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Abstract (118 words, max 120)

The chromosome periphery, which was first described over 130 years ago, is a complex network of proteins and RNA molecules (many derived from nucleoli) that covers the outer surface of chromosomes like a snow-drift and whose function remains mysterious. Technological advances and the recent discovery that Ki-67 acts as an organizer of this region has allowed the chromosome periphery to be dissected in previously unattainable detail, leading to a revival of interest in this obscure chromosomal compartment. Here we review the most recent advances into the composition, structure and function of the chromosome periphery, discuss possible roles of Ki-67 during mitosis and consider why this structure is likely to remain the focus of ongoing attention in the future.

Major Components of Mitotic Chromosomes

Duplication and segregation of the genome into two daughter cells, requires dramatic reorganization of the nucleus, with the transformation of the chromatin network into characteristic condensed mitotic chromosomes, accompanied by disassembly of the nucleolus. Mitotic chromosomes are generally considered to consist of five major regions/components (Figure 1A-E); nucleosomes/chromatin (grey), the chromosome scaffold (cyan), the centromere/kinetochore (green/magenta), and telomeres (orange). Each of these regions has been studied extensively, yielding strong evidence of their essential roles in chromosome structure and cell division [1-7]. Furthermore,

defects in these regions have been linked with aneuploidy, genetic instability and as a consequence, cancer [8-13] or fertility issues [14-18].

The fifth chromosomal compartment has received much less attention and is therefore far less understood. This mitotic chromosome periphery compartment covers the surface of mitotic chromosomes (Figure 1E – red).

A Non-Chromatin Structure that Covers Mitotic Chromosomes

A structure that originated in the nucleolus and was associated with mitotic chromosomes (referred to at the time as nuclear filaments) was first clearly described in 1882 [19]. Three years later, double staining of *Lilium* and *Clematis* cells using methyl green (chromatin) and fuchsin (nucleoli) revealed that the nucleolus disappears during spindle formation and reappears associated with metaphase chromosomes [20, 21] (for review see Montgomery, 1898 [21]). Even now this finding remains thought provoking, given that the nucleolus is essentially a ribosomal biogenesis and RNA processing factory that has no obvious functional ties with mitotic chromosome structure, apart from acting as the site of a secondary constriction known as the nucleolus organizer region (NOR).

The "transparent and gelatinous" structure was later termed the chromosome sheath [22, 23]. Subsequent names have included: the chromosome matrix [24], surface [25], periphery [26-28] or coat [29]; the perichromosomal layer [30] or region [31]; and a pellicle [32, 33] or halo [34] that surrounds chromosomes. We will refer to the 'mitotic chromosome

periphery compartment' which will be shortened to 'chromosome periphery' throughout this review.

A historical time-line of images for the chromosome periphery (Figure 2 – Key Figure) includes one of the earliest detailed sketches (Figure 2A) [35], early photographic evidence (Figure 2B, C) [24, 36] and an early micrograph obtained using fluorescent dyes (Figure 2D) [29]. During the next 30 years, technological advances permitted the development of the first antibody capable of recognizing a chromosome periphery protein (Figure 2E) [37] and an electron microscopy study providing ultra-structural details of the chromosome surface (Figure 2F) [38]. Most recently, further technological advances such as correlative light electron microscopy (CLEM) (Figure 2G) [39] and high-resolution light microscopy (Figure 2H) have provided an opportunity to explore the organization, function and composition of the chromosome periphery in unprecedented detail. Central to the recent surge of interest in this enigmatic compartment has been the discovery that Ki-67, a highly cited cancer prognostic marker [40], appears to be a key organizer of the chromosome periphery. This finding, together with several other important developments will be the focus of this review.

Chromosome Periphery Composition – The *Peripherome*

The mitotic chromosome periphery consists of numerous proteins, RNA species and ribosomal RNP complexes [26-28, 38, 41-43]. In 1994 the chromosome periphery was reported to consist of ~10 proteins [38]. 10 years later, this number had grown to over 50 characterised proteins, antigens and

RNAs [43]. This number is clearly an underestimate, as it does not include more recently described chromosome periphery proteins identified during shotgun proteomics screens of isolated chromosomes [44, 45]. The ever-growing list of components suggests that the *peripherome*, has yet to be fully characterised. Given the variety and diversity of components described, it is likely that this is a complex compartment or phase (see below).

Some of the best-known periphery components include the nucleolar proteins fibrillarin, nucleolin, nucleophosmin, peripherin and Ki-67 [25, 38, 43, 46-48]. Although the role of most of these proteins at the mitotic chromosome periphery has received little attention, Ki-67 has recently emerged as a particularly active subject for studies [39, 49-53].

Human Ki-67 is a 3256 amino acid protein recognized by a monoclonal antibody generated by injecting mice with nuclei isolated from Hodgkin lymphoma cells [40]. Major regions of the Ki-67 protein include; the N-terminal FHA domain; a PP1 binding domain; a large unstructured central region comprising 16 tandom repeats of 122 residues (in primates); and a C-terminal LR domain [54]. The function of these repeats, which are encoded by a single huge exon, remains unclear. Ki-67 redistributes from the nucleolar cortex and dense fibrillar compartments during interphase [55-57], to the chromosome periphery during mitosis [58, 59]. Critically, the antigen is not detected in non-cycling cells [40].

Ki-67 is widely regarded as one of the best markers to assess cell proliferation [60] and as a reagent to aid in determining patient prognosis for several tumour types, including breast cancer [61-63]. The protein is such a

successful marker because it is highly abundant and the epitope recognized by the Ki-67 monoclonal antibody (FKELF) is naturally amplified, being present on 9 of the 16 Ki-67 repeats that comprise much of the polypeptide.

Ki-67 and assembly of the peripherome

It has recently been proposed that the Chromatin Assembly Factor 1 (CAF-1) functions as a chaperone for Ki-67 [53]. The p150 subunit of CAF-1 promotes the localization of specific proteins to the nucleolus in interphase [64]. p150 co-localises with Ki-67 during all stages of mitosis and also in G_1 foci. A subset of p150 also associates with chromosomes [44], and depletion of p150 reduced the recruitment of Ki-67 to the periphery of metaphase chromosomes. This explains, at least in part, how Ki-67 might be recruited to the chromosome periphery. Ki-67 can also bind to DNA directly [65].

Using label-free mass spectrometry a recent study analysed Ki-67_FLAG, affinity purified from U2OS cells [51]. Gene ontology analyses of 406 proteins identified several nodes of enrichment: chromatin modification and transcription; ribosomal subunit biogenesis, pre-rRNA processing; protein translation; and splicing. Further analysis of protein complexes present in the spectra led to speculation that Ki-67 has a role in heterochromatin organization and compaction. This was supported by a reduction in perinucleolar staining for DNA, H3K9me3 and H4K20me3 in Ki-67-depleted HeLa, U20S and BJ-hTERT fibroblasts [51]. Ki-67 depleted cells showed a reduction in total chromatin compaction and loss of heterochromatin in a FLIM- FRET assay. These findings are consistent with previous reports that

6

Ki-67 interacts with HP1 [66, 67]. Interestingly, although the same study found that depletion of Ki-67 had no effect on the distribution of any of the three HP1 isoforms, it also observed that the overexpression of Ki-67 leads to ectopic heterochromatin formation with HP1 [51]. These results suggest that although Ki-67 has the ability to influence HP1 localization, its relationship with heterochromatin is complex.

Ki-67 is required for many (perhaps most) components to assemble to the mitotic chromosome periphery [39, 51]. Interestingly, this mammoth protein is predicted to be largely disordered, a property of proteins that have been shown to undergo phase separation [68-70]. Therefore, given recent insights into the role of phase separation in the structure of P granules [71], nucleoli [72], stress granules [73] and heterochromatin [74, 75], it is tempting to speculate that the mitotic chromosome periphery may be a liquid-like phase that coats the chromosome surface.

The role of Ki-67 in the formation of the chromosome periphery raises an interesting question about the evolutionary conservation of this compartment. For example, although nucleolin homologues have been identified in mouse, *Xenopus laevis* [76] and even *Schizosaccharomyces pombe* [77], Ki-67 is apparently only conserved in vertebrates, and even in vertebrates, the primary structure of the protein is remarkably variable. In the sole exception to this, Ki-67 has been reported in the dividing cells of *Schistosoma mansoni [78]*.

Chromosome Periphery Architecture

Measurements of the periphery as being between 87 and 150 nm thick [39, 50] were interpreted to imply that this is a relatively thin and therefore inconsequential component of mitotic chromosomes. However, volume varies with the cube of the radius, so even small radial differences can have large implications for the volumes included.   This point was recognized in a study, using advanced imaging (3D-CLEM – correlative light and electron microscopy) to model and define the architecture of all 46 human chromosomes in prophase and metaphase cells, and in the absence of Ki-67 [49]. Those experiments revealed that the volume of prophase chromosomes was only about 2/3 that of metaphase chromosomes. Interestingly, the "missing" volume corresponded almost exactly to the volume of the nucleolus, which was still intact in the prophase cell examined [49]. This led to the suggestion that roughly 1/3 of the volume of mitotic chromosomes might be found in the chromosome periphery – a calculation that is consistent with the previously measured thicknesses for the periphery.

Importantly, if the shotgun proteome of mitotic chromosomes is classified according to its functional characteristics, proteins associated with the periphery, nucleolus and RNA make up roughly 1/3 of the total mass, consistent with the volume measurements [49]. Using the data sets obtained from this study the scaled model of Figure 3 compares the overall size of metaphase chromosomes with and without the chromosome periphery.

3d-CLEM analysis revealed that both prophase chromosomes (in a nucleus with nucleolus not yet disassembled) and Ki-67-depleted chromosomes (lacking most periphery components) have a very 'rough'

surface appearance, whereas metaphase chromosomes have a smooth surface [49]. The fact that the surface of metaphase chromosomes is covered by the periphery compartment likely explains why scanning electron micrographs of isolated mitotic chromosomes do not show the nucleosome organisation of chromatin [79-82].

Chromosome Periphery Function(s)

Attempts to elucidate a role for nucleolar proteins in mitosis, distinct from their interphase functions have met with little success. For example no mitotic defects were reported following RNAi of fibrillarin or nucleophosmin [83]. Nucleolin depletion was reported to result in chromosome congression defects [84]. However this could be attributed to a role of nucleolin at the centrosome, as the same study also observed diminished spindle integrity [84]. Another study implicated Ki-67 in the regulation of the spindle microtubule stabilizing protein Hklp2 [85]. However, since Ki-67 is not localized to microtubules or microtubule plus-ends this may merit further investigation.

An obvious handicap limiting these studies from providing more convincing functional evidence, was the lack of ability to manipulate or remove the chromosome periphery.

The PP1γ-targeting protein Repo-man [86, 87] shares a highly conserved CDK-regulated PP1 binding motif with Ki-67 and is likely derived from Ki-67 by a gene duplication event [39]. This binding motif is significant, as Ki-67 was required for efficient recruitment of PP1γ to the periphery of mitotic chromosomes, but not to interphase nuclei [88]. Thus, both Repo-man

and Ki-67 cooperate in PP1γ targeting during anaphase. Furthermore, biochemical analysis showed that depletion of Ki-67 resulted in an increase in phosphorylation of B23/nucleophosmin, a nucleic acid-binding protein important for ribosome biogenesis and a component of the chromosome periphery [39] as well as increased phosphorylation of Ki-67 itself [88]. Ki-67 is hyperphosphorylated by Cdk1-cyclin B in mitosis [89].

PES1 [51], Nucleolin and NIFK all fail to localize correctly to the mitotic chromosome periphery in the absence of Ki-67, as do several novel cPERP proteins [39] (cPERPs B, C, D and F) identified in a major shotgun analysis of the mitotic chromosome proteome [44]. Reciprocal experiments revealed that Ki-67 itself did not rely on the presence of any these proteins or PP1 for its recruitment to the periphery [39]. Furthermore, correlative light electron microscopy (CLEM) strongly suggested that depletion of Ki-67 resulted in the loss of most, if not all, of the periphery compartment [39]. Interestingly, in the absence of Ki-67, many of the periphery proteins form domains of ~1-5 microns, which tend to be located at one end of the mass of chromosomes aligned at the metaphase plate [39]. It will be interesting in the future to determine whether these domains (which have the electron density of cytoplasm [39]) represent a phase separation of the chromosome periphery components when not associated with chromosomes.

These results suggested that Ki-67 might be required for the assembly of the chromosome periphery [39, 51], thus providing an opportunity for systematic testing for functional relevance of the compartment. Four classical functions have been suggested for the chromosome periphery [21-24, 90].

A Physical Barrier

The mitotic chromosome periphery could protect chromatin and prevent DNA damage following nuclear envelope breakdown. However, no obvious differences were found in the number of 53BP1 foci on mitotic chromosomes in the presence or absence of Ki-67, suggesting that the periphery is not required to protect mitotic chromosomes from damage induced in the cytoplasm of mitotic cells [39].

Maintenance of Chromosome Structure/Organisation

It is possible that the chromosome periphery has a more general role in the maintenance of chromosome structure/organization. Indeed one study reasoned that although condensin and Topo IIα are essential in many model organisms, in others systems, such as yeast and Drosophila oocytes, condensed chromosomes are still formed in their absence [91, 92]. This may suggest that other chromosomal proteins could play a similar functional role. To explore the function(s) of Ki-67 in interphase and mitosis, a Ki-67-mAID-mClover cell line that allows endogenous Ki-67 to be both visualized (via mClover) and targeted for rapid proteasomal degradation (via the mAID degron) was generated [52]. The rapid removal of Ki-67, specifically during mitosis, resulted in chromosomes not only becoming coalesced, but also structurally aberrant or "swollen". This phenotype was only observed following nuclear envelope breakdown at prometaphase. Further experiments revealed that Ki-67 depletion resulted in the mislocalisation of both Topo IIα and

11

condensin II complex member hCAP-H2, suggesting collaboration between chromosome periphery proteins and chromosome structure proteins [52]. Indeed, overexpression of Chmadrin (the marsupial homolog of Ki-67) truncation mutants has been previously shown to change interphase chromatin architecture [93], although no global changes to mitotic chromosome morphology were reported.

In contrast, a second study using an assay designed to test the intrinsic architecture of metaphase chromosomes, observed no clear difference between control and Ki-67 depleted chromosomes [39]. However, mitotic chromosomes show a strong tendency to be abnormally clumped together when Ki-67 is depleted [39, 49, 50].

Given these findings one might expect that loss of Ki-67 would be lethal, however a knock-out mouse for the Mki67 gene (the gene encoding Ki-67) exhibited normal growth and fertility [51]. Furthermore, MEFs showed that mutant cells could proliferate and divide like their WT counterparts. It remains to be determined whether Ki-67-null animals utilize another mechanism to target peripherome components to the mitotic chromosome surface.

The chromosome periphery maintains individualization of chromosomes
The chromosome periphery covers the entire outer surface of chromosomes, therefore it is feasible that it functions to keep chromosomes separate from one another, thereby preventing "tangling" with other chromosomes at a crowded metaphase plate. Evidence supporting this theory used FRET biosensors where Ki-67 was identified as the only candidate to contribute to

the spatial separation of mitotic chromosomes [50]. Further characterization suggested that metaphase chromosomes in cells depleted of Ki-67 appeared as a single contiguous and relatively immobile mass. Similar findings have been reported in other studies [49]. Chromosomes in these cells were significantly impaired in their ability to undergo normal segregation at anaphase, but ultimately segregate. The lack of movement of the metaphase chromosomes was confirmed by tracking the centromere-specific histone CENP-A.

It was hypothesized that Ki-67 acts as a steric and electrostatic coating on chromosomes, forming a sort of "biological surfactant" with an essential function in mitotic chromosome dispersion [50]. The authors then went on to test a panel of Ki-67 truncation mutants to elucidate the domains required for this proposed function, reporting that most, if not all, regions of the Ki-67 protein can restore chromosome separation, as long as they are fused to the C-terminal LR (chromatin binding) domain.

One might consider that structural defects or multi-chromosomal "clumping" would result in mitotic delay or an altered cell-cycle. Indeed this has been reported by some studies [50, 84], but others have reported normal proliferation in cells depleted of Ki-67 [51, 94]. Despite these defects, a Ki-67 knockout cell line was found to be viable, suggesting that although depletion of the protein causes a dramatic phenotype, cells can adapt to the loss of this protein, albeit with an increased sensitivity to stress [50].

The chromosome periphery is a landing pad for hitchhiker proteins

A large number of nucleolar components "piggy-back" on chromosomes during cell division. The reason for this is unknown, but one study found that the important nucleolar proteins B-23 and nucleolin were unevenly distributed to Ki-67-depleted daughter cells at cytokinesis [39]. Perhaps as a result of its role in keeping chromosomes separate, depletion of Ki-67 has significant effects on the distribution and morphology of nucleoli and nucleolar components at mitosis. Indeed, Ki-67-depleted HeLa cells tended to exhibit a single large, spherical nucleolus rather than several irregularly-shaped structures. This correlated with a decrease in the efficiency of nucleolar reactivation during mitotic exit [39]. One functional correlate of these changes was that rRNA transcription appeared less efficient in Ki-67-depleted cells [39, 95] (though this is debated, see below). This suggested that the large spherical nucleoli are less functional than their normal counterparts [39]. Similarly, Ki-67-depleted cells were often smaller than their control counterparts. Paradoxically, although other studies have reported reproducible (albeit marginal) support for delays in the early nucleolar pre-rRNA cleavage hierarchy, their data did not support a role for Ki-67 in rRNA transcription [51], as had been suggested by a previous study [95]. Interestingly, the selective loss of mitotic pre-rRNAs from the chromosome surface, has been shown to mislocalise other periphery components, including fibrillarin [96].

Implementing a dual tagging system, fusing fluorescent reporters to both the C and N terminus of Ki-67 lead one study to propose that a Ki-67 "brush" extends out 87 nm, from an anchor point at the chromosome into the

cytoplasm [50]. Interestingly the distal (N-terminal) portion of the Ki-67 "brush" contains an FHA (phosphopeptides-binding) and PP1-binding domain, so Ki-67 can act as a long flexible "dog leash" allowing PP1 to explore the surface of mitotic chromosomes (Figure 4). This PP1-binding domain is negatively regulated by CDK-phosphorylation, so that the phosphatase activity is likely to be most active during mitotic exit [39]. This could allow for the efficient dephosphorylation of diverse nucleolar proteins in the perichromosomal layer, thus helping to promote efficient nucleolar reassembly and re-activation.

Concluding remarks and future perspectives

A collection of recent publications has advanced our understanding of the structure, composition and function of the chromosome periphery, focusing primarily on the role of its important organizer, Ki-67. The physical parameters of the chromosome periphery are better defined, revealing it to occupy a much larger proportion of mitotic chromosomes than had been previously assumed [39, 49, 50]. Additions to the *peripherome* list such as CAF-1 and the cPERPs suggest that the list of components is far from complete.

Experimental observations now support three of the four classical hypothesized functions for the periphery: i) the maintenance of chromosome structure, particularly in the formation of heterochromatin, ii) a sheath to promote individualization (the "gelatinous layer" proposed in 1934 -[23]) and iii) a landing pad to carry and distribute client proteins and RNAs following cell division. The second and third of these functions together appear to contribute

(at least in some studies) to a role for the chromosome periphery in the assembly and function of individual nucleoli during mitotic exit. Given the apparent complexity of the chromosome periphery, it is possible that it performs multiple roles, although whether it is essential for mitotic progression remains a major question for future study [50,94]. The only of the original hypothetical functions that appears to have been ruled out by new data is the role of the periphery as protector from DNA damage during mitosis [39].

The recent revival of interest in the chromosome periphery, together with the new-found ways to manipulate it, have led to a number of unexpected insights, and experimental conflicts that are yet to be reconciled. Indeed, there is still much to do before the entire peripherome can be comprehensively mapped, and its function(s) definitively verified (see "Outstanding Questions"). The links of Ki-67 with cell proliferation, the nucleolus and the chromosome periphery ensure that this emerging area of chromosome biology is one to watch for the future.

Glossary

- **53-BP1:** Also known as tumour suppressor p53 binding protein 1
- **B23 (nucleophosmin):** nucleic acid-binding nucleolar protein involved in ribosomal biogenesis
- **CDK:** Cyclin Dependent Kinase
- **CLEM:** Correlative Light Electron Microscopy
- **cPERP:** chromosome periphery protein
- **FHA:** Forkhead-Associated domain

16

- **HP:** Heterochromatin Protein

- **Ki-67:** Kiel-67

- **LR domain:** a region containing multiple leucine (L) and arginine (R) pairs.

- **MEF:** Mouse Embryonic Fibroblast

- **NOR:** Nucleolar Organising Region

- **PES1:** Pescadillo homologue 1

- **PP1:** Protein Phosphatase 1

- **Repo-man:** Chromatin targeting chaperone of PP1.

- **RNP:** RiboNucleoProtein

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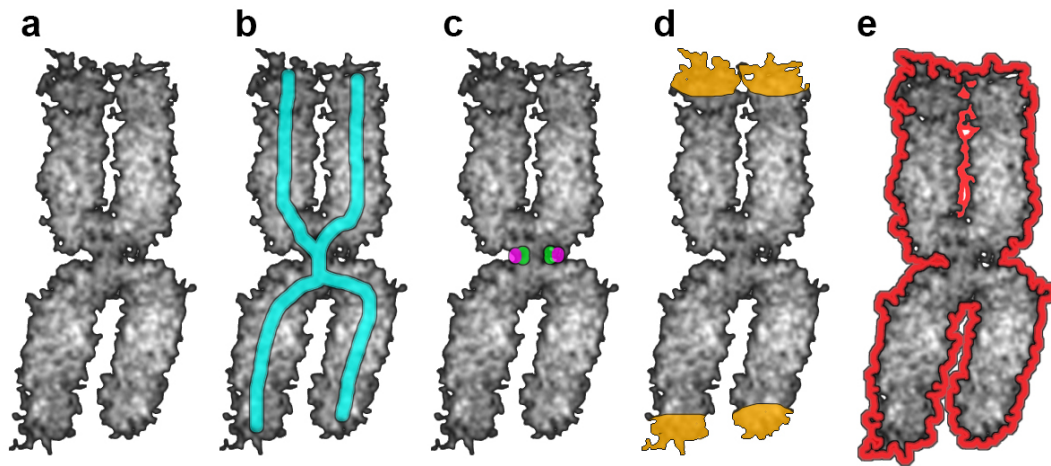
Figure 1 – Schematic highlighting the core components of a chromosome. A) Chromatin (grey) consisting of nucleosome units. B) The chromosome scaffold (cyan) consisting of KIF4, condensing and TopoII. C) The centromere (green) and kinetochore (magenta). D) Telomeres (orange). E) The chromosome periphery (red), consisting of an as of yet, incomplete list of proteins and RNA's.
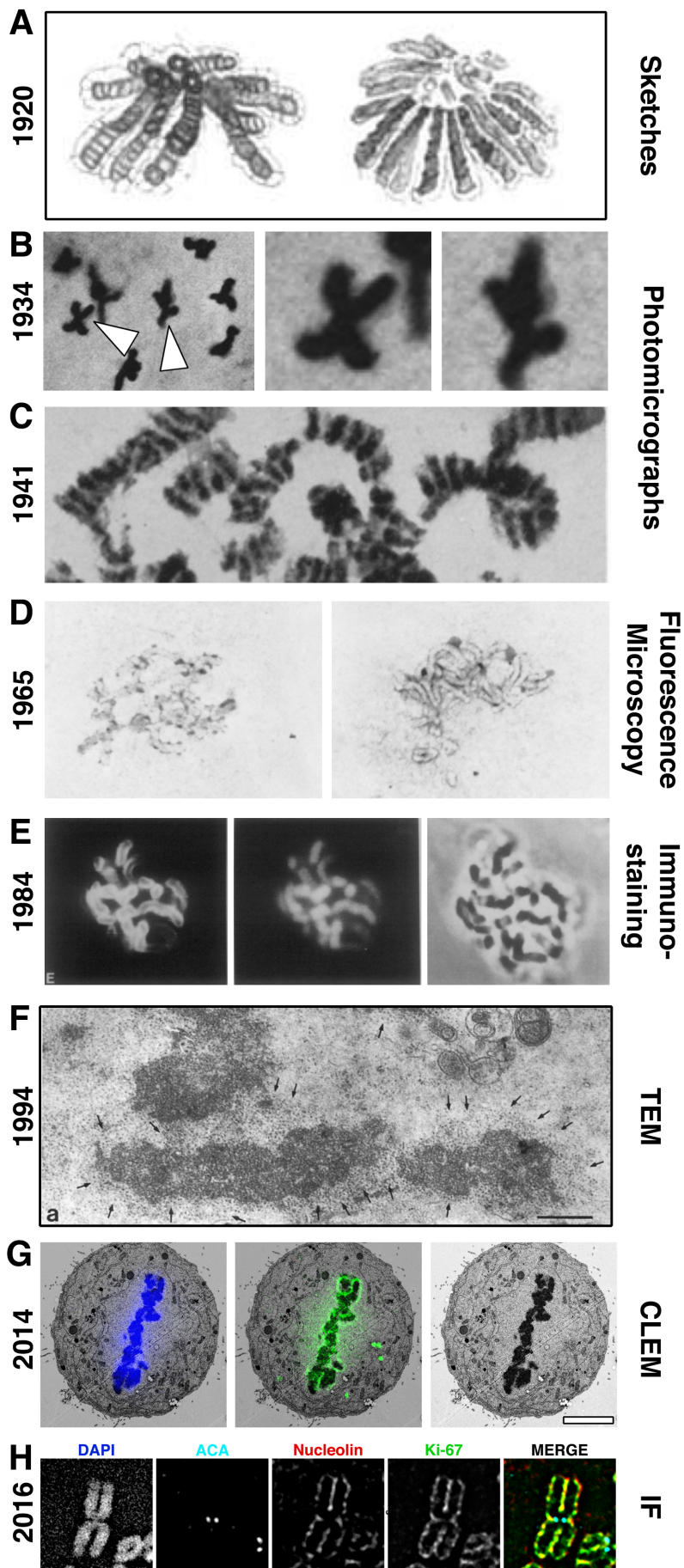
A 1920 — Sketches

B 1934 — Photomicrographs

C 1941 — Photomicrographs

D 1965 — Fluorescence Microscopy

E 1984 — Immuno-staining

F 1994 — TEM

G 2014 — CLEM

H 2016 — IF

DAPI   ACA   Nucleolin   Ki-67   MERGE

24

Figure 2 – A pictorial guide to some of the most historically important biological and technical advances associated with the chromosome periphery. A) One of the earliest sketches clearly depicting the chromosome periphery. This "anaphase" from the pollen grain of *Paris quadrifolia* has chromosomes that are " with sheath" [35]. B, C) Two of the earliest photomicrographs describing a "matrix substance" visible surrounding the chromosome arms [24]. Later the matrix was also described as being optically homogenous, when observing metaphase chromosomes of *Tradescantia congesta*, prepared using the Feulgen technique [36]. D) One of the first fluorescence microscopy images of the chromosome periphery. Using pyronin-methyl green staining, the pyronin revealed "RNA-rich bodies detected along chromosomes" of Chinese hamster metaphase chromosomes [29]. E) An early micrograph immuno-staining the chromosome periphery of CHO cells, using sera from a systemic rheumatic disease patient [37]. F) An early TEM image of HeLa cell chromosomes cryo-fixed in liquid helium and post-fixed in osmic acid. The "perichromosomal layer" can be seen around the chromosomes "forming a grainy structure" [38]. G) CLEM preparation of a mitotic HeLa cell investigating the chromosome periphery. The control cell (expressing GFP-cPERPC) was used as part of an experiment to show that Ki-67 RNAi leads to the physical loss of the chromosome periphery [39]. H) High-resolution immuno-fluorescence microscopy of a single mitotic chromosome stained with two chromosome periphery proteins (Ki-67 and nucleolin) [49].
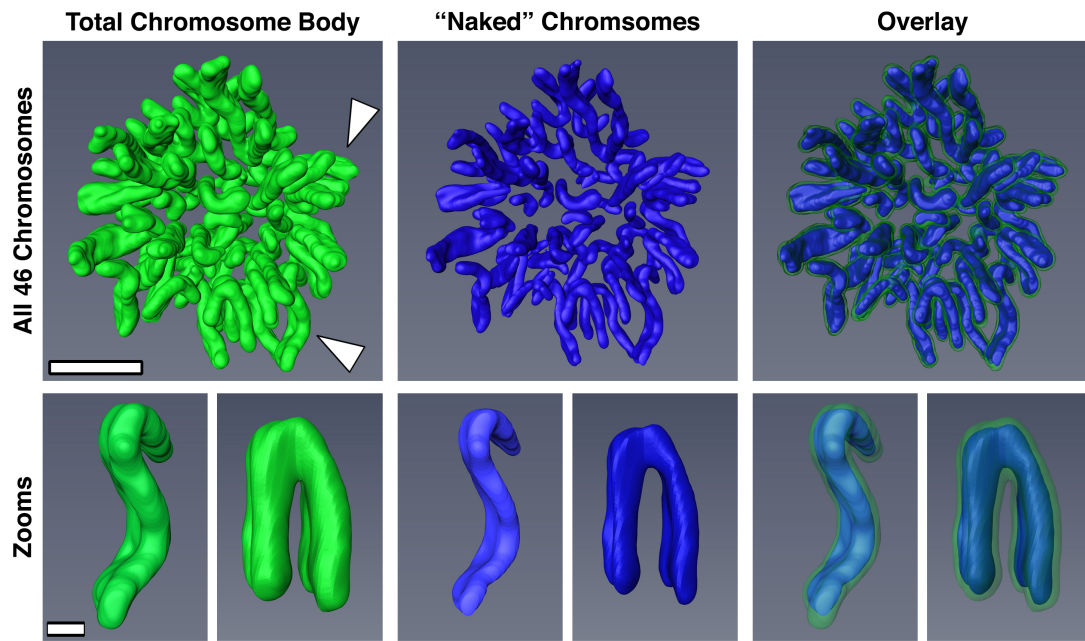
Figure 3 – Scaled representation of "naked" chromosomes. A 12 nm resolution digital model of all 46 human metaphase chromosomes, reconstructed from a 3D-CLEM data-set (Original data-set taken from Booth *et al* 2016). The 3D data was digitally edited to reduce the total volume of each chromosome (right panels - green) by 33%, the volume of the periphery estimated by protein mass [49], physical structure [49] and extrapolations of diameter measurements taken by light microscopy and TEM [27, 39, 50]. The resulting model (central panels - blue) represents "naked" chromosomes, presumably now only consisting almost entirely of chromatin. Right panels show an overlay of the translucent periphery (green) and opaque chromatin (blue). Scale bar – top panel 5 µm, bottom panel 2 µm.
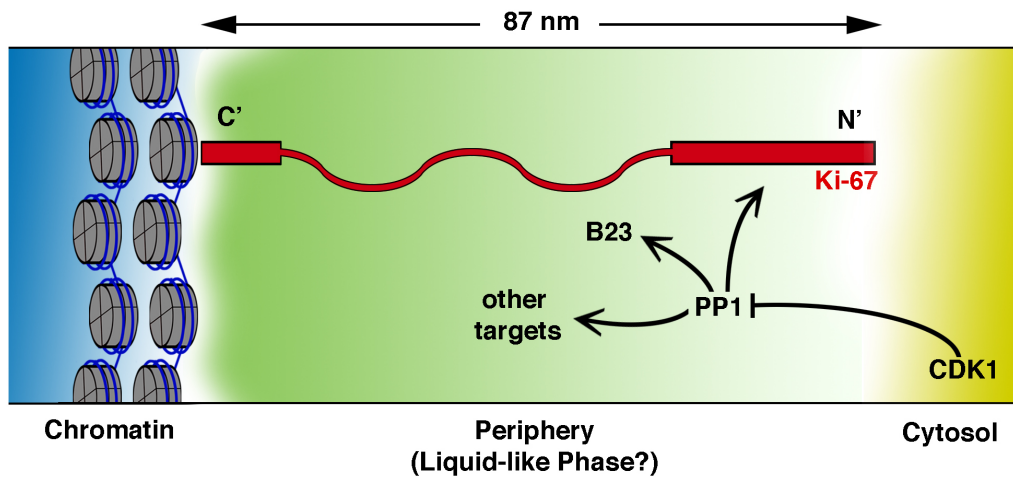
Figure 4 – Schematic of Ki-67 at the mitotic chromosome periphery. The C terminus of Ki-67 (red) is required for chromatin (blue) binding. The N-terminus extends 87 nm out from the chromatin [50] contains a binding site for protein phosphatase 1γ that can be negatively regulated by CDK1. Ki-67/PP1γ is required to dephosphorylate nucleolar protein B23 as well as Ki-67 itself during mitotic exit. Other targets remain to be determined. Ki-67 binding is required for efficient assembly of the chromosome periphery compartment [39, 51]