitotically stable minichromosomes were formed by chromosome truncation with

telomeric sequences…”

6. On page 8, line 16, when authors cite "top-down", they should cite works from William Brown (for example, PMID: 7987296; PMID: 8692956).

References were added (p. 8).

7. On page 10, line42-43, when author mentioned about knockout experiments, please cite Okada et al. (2006, Nature cell Biol.).

Reference was added in the text (p. 11).

8. On page 17, line 33, please add CENP-S/X and cite Amano et al. (2009, JCB, PMID: 19620631).

Reference was added in the text (p. 17 and 18).

Reviewer #3:
In the article entitled 'Using Human Artificial Chromosomes To Study Centromere Assembly And Function' authors review the use of HAC technology to understand centromere assembly and function. The article mainly focuses on studies using the alphoidtetO HAC, whose alpha-satellite containing chromatin can be specifically modified engineer epigenetic unique state and determine their effect of centromere establishment and function. The authors provide a comprehensive historical view on the development of human artificial chromosomes and the synthetic alphoidtetO HAC for epigenetic engineering of the centromere. Following this, the role of CENP-B and posttranslational modifications of in kinetochore assembly and the epigenetic balance between heterochromatin and acetylated chromatin is discussed. Authors discussed heterochromatin versus centrochromatin in centromere assembly and maintenance and provide concluding remarks with future perspective.

Overall this is an expertly written review that will provide a fantastic resource for those interested in the development of the HAC system and its applications in understanding chromosome biology. This is not surprising given that the authors are the major contributors to the success of this system. Although, at times, the focus on the HAC system comes at the expense of providing a more holistic description of centromere biology. Below are several specific suggestions and comments on the review.

We thank the reviewer for their thoughtful, which we believe substantially improved the MS.

Major Concerns

1. While very powerful, the HAC system has not thus far been able to dissect the contributions of alphoid DNA to centromere assembly and recruitment. Several systems in tissue culture and Drosophila have been used to create de novo centromeres that are independent of the HAC system. While these systems have not been as elegantly manipulated as the HAC system they have provided important about the sufficiency of centromere structure and assembly proteins and as such are worth mentioning in the review.

We added citations of advances in the centromere field using other systems apart from the HAC, such as Drosophila and yeast (p. 5)

2. The authors should comment on the fact that, although the alphoidtetO HAC tethering experiments have shed immense light on states of centromeric chromatin and how they relate to centromere function, the specific enzymes responsible for created and removing the marks at the centromere are less clear.

We included a sentence clarifying this point in pg 22

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| | 3. Authors provide a detailed historical view of the development of the HAC technology. Since this information is in great detail providing a timeline through a figure may make this clearer.<br><br>Since the review is focused mainly on the use of HACs for centrochromatin studies, we included a table providing a timeline with the progress made using the alphoidtetO HAC (Table 1).<br><br>4. The review is unique in that all the figures contain some primary data (Figure 1a, 1c, enlarged part of 1b and 1d, 2c, enlarged part of 2b, 3b, 3c, 4b). While I think this helps connect the models to the real data on which they are based, I do not think that is substantially increases the readability or impact of the review. I am also concerned because these primary data presented here are no presented as part of a complete and well controlled dataset. Presumably these are additional panels from the experiments which were already published, but that is an assumption. If the authors decide to keep these panels in the review, I strongly suggest that they provide direct references for each panel in which the controlled experiments have been already reviewed and published.<br><br>In the instances shown in Figure 1a,1b and 2b – these are just stock files of mitotic chromosomes and we feel that no other experimental attribution is necessary. In the case of the other panels, we have included attributions in the figure legends as follows – “Panel 1c shows unpublished data from the experiment presented in Molina et al., 2016b, Figure S1c”.<br><br>Minor Concerns<br><br>1. There are grammatical and formatting mistakes at several places throughout the manuscript. Some examples include, page#20, line#17 "Classic studies had showed….." should be replaced with "Classic studies have shown….." Page#15, line #14 and line #40 the cell cycle phase G1 is written in two different ways. There should be a consistent pattern.<br><br>The typos have been corrected.<br><br>2. Page 17…CENP-S and X not included in biochemical description of centromere.<br><br>We included the CENP-X/-S complex in the biochemical description of the centromere and we added the corresponding reference for it.<br><br>3. Page #5 line#38, the term READER is directly used for the first time and its description is given in subsequent section on page #17.<br><br>We believe that the term READER is in common usage, and so feel that it is acceptable to use it on p. 5 and define the other terms that we specifically associate with it on p. 17. It would be very clumsy to move the discussion of those terms forward to p. 5.<br><br>4. Page#12 & line#21, it is unclear why did the authors refer chromosome X? If the authors are suggesting that the X, and 17 and 21 have a higher density of CENP-B boxes, this should be stated more explicitly.<br><br>We refer to chromosome X because it is among the satellite DNAs from which HACs could be generated de novo, together with satellite DNAs from chromosomes 17 and 21. For this reason, taking into account this information we speculate about the density of CENP-B boxes as a possible cause for this. |

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Prof. Erich Nigg
Editor-in-chief
***Chromosoma***

Dear Erich,

It is our pleasure to submit a revised MS entitled “Using human artificial chromosomes to study centromere assembly and function” for consideration at ***Chromosoma***. The review is by Oscar Molina, Natalay Kouprina, Hiroshi Masumoto, Vladimir Larionov and William C Earnshaw.

We are very pleased that your referees in general were highly supportive of our review, and were grateful for their helpful comments, which we have addressed completely in our revision, as detailed in our response to referees. We believe that we have adequately addressed all comments, and hope that you will agree that our MS is now suitable for acceptance. It is our hope that this review will be regarded as a useful contribution to the scientific literature in this very interesting area.

Thank you very much for inviting us to write this review. We look forward to hearing from you when your deliberations are complete.

Best regards,

# USING HUMAN ARTIFICIAL CHROMOSOMES TO STUDY CENTROMERE ASSEMBLY AND FUNCTION

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*Keywords*: Human artificial chromosomes, centromere, kinetochore, CENP-A, mitosis

**Abstract**

Centromeres are the site of assembly of the kinetochore, which directs chromosome segregation during cell division. Active centromeres are characterized by the presence of nucleosomes containing CENP-A and a specific chromatin environment that resembles that of active genes. Recent work using Human Artificial Chromosomes (HAC) sheds light on the fine balance of different histone post-translational modifications and transcription that exists at centromeres for kinetochore assembly and maintenance. Here, we review the use of HAC technology to understand centromere assembly and function. We put particular emphasis on studies using the alphoid$^{tetO}$ HAC, whose centromere can be specifically modified for epigenetic engineering studies.

**Introduction**

Centromeres are defined cytologically as the primary constriction of mitotic chromosomes (Figure 1a) and have long been cytogenetically recognized as dark staining loci by C-banding. Centromeres define the site of assembly of the kinetochore, originally observed by electron microscopy as dark plates on the centromere surface (Luykx 1965; Brinkley and Stubblefield 1966; Jokelainen 1967). The kinetochore is a complex structure, containing more than 100 proteins that direct chromosome segregation by binding microtubules and regulating this process via interactions with mitotic checkpoint proteins (Cleveland et al. 2003; Allshire and Karpen 2008; Fukagawa and Earnshaw 2014a).

Centromeres of humans and other primates are characterized by the presence of alpha satellite (α-satellite) DNA sequences that span hundreds of kilobases up to five megabases (Willard 1985; Willard 1990). Alpha-satellite DNA is an AT-rich highly repetitive sequence that is based on a 171 bp monomer arranged in a tandem head-to-tail fashion (Willard 1990) (Figure 1b). These monomers are organized in higher-order repeats (HORs), each containing a characteristic number and sequence class of monomers. HORs are then repeated throughout the core region of each centromere (Choo et al. 1991; Aldrup-Macdonald and Sullivan 2014). Individual monomers share 50-70% homology with each other but corresponding monomers within a HOR share more than 90% identity (Figure 1b) (Waye and Willard 1989; Aldrup-Macdonald and Sullivan 2014). With the exception of the Y chromosome, the HORs comprising the centromere core all have at least one monomer containing the conserved 17 bp CENP-B box sequence. This is the binding site for the centromere protein CENP-B (Masumoto et al. 1989). Different classes of HORs are observed on various chromosomes, where they create chromosome-specific arrays (Aldrup-Macdonald and Sullivan 2014). The centromeric region with HORs (also termed the type I alphoid locus) is the core centromere on which kinetochores assemble (Figure 1c and d) (Choo et al. 1991; Ikeno et al. 1994; Aldrup-Macdonald and Sullivan

2014). Flanking the HORs, monomers lack CENP-B box sequences and are randomly arranged without high-order organizations (Ikeno et al. 1994). This monomeric α-satellite DNA (also termed the type II alphoid locus) comprises the pericentromeric regions linking the centromere to the chromosome arms (Ikeno et al. 1994; Schueler et al. 2001) (Figure 1d).

Although human centromeres consist of α-satellite repeats, a variety of evidence suggested that the DNA sequence is not the ultimate requirement for stable centromere formation. Stable dicentric chromosomes contain two α-satellite domains, one of which does not nucleate an active centromere (Earnshaw and Migeon 1985; Merry et al. 1985), except in those cases when the two centromeres are very close (Sullivan and Willard 1998). Typically, dicentric chromosomes are thought to be generated from arm breakage or shortening of telomeres, and consecutive rearrangements (Frias et al. 2012). These chromosomes are unstable due to the presence of two active centromeric regions that may attach to opposite spindle poles, resulting in chromosome bridges and breakage in anaphase (Stimpson et al. 2012). In rare instances, dicentric chromosomes can stabilize due to the inactivation of one of the two centromeres by deletion or by epigenetic silencing (Earnshaw and Migeon 1985; Merry et al. 1985; Earnshaw and Cooke 1989; Stimpson et al. 2012).

Further evidence for the epigenetic control of centromeres came with the discovery of neocentromeres that assemble on non-satellite DNA sequences in rare instances where a centromere has been lost or inactivated (Voullaire et al. 1993; du Sart et al. 1997; Warburton et al. 1997; Saffery et al. 2000; Lo et al. 2001; Warburton 2001; Alonso et al. 2003; Alonso et al. 2007; Fukagawa and Earnshaw 2014a; Nishino et al. 2012; Fukagawa and Earnshaw 2014b). Together, these observations led to the suggestion that centromere assembly and maintenance is regulated by epigenetic mechanisms (Earnshaw and Migeon 1985; Sullivan and Schwartz 1995; Karpen and Allshire 1997; Vafa and Sullivan 1997; Sugata et al. 2000).

One clue to the epigenetic regulation of centromeres came with the discovery of CENP-A, a centromere-specific histone H3 subtype that is concentrated at centromeres (Figure

1c and d), though it also binds at much lower concentrations throughout the rest of the genome (Earnshaw and Rothfield 1985; Palmer et al. 1987; Palmer et al. 1991; Sullivan et al. 1994; Bodor et al. 2014; Nishino et al. 2012; Hori et al. 2014). In eukaryotes, apart from Trypanosomatids (Akiyoshi and Gull 2013) and some holocentric insects (Drinnenberg et al. 2014), CENP-A is concentrated exclusively at active centromeres (Earnshaw et al. 1989; Warburton et al. 1997; Lo et al. 2001; Alonso et al. 2007) , where it forms the foundation for the kinetochore. Studies on stretched chromatin fibers from human and chicken DT-40 cells found that nucleosomes containing CENP-A are interspersed with nucleosomes containing the canonical histone H3 (Sullivan et al. 1994; Sullivan and Karpen 2004; Ribeiro et al. 2010; Nishino et al. 2012)). The nucleosomes containing the canonical histone H3 bear histone modifications that are typically found in the bodies of active genes, such as H3K4me2 and H3K36me2, thus, with CENP-A, forming a specific chromatin domain that has been termed "centrochromatin" (Blower et al. 2002; Sullivan and Karpen 2004; Bergmann et al. 2011; Hori et al. 2014). CENP-A-containing nucleosomes occupy a subset of the α-satellite HOR that ranges between 200 and up to a limit of 2,000 kb on different chromosomes and in different individuals (Sullivan et al. 2011). Centrochromatin in the core centromere is flanked by extensive regions of constitutional heterochromatin, containing marks such as H3K9me3 and its READER HP1 (Allshire and Karpen 2008) (Figure 1c and d).

Although some aspects of centromere biology are well studied, the organization of centromeric chromatin and its relevance for kinetochore assembly and chromosome segregation are less understood. Significant efforts in recent years have revealed much about the CENP-A nucleosome (Maiato et al. 2004; Cheeseman and Desai 2008; Santaguida and Musacchio 2009; Perpelescu and Fukagawa 2011; Schalch and Steiner 2016) and begun to reveal the epigenetic requirements for kinetochore formation and centromere function (Black and Cleveland 2011; Olszak et al. 2011; Roy and Sanyal 2011). In particular, our understanding of centromere assembly and function has improved greatly, in part due to the use of Human

Artificial Chromosomes (HACs), which have helped in our understanding of the minimal requirements for *de novo* kinetochore formation and stable maintenance throughout cell division.

HACs are small extrachromosomal elements that replicate autonomously and segregate accurately during cell division due to the presence of a functional centromere (Kouprina et al. 2014). In this review, we focus on the use of Human Artificial Chromosomes as models for *de novo* kinetochore formation and discuss how the HAC technology improved our understanding of centromere/kinetochore assembly and function.

**Human Artificial Chromosomes development: a historic view.**

The first eukaryotic artificial chromosomes were generated in the yeast *S. cerevisiae* (YACs) (Clarke and Carbon 1980; Murray and Szostak 1983). Those studies showed that stable linear YAC formation required at least three chromosomal elements – centromeres, telomeres and origins of replication (Murray and Szostak 1983; Young et al. 1998). The budding yeast point centromere (Pluta et al. 1995) is defined by the presence of a 125 bp sequence and is not dependent on epigenetic mechanisms for kinetochore assembly (Clarke and Carbon 1980; Cottarel et al. 1989; Spencer et al. 1990; Spencer and Hieter 1992; Doheny et al. 1993; Hegemann and Fleig 1993). In contrast, the much larger regional centromeres of *S. pombe* consist of a central core flanked by heterochromatin, and epigenetic regulation is critical for their assembly. Assembly of kinetochores *de novo* on artificial chromosomes in *S. pombe* was challenging (Clarke and Baum 1990) and appears to require both heterochromatin and stalled RNA polymerase (Folco et al. 2008; Kagansky et al. 2009; Catania and Allshire 2014; Catania et al. 2015; Allshire and Ekwall 2015). As will be seen below, this exhibits both similarities and differences from centromere assembly in human cells.

*De novo* artificial chromosome construction in mammalian cells was initially hampered by a poor understanding of the nature of the corresponding centromeres and origins of

replication in mammals, and also by the difficulty of cloning large stable fragments of centromere-repeat arrays (Neil et al. 1990). However, two groups succeeded in solving this problem in the 1990s. Harrington et al. developed a method for cloning large arrays of human α-satellite DNA based on the multimerization of single HOR units from chromosomes 17 and Y. These long arrays of α-satellite DNA (up to 1 Mb in size) were used to construct human artificial chromosomes (HACs) by co-transfecting them into human HT1080 cells together with telomeric DNA and random genomic DNA (Harrington et al. 1997). Cytogenetically and mitotically stable HACs were observed in 9 out of 26 clones obtained, however, most of these formed by either a chromosome truncation event or by rescue of an acentric fragment, and only one of the HACs was found to be formed *de novo*.

An alternative approach performed at the same time involved cloning α-satellite DNA type-I sequences derived from the human chromosome 21 HOR retrofitted with telomere sequences in YAC vectors (YAC-MAC system) (Ikeno et al. 1998). Introduction of YAC vectors with this α-satellite DNA into HT1080 cells efficiently formed HACs in 11 of 13 clones analyzed. However, positive clones showed a minor proportion of cells with HACs and only 4 of them had HACs in more than 50% of cells (Ikeno et al. 1998). Importantly, no HACs were observed when the YAC vector containing the divergent monomeric type-II α-satellite DNA from chromosome 21 was used. This suggested that CENP-B box sequences present in the homogeneous type-I α-satellite DNA sequences (but missing from the type-II α-satellite DNA arrays) might be necessary for *de novo* kinetochore assembly (Ohzeki et al. 2002; Basu et al. 2005). As will be discussed below, the role/s of CENP-B box sequences at centromeres is still not clear. These motifs are not present at the human Y centromere or in the α-satellite DNAs of African green monkey, which paradoxically does express CENP-B protein (Goldberg et al. 1996) (S. Kasinathan and S. Henikoff, personal communication).

Together, these two initial studies suggested that α-satellite DNA and telomeric sequences were required for HAC formation. However, a study by Ebersole and collaborators

later showed that circular vectors containing only α-satellite DNA (α-21-I) were as competent for *de novo* HAC formation as linear vectors after transfection in HT1080 cells. Furthermore, HACs formed from circular and linear vectors showed similar mitotic stability (Ebersole et al. 2000). Therefore, the only chromosomal components essential for *de novo* HAC formation are regular α-satellite DNA arrays with CENP-B boxes and origins of replication - telomeric sequences are only required to maintain the integrity of linear HACs.

Numerous other HACs have been developed since these first studies (Mills et al. 1999; Mejia and Larin 2000; Grimes et al. 2001; Kouprina et al. 2003; Kazuki et al. 2011; Mandegar et al. 2011; Iida et al. 2014; Takiguchi et al. 2014). They have been constructed by either a "top-down" approach, by which the first mitotically stable minichromosomes were formed by chromosome truncation with telomeric sequences (Brown et al. 1994; Farr et al. 1995; Heller et al. 1996; Mills et al. 1999; Kazuki et al. 2011); or a "bottom-up" approach (Figure 2), in which naked DNA is introduced into cells either by transfection (Henning et al. 1999; Ikeno et al. 2002; Kouprina et al. 2003; Suzuki et al. 2006) or by transduction with herpex simplex virus 1 (Moralli et al. 2006; Mandegar et al. 2011), thus generating *de novo* artificial chomosomes.

The diverse potentialities of HAC technology have been reviewed extensively in recent years (Mills et al. 1999; Saffery and Choo 2002; Basu and Willard 2006; Bergmann et al. 2012b; Kouprina et al. 2013; Moralli and Monaco 2015; Oshimura et al. 2015; Ohzeki et al. 2015). HACs represent potential vectors for delivery of large-genomic DNA regions and they have been proposed as new gene-delivery vectors for gene therapy that overcome some of the limitations of the current viral-based vectors (Kouprina et al. 2014). HACs offer: (i) stable maintenance at low-copy number, (ii) avoidance of an immunogenic response associated with adenoviral vectors and (iii) suitability for carrying even the largest full-length genes together with all regulatory regions. Indeed, HACs containing full genes have been shown to complement gene deficiencies in human cells (Mejia et al. 2001; Basu et al. 2005; Kim et al. 2011; Kononenko et al. 2014). However, before the HAC technology can be implemented

clinically as a gene delivery vector some limitations must be overcome (Kouprina et al. 2014): (i) low efficiency of HAC formation, (ii) complex repeated DNA structure that limits HAC characterization, (iii) impossibility of amplification of large amounts of vector outside eukaryotic cells, (iv) lack of efficient systems for HAC-delivery into target tissues or organs. Beyond their use as gene-delivery vectors, HACs have proven especially important in chromosome biology research, to quantify chromosome instability in cancer cells (Lee et al. 2013; Duffy et al. 2016; Kim et al. 2016; Lee et al. 2016), to study telomere maintenance (Wakai et al. 2014) and to study the chromatin requirements for centromere function (Nakano et al. 2008; Cardinale et al. 2009; Bergmann et al. 2011; Bergmann et al. 2012a; Ohzeki et al. 2012; Shono et al. 2015; Ohzeki et al. 2016; Martins et al. 2016; Molina et al. 2016b) (Table 1). In this last regard, a big step forward was the development of the alphoid$^{tetO}$ HAC (Nakano et al. 2008), whose kinetochore can be specifically targeted with chromatin modifiers, thus allowing epigenetic engineering of the centromere for functional studies.

**The synthetic alphoid$^{tetO}$ HAC for epigenetic engineering of the centromere**

Prior to the alphoid$^{tetO}$ HAC, all previous HACs were constructed using native HORs as the basic repeat structure for *de novo* centromere formation. Ebersole and collaborators described a method to amplify human alphoid repeats of a few hundred base pairs up to 120 Kb. This method included the use of rolling-circle amplification (RCA) of alphoid repeats *in vitro* and assembly of those repeats *in vivo* by recombination in yeast (transformation-associated recombination – TAR) into long arrays (Ebersole et al. 2005; Kouprina and Larionov 2016). Using RCA-TAR cloning, they successfully obtained alphoid-DNA arrays of 30-120 Kb from type-I HORs from chromosome 21 (with a 343 bp dimer HOR repeat as starting material) (Figure 2a). These synthetic arrays formed HACs in 10% of cell lines analyzed after transfection into human HT1080 cells (Ebersole et al. 2005).

The alphoid$^{tetO}$ HAC was the first of a new generation of synthetic HACs whose centromeric chromatin can be specifically modified (Nakano et al. 2008) (Figure 2). An α-satellite DNA array of 50 Kb was generated by RCA-TAR technology starting with a synthetic dimer consisting of a natural monomer from a chromosome 17 alphoid HOR with a CENP-B box fused to a completely synthetic monomer in which the CENP-B box had been replaced by a tetracycline operator (tetO), the binding site of the *E. coli* tetracycline repressor (tetR) (Figure 2a-c). This synthetic α-satellite DNA array – termed the alphoid$^{tetO}$ array- formed HACs after transfection into HT1080 cells. The presence of the tetO sequences enables targeting any desired protein into the active centromere of the alphoid$^{tetO}$ HAC as a tetR-fusion protein (Figure 2d). As a control, targeting of tetR-EYFP on its own is not detrimental for HAC kinetochore structure and function (Nakano et al. 2008). More recently, other synthetic alphoid$^{tetO}$ HACs were generated based on a native dimer from chromosome 21 α-satellite type-I DNA, in which a tetO was inserted into the place of the counter CENP-B box position into one of the monomers (Ohzeki et al. 2012).

In humans, *Drosophila*, and chicken DT40 cells CENP-A is interspersed with canonical histone H3 that is hypoacetylated and contains different levels of histone H3 lysine 4 di-methylation (H3K4me2) and lysine 36 di- and tri-methylation (H3K36me2/3) (Sullivan and Karpen 2004; Ribeiro et al. 2010; Bergmann et al. 2011). As described above, this specialized chromatin environment has been termed “centrochromatin” (Sullivan and Karpen 2004). The contribution of centrochromatin to kinetochore assembly and centromere function has been a topic of intense speculation, and raises several questions: (i) What are the role/s of specific histone modifications for CENP-A assembly and centrochromatin specification? (ii) Do the chromatin modifications vary throughout the cell cycle, possibly playing a regulatory role on kinetochore assembly? (iii) What maintains an “open” chromatin region such as centrochromatin embedded within large heterochromatin blocks at human centromeres?

Prior to the availability of the alphoid$^{tetO}$ HAC, attempts to manipulate centromere chromatin involved cell treatments with drugs (i.e. chemical inhibitors), protein over-expression or knockdown experiments (Desai et al. 2003; Sumer et al. 2004; Okada et al. 2006; Kwon et al. 2007; Cheeseman et al. 2008). Such approaches affect all chromosomes of the cell and potentially affect cellular physiology, which may generate off-target effects, thus hampering the interpretation of the results. Indeed, even if the reagents used are highly specific, most of the target enzymes act on non-chromatin substrates as well, thus confounding the interpretation of such experiments. The alphoid$^{tetO}$ HAC represents a precise tool that allows direct manipulation of one single kinetochore, whilst leaving the remainder of the cell unaffected.

Whereas none of the *de novo* HACs constructed before were physically mapped in molecular detail, largely due to the fact that they contain huge blocks of repeated DNA, the alphoid$^{tetO}$ HAC has been shown to consist of 1.1 Mb of continuous α-satellite DNA sequences assembled from tandem and inverted repeats that range in size from 25 to 150 Kb and an approximately 4 Mb fragment from the arm of chromosome 13 (Kouprina et al. 2012). A recent microscopy analysis revealed that the alphoid$^{tetO}$ HAC resembles a natural chromosome, containing, in addition to its kinetochore, chromosome scaffold (revealed by staining for condensin subunit SMC2) and a chromosome periphery compartment (revealed by staining for Ki-67) (Booth et al. 2016). Use of 3D correlative light electron microscopy (3d-CLEM) allowed the determination of the HAC volume at prometaphase. Assuming a normal density of chromatin packing, this yielded an estimated size of 5.5 MB for the HAC, essentially identical to the 5.1 MB calculated from the molecular cloning analysis (Kouprina et al. 2012; Booth et al. 2016). This agreement confirmed that the packing density of the alphoid$^{tetO}$ HAC is comparable to the other endogenous chromosomes. Thus, the mechanisms used to condense mitotic chromosomes are apparently independent of the chromosome shape and size (Booth et al. 2016).

In HAC formation experiments, the most common fate of the input DNA constructs is integration into the chromosome arm of a host chromosome (Figure 2b) (Ikeno et al. 1998; Masumoto et al. 1998; Nakano et al. 2008). Although this is not the desired outcome for HAC formation studies, the integrant clones have also proven to be useful for centromere formation studies, e.g. identification of chromatin states that promote CENP-A recruitment (Hori et al. 2014; Ohzeki et al. 2016). We will discuss below the use of the alphoid$^{tetO}$ HAC and the alphoid$^{tetO}$ integrations to study *de novo* centromere assembly and kinetochore maintenance.

**The role of CENP-B and histone posttranslational modifications in *de novo* kinetochore assembly**

One important requirement for *de novo* HAC formation is the presence of CENP-B box sequences - the binding site for the centromere protein CENP-B - in the input α-satellite DNA (Ohzeki et al. 2002; Basu et al. 2005). The density of CENP-B boxes in the input alphoid-DNA is an important factor for *de novo* centromere and HAC formation. Decreasing the density of CENP-B boxes in cloned chromosome 21 HORs abolished HAC formation (Okamoto et al. 2007), while increasing it in chromosome 17 HORs significantly raised the frequency of *de novo* HAC formation in HT1080 cells (Basu et al. 2005). These observations may explain why HACs could only initially be obtained using alphoid-DNA sequences from chromosomes 21, X and 17. These arrays apparently have the proper number and density of CENP-B boxes to sustain *de novo* centromere formation.

CENP-B is a highly conserved protein that binds to CENP-B-boxes in human α-satellite DNA and mouse minor satellites. CENP-B binds DNA via its amino-terminal domain and dimerizes via its carboxy-terminal domain (Masumoto et al. 1989; Pluta et al. 1992). The function(s) of CENP-B in centromere assembly and maintenance has long been unclear. In human cells, CENP-B boxes are present at almost all centromeres but not on the Y

chromosome or at neocentromeres (Masumoto et al. 1989; Earnshaw and Rattner 1991). Importantly, the absence of CENP-B boxes has been demonstrated at some centromeres of other species, such as African green monkey, chicken and mice (Goldberg et al. 1996; Pertile et al. 2009; Kugou et al. 2016) (S. Kasinathan and S. Henikoff, personal communication). CENP-B knockout mice are viable and fertile with normal centromere function (Hudson et al. 1998; Perez-Castro et al. 1998; Kapoor et al. 1998). It was thus suggested that CENP-B is not required for kinetochore maintenance but it might be important for *de novo* kinetochore assembly (Ohzeki et al. 2015).

In one important study that shed light on the role of CENP-B during *de novo* centromere assembly, BAC constructs carrying human α-satellite DNA with wild-type or mutant CENP-B boxes were transfected into wild-type or CENP-B-deficient mouse embryonic fibroblasts (MEFs) with or without exogenous CENP-B expression (Okada et al. 2007). The results indicated that CENP-B has a dual antagonistic role on centromere satellite DNA, balancing *de novo* CENP-A assembly versus heterochromatin-induced inactivation depending on the surrounding chromatin context. On the one hand, CENP-B was essential for *de novo* CENP-A assembly, which was abolished when the CENP-B gene was knocked out. CENP-A assembly was restored when exogenous CENP-B was overexpressed in CENP-$B^{-/-}$ MEFs. On the other hand, the same study also reported an antagonistic role of CENP-B, which could induce strong heterochromatin assembly, detected by incorporation of H3K9me3, at sites of ectopic integration of the α-satellite DNA on the endogenous chromosome arms. CENP-A assembly was suppressed at these ectopic sites, apparently as a result of the heterochromatin assembly (Okada et al. 2007).

More recent studies showed that CENP-A binds to CENP-B and CENP-C through its N-terminal and C-terminal tails, respectively (Carroll et al. 2010; Fachinetti et al. 2013). Available data suggest the existence of two kinetochore assembly pathways: one involving the CENP-A C-terminus binding to CENP-C and the other involving the CENP-A N-terminus binding to CENP-

B. Perhaps both pathways are essential for *de novo* HAC formation but not required for the maintenance of established centromeres (Ohzeki et al. 2015). *In vitro* experiments demonstrated that the CENP-B DNA binding domain specifically bound to the CENP-A-H4 complex, but not the H3.1-H4 complex. Moreover, CENP-B binding to the CENP-B box enhanced the retention of preassembled CENP-A nucleosomes on alphoid DNA *in vivo* (Fujita et al. 2015). This data is consistent with previous observations that CENP-B promotes *de novo* formation of stable CENP-A-chromatin during HAC formation (Okada et al. 2007). While these studies have demonstrated that CENP-B has a significant role in kinetochore assembly and function, its exact function/s are still under investigation.

**Breaking the HAC Barrier**

Formation of HACs initially appeared to be limited to the fibrosarcoma-derived human cell line HT1080. A ground-breaking study using the alphoid$^{tetO}$ HAC tested the hypothesis that this cell line restriction might be due to epigenetic effects such as modifications of the canonical H3 nucleosomes that are interspersed between CENP-A nucleosomes in centrochromatin (Blower et al. 2002; Sullivan and Karpen 2004; Ribeiro et al. 2010). That study demonstrated that *de novo* centromere assembly and maintenance is dependent on a balance between the levels of histone H3 lysine-9 methylation (H3K9me3) and acetylation on the α-satellite chromatin (Ohzeki et al. 2012). The authors found that the chromosome 21-derived alphoid$^{tetO}$ array rapidly assembled CENP-A when it was introduced into HeLa and other cell lines. However, consistent with previous data (Okada et al. 2007), the CENP-A molecules were lost from the array over the next few days concomitant with an accumulation of H3K9me3 on the array.

Ohzeki et al. found that HT1080 cells show much reduced levels of H3K9me3 due to decreased expression of the H3K9-specific methyltransferase SUV39H1 compared to other commonly used mammalian cell lines, such as HeLa (Okada et al. 2007; Ohzeki et al. 2012).

Strikingly, knockdown of the SUV39H1 methyltransferase in HeLa cells promoted both the initial assembly of CENP-A and the maintenance of CENP-A chromatin at the alphoid$^{tetO}$ array (Ohzeki et al. 2012). These data strongly suggested that heterochromatin formation antagonizes CENP-A maintenance.

To further explore this hypothesis, they exploited the alphoid$^{tetO}$ system by introducing into HeLa cells alphoid$^{tetO}$ DNA together with DNA encoding a fusion of either the PCAF or p300 histone acetyltransferase (HAT) domains to tetR. Remarkably, binding of either tetR-HAT domain fusion led to stable CENP-A deposition on the alphoid$^{tetO}$ array. This resulted in the first formation of mitotically stable HACs in any human cell line other than HT1080 (Ohzeki et al. 2012).

Consistent with the HAT requirement for *de novo* HAC formation, Ohzeki et al. found a transient increase of histone H3 lysine 9 acetylation (H3K9ac) at endogenous centromeres in a small temporal window following release from mitotic arrest. This suggested that histone acetylation is required for *de novo* establishment of CENP-A chromatin. These results were consistent with previous work showing that the Mis18 complex, a key protein complex involved in the CENP-A deposition pathway, associates with centromeres from anaphase to early $G_1$ in human cells (Fujita et al. 2007; Maddox et al. 2007). Furthermore, although the chromatin modifications required for Mis18 complex recruitment to centromeres are unknown, loss of CENP-A assembly following Mis18α knockdown could be restored by treatment with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) (Fujita et al. 2007). Altogether, these data suggest that intrinsic HAT activity may be involved in the maintenance of established CENP-A chromatin, possibly by preventing heterochromatin spreading into centromeric α-satellite DNA.

More recently, Ohzeki and collaborators used an ectopic non-centromeric alphoid$^{tetO}$ array inserted in a chromosome arm to develop a protein-protein interaction assay to identify which of the 17 human HATs is involved in α-satellite DNA licensing for *de novo* centromere

formation. They found that the KAT7/HBO1/MYST2 complex interacts with Mis18BP1 and that it localizes to centromeres in early $G_1$ (Ohzeki et al. 2016). Consistently, when KAT7 was tethered to the ectopic alphoid$^{tetO}$ array integration as a TetR fusion, it reduced H3K9me3 and provided competence for CENP-A assembly and maintenance (Ohzeki et al. 2016).

Altogether, these data suggest that *de novo* centromere assembly in human cells is dependent on an epigenetic balance between heterochromatin and acetylated chromatin, upstream of the CENP-A deposition pathway. More detailed knowledge of the processes that promote *de novo* centromere assembly and identification of the factors required should ultimately allow the generation of HACs in any cell line. This will increase the versatility and the efficiency of the HAC technology, thereby overcoming one of the limitations that remain before HACs can be used as gene delivery vectors for gene therapy.

**Heterochromatin versus centrochromatin in centromere assembly and maintenance.**

The relationship between centromeres and heterochromatin is complex. As described above, the core of each centromere is composed of a specialized class of centrochromatin. At natural centromeres, this relatively open chromatin domain is flanked by inactive chromatin – typically constitutive heterochromatin rich in H3K9me3 (Figure 1), but in some cases facultative heterochromatin containing polycomb-associated H3K27me3 chromatin marks (Martins et al. 2016). In *S. pombe* the border between the two regions may be delimited by tRNA genes (Takahashi et al. 1991; Takahashi et al. 1992), but in humans it is less clear how the heterochromatin is prevented from invading the centrochromatin domain.

Heterochromatin is important for normal chromosome segregation and maintaining genomic stability (Peters et al. 2001; Slee et al. 2012; Molina et al. 2016a). It facilitates sister chromatid cohesion by recruiting cohesin complexes (Bernard et al. 2001; Nonaka et al. 2002; Yamagishi et al. 2008; Gartenberg 2009) and, at least *in S. pombe*, it was reported to be necessary for *de novo* kinetochore formation (Folco et al. 2008; Kagansky et al. 2009). Indeed,

the impact of removing heterochromatin on kinetochore structure and function was recently explored using the novel synthetic biology approach PREdiTOR (Protein READING and EDITING of Residues), in which the H3K9me-specific demethylase JMJD2D was tethered to all centromeres (Molina et al. 2016a). Heterochromatin removal led to chromosome segregation defects as a result of disrupted kinetochore structure, chromosome passenger complex mislocalization and decreases in centromeric stiffness in metaphase (Molina et al. 2016a).

During *de novo* centromere assembly, heterochromatin appears to drive newly assembled CENP-A off of α-satellite DNA that has been transfected into human cells unless the newly assembling chromatin is acetylated (Ohzeki et al. 2012). However, in *S. pombe* it is precisely heterochromatin that is needed to render newly introduced minichromosome DNA capable of assembling a stable centromere (Folco et al. 2008; Kagansky et al. 2009).

The alphoid$^{tetO}$ HAC has been used to study interactions between heterochromatin and centromeres, and even with this system, the relationship has proven to be complex. To discuss this, we will use the terminology EDITOR → MARK → READER → CHROMATIN STATE (E → M → R → C) to describe chromatin states (Molina et al. 2016a). The term EDITOR refers to both writers and erasers of chromatin marks. As we will show, the alphoid$^{tetO}$ centromere, once established, appears to be able to resist the effects of EDITORS that promote inactive chromatin states, but it is not able to resist the, presumably stronger, effects caused by the tethering of READERS (MARTINS ET AL. 2016).

The functional core of the inner kinetochore consists of a group of 16 proteins that are associated with centromeres throughout the cell cycle. This group, known as the constitutive centromere associated network (CCAN) (Foltz et al. 2006; Okada et al. 2006; Hori et al. 2008) includes CENP-A, CENP-C and five multi-subunits complexes grouped based on their functions and biochemical features: CENP-L/-N, CENP-H/-I/-K/-M, CENP-O/-P/-Q/-R/-U, CENP-S/-X and CENP-T/-W (Hori et al. 2008; Amano et al. 2009; Przewloka et al. 2011; Screpanti et al. 2011;

Nishino et al. 2013). The different members of the CCAN bridge the inner kinetochore plate to the microtubule-associated outer kinetochore plate assembled in mitosis.

The proper assembly of CCAN components, and in turn the outer kinetochore proteins, is dependent on the presence of CENP-A (Oegema et al. 2001; Goshima et al. 2003; Liu et al. 2006; Hori et al. 2013; Shono et al. 2015). This suggested that CENP-A might act as an epigenetic mark responsible for the maintenance of centromere identity (Vafa and Sullivan 1997; Warburton et al. 1997). Importantly, unlike the canonical histone H3.1 and H3.2, CENP-A deposition is not coupled with DNA replication (except in budding yeast) (Pearson et al. 2004). Instead, it takes place in humans during early $G_1$ phase, following the loss of CDK activity (Jansen et al. 2007; Silva et al. 2012; Spiller et al. 2017). In most vertebrates, the Mis18 complex of Mis18α, Mis18β and Mis18BP1, apparently licenses the centrochromatin for CENP-A deposition (Fujita et al. 2007; Barnhart et al. 2011), mediated by the CENP-A-specific chaperone HJURP (Foltz et al. 2009; Dunleavy et al. 2009).

The contributions of centrochromatin to CENP-A deposition and kinetochore maintenance have been of great interest, since it is well-documented that histone post-translational modifications play crucial roles in the regulation of diverse cell processes, such as DNA replication (Alabert and Groth 2012), DNA repair (Dinant et al. 2008; Lahtz and Pfeifer 2011), gene expression (Berger 2007) and telomere maintenance (Schoeftner and Blasco 2010).

The ability to engineer the alphoid$^{tetO}$ HAC centromeric chromatin by targeting tetracycline repressor fusion chimeras make this a suitable system to dissect the epigenetic factors that control kinetochore maintenance and function at an established centromere (Nakano et al. 2008) (Table 1). Initial studies involved the tethering of a heterochromatin-seeding transcriptional repressor (tTS) to the alphoid$^{tetO}$ centromere. This increased the levels of H3K9me3, leading to the recruitment of heterochromatin protein 1 (HP1α), the loss of CENP-A and destabilization of the HAC. This experiment showed that heterochromatinization

of centrochromatin is incompatible with kinetochore maintenance and function (Nakano et al. 2008).

Subsequent studies further explored the events that occur during tTS-mediated kinetochore disruption (Cardinale et al. 2009). To understand how heterochromatin inactivates the kinetochore, Cardinale et al. used a tetR chimeric protein containing the multidomain scaffolding transcriptional silencer KAP1, a downstream effector of the tTS (Friedman et al. 1996). Tethering KAP1 to the alphoid$^{tetO}$ HAC caused levels of CENP-C and CENP-H to decrease faster than levels of CENP-A. Thus, heterochromatin-induced loss of kinetochore structure follows a hierarchical process, with CENP-C and CENP-H being displaced independently from CENP-A, which showed a more gradual loss (Cardinale et al. 2009). Other studies tethering the H3K9-methyltransferase SUV39H1 to the alphoid$^{tetO}$ HAC further confirmed that heterochromatin nucleation is incompatible with kinetochore assembly (Ohzeki et al. 2012).

Together, these data strongly suggest that a balance between an open euchromatin signature and a flanking heterochromatin domain is necessary for proper chromosome segregation. The nature and dynamics of the boundary between these two mutually exclusive chromatin domains at centromeres is currently an open question that is under active investigation.

**Kinetochores are maintained by a balance of histone post-translational modifications and transcription.**

The finding that excessive heterochomatin is detrimental to kinetochore function was originally a surprise, as centromeres are embedded in constitutive heterochromatin regions in most eukaryotes and therefore, were classically regarded as heterochromatic themselves. However, initial studies on stretched chromatin fibers showed that CENP-A domains show chromatin marks typically associated with transcriptionally active regions, such as H3K4me2 or H3K36me2 (Sullivan and Karpen 2004; Ribeiro et al. 2010; Bergmann et al. 2011). Consistent

with the presence of active marks at centromeres, recent studies have revealed that centromeres undergo low levels of RNAP II-mediated transcription during mitosis (Figure 3) (Chan et al. 2012; Quenet and Dalal 2014; Rosic et al. 2014; Liu et al. 2015; Catania et al. 2015). Importantly, the low levels of RNAP II transcription at centromeres are found in yeast, maize, *Drosophila melanogaster*, mice and humans, thus suggesting a conserved role in kinetochore maintenance (Topp et al. 2004; Kanellopoulou et al. 2005; Bergmann et al. 2011; Chan et al. 2012; Quenet and Dalal 2014; Catania and Allshire 2014; Liu et al. 2015; Molina et al. 2016b).

H3K4me2, present at centrochromatin (Sullivan and Karpen 2004), is a modification typically found in the 5' region of poised and actively transcribing genes. It is considered to mark transcriptionally permissive chromatin (Ernst and Kellis 2010). The consequences for kinetochore function of removing H3K4me2 from centrochromatin were tested using the alphoid$^{tetO}$ HAC by tethering the H3K4me2-specific demethylases LSD1 and LSD2 (Bergmann et al. 2011; Molina et al. 2016b). Both studies found that H3K4me2 removal inhibits centromeric transcription and ultimately leads to kinetochore destabilization. Strikingly, H3K4me2 removal resulted in a failure to recruit HJURP to the HAC, thus explaining the observed decrease in CENP-A levels at the HAC centromere (Bergmann et al. 2011). The repression of transcription after removal of H3K4me2 was coupled by a decrease of other transcription-associated marks such as H3K36me2 at centromeres (Bergmann et al. 2011; Molina et al. 2016b).

Classic studies have shown that strong transcriptional activation through the centromere in budding yeast could inactivate the kinetochore, and this was the basis for the first ever conditional kinetochore (Hill and Bloom 1987). In order to study how transcription and open chromatin influence kinetochore maintenance, a mild transcriptional activator that increased transcription by 10-fold (the minimal activation domain of p65 of NF-κB), was tethered to the alphoid$^{tetO}$ HAC as a tetR-fusion protein (Bergmann et al. 2012a). Despite the induction of local histone H3K9 acetylation, the kinetochore remained functional. In contrast, tethering the potent chimeric transcriptional activator tetR-EYFP-VP16 (a strong activation

domain from Herpes simplex virus), increased transcription of the alphoid$^{tetO}$ array >150-fold and rapidly disrupted the HAC kinetochore (Bergmann et al. 2012a). Together, these data suggest that functional centrochromatin requires a tightly regulated balance of local transcriptional activity.

To further explore the role of transcription and its links with specific histone post-translational modifications, a recent study designed "in situ" epistasis analysis, in which pairs of antagonistic chromatin modifying activities were targeted simultaneously to the alphoid$^{tetO}$ HAC array (Figure 4a) (Molina et al. 2016b). These assays allowed investigators to uncouple centromeric transcription from histone modifications at the HAC centromere. Simultaneous tethering of LSD2, which demethylates H3K4 and decreases transcription, together with two different factors that promote transcription revealed that only transcription associated with H3K9ac could render the centrochromatin resistent to H3K4me2 removal. Co-tethering of CENP-28/Eaf6, which increased transcription associated with histone H4 acetylation did not rescue the centromere, whereas co-thering of p65, which increased transcription associated with histone H3K9 acetylation did rescue (Figure 4b). Subsequent Halo-CENP-A pulse-chase experiments coupled with *in situ* epistasis assays, showed that transcription is linked to CENP-A deposition (Molina et al. 2016b). This is consistent with previous data suggesting an interaction between centromeric transcripts and preassembled HJURP-CENP-A complexes (Quenet and Dalal 2014).

H3K4me2-associated transcription together with H3K9ac prevents heterochromatin spreading into centrochromatin (Molina et al. 2016b). This suggested that the euchromatin-heterochromatin barrier at human centromeres might be determined by chromatin modifications rather than specific genes or sequences as found in *S. pombe*, where tRNA sequences separate the core centromeric sequences from the outer repeats (Scott et al. 2007).

The fine balance between centrochromatin and heterochromatin was further explored in a study in which EDITORS that lay down MARKS characteristic of heterochromatin were

targeted into the alphoid$^{tetO}$ centromere as tetR fusions. Interestingly targeting of the EDITOR EZH2, which deposited the MARK H3K27me3 and induced binding of members of the polycomb PRC1 complex neither inactivated centromeric transcription nor did it inactivate the centromere (Martins et al. 2016). In contrast, if a READER from the PRC1 complex was directly tethered, giving a much stronger polycomb response, this did inactivate the kinetochore. Thus, the centromere appeared to be able to resist the more-or-less physiological initiation of a silent chromatin state but not to resist such a state when that was stably imposed within heterochromatin.

Together, these studies reveal that chromatin modifications and centromeric transcription operate side by side in kinetochore maintenance. Furthermore, centromeres appear to have as-yet unknown mechanisms that allow them to "buffer" and resist incursions by silent chromatin states. One possibility is that the presence of CENP-A nucleosomes, which resist many of these modifications, lowers the concentration of responding H3 nucleosomes and thereby weakens the establishment of silent states.

**Concluding remarks and future perspective**

HACs provide a powerful tool for studying the role of centromere chromatin on kinetochore assembly and function. Studies with the alphoid$^{tetO}$ HAC allowed tests of the long-standing hypothesis that the chromatin environment is integral to centromere identity. However, the specific enzymes responsible to create and delete these epigenetic marks at centromeres are less clear. The alphoid$^{tetO}$ arrays have the potential of being used for *de novo* HAC formation in different cell lines as they can be engineered to overcome the epigenetic barrier for HAC formation. This will be important to explore kinetochore stability in different backgrounds and may help us to understand mechanisms of CIN (chromosomal instability) in some cell types. Importantly, the possibility of generating HACs in any cell type overcame an important limitation to the use of HACs as gene delivery vectors for gene therapy.

One limitation of the current alphoid$^{tetO}$ HAC is the impossibility of separately engineer the centromere to study functional interactions between the kinetochore and the pericentromeric heterochromatin. Next-generation synthetic HACs are currently being developed containing separate centrochromatin and heterochromatin domains that can be independently targeted, thus more closely resembling endogenous centromeres (Molina and Earnshaw, *unpublished*). These new HACs will potentially offer insights into the role/s of pericentromeric heterochromatin in kinetochore maintenance. Ultimately, they may allow us to understand and manipulate the epigenetic balance between euchromatin and heterochromatin domains that governs centromere function and stability.

Other limitations of the HAC technology include the low efficiency of HAC formation. Although important advances have been made in this regard (Ohzeki et al. 2012) (Molina and Earnshaw, *unpublished*), it is still necessary to develop methodologies for efficient HAC formation in different cellular models before the HAC technology can be widely implemented in research laboratories.

**Compliance with ethical standards:**

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**REFERENCES:**


Akiyoshi B, Gull K (2013) Evolutionary cell biology of chromosome segregation: insights from trypanosomes Open biology 3:130023 doi:10.1098/rsob.130023

Alabert C, Groth A (2012) Chromatin replication and epigenome maintenance Nature reviews Molecular cell biology 13:153-167 doi:10.1038/nrm3288

Aldrup-Macdonald ME, Sullivan BA (2014) The past, present, and future of human centromere genomics Genes 5:33-50

Alonso A et al. (2007) Co-localization of CENP-C and CENP-H to discontinuous domains of CENP-A chromatin at human neocentromeres Genome biology 8:R148 doi:10.1186/gb-2007-8-7-r148

Alonso A, Mahmood R, Li S, Cheung F, Yoda K, Warburton PE (2003) Genomic microarray analysis reveals distinct locations for the CENP-A binding domains in three human chromosome 13q32 neocentromeres Human molecular genetics 12:2711-2721 doi:10.1093/hmg/ddg282

Allshire RC, Ekwall K (2015) Epigenetic Regulation of Chromatin States in Schizosaccharomyces pombe Cold Spring Harbor perspectives in biology 7:a018770 doi:10.1101/cshperspect.a018770

Allshire RC, Karpen GH (2008) Epigenetic regulation of centromeric chromatin: old dogs, new tricks? Nature reviews Genetics 9:923-937 doi:10.1038/nrg2466

Amano M, Suzuki A, Hori T, Backer C, Okawa K, Cheeseman IM, Fukagawa T (2009) The CENP-S complex is essential for the stable assembly of outer kinetochore structure The Journal of cell biology 186:173-182 doi:10.1083/jcb.200903100

Barnhart MC, Kuich PH, Stellfox ME, Ward JA, Bassett EA, Black BE, Foltz DR (2011) HJURP is a CENP-A chromatin assembly factor sufficient to form a functional de novo kinetochore The Journal of cell biology 194:229-243 doi:10.1083/jcb.201012017

Basu J, Stromberg G, Compitello G, Willard HF, Van Bokkelen G (2005) Rapid creation of BAC-based human artificial chromosome vectors by transposition with synthetic alpha-satellite arrays Nucleic acids research 33:587-596 doi:10.1093/nar/gki207

Basu J, Willard HF (2006) Human artificial chromosomes: potential applications and clinical considerations Pediatric clinics of North America 53:843-853, viii doi:10.1016/j.pcl.2006.08.013

Berger SL (2007) The complex language of chromatin regulation during transcription Nature 447:407-412 doi:10.1038/nature05915

Bergmann JH et al. (2012a) Epigenetic engineering: histone H3K9 acetylation is compatible with kinetochore structure and function Journal of cell science 125:411-421 doi:10.1242/jcs.090639

Bergmann JH, Martins NM, Larionov V, Masumoto H, Earnshaw WC (2012b) HACking the centromere chromatin code: insights from human artificial chromosomes Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology 20:505-519 doi:10.1007/s10577-012-9293-0

Bergmann JH et al. (2011) Epigenetic engineering shows H3K4me2 is required for HJURP targeting and CENP-A assembly on a synthetic human kinetochore The EMBO journal 30:328-340 doi:10.1038/emboj.2010.329

Bernard P, Maure JF, Partridge JF, Genier S, Javerzat JP, Allshire RC (2001) Requirement of heterochromatin for cohesion at centromeres Science 294:2539-2542 doi:10.1126/science.1064027

Black BE, Cleveland DW (2011) Epigenetic centromere propagation and the nature of CENP-a nucleosomes Cell 144:471-479 doi:10.1016/j.cell.2011.02.002

Blower MD, Sullivan BA, Karpen GH (2002) Conserved organization of centromeric chromatin in flies and humans Developmental cell 2:319-330
Bodor DL et al. (2014) The quantitative architecture of centromeric chromatin eLife 3:e02137 doi:10.7554/eLife.02137
Booth DG et al. (2016) 3D-CLEM Reveals that a Major Portion of Mitotic Chromosomes Is Not Chromatin Molecular cell 64:790-802 doi:10.1016/j.molcel.2016.10.009
Brinkley BR, Stubblefield E (1966) The fine structure of the kinetochore of a mammalian cell in vitro Chromosoma 19:28-43
Brown KE, Barnett MA, Burgtorf C, Shaw P, Buckle VJ, Brown WR (1994) Dissecting the centromere of the human Y chromosome with cloned telomeric DNA Human molecular genetics 3:1227-1237
Cardinale S et al. (2009) Hierarchical inactivation of a synthetic human kinetochore by a chromatin modifier Molecular biology of the cell 20:4194-4204 doi:10.1091/mbc.E09-06-0489
Carroll CW, Milks KJ, Straight AF (2010) Dual recognition of CENP-A nucleosomes is required for centromere assembly The Journal of cell biology 189:1143-1155 doi:10.1083/jcb.201001013
Catania S, Allshire RC (2014) Anarchic centromeres: deciphering order from apparent chaos Current opinion in cell biology 26:41-50 doi:10.1016/j.ceb.2013.09.004
Catania S, Pidoux AL, Allshire RC (2015) Sequence features and transcriptional stalling within centromere DNA promote establishment of CENP-A chromatin PLoS Genet 11:e1004986 doi:10.1371/journal.pgen.1004986
Clarke L, Baum MP (1990) Functional analysis of a centromere from fission yeast: a role for centromere-specific repeated DNA sequences Molecular and cellular biology 10:1863-1872
Clarke L, Carbon J (1980) Isolation of a yeast centromere and construction of functional small circular chromosomes Nature 287:504-509
Cleveland DW, Mao Y, Sullivan KF (2003) Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling Cell 112:407-421
Cottarel G, Shero JH, Hieter P, Hegemann JH (1989) A 125-base-pair CEN6 DNA fragment is sufficient for complete meiotic and mitotic centromere functions in Saccharomyces cerevisiae Molecular and cellular biology 9:3342-3349
Chan FL, Marshall OJ, Saffery R, Kim BW, Earle E, Choo KH, Wong LH (2012) Active transcription and essential role of RNA polymerase II at the centromere during mitosis Proceedings of the National Academy of Sciences of the United States of America 109:1979-1984 doi:10.1073/pnas.1108705109
Cheeseman IM, Desai A (2008) Molecular architecture of the kinetochore-microtubule interface Nature reviews Molecular cell biology 9:33-46 doi:10.1038/nrm2310
Cheeseman IM, Hori T, Fukagawa T, Desai A (2008) KNL1 and the CENP-H/I/K complex coordinately direct kinetochore assembly in vertebrates Molecular biology of the cell 19:587-594 doi:10.1091/mbc.E07-10-1051
Choo KH, Vissel B, Nagy A, Earle E, Kalitsis P (1991) A survey of the genomic distribution of alpha satellite DNA on all the human chromosomes, and derivation of a new consensus sequence Nucleic acids research 19:1179-1182
Desai A, Rybina S, Muller-Reichert T, Shevchenko A, Shevchenko A, Hyman A, Oegema K (2003) KNL-1 directs assembly of the microtubule-binding interface of the kinetochore in C. elegans Genes & development 17:2421-2435 doi:10.1101/gad.1126303
Dinant C, Houtsmuller AB, Vermeulen W (2008) Chromatin structure and DNA damage repair Epigenetics & chromatin 1:9 doi:10.1186/1756-8935-1-9
Doheny KF, Sorger PK, Hyman AA, Tugendreich S, Spencer F, Hieter P (1993) Identification of essential components of the S. cerevisiae kinetochore Cell 73:761-774

Drinnenberg IA, deYoung D, Henikoff S, Malik HS (2014) Recurrent loss of CenH3 is associated with independent transitions to holocentricity in insects eLife 3 doi:10.7554/eLife.03676

du Sart D et al. (1997) A functional neo-centromere formed through activation of a latent human centromere and consisting of non-alpha-satellite DNA Nature genetics 16:144-153 doi:10.1038/ng0697-144

Duffy S et al. (2016) Overexpression screens identify conserved dosage chromosome instability genes in yeast and human cancer Proceedings of the National Academy of Sciences of the United States of America 113:9967-9976 doi:10.1073/pnas.1611839113

Dunleavy EM et al. (2009) HJURP is a cell-cycle-dependent maintenance and deposition factor of CENP-A at centromeres Cell 137:485-497 doi:10.1016/j.cell.2009.02.040

Earnshaw WC, Cooke CA (1989) Proteins of the inner and outer centromere of mitotic chromosomes Genome / National Research Council Canada = Genome / Conseil national de recherches Canada 31:541-552

Earnshaw WC, Migeon BR (1985) Three related centromere proteins are absent from the inactive centromere of a stable isodicentric chromosome Chromosoma 92:290-296

Earnshaw WC, Ratrie H, 3rd, Stetten G (1989) Visualization of centromere proteins CENP-B and CENP-C on a stable dicentric chromosome in cytological spreads Chromosoma 98:1-12

Earnshaw WC, Rattner JB (1991) The use of autoantibodies in the study of nuclear and chromosomal organization Methods in cell biology 35:135-175

Earnshaw WC, Rothfield N (1985) Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma Chromosoma 91:313-321

Ebersole T et al. (2005) Rapid generation of long synthetic tandem repeats and its application for analysis in human artificial chromosome formation Nucleic acids research 33:e130 doi:10.1093/nar/gni129

Ebersole TA, Ross A, Clark E, McGill N, Schindelhauer D, Cooke H, Grimes B (2000) Mammalian artificial chromosome formation from circular alphoid input DNA does not require telomere repeats Human molecular genetics 9:1623-1631

Ernst J, Kellis M (2010) Discovery and characterization of chromatin states for systematic annotation of the human genome Nature biotechnology 28:817-825 doi:10.1038/nbt.1662

Fachinetti D et al. (2013) A two-step mechanism for epigenetic specification of centromere identity and function Nature cell biology 15:1056-1066 doi:10.1038/ncb2805

Farr CJ, Bayne RA, Kipling D, Mills W, Critcher R, Cooke HJ (1995) Generation of a human X-derived minichromosome using telomere-associated chromosome fragmentation The EMBO journal 14:5444-5454

Folco HD, Pidoux AL, Urano T, Allshire RC (2008) Heterochromatin and RNAi are required to establish CENP-A chromatin at centromeres Science 319:94-97 doi:10.1126/science.1150944

Foltz DR et al. (2009) Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP Cell 137:472-484 doi:10.1016/j.cell.2009.02.039

Foltz DR, Jansen LE, Black BE, Bailey AO, Yates JR, 3rd, Cleveland DW (2006) The human CENP-A centromeric nucleosome-associated complex Nature cell biology 8:458-469 doi:10.1038/ncb1397

Frias C, Pampalona J, Genesca A, Tusell L (2012) Telomere dysfunction and genome instability Frontiers in bioscience 17:2181-2196

Friedman JR, Fredericks WJ, Jensen DE, Speicher DW, Huang XP, Neilson EG, Rauscher FJ, 3rd (1996) KAP-1, a novel corepressor for the highly conserved KRAB repression domain Genes & development 10:2067-2078

Fujita R et al. (2015) Stable complex formation of CENP-B with the CENP-A nucleosome Nucleic acids research 43:4909-4922 doi:10.1093/nar/gkv405

Fujita Y, Hayashi T, Kiyomitsu T, Toyoda Y, Kokubu A, Obuse C, Yanagida M (2007) Priming of centromere for CENP-A recruitment by human hMis18alpha, hMis18beta, and M18BP1 Developmental cell 12:17-30 doi:10.1016/j.devcel.2006.11.002

Fukagawa T, Earnshaw WC (2014a) The centromere: chromatin foundation for the kinetochore machinery Developmental cell 30:496-508 doi:10.1016/j.devcel.2014.08.016

Fukagawa T, Earnshaw WC (2014b) Neocentromeres Current biology : CB 24:R946-947 doi:10.1016/j.cub.2014.08.032

Gartenberg M (2009) Heterochromatin and the cohesion of sister chromatids Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology 17:229-238 doi:10.1007/s10577-008-9012-z

Goldberg IG, Sawhney H, Pluta AF, Warburton PE, Earnshaw WC (1996) Surprising deficiency of CENP-B binding sites in African green monkey alpha-satellite DNA: implications for CENP-B function at centromeres Molecular and cellular biology 16:5156-5168

Goshima G, Kiyomitsu T, Yoda K, Yanagida M (2003) Human centromere chromatin protein hMis12, essential for equal segregation, is independent of CENP-A loading pathway The Journal of cell biology 160:25-39 doi:10.1083/jcb.200210005

Grimes BR, Schindelhauer D, McGill NI, Ross A, Ebersole TA, Cooke HJ (2001) Stable gene expression from a mammalian artificial chromosome EMBO reports 2:910-914 doi:10.1093/embo-reports/kve187

Harrington JJ, Van Bokkelen G, Mays RW, Gustashaw K, Willard HF (1997) Formation of de novo centromeres and construction of first-generation human artificial microchromosomes Nature genetics 15:345-355 doi:10.1038/ng0497-345

Hegemann JH, Fleig UN (1993) The centromere of budding yeast BioEssays : news and reviews in molecular, cellular and developmental biology 15:451-460 doi:10.1002/bies.950150704

Heller R, Brown KE, Burgtorf C, Brown WR (1996) Mini-chromosomes derived from the human Y chromosome by telomere directed chromosome breakage Proceedings of the National Academy of Sciences of the United States of America 93:7125-7130

Henning KA, Novotny EA, Compton ST, Guan XY, Liu PP, Ashlock MA (1999) Human artificial chromosomes generated by modification of a yeast artificial chromosome containing both human alpha satellite and single-copy DNA sequences Proceedings of the National Academy of Sciences of the United States of America 96:592-597

Hill A, Bloom K (1987) Genetic manipulation of centromere function Molecular and cellular biology 7:2397-2405

Hori T et al. (2008) CCAN makes multiple contacts with centromeric DNA to provide distinct pathways to the outer kinetochore Cell 135:1039-1052 doi:10.1016/j.cell.2008.10.019

Hori T, Shang WH, Takeuchi K, Fukagawa T (2013) The CCAN recruits CENP-A to the centromere and forms the structural core for kinetochore assembly The Journal of cell biology 200:45-60 doi:10.1083/jcb.201210106

Hori T et al. (2014) Histone H4 Lys 20 monomethylation of the CENP-A nucleosome is essential for kinetochore assembly Developmental cell 29:740-749 doi:10.1016/j.devcel.2014.05.001

Hudson DF et al. (1998) Centromere protein B null mice are mitotically and meiotically normal but have lower body and testis weights The Journal of cell biology 141:309-319

Iida Y et al. (2014) Bi-HAC vector system toward gene and cell therapy ACS synthetic biology 3:83-90 doi:10.1021/sb400166j

Ikeno M et al. (1998) Construction of YAC-based mammalian artificial chromosomes Nature biotechnology 16:431-439 doi:10.1038/nbt0598-431

Ikeno M, Inagaki H, Nagata K, Morita M, Ichinose H, Okazaki T (2002) Generation of human artificial chromosomes expressing naturally controlled guanosine triphosphate cyclohydrolase I gene Genes to cells : devoted to molecular & cellular mechanisms 7:1021-1032

Ikeno M, Masumoto H, Okazaki T (1994) Distribution of CENP-B boxes reflected in CREST centromere antigenic sites on long-range alpha-satellite DNA arrays of human chromosome 21 Human molecular genetics 3:1245-1257
Jansen LE, Black BE, Foltz DR, Cleveland DW (2007) Propagation of centromeric chromatin requires exit from mitosis The Journal of cell biology 176:795-805 doi:10.1083/jcb.200701066
Jokelainen PT (1967) The ultrastructure and spatial organization of the metaphase kinetochore in mitotic rat cells Journal of ultrastructure research 19:19-44
Kagansky A et al. (2009) Synthetic heterochromatin bypasses RNAi and centromeric repeats to establish functional centromeres Science 324:1716-1719 doi:10.1126/science.1172026
Kanellopoulou C et al. (2005) Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing Genes & development 19:489-501 doi:10.1101/gad.1248505
Kapoor M et al. (1998) The cenpB gene is not essential in mice Chromosoma 107:570-576
Karpen GH, Allshire RC (1997) The case for epigenetic effects on centromere identity and function Trends in genetics : TIG 13:489-496
Kazuki Y et al. (2011) Refined human artificial chromosome vectors for gene therapy and animal transgenesis Gene therapy 18:384-393 doi:10.1038/gt.2010.147
Kim JH et al. (2011) Human artificial chromosome (HAC) vector with a conditional centromere for correction of genetic deficiencies in human cells Proceedings of the National Academy of Sciences of the United States of America 108:20048-20053 doi:10.1073/pnas.1114483108
Kim JH et al. (2016) Development of a novel HAC-based "gain of signal" quantitative assay for measuring chromosome instability (CIN) in cancer cells Oncotarget 7:14841-14856 doi:10.18632/oncotarget.7854
Kononenko AV et al. (2014) A portable BRCA1-HAC (human artificial chromosome) module for analysis of BRCA1 tumor suppressor function Nucleic acids research 42 doi:10.1093/nar/gku870
Kouprina N, Earnshaw WC, Masumoto H, Larionov V (2013) A new generation of human artificial chromosomes for functional genomics and gene therapy Cellular and molecular life sciences : CMLS 70:1135-1148 doi:10.1007/s00018-012-1113-3
Kouprina N et al. (2003) Cloning of human centromeres by transformation-associated recombination in yeast and generation of functional human artificial chromosomes Nucleic acids research 31:922-934
Kouprina N, Larionov V (2016) Transformation-associated recombination (TAR) cloning for genomics studies and synthetic biology Chromosoma 125:621-632 doi:10.1007/s00412-016-0588-3
Kouprina N et al. (2012) Organization of synthetic alphoid DNA array in human artificial chromosome (HAC) with a conditional centromere ACS synthetic biology 1:590-601 doi:10.1021/sb3000436
Kouprina N, Tomilin AN, Masumoto H, Earnshaw WC, Larionov V (2014) Human artificial chromosome-based gene delivery vectors for biomedicine and biotechnology Expert opinion on drug delivery 11:517-535 doi:10.1517/17425247.2014.882314
Kugou K, Hirai H, Masumoto H, Koga A (2016) Formation of functional CENP-B boxes at diverse locations in repeat units of centromeric DNA in New World monkeys Scientific reports 6:27833 doi:10.1038/srep27833
Kwon MS, Hori T, Okada M, Fukagawa T (2007) CENP-C is involved in chromosome segregation, mitotic checkpoint function, and kinetochore assembly Molecular biology of the cell 18:2155-2168 doi:10.1091/mbc.E07-01-0045
Lahtz C, Pfeifer GP (2011) Epigenetic changes of DNA repair genes in cancer Journal of molecular cell biology 3:51-58 doi:10.1093/jmcb/mjq053

Lee HS et al. (2013) A new assay for measuring chromosome instability (CIN) and identification of drugs that elevate CIN in cancer cells BMC cancer 13:252 doi:10.1186/1471-2407-13-252

Lee HS et al. (2016) Effects of Anticancer Drugs on Chromosome Instability and New Clinical Implications for Tumor-Suppressing Therapies Cancer research 76:902-911 doi:10.1158/0008-5472.CAN-15-1617

Liu H, Qu Q, Warrington R, Rice A, Cheng N, Yu H (2015) Mitotic Transcription Installs Sgo1 at Centromeres to Coordinate Chromosome Segregation Molecular cell 59:426-436 doi:10.1016/j.molcel.2015.06.018

Liu ST, Rattner JB, Jablonski SA, Yen TJ (2006) Mapping the assembly pathways that specify formation of the trilaminar kinetochore plates in human cells The Journal of cell biology 175:41-53 doi:10.1083/jcb.200606020

Lo AW et al. (2001) A 330 kb CENP-A binding domain and altered replication timing at a human neocentromere The EMBO journal 20:2087-2096 doi:10.1093/emboj/20.8.2087

Luykx P (1965) The structure of the kinetochore in meiosis and mitosis in Urechis eggs Experimental cell research 39:643-657

Maddox PS, Hyndman F, Monen J, Oegema K, Desai A (2007) Functional genomics identifies a Myb domain-containing protein family required for assembly of CENP-A chromatin The Journal of cell biology 176:757-763 doi:10.1083/jcb.200701065

Maiato H, Sampaio P, Sunkel CE (2004) Microtubule-associated proteins and their essential roles during mitosis International review of cytology 241:53-153 doi:10.1016/S0074-7696(04)41002-X

Mandegar MA et al. (2011) Functional human artificial chromosomes are generated and stably maintained in human embryonic stem cells Human molecular genetics 20:2905-2913 doi:10.1093/hmg/ddr144

Martins NM, Bergmann JH, Shono N, Kimura H, Larionov V, Masumoto H, Earnshaw WC (2016) Epigenetic engineering shows that a human centromere resists silencing mediated by H3K27me3/K9me3 Molecular biology of the cell 27:177-196 doi:10.1091/mbc.E15-08-0605

Masumoto H, Ikeno M, Nakano M, Okazaki T, Grimes B, Cooke H, Suzuki N (1998) Assay of centromere function using a human artificial chromosome Chromosoma 107:406-416

Masumoto H, Masukata H, Muro Y, Nozaki N, Okazaki T (1989) A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite The Journal of cell biology 109:1963-1973

Mejia JE, Larin Z (2000) The assembly of large BACs by in vivo recombination Genomics 70:165-170 doi:10.1006/geno.2000.6372

Mejia JE, Willmott A, Levy E, Earnshaw WC, Larin Z (2001) Functional complementation of a genetic deficiency with human artificial chromosomes American journal of human genetics 69:315-326 doi:10.1086/321977

Merry DE, Pathak S, Hsu TC, Brinkley BR (1985) Anti-kinetochore antibodies: use as probes for inactive centromeres American journal of human genetics 37:425-430

Mills W, Critcher R, Lee C, Farr CJ (1999) Generation of an approximately 2.4 Mb human X centromere-based minichromosome by targeted telomere-associated chromosome fragmentation in DT40 Human molecular genetics 8:751-761

Molina O, Carmena M, Maudlin IE, Earnshaw WC (2016a) PREditOR: a synthetic biology approach to removing heterochromatin from cells Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology 24:495-509 doi:10.1007/s10577-016-9539-3

Molina O et al. (2016b) Epigenetic engineering reveals a balance between histone modifications and transcription in kinetochore maintenance Nature communications 7:13334 doi:10.1038/ncomms13334

Moralli D, Monaco ZL (2015) Developing de novo human artificial chromosomes in embryonic stem cells using HSV-1 amplicon technology Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology 23:105-110 doi:10.1007/s10577-014-9456-2

Moralli D, Simpson KM, Wade-Martins R, Monaco ZL (2006) A novel human artificial chromosome gene expression system using herpes simplex virus type 1 vectors EMBO reports 7:911-918 doi:10.1038/sj.embor.7400768

Murray AW, Szostak JW (1983) Construction of artificial chromosomes in yeast Nature 305:189-193

Nakano M et al. (2008) Inactivation of a human kinetochore by specific targeting of chromatin modifiers Developmental cell 14:507-522 doi:10.1016/j.devcel.2008.02.001

Neil DL, Villasante A, Fisher RB, Vetrie D, Cox B, Tyler-Smith C (1990) Structural instability of human tandemly repeated DNA sequences cloned in yeast artificial chromosome vectors Nucleic acids research 18:1421-1428

Nishino T, Rago F, Hori T, Tomii K, Cheeseman IM, Fukagawa T (2013) CENP-T provides a structural platform for outer kinetochore assembly The EMBO journal 32:424-436 doi:10.1038/emboj.2012.348

Nishino T et al. (2012) CENP-T-W-S-X forms a unique centromeric chromatin structure with a histone-like fold Cell 148:487-501 doi:10.1016/j.cell.2011.11.061

Nonaka N, Kitajima T, Yokobayashi S, Xiao G, Yamamoto M, Grewal SI, Watanabe Y (2002) Recruitment of cohesin to heterochromatic regions by Swi6/HP1 in fission yeast Nature cell biology 4:89-93 doi:10.1038/ncb739

Oegema K, Desai A, Rybina S, Kirkham M, Hyman AA (2001) Functional analysis of kinetochore assembly in Caenorhabditis elegans The Journal of cell biology 153:1209-1226

Ohzeki J et al. (2012) Breaking the HAC Barrier: histone H3K9 acetyl/methyl balance regulates CENP-A assembly The EMBO journal 31:2391-2402 doi:10.1038/emboj.2012.82

Ohzeki J, Larionov V, Earnshaw WC, Masumoto H (2015) Genetic and epigenetic regulation of centromeres: a look at HAC formation Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology 23:87-103 doi:10.1007/s10577-015-9470-z

Ohzeki J, Nakano M, Okada T, Masumoto H (2002) CENP-B box is required for de novo centromere chromatin assembly on human alphoid DNA The Journal of cell biology 159:765-775 doi:10.1083/jcb.200207112

Ohzeki J et al. (2016) KAT7/HBO1/MYST2 Regulates CENP-A Chromatin Assembly by Antagonizing Suv39h1-Mediated Centromere Inactivation Developmental cell 37:413-427 doi:10.1016/j.devcel.2016.05.006

Okada M et al. (2006) The CENP-H-I complex is required for the efficient incorporation of newly synthesized CENP-A into centromeres Nature cell biology 8:446-457 doi:10.1038/ncb1396

Okada T, Ohzeki J, Nakano M, Yoda K, Brinkley WR, Larionov V, Masumoto H (2007) CENP-B controls centromere formation depending on the chromatin context Cell 131:1287-1300 doi:10.1016/j.cell.2007.10.045

Okamoto Y, Nakano M, Ohzeki J, Larionov V, Masumoto H (2007) A minimal CENP-A core is required for nucleation and maintenance of a functional human centromere The EMBO journal 26:1279-1291 doi:10.1038/sj.emboj.7601584

Olszak AM et al. (2011) Heterochromatin boundaries are hotspots for de novo kinetochore formation Nature cell biology 13:799-808 doi:10.1038/ncb2272

Oshimura M, Uno N, Kazuki Y, Katoh M, Inoue T (2015) A pathway from chromosome transfer to engineering resulting in human and mouse artificial chromosomes for a variety of applications to bio-medical challenges Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology 23:111-133 doi:10.1007/s10577-014-9459-z

Palmer DK, O'Day K, Trong HL, Charbonneau H, Margolis RL (1991) Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone Proceedings of the National Academy of Sciences of the United States of America 88:3734-3738

Palmer DK, O'Day K, Wener MH, Andrews BS, Margolis RL (1987) A 17-kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones The Journal of cell biology 104:805-815

Pearson CG, Yeh E, Gardner M, Odde D, Salmon ED, Bloom K (2004) Stable kinetochore-microtubule attachment constrains centromere positioning in metaphase Current biology : CB 14:1962-1967 doi:10.1016/j.cub.2004.09.086

Perez-Castro AV, Shamanski FL, Meneses JJ, Lovato TL, Vogel KG, Moyzis RK, Pedersen R (1998) Centromeric protein B null mice are viable with no apparent abnormalities Developmental biology 201:135-143 doi:10.1006/dbio.1998.9005

Perpelescu M, Fukagawa T (2011) The ABCs of CENPs Chromosoma 120:425-446 doi:10.1007/s00412-011-0330-0

Pertile MD, Graham AN, Choo KH, Kalitsis P (2009) Rapid evolution of mouse Y centromere repeat DNA belies recent sequence stability Genome research 19:2202-2213 doi:10.1101/gr.092080.109

Peters AH et al. (2001) Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability Cell 107:323-337

Pluta AF, Mackay AM, Ainsztein AM, Goldberg IG, Earnshaw WC (1995) The centromere: hub of chromosomal activities Science 270:1591-1594

Pluta AF, Saitoh N, Goldberg I, Earnshaw WC (1992) Identification of a subdomain of CENP-B that is necessary and sufficient for localization to the human centromere The Journal of cell biology 116:1081-1093

Przewloka MR, Venkei Z, Bolanos-Garcia VM, Debski J, Dadlez M, Glover DM (2011) CENP-C is a structural platform for kinetochore assembly Current biology : CB 21:399-405 doi:10.1016/j.cub.2011.02.005

Quenet D, Dalal Y (2014) A long non-coding RNA is required for targeting centromeric protein A to the human centromere eLife 3:e03254 doi:10.7554/eLife.03254

Ribeiro SA et al. (2010) A super-resolution map of the vertebrate kinetochore Proceedings of the National Academy of Sciences of the United States of America 107:10484-10489 doi:10.1073/pnas.1002325107

Rosic S, Kohler F, Erhardt S (2014) Repetitive centromeric satellite RNA is essential for kinetochore formation and cell division The Journal of cell biology 207:335-349 doi:10.1083/jcb.201404097

Roy B, Sanyal K (2011) Diversity in requirement of genetic and epigenetic factors for centromere function in fungi Eukaryotic cell 10:1384-1395 doi:10.1128/EC.05165-11

Saffery R, Choo KH (2002) Strategies for engineering human chromosomes with therapeutic potential The journal of gene medicine 4:5-13

Saffery R, Irvine DV, Griffiths B, Kalitsis P, Wordeman L, Choo KH (2000) Human centromeres and neocentromeres show identical distribution patterns of >20 functionally important kinetochore-associated proteins Human molecular genetics 9:175-185

Santaguida S, Musacchio A (2009) The life and miracles of kinetochores The EMBO journal 28:2511-2531 doi:10.1038/emboj.2009.173

Scott KC, White CV, Willard HF (2007) An RNA polymerase III-dependent heterochromatin barrier at fission yeast centromere 1 PloS one 2:e1099 doi:10.1371/journal.pone.0001099

Screpanti E, De Antoni A, Alushin GM, Petrovic A, Melis T, Nogales E, Musacchio A (2011) Direct binding of Cenp-C to the Mis12 complex joins the inner and outer kinetochore Current biology : CB 21:391-398 doi:10.1016/j.cub.2010.12.039

Schalch T, Steiner FA (2016) Structure of centromere chromatin: from nucleosome to chromosomal architecture Chromosoma doi:10.1007/s00412-016-0620-7
Schoeftner S, Blasco MA (2010) Chromatin regulation and non-coding RNAs at mammalian telomeres Seminars in cell & developmental biology 21:186-193 doi:10.1016/j.semcdb.2009.09.015
Schueler MG, Higgins AW, Rudd MK, Gustashaw K, Willard HF (2001) Genomic and genetic definition of a functional human centromere Science 294:109-115 doi:10.1126/science.1065042
Shono N et al. (2015) CENP-C and CENP-I are key connecting factors for kinetochore and CENP-A assembly Journal of cell science 128:4572-4587 doi:10.1242/jcs.180786
Silva MC, Bodor DL, Stellfox ME, Martins NM, Hochegger H, Foltz DR, Jansen LE (2012) Cdk activity couples epigenetic centromere inheritance to cell cycle progression Developmental cell 22:52-63 doi:10.1016/j.devcel.2011.10.014
Slee RB et al. (2012) Cancer-associated alteration of pericentromeric heterochromatin may contribute to chromosome instability Oncogene 31:3244-3253 doi:10.1038/onc.2011.502
Spencer F, Gerring SL, Connelly C, Hieter P (1990) Mitotic chromosome transmission fidelity mutants in Saccharomyces cerevisiae Genetics 124:237-249
Spencer F, Hieter P (1992) Centromere DNA mutations induce a mitotic delay in Saccharomyces cerevisiae Proceedings of the National Academy of Sciences of the United States of America 89:8908-8912
Spiller F, Medina-Pritchard B, Abad MA, Wear MA, Molina O, Earnshaw WC, Jeyaprakash AA (2017) Molecular basis for Cdk1-regulated timing of Mis18 complex assembly and CENP-A deposition EMBO reports doi:10.15252/embr.201643564
Stimpson KM, Matheny JE, Sullivan BA (2012) Dicentric chromosomes: unique models to study centromere function and inactivation Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology 20:595-605 doi:10.1007/s10577-012-9302-3
Sugata N et al. (2000) Human CENP-H multimers colocalize with CENP-A and CENP-C at active centromere--kinetochore complexes Human molecular genetics 9:2919-2926
Sullivan BA, Karpen GH (2004) Centromeric chromatin exhibits a histone modification pattern that is distinct from both euchromatin and heterochromatin Nature structural & molecular biology 11:1076-1083 doi:10.1038/nsmb845
Sullivan BA, Schwartz S (1995) Identification of centromeric antigens in dicentric Robertsonian translocations: CENP-C and CENP-E are necessary components of functional centromeres Human molecular genetics 4:2189-2197
Sullivan BA, Willard HF (1998) Stable dicentric X chromosomes with two functional centromeres Nature genetics 20:227-228 doi:10.1038/3024
Sullivan KF, Hechenberger M, Masri K (1994) Human CENP-A contains a histone H3 related histone fold domain that is required for targeting to the centromere The Journal of cell biology 127:581-592
Sullivan LL, Boivin CD, Mravinac B, Song IY, Sullivan BA (2011) Genomic size of CENP-A domain is proportional to total alpha satellite array size at human centromeres and expands in cancer cells Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology 19:457-470 doi:10.1007/s10577-011-9208-5
Sumer H, Saffery R, Wong N, Craig JM, Choo KH (2004) Effects of scaffold/matrix alteration on centromeric function and gene expression The Journal of biological chemistry 279:37631-37639 doi:10.1074/jbc.M401051200
Suzuki N, Nishii K, Okazaki T, Ikeno M (2006) Human artificial chromosomes constructed using the bottom-up strategy are stably maintained in mitosis and efficiently transmissible to

progeny mice The Journal of biological chemistry 281:26615-26623 doi:10.1074/jbc.M603053200
Takahashi K, Murakami S, Chikashige Y, Funabiki H, Niwa O, Yanagida M (1992) A low copy number central sequence with strict symmetry and unusual chromatin structure in fission yeast centromere Molecular biology of the cell 3:819-835
Takahashi K, Murakami S, Chikashige Y, Niwa O, Yanagida M (1991) A large number of tRNA genes are symmetrically located in fission yeast centromeres Journal of molecular biology 218:13-17
Takiguchi M et al. (2014) A novel and stable mouse artificial chromosome vector ACS synthetic biology 3:903-914 doi:10.1021/sb3000723
Topp CN, Zhong CX, Dawe RK (2004) Centromere-encoded RNAs are integral components of the maize kinetochore Proceedings of the National Academy of Sciences of the United States of America 101:15986-15991 doi:10.1073/pnas.0407154101
Vafa O, Sullivan KF (1997) Chromatin containing CENP-A and alpha-satellite DNA is a major component of the inner kinetochore plate Current biology : CB 7:897-900
Voullaire LE, Slater HR, Petrovic V, Choo KH (1993) A functional marker centromere with no detectable alpha-satellite, satellite III, or CENP-B protein: activation of a latent centromere? American journal of human genetics 52:1153-1163
Wakai M, Abe S, Kazuki Y, Oshimura M, Ishikawa F (2014) A human artificial chromosome recapitulates the metabolism of native telomeres in mammalian cells PloS one 9:e88530 doi:10.1371/journal.pone.0088530
Warburton PE (2001) Epigenetic analysis of kinetochore assembly on variant human centromeres Trends in genetics : TIG 17:243-247
Warburton PE et al. (1997) Immunolocalization of CENP-A suggests a distinct nucleosome structure at the inner kinetochore plate of active centromeres Current biology : CB 7:901-904
Waye JS, Willard HF (1989) Chromosome specificity of satellite DNAs: short- and long-range organization of a diverged dimeric subset of human alpha satellite from chromosome 3 Chromosoma 97:475-480
Willard HF (1985) Chromosome-specific organization of human alpha satellite DNA American journal of human genetics 37:524-532
Willard HF (1990) Centromeres of mammalian chromosomes Trends in genetics : TIG 6:410-416
Yamagishi Y, Sakuno T, Shimura M, Watanabe Y (2008) Heterochromatin links to centromeric protection by recruiting shugoshin Nature 455:251-255 doi:10.1038/nature07217
Young DJ, Nimmo ER, Allshire RC (1998) A Schizosaccharomyces pombe artificial chromosome large DNA cloning system Nucleic acids research 26:5052-5060

**Figure legends:**

**Figure 1:** Genomic and epigenetic organization of human centromeres. (a) Metaphase chromosome spread of the human HCT116 cell line. DNA is stained with DAPI. (O. Molina, unpublished data) (b) Human centromeres (yellow arrowheads) contain α-satellite sequences. In the inner core of the centromere 171 bp α-satellite monomers are organized in HORs that span up to 5 Mb. Unordered monomeric α-satellite repeats are flanking the HORs. (c) Human metaphase chromosome spread immunostained for centromeres using antibodies recognizing CENP-A (red) and histone H3 trimethylated on lysine 9 (green). DNA is stained with DAPI (blue). Panel 1c shows unpublished data from the experiment presented in Molina et al. 2016b, Figure 8c. (d) Epigenetic organization of human centromeres. CENP-A nucleosomes are localized in the outer centromere (core centromere region) and they are flanked by nucleosomes containing the canonical histone H3 bearing heterochromatin marks. Scale bars=10 µm

**Figure 2:** The synthetic alphoid$^{tetO}$ HAC for epigenetic engineering of the centromere. (a) Diagram showing RCA-TAR cloning steps for HAC construction. (b) *Bottom-up* approach for HAC construction in HT1080 cells using the alphoid$^{tetO}$ array. FISH images show examples of possible outcomes of alphoid$^{tetO}$ array transfection in HT1080 cells, ectopic integration into a chromosome arm (top) and *de novo* HAC formation (bottom). The vector backbone (TAR cloning vector) was used as DNA-probe for FISH (red). (O. Molina, unpublished data) (c) Metaphase chromosome spread of a HeLa cell line containing the alphoid$^{tetO}$ HAC. The chromosomes were cytospun onto a glass slide and subsequently immunostained using antibodies recognizing CENP-A (red) and histone H3 dimethylated on lysine 4 (green). DNA was stained with DAPI (blue). Yellow arrowhead indicates the alphoid$^{tetO}$ HAC. Panel 2c shows unpublished data from the experiment presented in Molina et al. 2016b, Figure S1C. (d) Schematic representation of the epigenetic engineering strategy using the alphoid$^{tetO}$ HAC. Scale bar=10 µm.

**Figure 3:** Detection of active RNAPII transcription at human centromeres in mitosis. (a) Diagram showing the presence of active RNAP II transcription in centrochromatin of metaphase chromosomes. CENP-A-containing nucleosomes are depicted in red and nucleosomes containing canonical histone H3 in blue. (b) Metaphase chromosome spread of a HeLa cell line containing the alphoid$^{tetO}$ HAC (yellow arrowhead) and immunostained for RNAPII phosphorylated on serine 2 (RNAPII S2P- red). DNA, stained with DAPI, is shown in white. Insets: 3X magnifications of the alphoid$^{tetO}$ HAC. Panel 3b shows unpublished data from the experiment presented in Molina et al. 2016b, Figure 2d. (c) *In situ* transcription assay showing nascent centromeric transcripts (red) on metaphase chromosomes (blue). Panel 3c shows unpublished data from the experiment presented in Molina et al. 2016b, Figure 2a, b. Scale bars=10 µm.

**Figure 4:** *In situ* epistasis assays in the alphoid$^{tetO}$ HAC centromere uncouple epigenetic marks from transcription. (a) Schematic representation of an *in situ* epistasis assay tethering two competing chromatin modifiers (LSD2 and p65) fused with different fluorescent proteins to the alphoid$^{tetO}$ HAC. (b) Representative images of 1C7 cells containing the alphoid$^{tetO}$ HAC and expressing the indicated tetR-fusion constructs. Cells were immunostained with antibodies recognizing CENP-A (third panel). Merged images represent the overlay of EYFP and TMR-Star signals with CENP-A. Yellow arrowheads depicts the alphoid$^{tetO}$ HAC. Panel 4b shows unpublished data from the experiment presented in Molina et al. 2016b, Figure 6c. Scale bar=10 µm.

**Table legend:**

**Table 1:** Timeline of the use of chimeric proteins to manipulate the centromeric chromatin of the alphoid$^{tetO}$ synthetic HAC

| tetR-fusion constructs | Catalytical function | Observed effects on $^{tetO}$arrays. *HAC outcome* | Findings | Reference |
|---|---|---|---|---|
| Transcriptional repressor (tTS) | Seeds H3K9me3 and recruits HP1 | Fast kinetochore destabilization. *HAC loss* | Heterochomatin is incompatible with kinetochore function | Nakano et al. 2008 |
| Transcripional transactivator (tTA) | Transcriptional activation or induction of transcriptionally-competent open chromatin. | Heterogeneous outcome. Some clones show kinetochore destabilization. *Partial HAC loss* | Kinetochores can resist a certain degree of chromatin "opening" | Nakano et al. 2008 |
| KAP1 | Heterochromatin formation (downstream effector of tTS) | Fast kinetochore destabilization. *HAC loss* | Heterochromatin-induced loss of the kinetochore follows a hierarchical pattern. CENP-H/I is displaced independently from CENP-A. | Cardinale et al. 2009 |
| LSD1 | H3K4me2 demethylase (**recruits HDACs*) | Decrease in centromeric transcription. Disruption of new CENP-A assembly. *HAC loss (long-term)* | H3K4me2 is necessary for HJURP targeting to centromeres and new CENP-A assembly. | Bergmann et al. 2011 |
| SUV39H1 | H3K9me2/3 methyltransferase | Kinetochore destabilization. *HAC loss* | Heterochomatin is incompatible with kinetochore function | Ohzeki et al. 2012 |
| P65 | Mild transcriptional activator (NF-KB). Seed H3K9ac | Increase transcription 10-fold. No kinetochore defects. *HAC maintenance* | Kinetochores tolerate low levels of transcription and chromatin "opening". | Bergmann et al. 2012 |
| VP16 | Strong transcriptional activator (HSV). Seed H3K9ac | Increase transcription 150-fold. Fast kinetochore destabilization. *HAC loss* | Kinetochores are incompatible with high transcriptional activity. | Bergmann et al. 2012 |
| EZH2 | H3K27me3 methyltransferase | Kinetochore structure and function is maintained. *HAC maintenance* | Kinetochore resists H3K27me3 seeding | Martins et al. 2016 |
| BMI | Subunit of the Polycomb Repressor Complex 1 | Recruitment of RING1A. CENP-A loss and kinetochore | Constitutive tethering of READERS is more effective in | Martins et al. 2016 |

| | | | | |
|---|---|---|---|---|
| | | destabilization. *HAC loss* | kinetochore destabilization than EDITORS | |
| LSD2 | H3K4me2 demethylase | Decrease in centromeric transcription. Kinetochore destabilization. *HAC loss (long-term)* | H3K4me2 is necessary to maintain centromeric transcription, histone turnover and prevents H3K9me3 spreading into centrochromatin | Molina et al., 2016b |
| LSD2 + CENP-28 | H3K4me2 demethylation + H4K12 acetylation | Maintenance of centromeric transcription. Kinetochore destabilization in the absence of H3K4me2. *HAC loss* | Transcription associated with H4ac does not prevent kinetochore inactivation in the absence of H3K4me2. | Molina et al. 2016b |
| LSD2 + P65 | H3K4me2 demethylation + H3K9ac acetylation | Maintenance of centromeric transcription. Recovery of kinetochore defects in the absence of H3K4me2. *HAC maintenance* | H3K9ac bypasses H3K4me2 requirement for kinetochore maintenance. It prevents heterochromatin spreading into centrochromatin. | Molina et al. 2016b |

**Timeline of the use of chimeric proteins to manipulate the centromeric chromatin of the alphoid$^{tetO}$ synthetic HAC**

| **tetR-fusion constructs** | **Catalytical function** | **Observed effects on $^{tetO}$arrays. *HAC outcome*** | **Findings** | **Reference** |
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| | | | | |
|---|---|---|---|---|
| | Repressor Complex 1 | kinetochore destabilization. *HAC loss* | effective in kinetochore destabilization than EDITORS | |
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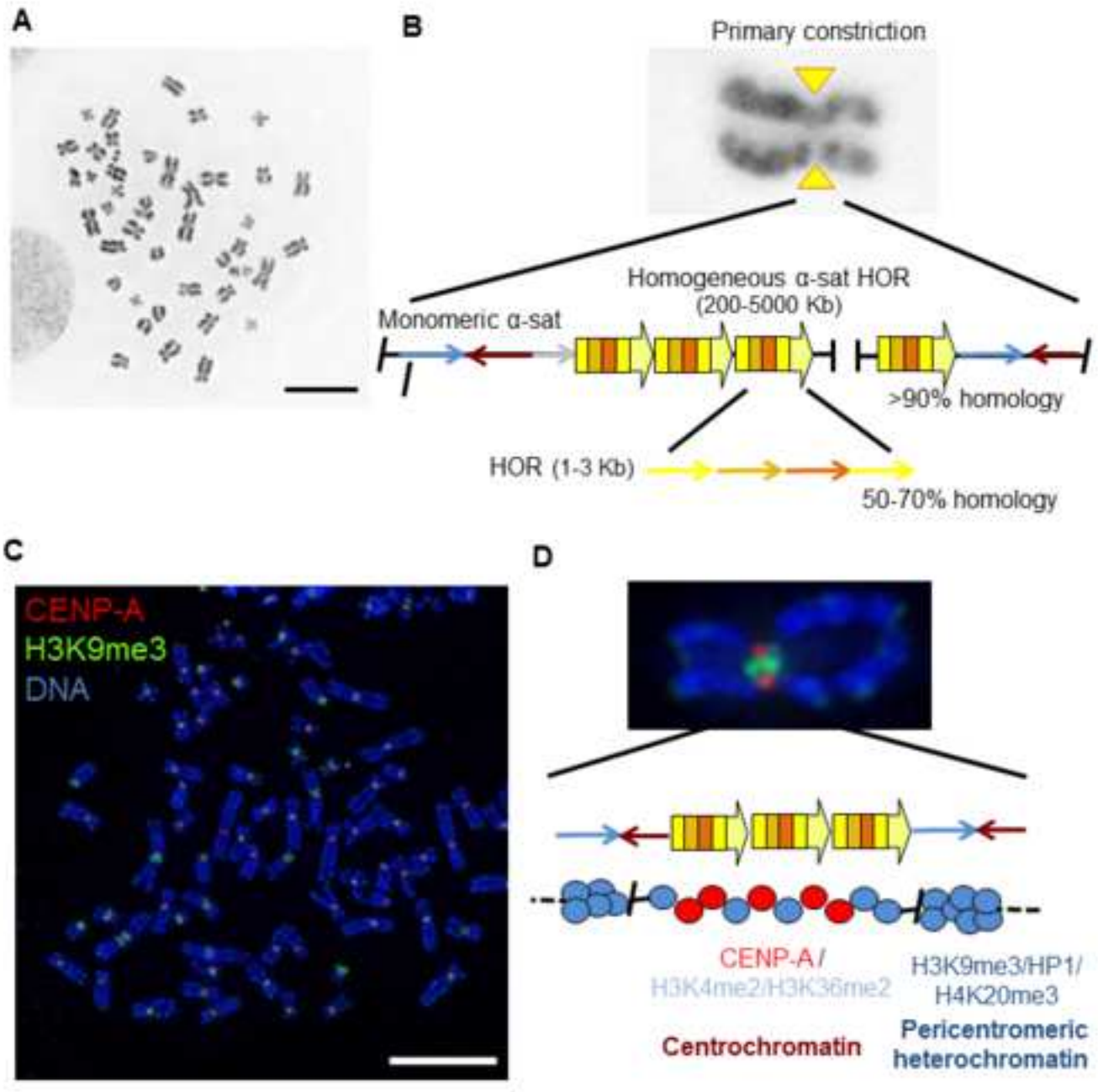
Figure 1






**Molina et al. Figure 1**

Figure 2

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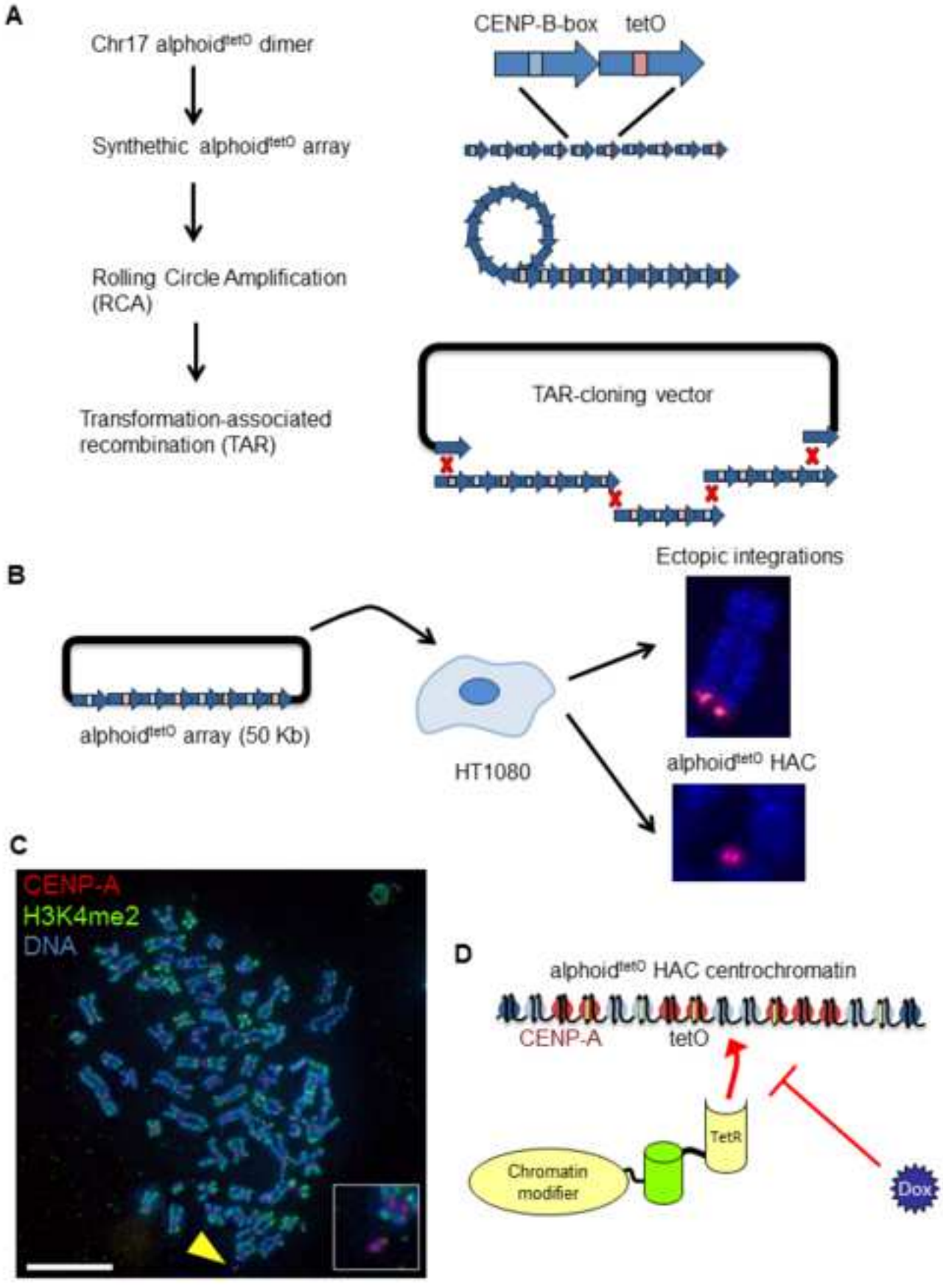


**Molina et al. Figure 2**

Figure 3

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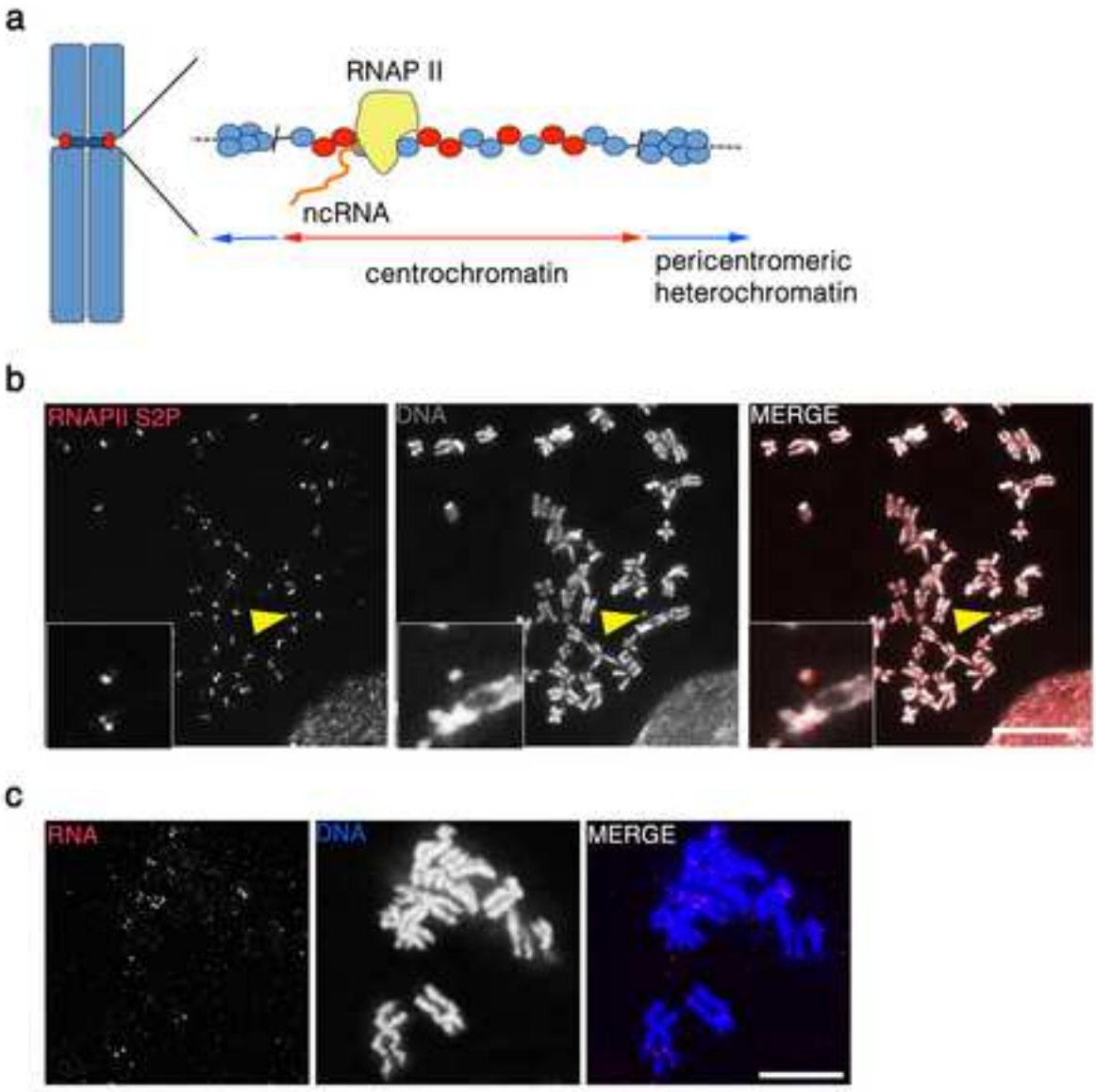


Molina et al. Figure 3

Figure 4

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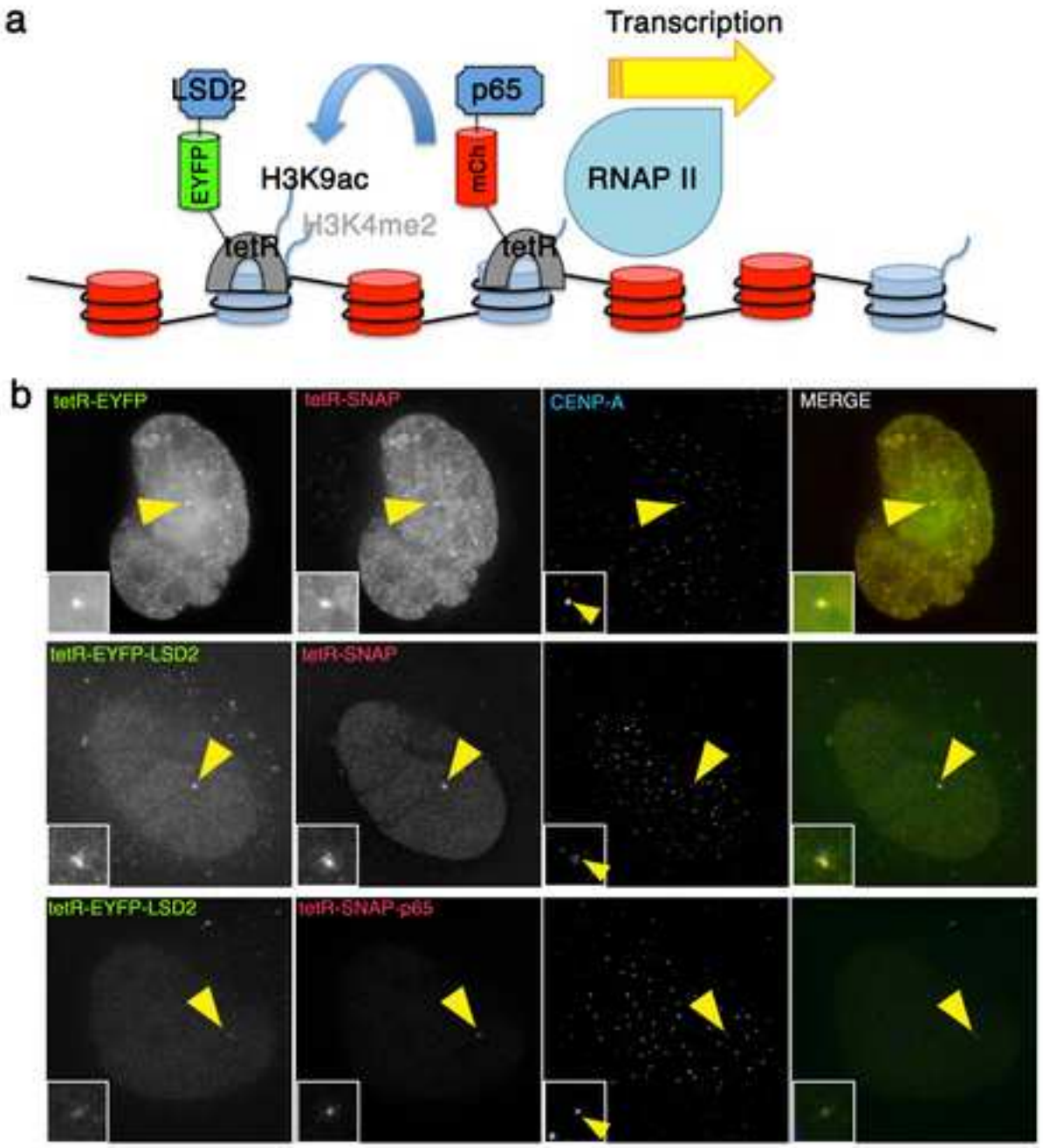


Molina et al. Figure 4