

Histone metabolic pathways and chromatin assembly factors as proliferation markers.

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Abstract

The structural organization of DNA into chromatin is of key importance to regulate genome function and stability. Maintenance of such an organization is thus crucial to preserve cellular identity. At each cell cycle, during S phase, this is achieved by duplication of chromatin structure in tight coordination with DNA replication. Such a coordinate process requires histone synthesis and their deposition onto DNA by chromatin assembly factors to be efficiently coupled to DNA synthesis. In this review, we highlight the intimate relationship between these chromatin-related events and DNA replication and we show how it is possible to take advantage of this coupling in order to identify cells with high replicative potential such as tumor cells. On the basis of recent data, we discuss the potential use of chromatin-associated factors as new proliferation markers of interest for cancer diagnosis and prognosis.

Keywords: histones; chaperones; chromatin assembly; DNA replication; proliferation marker; cancer.

1. Introduction

The functional organization of DNA in the nucleus of eukaryotic cells is chromatin (from the Greek *khroma* meaning coloured), a name given by Flemming based on the property to retain basic dyes [1]. This nucleoproteic structure is organized periodically and its basic unit is the nucleosome [2]. The nucleosome core particle now described in great details at the structural level [3] consists of 146 bp of DNA wrapped around a histone octamer, comprising two copies of each core histone H2A, H2B, H3 and H4. Such an organization can provide, in addition to the DNA sequence, another layer of information, which is termed 'epigenetic' since it is not genetically encoded, yet can be transmitted throughout multiple cell generations as defined by Russo et al. [4]. Indeed, in addition to DNA methylation, histone composition (variants and combination of covalent modifications) up to higher order chromatin organization constitute major epigenetic marks. Histone modifications have been hypothesized to participate in a so-called "histone code" [5,6] whereby combinations of histone modifications define different chromatin states through the recruitment of specific proteins. Epigenetic information being a key component in the regulation of gene expression, it has to be maintained to preserve cellular identity. Notably, disruption of epigenetic integrity has been associated with numerous diseases including cancers and cancer-prone disorders [7-9]. A crucial time for the maintenance of chromatin integrity during cellular life is obviously S phase, the stage at which a faithful duplication of chromatin organization has to be achieved in tight coordination with DNA replication. In this review, we emphasize how histone synthesis and their subsequent deposition onto DNA stimulated by histone chaperones are coupled to DNA replication during cell cycle progression and we discuss the potential use of chromatin-associated factors as new tools to identify proliferating cells.

2. Chromatin integrity and cell cycle progression

A growing body of evidence now clearly integrates chromatin into the control of cell cycle progression (reviewed in [10]). Not only is chromatin a major target of cell cycle checkpoint responses, but aberrant chromatin structures can also be detected by checkpoint mechanisms leading to cell cycle arrest, highlighting that stability of chromatin structure ensures normal cell cycle progression. Chromatin integrity is threatened upon DNA damage or any destabilization event. At each cell cycle, doubling of the DNA material in S phase requires additional histones in order to maintain a proper DNA-histone ratio. Chromatin stability is particularly challenged during this replicative process, which involves large-scale chromatin modifications. Upon replication fork passage, chromatin structure is disrupted and parental histones are randomly distributed onto both DNA strands [11] providing half of the histones that are required to duplicate chromatin structure. The additional supply of histones that is necessary for completion of chromatin restoration is achieved by histone neosynthesis and incorporation of newly synthesized histones into nucleosomes, which is termed *de novo* nucleosome assembly. Importantly, maintenance of chromatin organization requires a tight coordination between *de novo* histone incorporation and transfer of parental histones. A major challenge is thus to coordinate efficiently histone synthesis and their deposition onto DNA with DNA replication in addition to maintain epigenetic marks.

3. Coupling between histone synthesis and DNA replication

The majority of histone synthesis is restricted to S phase, in a way that is strictly coupled to DNA replication [12] in order to provide material necessary to assemble chromatin onto newly synthesized DNA. Notably, one major contribution to this cell cycle regulation of histone biosynthesis occurs at the mRNA level in all eukaryotes [13]. Histone expression is dependent upon ongoing DNA synthesis as attested by the use of replication inhibitors, which elicits an evolutionarily conserved response leading to a decrease in histone mRNA levels though using slightly different strategies among eukaryotes. In yeast it is based essentially on transcription regulation [14] whereas an important additional contribution at the level of mRNA stability has been described in higher eukaryotes [15,16]. While affecting DNA replication has a strong effect on histone synthesis, conversely depletion of histone pools by repression of histone gene transcription can itself cause cell cycle arrest in yeast, which arrests after completion of one round of DNA replication when histone synthesis is blocked [17]. Recently, a comparable mechanism was proposed in human cells that showed an S phase arrest under conditions affecting histone mRNA level [18]. Taken together, these data highlight how tightly histone expression can be coupled to cell cycle progression. This coupling ensures a delicate balance between histone synthesis and their incorporation into chromatin during DNA replication, minimizing the amount of free histones, which could potentially trigger cellular defects [19,20]. Interestingly, recent data have evidenced a mechanism in yeast, which leads to the degradation of excess histones that are not packaged into chromatin [21]. Whether a similar surveillance mechanism exists in higher eukaryotes will be important to examine.

Besides S phase related histones, minor forms of histones have been found expressed also outside of S phase [22]. These histone variants, called replacement histones, have been identified for all histones except H2B. They are structurally similar to replication-associated histones but functionally distinct due to limited differences in their amino-acid sequences. Replacement histones are generally constitutively synthesized at low amount throughout interphase in cycling cells but also in non-proliferating cells during differentiation or quiescence [23]. The ratio between the amount of S phase-related and replacement histones

can thus be a good indication of the proliferative state of the cells. The H3 variants H3.1 and H3.3 (fig. 1A) are interesting in that respect since regulation of their deposition onto DNA parallels the control of their synthesis in terms of coupling with DNA replication. Nucleosome assembly of the replication-associated histone H3.1 is coupled to DNA synthesis and occurs almost essentially at replication forks in S phase cells whereas the replacement variant H3.3 can be deposited at any stage of the cell cycle independently of DNA synthesis [24,25]. Therefore, this example illustrates how different histone variants can be incorporated into chromatin through distinct nucleosome assembly pathways. In light of this distinction, an important issue was to determine whether the deposition mechanism of such histone variants onto DNA involves dedicated histone chaperones, which could help to determine a specific deposition pathway.

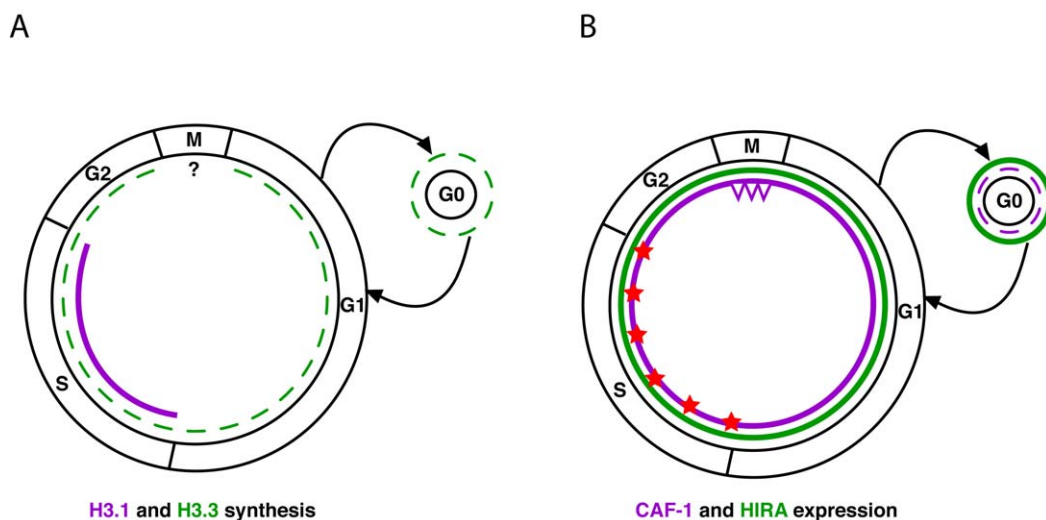


Fig.1: Parallel between histone variant synthesis and expression of the corresponding chromatin assembly factors. A, Synthesis of the canonical H3 variant H3.1 is tightly coupled to DNA replication thus restricted to S-phase whereas the replacement variant H3.3 is constitutively synthesized even in quiescent cells (G0). B, The chromatin assembly factor HIRA, which is specifically involved in H3.3 deposition onto DNA, is also constitutively expressed independently of the proliferative state of the cells. Conversely, the chromatin assembly factor CAF-1, which promotes nucleosome assembly of H3.1, is massively downregulated in quiescent cells. It is expressed throughout the cell cycle but more specifically required for S phase progression (stars) and its chromatin assembly activity is lost in mitosis (triangles).

4. Histone chaperones and chromatin assembly

Histones are escorted by histone chaperone proteins (reviewed in [26]) that are usually defined as histone interacting factors that stimulate histone transfer reactions without being part of the final product. They accompany histones from their point of synthesis up to their deposition onto DNA, which, schematically, proceeds in two steps: the loading of H3 and H4 histones occurs first, then the deposition of H2A and H2B follows (fig. 2A). Numerous histone chaperones have already been identified building up a complex network of histone associated factors playing different roles within the histone metabolic pathway, including histone storage, histone translocation to the nucleus, histone exchange or histone deposition onto DNA to form nucleosomes. In such an assembly line, only the chaperones directly involved in the latter step will be referred to as physiological chromatin assembly factors. *In vivo* these factors are crucial to control the deposition of histones onto DNA, allowing proper formation of a nucleosome. The chromatin assembly factors CAF-1 (Chromatin Assembly Factor-1) and HIRA (Histone Regulator A) are both involved in the initial step of nucleosome formation, *i. e.* deposition of H3 and H4 histones onto DNA. Yet these factors do not display

strictly redundant functions as they promote distinct nucleosome assembly pathways: one which is coupled to DNA synthesis via CAF-1 [27] and the other one being independent of DNA synthesis via HIRA [28] (fig. 2B). It is worth noting the parallel between these nucleosome assembly pathways and the way H3 variants are deposited onto DNA as attested in a recent study that revealed that H3.1 and H3.3 variants are specifically associated with CAF-1 and HIRA, respectively [25]. Furthermore, CAF-1 can promote nucleosome assembly of the S-phase related variant H3.1 whereas HIRA stimulates deposition onto DNA of the replacement variant H3.3 [25] (fig. 2B).

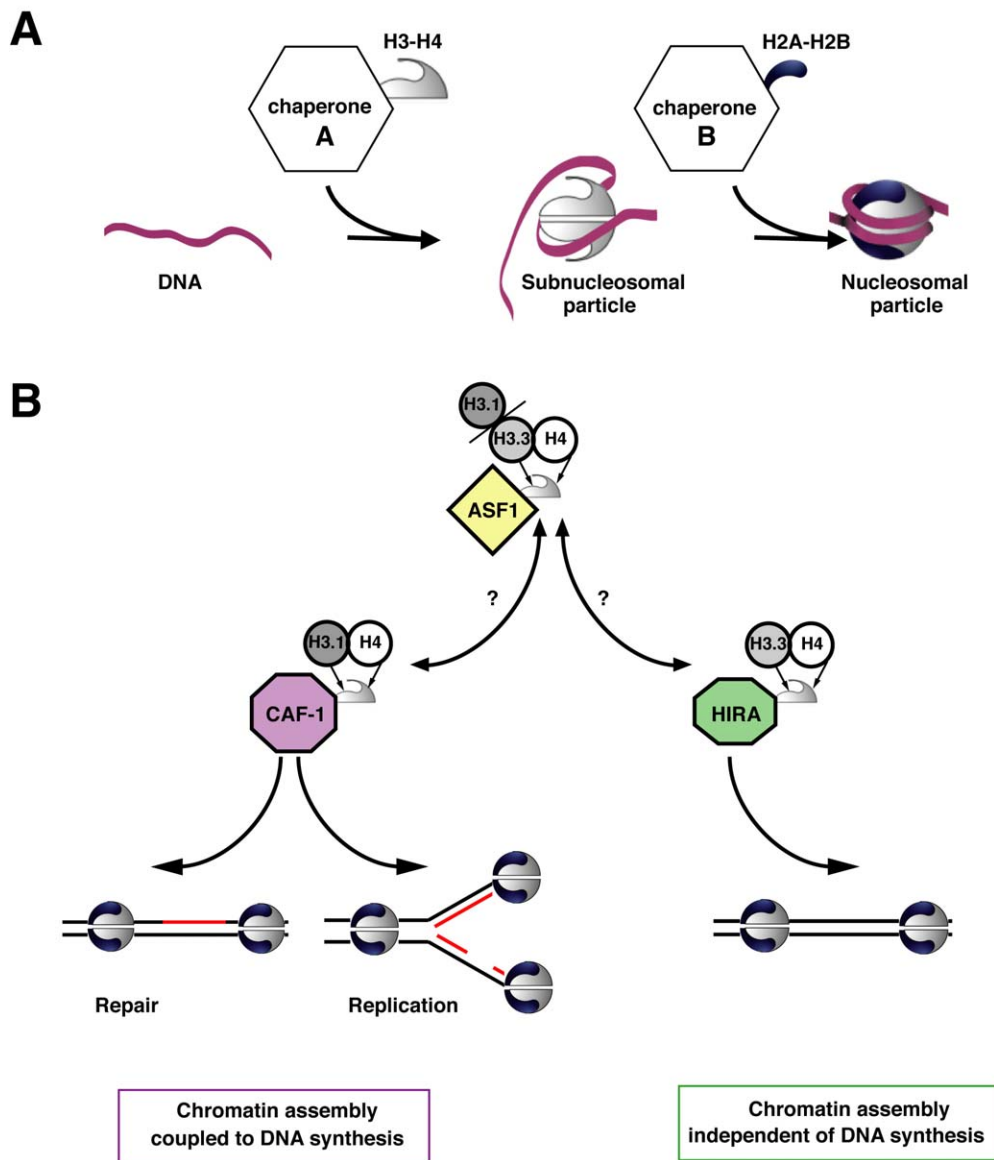


Fig. 2: Histone variants, their chaperones and specific chromatin assembly pathways. A, General scheme for histone deposition onto DNA in a two step mechanism: H3 and H4 histones are loaded first giving rise to a subnucleosomal particle. The subsequent adjunction of H2A and H2B allows formation of a complete nucleosomal particle. At each step, histones are escorted by distinct chaperone proteins (designated by the letters A and B, respectively). B, Scheme depicting how H3-H4 histones are escorted by a network of histone chaperones to promote deposition onto DNA. CAF-1 and HIRA act as nucleosome assembly factors that are associated with specific H3 variants (H3.1 and H3.3 respectively) and involved in distinct nucleosome assembly pathways (coupled to and independent of DNA synthesis respectively). Red bars correspond to DNA synthesis. ASF-1, which interacts with CAF-1 and HIRA, can equally associate with H3.1 and H3.3 and could act as a histone exchange factor upstream in the pathway. Arrows indicate histone transfer reactions.

In addition, other histone chaperones are likely to cooperate with nucleosome assembly factors to promote chromatin assembly as exemplified with ASF1 (Anti-Silencing Function1), another H3 and H4 chaperone that interacts with CAF-1 and HIRA [29,30]. Furthermore, ASF1 was shown to stimulate CAF-1 activity to assemble nucleosomes *in vitro*, potentially acting as a histone donor for CAF-1 [29,31]. Although *in vitro* studies are lacking at the moment for ASF1-HIRA synergy, genetic evidence in yeast, showing that HIRA and ASF1 counterparts belong to a same genetic pathway to create a repressive chromatin structure [32,33], are in favor of a similar cooperation between ASF1 and HIRA for nucleosome assembly. A recent study also proposed that ASF1 could play the additional role of a histone acceptor, which can promote chromatin disassembly during transcription activation in yeast [34]. Thus ASF1 could perhaps act as a histone exchange factor, which would accept histones and transfer them to the nucleosome assembly factors CAF-1 and HIRA for loading onto DNA (fig. 2B). Interestingly, several lines of evidence suggest that, besides their role in the nucleosome assembly pathway, the histone chaperones ASF1 and HIRA could be involved in the control of histone synthesis. Yeast ASF1 is a silencing factor required for histone gene repression during the cell cycle [33]. HIRA is the human homologue of yeast Hir proteins [35], which are negative regulators of histone gene transcription [36]. HIRA is proposed to display the same activity in higher eukaryotes as its ectopic expression in human cells leads to an S phase arrest [37,38], which has been attributed to repression of histone transcription that in turn would inhibit DNA replication [18]. Taken together these data highlight how intimately histone chaperones can be involved in histone metabolism, from regulation of histone synthesis to histone deposition onto DNA. They can promote nucleosome assembly of specific histone variants by distinct pathways. Among the different histone chaperones identified to date, the chromatin assembly factor CAF-1 distinguishes itself by its tight coupling with DNA synthesis.

5. Chromatin Assembly Factor-1: a key component in chromatin assembly during DNA replication

CAF-1 is the only chromatin assembly factor known to date that is able to promote nucleosome assembly on newly synthesized DNA [39]. This highly conserved protein complex has been first identified *in vitro* by its ability to stimulate histone deposition on plasmid DNA during DNA replication [27,40]. Its chromatin assembly activity has later been associated with DNA repair *in vitro* in the context of nucleotide excision repair [41] and repair of single-strand breaks and gaps [42]. These data have been further confirmed by *in vivo* experiments showing a colocalization between CAF-1 and replication foci in S phase [43,44] and between CAF-1 and repair sites [45-47]. The coupling between CAF-1 activity and DNA synthesis is mediated in each case by the polymerase-sliding clamp PCNA (Proliferating Cell Nuclear Antigen), which directly interacts with the largest subunit of CAF-1 and thus would serve for a targeting to sites of DNA synthesis [42,48]. CAF-1 is expressed throughout the cell cycle and is active for chromatin assembly in *in vitro* assays throughout interphase, its chromatin assembly activity being lost in mitosis [49] (fig. 1B). CAF-1 is present in the cells in a complex with newly synthesized H3 and H4 histones, as identified by covalent modifications on their amino-terminal tails [50]. Recent data have further revealed that CAF-1 is associated with, and specifically involved in the deposition of, the histone variant H3.1, which is S phase related [25]. Furthermore, disruption of CAF-1 function in higher eukaryotes including plants [51], *Xenopus* [52] and human [38] [53] [54] has evidenced its crucial involvement in proliferation events (fig. 1B). More specifically, a dominant negative interference targeting the largest subunit of CAF-1 in human cells triggers an S phase arrest [38] and downregulation of this same subunit shows an accumulation of

cells in early S phase [53], highlighting the fact that CAF-1 plays an essential role in mid-late S phase. Importantly, the largest subunit of CAF-1 can interact with HP1 (Heterochromatin Protein 1) [55]. This is particularly interesting given that HP1 proteins are highly enriched in pericentric heterochromatin domains, which replicate precisely during mid S phase. Recently, a dedicated role of CAF-1 in duplication of pericentric heterochromatin was evidenced in mammalian cells. CAF-1 function in these domains would participate in the maintenance of specific HP1 isoforms [56]. Taken together, these data provide compelling evidence for CAF-1 role in chromatin assembly of S phase histones coupled to DNA replication and heterochromatin inheritance.

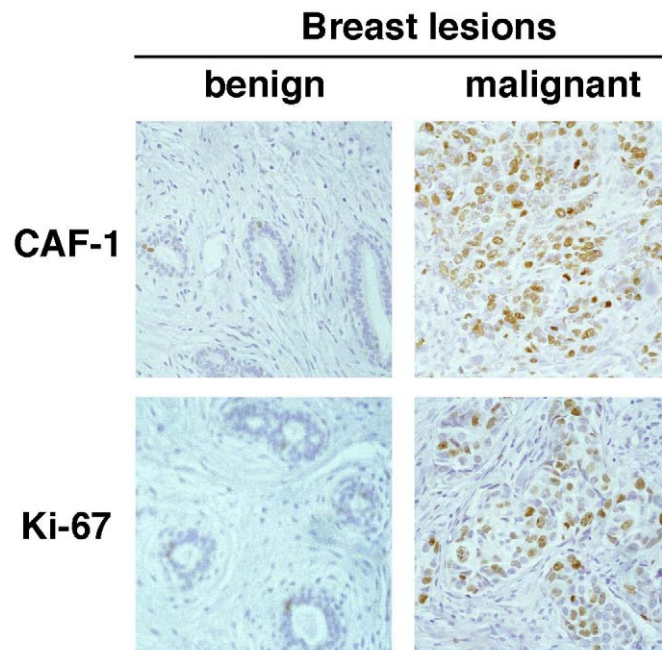


Fig. 3: Chromatin Assembly Factor-1: a new proliferation marker in breast cancer. Immunohistochemical stainings for CAF-1 and the routinely used proliferation marker Ki-67 in tissue sections from benign and malignant breast lesions as indicated (magnification, 200X). Highly proliferating cells within malignant breast lesions display strong positivity in each case. (Reprinted, with permission, from Polo et al., 2004 [57]).

6. Chromatin Assembly Factor-1: a new marker to assess cellular proliferation

Considering the crucial requirement of CAF-1 for nucleosome assembly coupled to DNA replication and S phase progression, this chromatin assembly factor could offer a convenient tool to discriminate between proliferating and quiescent states. Indeed, the high sensitivity of CAF-1 expression to the proliferative state of the cells was recently revealed. We found that CAF-1 is massively downregulated both at the RNA and protein levels upon cell cycle exit when cells enter quiescence contrary to the chromatin assembly factor HIRA which remains highly expressed in quiescent cells [57] (fig. 1B). These data further strengthen the parallel behaviour between both chromatin assembly factors and their corresponding histone H3 variants (fig. 1). In addition, CAF-1 was found to be overexpressed in cultured cells derived from breast tumors compared to cells from normal breast tissue. Furthermore, a study of clinical samples from breast lesions evidenced a strong correlation between the expression of CAF-1 and the routinely used proliferation marker Ki-67 (fig. 3). This study also showed an association of CAF-1 expression with several prognostic indicators, which validated CAF-1 as a powerful proliferation marker with potential prognostic value in breast cancer [57]. Notably, the wide range of expression of CAF-1 as well as its essential role opens up the

possibility that its use could be extended to cancers affecting other tissues. It would be interesting to compare CAF-1 properties with other proliferation markers such as the MCM (MiniChromosome Maintenance) proteins [58] that are involved in the regulation of DNA replication. In any case, to date, CAF-1 is the first example of a chromatin-associated factor shown to be helpful to assess cellular proliferation.

7. Conclusion/ Future directions

Histone synthesis and chromatin assembly activity are intimately linked to DNA replication and cell cycle progression. Thus they should be considered as integral part of cellular proliferation. Accordingly, the expression of chromatin-associated factors such as histones or histone chaperones can be used to assess cellular proliferation and help to identify cancer cells, as documented here for the chromatin assembly factor CAF-1. Beyond their interest as proliferation markers, deregulated histone synthesis and chromatin assembly could be integrated into a pathway leading to tumorigenesis considering their involvement in sustained cellular proliferation. Future works should help to unveil the position of these epigenetic events within the tumorigenesis cascade in order to gain more insights into their contribution to tumor phenotype. Considering their essential requirement for cell cycle progression, they could also represent interesting new therapeutic targets with cytostatic and potentially cytotoxic effect that deserve to be investigated in the future.

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