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Mammalian chromosomes contain cis-acting elements that control replication timing, mitotic condensation and stability of entire chromosomes

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Abstract

Recent studies indicate that mammalian chromosomes contain discrete cis-acting loci that control replication timing, mitotic condensation and stability of entire chromosomes. Disruption of the large non-coding RNA gene ASAR6 results in late replication, an under-condensed appearance during mitosis, and structural instability of human chromosome 6. Similarly, disruption of the mouse Xist gene in adult somatic cells results in a late replication and instability phenotype on the X chromosome. ASAR6 shares many characteristics with Xist, including random mono-allelic expression and asynchronous replication timing. Additional ‘chromosome engineering’ studies indicate that certain chromosome rearrangements affecting many different chromosomes display this abnormal replication and instability phenotype. These observations suggest that all mammalian chromosomes contain ‘inactivation/stability centers’ that control proper replication, condensation and stability of individual chromosomes. Therefore, mammalian chromosomes contain four types of cis-acting elements, origins, telomeres centromeres, and ‘inactivation/stability centers’, all functioning to ensure proper replication, condensation, segregation and stability of individual chromosomes.

Introduction

Mammalian chromosomes contain three distinct types of cis-acting elements that function during the cell cycle to maintain the proper number and structure of chromosomes. These cis-acting elements are: 1) origins of replication, which serve as the sites of initiation of DNA synthesis and are activated during a ‘temporal replication program’ along each chromosome during S phase; 2) telomeres, which function during all phases of the cell cycle to protect the ends of linear chromosomes; and 3) centromeres, which function as sites of mitotic spindle attachment and are critical for the proper separation of sister chromatids during cytokinesis. Recent genetic evidence suggests that mammalian chromosomes also contain a fourth type of cis-acting chromosomal element. For example, a recent series of “chromosome-engineering” studies led to the identification of a discrete cis-acting locus that controls chromosome-wide replication timing, mitotic chromosome condensation, and stability of human chromosome 6 [1]. Molecular characterization of this chromosome 6 locus identified a large intergenic non-coding RNA gene, which was named *asynchronous replication and autosomal RNA on chromosome 6* (ASAR6). Cre/loxP-mediated disruption of the ASAR6 gene results in extremely late replication, an under-condensed appearance during mitosis, and structural instability of human chromosome 6 [1,2]. In a separate series of experiments, it was found that disruption of the large non-coding RNA gene Xist, results

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in extremely late replication, abnormal chromatin structure and instability of the X chromosome [3,4]. The Xist gene resides within the X inactivation center, and is known to participate in the silencing of genes during dosage compensation in female cells [5]. Interestingly, ASAR6 shares many characteristics with Xist, including random mono-allelic expression, asynchronous replication timing, and regulation of the expression of linked mono-allelic genes [1]. Additional studies have indicated that ~5% of random translocations in human chromosomes, induced by either Cre/loxP or exposure to ionizing radiation (IR), display the under-condensed and late replicating phenotype affecting individual chromosomes [2,6]. The under-condensed and late replicating phenotype occurs on certain chromosome rearrangements and not on others and is therefore regulated by a cis-acting mechanism. In addition, the under-condensed and late replicating phenotype can occur on rearrangements involving most if not all human chromosomes [2,6]. The under-condensed and late replicating phenotype causes an ~50 fold increase in the rate at which secondary rearrangements occur [2]. Therefore this abnormal chromosomal phenotype causes structural instability of individual chromosomes. Taken together, these studies suggest that all mammalian chromosomes contain cis-acting loci that function to maintain proper replication timing, mitotic condensation and stability of individual chromosomes.

Mammalian cells replicate segments of their genomes in a defined temporal sequence. It is clear that the determinants of replication timing are not encoded within the sequence of the origins of replication, but rather the timing of origin firing is dictated by chromosomal location [7,8]. Recent studies indicate that at least half of the genome is subject to changes in the temporal sequence of origin activation during development [9,10]. The current thinking is that replication timing is directly predictive of, if not directly linked to, complex higher-order features of chromosome architecture [11,12]. It is also becoming clear that alterations in the replication-timing program are common features of cancer cells [11,13,14]. Unfortunately, both the mechanisms and the significance of this temporal replication program remain poorly defined.

Numerous reports over the past 40+ years have described an abnormal chromosomal phenotype affecting single or a few chromosomes in mitotic preparations from mammalian cells. For example, one or a few under-condensed chromosomes have been observed following acute exposure to mitotic spindle poisons or DNA damage [6,15–26]. In addition, these under-condensed chromosomes also display extremely late replication, with DNA synthesis continuing into the G2 phase. Other studies have documented individual under-condensed and late replicating chromosomes in tumor-derived cell lines, primary tumor samples, established cell lines, and primary lymphocytes from patients containing ring chromosomes [17,19,27–29]. While the morphological appearance of individual under-condensed and late replicating chromosomes observed in these various cellular contexts appears to be quite similar, the mechanisms underlying these phenotypes are likely to be distinct. An additional complication in the literature is that this phenotype has been referred to by many different descriptors, including: extended [15], incompletely condensed [27], pulverized [16–19], prematurely condensed [20–23], allocyclic [24,25], or delayed [1,2,6,29,30]. I have provided a discussion of these early observations in this review, and described how new genetic data has clarified some of the ambiguities associated with this abnormal chromosomal phenotype. For the sake of simplicity I have referred to this abnormal phenotype affecting individual chromosomes as “under-condensed and late replicating” throughout this review.

In addition to cis-acting chromosomal elements, mammalian cells also contain many cellular processes that function to maintain the structural integrity of chromosomes. These processes include: an exquisitely regulated temporal replication program that controls the timing and location of DNA synthesis; numerous cell cycle checkpoint pathways that function to delay

cell cycle progression until the damage is repaired; and DNA repair pathways that repair many different types of damage. Disruption of these processes, resulting in genomic instability, is thought to be a hallmark of cancer cells [31]. The genomic instability that is present in cancer has traditionally been thought to generate numerous mutations throughout the genome in a random process [14,32,33]. Thus, alterations in the cellular surveillance systems that monitor proper DNA replication, microtubule-chromosome attachment, cell cycle progression, and repair of DNA damage are expected to affect all chromosomes equally [33,34]. However, recent sequencing studies of tumor cell genomes indicate that in some cancers individual chromosomes can experience numerous rearrangements in a process called chromothripsis [35]. The discovery of chromothripsis raises intriguing questions about how large numbers of rearrangements can be confined to individual chromosomes. In this review, I have described recent evidence that supports a model in which all mammalian chromosomes contain discrete cis-acting loci that function to maintain proper replication timing, mitotic condensation, and consequently the structural stability of individual chromosomes.

The first description of individual under-condensed and late replicating chromosomes

In 1964 Dr. E. Stubblefield described an abnormal condensation and replication timing phenotype affecting individual mammalian chromosomes [15]. Using time-lapse photography he found that cells treated with the mitotic spindle poison colcemid arrested cell cycle progression in metaphase for approximately 12 hours. Following this metaphase arrest the cells experienced an abnormal anaphase, de-condensed their chromosomes, and reentered interphase without completing cytokinesis. The resulting cells contained multiple nuclei or perhaps more appropriately micronuclei as these nuclear structures did not contain full sets of chromosomes. During the next S-phase the micronuclei occasionally displayed asynchronous replication. In addition, single or groups of chromosomes displaying “late replication” were often abnormally condensed at the next metaphase [15].

Dr. Stubblefield proposed a model in which the segregation of chromosomes into different compartments (i.e. micronuclei) results in DNA synthesis beginning at different times or at different rates [15]. The under-condensed and late replicating chromosomes were observed in fixed metaphase spreads, however. Therefore it was not possible to determine whether or not the under-condensed chromosomes were in micronuclei prior to mitosis. In addition, subsequent work from the Debatisse lab found that the replication asynchrony associated with exposure to spindle poisons occurs equally in fragmented and non-fragmented nuclei, indicating that asynchronous replication is not dependent on physical separation of the chromosomes into isolated compartments [36]. Regardless, the under-condensed and late replicating phenotype induced by spindle poisons could affect many different chromosomes and was originally referred to simply as “extended chromosomes” [15]. These observations have been expanded to include numerous cell types from many different mammalian species treated with mitotic spindle poisons [16,20,36–38].

Chromosome Pulverization and Premature Chromosome Condensation

The morphological appearance of individual under-condensed and late replicating chromosomes described by Stubblefield is quite similar to an abnormal chromosome condensation phenotype induced by infection of mammalian cells with certain viruses. The phrase “chromosome pulverization” is a descriptive term that was first used to describe the fragmented appearance of complete sets of chromosomes in human cells infected with the measles virus [39]. The authors of this paper made two important observations: 1) infection of cells with the measles virus results in “a striking and extreme chromosome fragmentation,

referred to as chromosome pulverization” and 2) “the phenomenon occurred in syncytial cells in which nuclei were at different mitotic stages” [39]. In addition, complete sets of chromosomes occasionally exhibited different degrees of damage, ranging from completely fragmented or pulverized chromatin to apparently undamaged metaphase chromosomes [39,40]. Initially, the chromosome damage was thought to be a direct result of the cytopathic effects of the virus [39–41]. However, it soon became clear that “chromosome pulverization” was caused by viruses that induce cell fusion [[41–44]; and see below].

Subsequently, in an elegant series of experiments using a virus-mediated cell fusion protocol the phenomenon of premature chromosome condensation (PCC) was described [45]. The fusion of interphase cells with metaphase cells was shown to lead to condensation of the interphase chromatin within 30–60 minutes. In addition, depending on the cell cycle phase of the interphase cell different chromosomal morphologies were observed. If the interphase cells were in G2 the chromosomes appeared similar to normal mitotic chromosomes with sister chromatid attachments and no breaks or gaps along the length of the chromatids. However, the chromatids of G2-PCC were longer than metaphase chromosomes, and appeared similar to chromosomes in prophase. If the interphase cells were in G1 the chromatids appeared similar to G2-PCC, except that there were no sister chromatids as replication had not yet started in the G1 nucleus. Like G2-PCC, the chromatids of G1-PCC resembled prophase chromosomes and did not contain breaks or gaps along their length. In contrast, if the interphase cell was in S-phase the chromosomes appeared as completely fragmented, partially condensed pieces of chromatin, or chromosomes with multiple breaks and gaps. Thus, S-PCC represents the extreme fragmentation of chromosomes previously described as “chromosome pulverization” [39,40].

Individual under-condensed and late replicating chromosomes occur in cells exposed to DNA damage

Individual under-condensed and late replicating chromosomes have also been observed in cells exposed to DNA damaging agents and in cells from the chromosome instability syndromes Fanconi’s Anemia and Bloom’s syndrome [20–22,24,25,46]. During the early studies of these under-condensed and late replicating chromosomes the prevailing view was that they arose from micronuclei under the influence of the main nucleus entering mitosis, in the same way that fusion of mitotic cells with interphase cells induces PCC [reviewed in [23]]. However, several observations made by the Therman group did not support the hypothesis that under-condensed and late replicating chromosomes originated from micronuclei [25]. Thus, the positioning on the metaphase plate, the number of under-condensed chromosomes within individual cells, the variable degree of condensation between different under-condensed chromosomes within the same cell, and the lack of the G1-type PCC suggested that micronuclei were derived from the under-condensed and late replicating chromosomes and not the other way around [24,25]. In addition, under-condensed and late replicating chromosomes have been detected in 25–50% of cells from many different tumor-derived cell lines (see below), but the frequency of micronuclei in these same cells was <5% [6,29]. Therefore, under-condensed and late replicating chromosomes are more frequent than micronuclei, which supports the notion that under-condensed and late replicating chromosomes can give rise to micronuclei [24,25]. The Therman group went on to propose that a more accurate description of individual under-condensed and late replicating chromosomes was that they were “left behind in their cycle” and consequently were the opposite of PCC [24]. Furthermore, they proposed that individual under-condensed and late replicating chromosomes were caused by the mutation or disruption of hypothetical “coiling centers” located on every chromosome [24,25]. In addition, they proposed that “the coiling centers of different chromosomes are equally likely

to be inactivated and that they act independently of each other” [25]. It would take nearly 25 years for the first examples of these “coiling centers” to be identified (see below).

Certain chromosome rearrangements display an under-condensed and late replicating phenotype

Shortly after the characterization of “extended chromosomes” and “chromosome pulverization” induced by spindle poisons and viruses, respectively, Dr. Harold zur Hausen described a morphologically similar cytogenetic abnormality affecting individual chromosomes in cell lines derived from 7 different leukemia patients [27]. All 7 cell lines contained one or a few abnormally condensed chromosomes in 0.3–7% of metaphase spreads. These under-condensed chromosomes synthesized DNA after the normally condensed chromosomes had finished replication, indicating that these under-condensed chromosomes were extremely late replicating, with DNA synthesis extending into the G2 phase [27]. These observations have been extended to include chromosomes present in numerous tumor-derived and permanent cell lines [16,17,19,27,29]. In addition, the under-condensed and late replicating phenotype present in several tumor-derived cell lines occurred on certain chromosome rearrangements and not on others, suggesting that the under-condensed and late replicating phenotype was associated with certain rearranged chromosomes [29]. Fig. 1 shows examples of individual tumor-derived chromosome rearrangements displaying this under-condensed phenotype.

In the same year that Dr. zur Hausen described “incomplete condensation”, Dr. Ulla Gripenberg described an under-condensed and late replicating phenotype occurring on a ring chromosome in primary blood lymphocytes from a patient with multiple developmental abnormalities [28]. Subsequent studies have described a similar under-condensed and late replicating phenotype on some but not all ring chromosomes in humans [47–55]. Taken together, these observations indicate that an under-condensed and late replicating phenotype can occur on structurally rearranged chromosomes present in cells that were not acutely exposed to viruses, mitotic spindle poisons or DNA damaging agents.

Short colcemid treatment interferes with the ability to detect the under-condensed phenotype

The majority of cytogenetic procedures designed to analyze mammalian chromosomes include a colcemid pretreatment step prior to mitotic harvest [56–59]. Therefore, the majority of studies describing individual under-condensed and late replicating chromosomes included a colcemid step, and the reported frequencies of this phenotype were typically between 0.1 and 5% [15–25,27]. However, even from the initial observation, Dr. zur Hausen noticed that the frequency of under-condensed and late replicating chromosomes was greater in “anaphase preparations” generated in the absence of colcemid than in mitotic harvests prepared with colcemid pretreatment [27]. More recently, short-term (1–3 hours) colcemid exposure was found to have a dramatic affect on the ability to detect the under-condensed phenotype in standard mitotic preparations [29]. Thus, an analysis of several tumor-derived cell lines indicated that individual under-condensed and late replicating chromosomes could be detected in >35% of mitotic spreads prepared in the absence of colcemid, but in <1% of the spreads prepared with a colcemid pretreatment step [29]. The mechanism by which a relatively short colcemid exposure interferes with the ability to detect the under-condensed phenotype is not known, but it may be related to the ability of colcemid to delay the G2-M transition [56,60]. Thus, the colcemid induced G2 delay may allow the late replicating chromosomes to complete DNA synthesis prior to the onset of chromosome condensation at the end of G2 [29,30]. Regardless, it is likely that the frequency of under-condensed and late

replicating chromosomes was underestimated in the earlier studies due to the use of colcemid prior to mitotic harvest. It should be pointed out that the relatively short exposure (1–3 hours) to colcemid during standard cytogenetic procedures is not long enough to induce the asynchronous replication phenotype first described by Stubblefield, where micronuclei and asynchronously replicating chromosomes were not detected for at least 12 hours of colcemid exposure [15,36].

DNA synthesis extending through G2 and into mitosis

Given the relatively high frequency of chromosomes displaying the under-condensed phenotype detected in certain cancer cell lines in the absence of colcemid, a detailed analysis of the replication phenotype was now possible. Thus, using pulses of BrdU followed by relatively long chase periods, it was found that individual under-condensed chromosomes displayed a delay in replication timing (DRT) of >2 hours ([29]; Fig. 2). This DRT phenotype affects both initiation and completion of DNA synthesis along the entire length of the chromosome. In addition, using an “*in situ* DNA replication” protocol [61] active DNA synthesis was detected on individual under-condensed chromosomes during mitosis, indicating that replication continued through G2 and into mitosis [30]. All of the previous studies that assayed replication timing of under-condensed chromosomes used exposure of proliferating cells to tritiated thymidine or BrdU. Furthermore, there is always a delay prior to fixation (at least as long as the exposure) using these protocols. Therefore, the analysis by Chang et al [30] described the first example of active DNA synthesis occurring during mitosis on individual under-condensed chromosomes.

Adaptation of the replication checkpoint leads to DNA synthesis in mitosis

The observation of DNA synthesis occurring on individual chromosomes during mitosis was a surprising observation [30]. The DNA replication checkpoint is thought to function to ensure that DNA synthesis does not occur during mitosis [62–64]. When DNA replication is stalled, the ATR kinase phosphorylates CHK1 on serine 345, which activates a signaling cascade that results in cell cycle arrest prior to mitosis [65]. Using an antibody against phosphorylated CHK1 it was found that “chromosome-sized domains” of phospho-CHK1 immunoreactivity were present on the under-condensed and late replicating chromosomes during interphase, indicating that the delayed replication on these chromosomes activated ATR leading to phosphorylation of CHK1 [30]. Therefore, cells containing under-condensed and late replicating chromosomes initially activate the replication checkpoint but subsequently experience “checkpoint adaptation”, thereby allowing cells to enter mitosis with active DNA synthesis [[30]; see Fig. 3]. Checkpoint adaptation occurs in cells that are arrested at a DNA structure checkpoint but eventually over-ride the arrest and re-enter the cell cycle without repair of the damage [66]. Interestingly, checkpoint adaptation in yeast has been shown to precede both spontaneous and damage induced genomic instability [67]. Therefore, adaptation of the replication checkpoint can help to explain the structural instability of individual mammalian chromosomes with extremely late replication [2,30].

Delayed recruitment of Aurora B leads to delayed mitotic condensation

Proper chromosome condensation during mitosis is associated with phosphorylation of histone H3 on serine 10 [68]. H3 phosphorylation is initiated in late G2, completed just prior to nuclear envelope breakdown, and is required for proper mitotic chromosome condensation [68,69]. Using an antibody specific to this modification, H3 phosphorylation was found to be undetectable on individual under-condensed chromosomes during mitosis [29,30], suggesting that the under-condensed chromosomes have an early-G2 state of condensation [29]. Therefore, the under-condensed phenotype was described as a delay in mitotic chromosome condensation (DMC) [29,30]. In addition, this delayed phosphorylation

of H3 was associated with delayed recruitment of Aurora B to the under-condensed chromosomes [30]. Because Aurora B kinase activity is responsible for the majority of serine 10 phosphorylation of H3 during mitosis [70], these observations indicate that the under-condensed phenotype involves delayed recruitment of Aurora B to the late replicating chromosomes [30]. Furthermore, Aurora B kinase activity is involved in proper chromosome alignment and mitotic spindle assembly checkpoint (SAC) function [71]. Therefore, the delayed recruitment of Aurora B can help to explain the misaligned and lagging nature of individual under-condensed and late replicating chromosomes [30].

Individual under-condensed and late replicating chromosomes activate the SAC

The SAC functions to delay entry into anaphase until all of the kinetochores have attached properly to spindle microtubules [reviewed in [72–74]]. One critical component of the SAC is MAD2 [75]. MAD2 binds to unattached kinetochores and participates in a signaling cascade that prevents the metaphase to anaphase transition until all kinetochores are attached to microtubules [reviewed in [76]]. The kinetochores of chromosomes with the under-condensed and late replicating phenotype are MAD2 positive when all of the other normally condensed metaphase chromosomes are MAD2 negative, indicating that chromosomes with the under-condensed and late replicating phenotype are delayed in spindle attachment, and consequently activate the SAC [30]. In addition, a recent live cell imaging study found that a specific type of improper microtubule-chromosome attachment, known as merotelic attachment, is a major source of lagging and missegregating chromosomes in chromosomally unstable tumor cells [77]. Merotelic attachments occur when single kinetochores form abnormal attachments to the microtubules of both spindle poles [78]. Furthermore, inhibition of Aurora B kinase leads to a significant increase in the frequency of merotelic attachments [79]. Additional studies have found that Aurora B drives mitotic error correction, promotes biorientation of chromatids, and directly regulates the SAC [71,74]. Therefore, the delayed recruitment of Aurora B to the under-condensed and late replicating chromosomes likely contributes to activation of the SAC, chromosome misalignment, and missegregation of the affected chromosomes [2,29,30].

A genetic basis for under-condensed and late replicating chromosomes

The first suggestion that chromosome rearrangements could be responsible for the under-condensed and late replicating phenotype came from the observation that under-condensed and late replicating chromosomes were more common in cells treated with DNA damaging agents and in cells from patients with DNA breakage syndromes [24,25]. However, the formal proof that chromosomal rearrangements could cause the under-condensed and late replicating phenotype was provided by a series of “chromosome-engineering” studies [1,2,6]. The chromosome-engineering strategy involved the random integration of two loxP containing cassettes into the genome of a human cell line. Subsequent expression of Cre in independent clones allowed for the isolation of cells containing specific chromosome translocations [2]. This analysis found that ~5% of random inter-chromosomal translocations displayed the under-condensed and late replicating phenotype. Furthermore, the vast majority of Cre/loxP-mediated translocations did not display the under-condensed and late replicating phenotype, even when generated in multiple independent events. Therefore, this abnormal phenotype is the result of a cis-acting mechanism [2]. This study led to the characterization of five cell lines that generate balanced translocations that display the under-condensed and late replicating phenotype in a Cre dependent manner [2]. Fig. 4A illustrates a Cre/loxP dependent t(6;10), which displays the under-condensed and late replicating phenotype on the chromosome 10 derivative and not on the chromosome 6 derivative [1,2]. Subsequently, generating alternative translocations with the original loxP

cassette integrations in chromosomes 6 and 10 indicated that the under-condensed and late replicating phenotype segregated with the chromosome 6 loxP cassette integration site of this t(6;10) [1]. Finally, generating a set of nested deletions anchored at the original chromosome 6 loxP integration site indicated that deletions as small as ~76 kilobases result in the under-condensed and late replicating phenotype on chromosome 6 [1]. This set of experiments identified a discrete cis-acting locus that when disrupted causes the under-condensed and late replicating phenotype on human chromosome 6 [1].

Disruption of chromosome “ginactivation/stability” centers results in the under-condensed and late replicating phenotype

Significant advances in fields of study are often associated with surprising observations. The Xist large non-coding RNA gene is known to mediate gene silencing and late replication of the inactive X chromosome during early development (18, 19). Therefore, disruption of Xist would be predicted to result in earlier replication of the X chromosome. However, recent reports from the Marahrens lab indicate that Cre/loxP-mediated deletion of the murine *Xist* gene, in adult somatic cells, causes a delay in replication timing of the entire X chromosome [3,4]. In addition, abnormal chromosomal morphologies and secondary rearrangements were observed on the Xist-deleted chromosomes [3]. Therefore, the phenotype of X chromosomes containing *Xist* deletions appears to be similar to the under-condensed and late replication phenotype affecting autosomes described above. A detailed analysis of the human chromosome 6 locus, described above, indicated that the deletions that cause delayed replication of chromosome 6 disrupt a large non-coding RNA gene, which was named ASynchronous replication and Autosomal RNA on chromosome 6 (ASAR6) ([1]; and see Fig. 4B). As the name implies, the ASAR6 gene displays asynchronous replication timing between alleles [1]. In addition, the ASAR6 RNA is subject to random mono-allelic expression. Furthermore, disruption of ASAR6 results in activation of the previously silent alleles of linked mono-allelically expressed genes on chromosome 6 ([1]; Fig. 4C). Therefore, ASAR6 shares many characteristics with Xist. However, there are also some differences. For example, the Xist non-coding RNA physically “coats” the inactive X chromosome, while the ASAR6 RNA does not coat chromosome 6 [1]. In addition, Xist appears to be expressed in all female tissues (reviewed in [5,80]), while expression of ASAR6 is limited to a subset of adult tissues [1]. Finally, disruption of ASAR6 results in an ~30 fold increase in the rate at which secondary rearrangements occur on the affected chromosome [1,2]. Similarly, disruption of Xist results in instability of the X chromosome [3,4]. Therefore, ASAR6 and Xist function to maintain the structural integrity of their respective chromosomes.

Chromothripsis and chromosome pulverization

Recently, the phenomenon of “chromothripsis” was described as a new mechanism for generating complex chromosome rearrangements in cancer cells [35]. Chromothripsis appears to be a cataclysmic event in which one or a few chromosomes or chromosome arms are fragmented and then reassembled in a haphazard manner. The sequences at the junctions showed either a lack of homology or microhomology between the joined segments, suggesting that the ends were joined by non-homologous end joining (NHEJ). Chromothripsis-type rearrangements were recently observed in several additional tumor types and appear to be more common than originally thought [81–84]. The discovery of chromothripsis raises intriguing questions about how rearrangements can be confined to an individual chromosome. The authors of the original chromothripsis paper proposed that either exposure to IR or telomere dysfunction was responsible for the multiple rearrangements affecting individual chromosomes [35]. Alternatively, recent work from the Pellman lab suggests that missegregation of chromosomes, caused by transient mitotic

spindle disruption, can result in lagging chromosomes, micronucleus formation, late replication, and ‘pulverization’ of individual chromosomes [26]. The model proposed by the Pellman group involves mitotic errors involving individual chromosomes leading to the formation of micronuclei that then display: late replication, abnormal condensation, and multiple rearrangements affecting a single chromosome. This model fits nicely with our model of structural instability of individual under-condensed and late replicating chromosomes ([29]; and see below). Thus, under-condensed and late replicating chromosomes are delayed in their recruitment of Aurora B, are frequently unattached to the mitotic spindle, activate the SAC, and are often seen as lagging chromosomes during mitosis [30]. Consequently, disruption of cis-acting loci that cause the under-condensed and late replicating phenotype can explain how cells acquire micronuclei and experience instability of individual chromosomes in the absence of mitotic spindle poisons (Fig. 5A).

Complex chromosome rearrangements are generated by replicative mechanisms

The complex chromosome rearrangements associated with genomic disorders in humans were recently found to resemble chromothripsis [85,86]. Sequencing the breakpoints at these complex rearrangements identified characteristic features, including small templated insertions of nearby sequences and microhomologies, suggestive of replicative processes [85]. These observations led the Lupski group to propose the term “chromoanasythesis” as an alternative descriptor to chromothripsis for the shattering and reassembly of chromosomes via replicative mechanisms [85]. The Lupski group proposed a microhomology mediated break induced replication (MMBIR) and a related fork stalling and template switching (FoSTeS) model for the origin of these complex rearrangements [85]. The distinction between MMBIR/FoSTeS and NHEJ is that the microhomology junctions in MMBIR/FoSTeS are followed by stretches of DNA sequence derived from elsewhere, usually nearby [87]. The MMBIR/FoSTeS models involve stalled DNA replication forks that are resolved by replication restart using short stretches of homology [88]. Therefore, the Lupski models also fit nicely with the under-condensed and late replicating chromosome model. Thus, the stalled replication forks of the MMBIR/FoSTeS pathways could potentially be caused by the premature condensation of partially replicated chromosomes as they enter mitosis. Thus, I propose a model for the instability of individual chromosomes that incorporates aspects of both the Pellman and Lupski models, and includes: 1) delayed replication timing of individual chromosomes caused by genetic disruption of inactivation/stability centers, 2) delayed recruitment of Aurora B resulting in delayed mitotic chromosome condensation, 3) delayed mitotic spindle attachment leading to chromosome missegregation and the formation of micronuclei, 4) checkpoint adaptation and the onset of mitotic chromosome condensation prior to the completion of DNA synthesis leading to stalled replication forks, and 5) multiple rearrangements generated at the stalled replication forks via NHEJ and/or MMBIR/FoSTeS type mechanisms (Fig. 5B).

Unanswered questions

There are several important questions that remain unanswered regarding these inactivation/stability centers. For example, do all eukaryotic chromosomes have them? Are there more than one per chromosome? In addition, the relationship between inactivation/instability centers and chromosome fragile sites is not known. Common fragile sites represent regions of chromosomes that are prone to breakage upon replication stress [89]. Common fragile sites are known to be extremely late replicating due to a regional paucity in initiation events [90,91]. Therefore, one prediction of the late replicating chromosome model described above is that common fragile sites located on an under-condensed and late replicating chromosome would become even later replicating and perhaps even more unstable. In

addition, whether or not ASAR6 regulates replication timing in tissues where ASAR6 is not expressed is not known. Then again, disruption of Xist on the active X chromosome, where Xist is silent, also delays replication timing of the X chromosome [4]. This observation argues that the Xist RNA is not involved in the replication timing delay associated with Xist gene disruption. Finally, perhaps the biggest unanswered question regarding under-condensed and late replicating chromosomes lies within a larger question concerning the mechanisms regulating initiation of DNA replication in mammals. Thus, where and when DNA replication initiates along mammalian chromosomes remains as one of the greatest unanswered questions in biology [12,92]. It has been argued that the late replicating inactive X chromosome and the early replicating active X chromosome use the same origins of replication, but that these origins are activated at different times on the two X chromosomes in female cells [93,94]. Therefore, because Xist regulates X chromosome replication timing, it is likely that Xist, and ASAR6 as well, control the timing of origin activation via a cis-acting mechanism. Characterization of the mechanisms regulating the spatiotemporal replication program on mammalian chromosomes is clearly an important goal for understanding genome duplication, development and chromosomal integrity.

Conclusions

The similarities in morphological appearance between individual under-condensed and late replicating chromosomes and the complete sets of chromosomes displaying “chromosome pulverization” or PCC led to the notion that individual under-condensed and late replicating chromosomes were caused by the segregation of chromosomes into different nuclear compartments. However, more recent studies indicate that disruption of discrete cis-acting loci can result in an under-condensed and late replicating phenotype affecting individual chromosomes. Furthermore, the under-condensed and late replicating phenotype has been detected on chromosome rearrangements involving many different human and mouse chromosomes [1–3,6,29]. Therefore, it seems likely that all mammalian chromosomes contain loci that function to regulate chromosome-wide replication timing, mitotic condensation and stability of individual chromosomes. Given the similarities in structure and function of the two loci characterized to date, Xist and ASAR6, it is proposed here that all mammalian chromosomes contain functional chromosome inactivation/stability centers that act to maintain proper replication timing, mitotic chromosome condensation and stability of individual chromosomes. Under this scenario every mammalian chromosome contains four cis-acting elements, origins of replication, centromeres, telomeres, and ‘inactivation/stability centers’, all functioning to ensure proper replication, segregation and stability of individual chromosomes.

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Abbreviations

ASAR6	asynchronous replication and autosomal RNA on chromosome 6
ATR	ataxia telangiectasia and Rad3 related
Chk1	checkpoint kinase 1
Cre/loxP	cyclic recombinase/locus of chromosomal crossover (x)
CSI	chromosome structure instability

DMC	delay in mitotic chromosome condensation
DRT	delay in replication timing
FoSTeS	fork stalling and template switching
IR	ionizing radiation
MAD2	mitotic arrest deficient 2
MMBIR	microhomology mediated break induced replication
NHEJ	non-homologous end joining
PCC	premature chromosome condensation
SAC	spindle-assembly checkpoint
S-CIN	segmental-chromosomal instability
TIN	translocation instability
Xist	inactive X-specific transcript

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Figure 1. Individual under-condensed chromosomes. **A–D:** Mitotic spreads from cells containing a tumor-derived i(3q) chromosome [29]. **A, B:** Black and white images of mitotic spreads stained with DAPI. Insets show the pseudo-color images of the DAPI staining plus a chromosome 3 centromere (CHR 3 cen) detected by fluorescence in situ hybridization using a chromosome 3 centromeric probe (Vysis). **D:** An expanded view of the boxed chromosomes from panel **C**. The arrows mark sites of breaks and gaps in the under-condensed chromosome. Mitotic spreads were prepared without a colcemid pretreatment step.

Figure 2.

Replication-timing analysis of chromosomes with the under-condensed phenotype. Asynchronously growing cells were pulsed with BrdU for 15 minutes (green arrows) and chased for increasing amounts of time (3–10 hours) prior to mitotic harvest. The under-condensed chromosomes show a delay in both initiation and completion of DNA synthesis [29].

Replication Checkpoint Activation and Adaptation

Figure 3.

Adaptation of the replication checkpoint in cells with individual under-condensed and late replicating chromosomes. The left panel illustrates a normal transition from S-phase to M-phase. Chromosomes complete replication prior to mitotic chromosome condensation. The middle panel illustrates the consequences of hydroxyurea (HU) or aphidicolin (Aph) exposure on the S-M phase transition. The replication checkpoint arrests cells with un-replicated DNA prior to mitotic entry. The right panel illustrates the consequences of delayed replication on a single chromosome. Individual late replicating chromosomes contain phosphorylated Chk1 (serine 345; red) indicating that the replication checkpoint has been activated [30]. However, DNA synthesis can be detected during mitosis, suggesting that checkpoint adaptation has occurred, which allows cells with active DNA synthesis to enter mitosis [30].

Figure 4.

A: A balanced translocation generated by Cre/loxP displays the under-condensed and late replicating phenotype. In the example shown, chromosomes 6 and 10 contain integrated cassettes and produce a balanced t(6;10) following Cre transient expression. The chromosome 10 derivative (der10) displays the under-condensed and late replicating phenotype, even when generated in multiple independent events [2]. The chromosome 6 derivative (der6) displays normal replication and condensation. **B:** Schematic illustration of the ASAR6 locus. An ~1.3 mb region of human chromosome 6 (96,000,000–97,300,000). Mono-allelic expression of the non-coding RNAs ASAR6 and FHL5OST and the protein-coding gene FUT9 (red arrows). The location of the loxP cassette integration site is

indicated. **C:** Deletion of part (~76 kb) of the expressed allele of ASAR6 results in bi-allelic expression of FHL5OST and FUT9 (red arrows). The FHL5 gene was not expressed, and the K0776 gene was bi-allelic in the cells used for these experiments [1].

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Figure 5.

A: Under-condensed and late replicating chromosomes lead to mitotic errors. An individual late replicating chromosome (red*) is delayed in condensation due to delayed recruitment of Aurora B and delayed phosphorylation of histone H3 (i). Delayed condensation leads to activation and subsequently adaptation of the SAC (ii). Adaptation of the SAC can lead to missegregation and micronucleus formation of the delayed chromosome (iii). Normally condensed chromosomes are shown in green, mitotic spindles are shown in blue. **B:** Model for structural instability of under-condensed and late replicating chromosomes. Disruption of an inactivation/stability center leads to delayed replication timing of an individual chromosome. Delayed replication timing leads to delayed recruitment of Aurora B and delayed mitotic chromosome condensation. The replication checkpoint experiences adaptation, which leads to the onset of mitotic chromosome condensation prior to the completion of DNA synthesis. This premature chromosome condensation leads to stalled replication forks, and multiple rearrangements (deletions, inversions, duplications, and translocations) on the affected chromosome via NHEJ and/or MMBIR/FoSTeS type mechanisms.