Repo-Man at the Intersection of Chromatin Remodelling, DNA Repair, Nuclear Envelope Organization, and Cancer Progression

Paola Vagnarelli

Abstract Nuclear structure and chromatin changes are very useful biomarkers in cancer diagnosis. Despite this, their biological significance and relevance to cancer progression are still not well understood. The identification of new proteins that link the nuclear envelope to chromatin organization and the understanding of the molecular mechanisms underlying these connections have begun to provide some important clues. This review discusses the role of the nuclear protein Repo-Man (*CDCA2*) in the maintenance of genome stability. Repo-Man (*CDCA2*) is a targeting subunit for the protein phosphatase 1 involved in the dephosphorylation of histone H3 during mitotic exit. In this role, it is important for the chromatin organization in post-mitotic nuclei. Repo-Man (*CDCA2*) is also essential for proper nuclear envelope reformation and the regulation of DNA damage responses. The relevance of this complex for cancer biology is also corroborated by emerging evidence that provides a correlation between Repo-Man (*CDCA2*) expression levels and cancer progression; several studies now suggest that Repo-Man (*CDCA2*) represents a very strong prognostic marker for poor patient survival.

Keywords Mitosis • Chromosome instability • DNA repair • Cancer progression • NUP153

Abbreviations

CDCA2	Cell division cycle associated 2
CPC	Chromosomal passenger complex
Cisplatin	*Cis*-diamminedichloroplatinum(II)
DDR	DNA damage response

P. Vagnarelli (✉)
Division of Biosciences, Brunel University, London UB83PH, UK
e-mail: Paola.Vagnarelli@brunel.ac.uk

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FRAP	Fluorescence recovery after photobleaching
MT	Microtubule
OSCC	Oral squamous cell carcinoma
PP1	Protein phosphatase 1
Repo-Man	Recruits PP1 onto mitotic chromatin at anaphase
RCA	Regulator of chromatin architecture
SS	Synovial sarcoma

Introduction

Nuclear structure changes are widely used by pathologists as they currently represent useful clinical biomarkers in cancer diagnosis for a number of cancer types. In fact, tumor cells are often distinguished by the presence of a lobulated nuclear envelope and abnormalities in chromatin organization [1]. Despite these criteria being of such general and important use in cancer diagnosis, we still do not have a clear picture of the causal-effect relationships of the observed changes and how or if any of the pathways that lead to such morphological changes could also be important as drug targets for cancer treatments.

Therefore, it is important to ultimately understand the molecular basis of alterations in nuclear structure that are associated with the clinical risk for disease recurrence and progression to metastasis. These changes may play an active role in cancer progression by contributing to many of the nuclear changes observed in the functionality of a cancer cell. Nuclear matrix changes appear to be a rich source for potential cancer biomarkers and may indeed reveal important cellular clues about the cancer process and its progression. Therefore the identification of the molecular pathways that link the nuclear envelope to chromatin organization and function has become of pivotal importance. Some components are well-known players in the process and currently under detailed investigation, while others have just emerged on the scene. In this review, we discuss the discovery and function of the novel chromatin binding and nuclear envelope interacting protein Repo-Man (*CDCA2*) and its connection with cancer progression.

Repo-Man Is a Cell-Cycle Associated Protein Phosphatase 1 Targeting Subunit

Cell division cycle associated 2 (CDCA2/Repo-Man) is localized on chromosome 8 (8p21.2) and was first identified by Walker [2] as a novel putative cell-cycle associated gene in a study that used co-expression analyses of several microarray databases to identify genes that were expressed in a similar way to known cell-cycle genes. However, the function for this protein remained unknown until Trinkle-Mulcahy and Lamond found CDCA2 in a proteomic analysis of protein

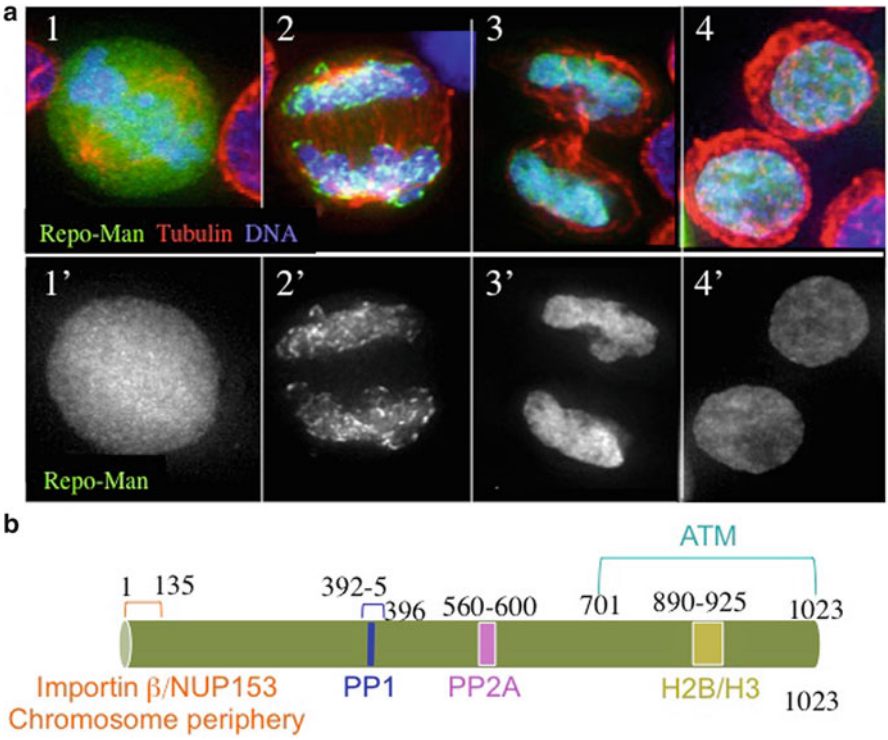

Fig. 1 Localization and interactions of Repo-Man (CDCA2). (**a**) Repo-Man is a nuclear protein in interphase (panel 4), it becomes dispersed in early mitosis (panel 1) and re-associates with the chromatin during mitotic exit (panels 2 and 3) where it localizes to the bulk of chromatin but has a separate population enriched at the periphery of the chromosomes. Repo-Man (*green*), α-Tubulin (*red*), DNA (*blue*). (**b**) Repo-Man schematic illustrating the binding domains for the interactors so far identified (see text for details)

phosphatase 1 (PP1) binding proteins. In this study, it was first shown that CDCA2 was a PP1 targeting subunit, responsible for the targeting of PP1 to chromatin in anaphase, and that it was essential for cell proliferation in vitro [3]. Because of these characteristics, it was renamed Repo-Man for "*Re*cruits *P*P1 *o*nto *M*itotic chromatin at *an*aphase."

Repo-Man is a nuclear protein in interphase that becomes dispersed in the cytoplasm at the onset of mitosis (Fig. 1a). In early mitosis (prometaphase/metaphase), it associates with chromatin in a highly dynamic manner as shown by fluorescence recovery after photobleaching analyses [4], and this dynamic behaviour is regulated by phosphorylation. At anaphase, Repo-Man becomes more tightly associated with chromatin, and it remains so during interphase where the chromosome-bound pool of Repo-Man has a very low turnover [4].

The biological importance of this protein was soon after revealed by a study of Vagnarelli and colleagues who showed that the Repo-Man/PP1 complex was a

critical component of the chromatin reorganization machinery responsible for chromosome de-condensation at the transition from mitosis to G1 [5]. The targeting of Repo-Man/PP1 to anaphase chromatin is responsible for the inactivation of an as yet unknown factor, functionally termed Regulator of Chromosome Architecture (RCA), that acts in parallel with Condensin, Topoisomerase II, and Kif4 to organize/form the highly structured condensed form of chromatin in mitotic chromosomes [6–8].

In order to identify the molecular pathways that are controlled by Repo-Man, a few groups have used different approaches. The consensus from all their published studies is that Repo-Man is the phosphatase that regulates Aurora B kinase localization during mitotic progression and that dephosphorylates some chromosome-associated Aurora B substrates [9–12]. It was also found that Repo-Man interacts with nuclear membrane components, thus contributing to post-mitotic nuclear reformation [9]. Moreover, it was shown that the Repo-Man/PP1 complex modulates ATM activation, thereby setting the threshold for checkpoint activation [13]. Thus Repo-Man has crucial functions not only during mitosis but also before and after. This marks Repo-Man as a crucial hub for the regulation of chromatin organization and the maintenance of genome stability. I will analyze separately these different functions, but we have to bear in mind that the importance of Repo-Man function could rely on the coordination of all of them.

Repo-Man Counteracts Aurora B Kinase in Mitosis

Aurora B is a very important kinase for the regulation of mitotic progression. It is the catalytic subunit of the Chromosomal Passenger Complex (CPC) (for a recent review see ref. 14). In mitosis it modulates the strength of kinetochore attachments to microtubules (MTs) by differentially phosphorylating components of the kinetochore in response to tension and it is necessary for the establishment of bi-orientation and to allow correct chromosome segregation. The CPC is localized at the inner centromere of the mitotic chromosomes in metaphase, and this is due to the ability of the complex to recognize specifically the tail of Histone H3 when it is phosphorylated at Thr3. (This modification occurs only in mitosis at the inner centromeric region and it is accomplished by Haspin kinase [15–21].) This interaction is mediated by the CPC scaffolding protein Survivin. In fact, a sustained Haspin activity even after anaphase onset compromises the ability of the CPC to transfer to the spindle [18], while a premature removal of this phosphorylation by a hyperactive form of the Repo-Man/PP1 phosphatase causes a displacement of the CPC from the centromere and the increased dephosphorylation blocks the cells ability to correct inappropriate kinetochore-microtubules attachments [9, 10]. The complex interplay of this network of kinases and phosphatases at the kinetochore has become even clear based on some recent results from the Bollen laboratory. Repo-Man itself appears to be a substrate of Aurora B kinase: phosphorylation of Repo-Man

at Ser893 decreases its affinity for chromatin. This site is then dephosphorylated by PP2A at anaphase onset which allows the targeting of the phosphatase back to the chromosomes where Repo-Man directs the dephosphorylation of H3Thr3ph by PP1 [12].

During mitotic exit the Repo-Man/PP1 complex also dephosphorylates Histone H3 at Ser10 and Ser28 (other Aurora B substrates) [9, 11]. This phosphatase has important functions also after cell division. Lack of Repo-Man/PP1 results in the inability to remove H3 mitotic phosphorylation which impairs binding of HP1. This in turn alters chromatin structural organization at the beginning of the new cell cycle [9]. All these results together clearly link Repo-Man to the maintenance of genome stability during cell division and they could explain why proper regulation of the expression levels of this PP1 targeting subunit is crucial for error-free mitoses.

Repo-Man Contributes to Nuclear Envelope Reassembly During Mitotic Exit

Beside its role as an important mitotic phosphatase, Repo-Man has a very peculiar localization during mitotic exit. As mentioned before, it targets to chromatin at anaphase onset where it localizes widely to the bulk of chromatin, but some becomes enriched at the periphery of the chromosomes (Figs. 1a and 2) in regions where both Importinβ and Nup153 are present. Mass spectrometry analyses of Repo-Man interactors have revealed that Repo-Man interacts directly with Importinβ and directly or indirectly with Nup153 [9] (Fig. 1b). These interactions are not present in early mitosis, but are established during mitotic exit and possibly maintained during much of interphase as well. Perturbation of these interactions by depleting Repo-Man causes major defects in nuclear envelope reorganization, thereby revealing yet another important function of the Repo-Man/PP1 complex in the establishment of a functional G1 nucleus after division.

The different chromatin sub-localizations are driven by two distinct chromatin-targeting domains in Repo-Man: the N-terminal domain is essential for the chromosome periphery targeting (aa 1–135), and the C-terminal domain (aa 560–9,250) is responsible for targeting to the bulk of chromatin (Fig. 1b). This enrichment of the protein in distinct compartments is established during mitotic exit but is maintained in interphase as well where a distinction in targeting is observed for the fragments between the nuclear periphery and the nucleoplasm (Fig. 2a, b). However, it is unclear whether the population of Repo-Man at the periphery of mitotic chromosomes directly corresponds to the same population/interactions responsible for targeting to the nuclear envelope/periphery in interphase. Another open question is whether these different enrichments in interphase have functional relevance. We presently do not know the chromatin binding sites for Repo-Man and the chromatin landscape that the binding of this phosphatase establishes; however, both aspects are pivotal toward the understanding of its complex functions in the cell cycle.

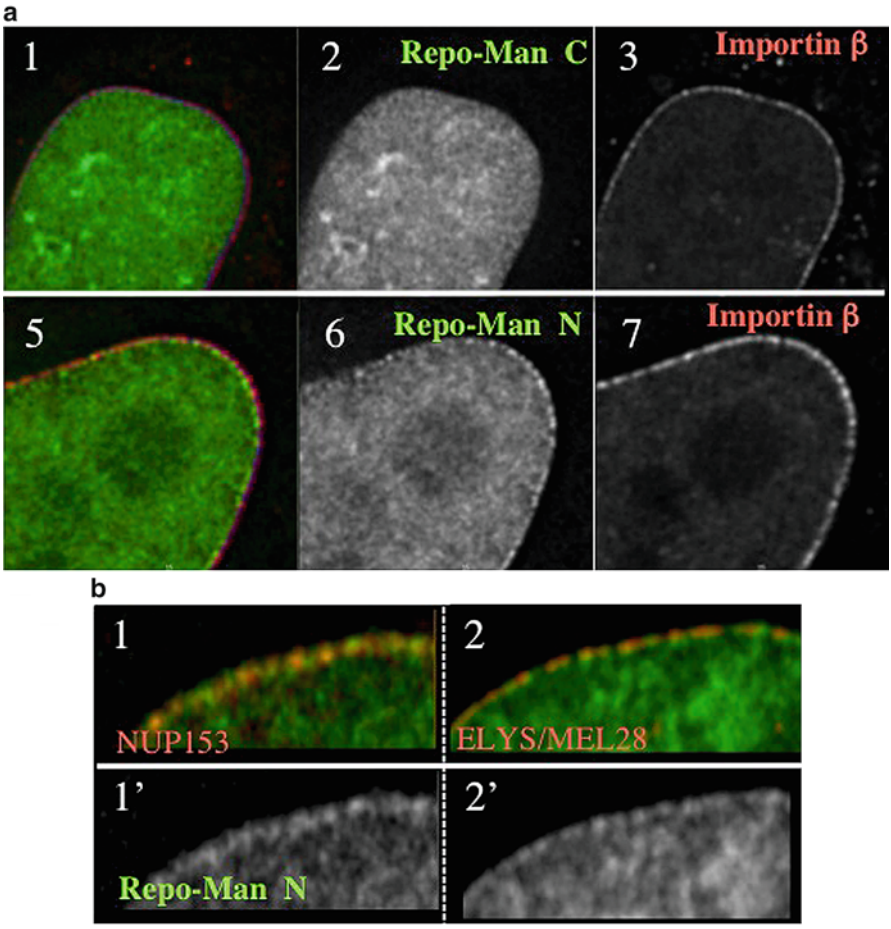

Fig. 2 Different chromatin-targeting domains localize Repo-Man in different nuclear compartments. (**a**) The C-terminal domain of Repo-Man targets the protein to the nuclear chromatin with exclusion of the nucleoli (panels 1–3), while the N-terminal domain (aa 1–135) targets the protein to the nuclear periphery (panels 5–7), as indicated by the proximity of Repo-Man (*green*) with Importinβ (*red*). (**b**) Co-staining of Repo-Man N-terminal domain (*green*) with the nucleoporins Nup153 and ELYS/MEL28

Repo-Man and the DNA Damage Response

The first link between Repo-Man/PP1 and DNA repair was provided by studies in *Xenopus* oocytes and egg extracts where it was shown to be required for setting the threshold for checkpoint activation after DNA damage. In this system, Repo-Man interacts with ATM (Fig. 1b) and co-localizes with ATM on chromatin [13]. However, the interaction between ATM and Repo-Man does not seem to be conserved in other systems as ATM was not found by proteomic analyses of the

Repo-Man interactome in DT40 and HeLa [9, 22], though it remains possible that other experimental conditions might reveal the interaction.

In the *Xenopus* system, Repo-Man appears to be responsible for targeting PP1γ to negatively regulate DNA damage-induced signal transduction. Further evidence indicates that Repo-Man overexpression reduces DNA damage-induced ATM activation, whereas a PP1 binding-deficient Repo-Man dominant-negative mutant enhances the response. By a mechanism that is not very clear at the moment (but possibly involves phosphorylation of Repo-Man itself) Repo-Man is released from the chromatin at DNA damage sites and dissociates from active ATM; this release presumably facilitates DNA damage response (DDR) activation.

These findings are very important since DDR is activated in early, pre-cancerous cells as a barrier to suppress cell proliferation and cancer progression [23], but it is reduced in late-stage cancer cells and the mechanism of this modulation in DNA damage responsiveness is unknown. In light of this, it is quite possible that overexpression of Repo-Man could result in desensitization of cells to DDR. Analyses of Repo-Man overexpression levels have revealed that indeed several but not all late-stage cancer cells have upregulated levels of Repo-Man. It has been proposed by J. Maller and collaborators that in the early stages of cancer progression DDR is activated in response to elevated genomic instability to prevent further cell proliferation [13]. If in some cells Repo-Man is upregulated, this will provide a selective growth advantage but also an increase of DNA damage, resulting in acquisition of additional mutations and further cancer progression. This implies that a reduction of Repo-Man levels should restore a normal DDR and this appears to be the case: Repo-Man depletion in advance stage cancer cells resensitizes them to the DDR and restrains their growth.

Additional support to these findings came from studies on oral squamous cell carcinoma (OSCC). Microarray analysis has shown that Repo-Man (*CDCA2*) is one of the genes upregulated in this cancer [24]. Depletion of Repo-Man in OSCC cell lines causes a decrease in cell proliferation due to cell-cycle arrest at the G1 phase. This is due to the upregulation of p21Cip1, p27Kip1, p15 INK4B, and p16INK4A and down-regulation of CDK4, CDK6, Cyclin D1, and Cyclin E [25]. In cells with wild-type p53, activated ATM phosphorylates p53 at Ser15 [26]. This stabilizes p53 so that it can induce transcription of p21Cip1 (CDK inhibitor) and prevent CDK4 and/or CDK6 and CDK2-mediated G1/S transition [27–30]. The activated ATM phosphorylates p53 at Ser46 as well, and this phosphorylation is essential for the induction of apoptosis after DNA damage [31].

The very important role of Repo-Man/PP1 in cancer progression and DNA damage signal transduction marks this complex a potentially very important target for cancer therapy. In fact, depletion of Repo-Man reactivates the DDR and blocks the abnormal proliferation of late-stage cancer cells [13]. This also suggests the possibility that lowering the levels of Repo-Man (in cancer where it is overexpressed) combined with the induction of DNA damage should drive the cells into the apoptotic pathway. This was proved to be correct, and it was shown that Repo-Man RNAi in OSCC (where Repo-Man is upregulated) combined with Cisplatin (cis-diamminedichloroplatinum(II); CDDP) treatments leads these cells into apoptosis

while the control cells did not enter the apoptotic pathway [25]. This suggests that Repo-Man suppression might have a considerable potential in enhancing the therapeutic effects of irradiation and anticancer drugs that cause DNA damage, though more models must be examined in order to determine if this pathway could represent a general hit for future drug development.

Repo-Man and Cancer Progression

From the studies mentioned above, it is quite clear that the Repo-Man/PP1 complex could have a potential role in cancer progression. However, the question is what the relevance of these biological findings is in the landscape of human cancer types. Since the first identification of the gene it was clear that Repo-Man was associated with proliferative markers, but one of the first correlations between levels of expression of Repo-Man and malignant transformation came by studies of neuroblastomas [32]. Expression profiling of 103 neuroblastoma tumors revealed that Repo-Man (*CDCA2*) is among the top-scored genes that are upregulated in stage 4 neuroblastoma cancers. In collaboration with A. Sala (Brunel University, UK), we have investigated these findings further, demonstrating an increase in Repo-Man also at the protein level and showing a correlation between Repo-Man level and Myc-N expression (Fig. 3e). The Kaplain–Meyer curve for survival of neuroblastoma patients with low and high levels of Repo-Man also shows that upregulation of Repo-Man correlates with a bad prognosis (Vagnarelli and Sala, unpublished). Although we still do not know the molecular aspects that are behind this correlation, it becomes quite clear that Repo-Man profiling could add some more diagnostic and prognostic value for this particular tumor.

Another study has compared gene expression profiles from a series of melanoma cell lines representing discrete stages of malignant progression that recapitulate critical characteristics of the primary lesions from which they were derived [33]. The reported analyses have identified expression signatures associated with melanoma progression that include principally the upregulation of activators of cell-cycle progression and DNA replication/repair; Repo-Man (*CDCA2*) is part of this cohort of 18 signature genes. However, no further studies have been conducted to understand the biological significance of this correlation and its relevance with the progression of melanoma.

The theme that Repo-Man is overexpressed in late-stage cancers and correlates to a bad prognosis stands true also in other cancer types. A recent study was carried out to identify gene signatures that could be prognostic for the metastatic behavior of Synovial sarcoma (SS) [34]. SS occurs in both children and adults, although metastatic events are much more common in adults. Whereas the importance of the t(X;18) translocation in SS oncogenesis is well established, the genetic basis of SS metastasis is not clear. By comparing expression profiles of tumors with or without metastasis Repo-Man (*CDCA2*) was identified as one of the two top-ranked genes

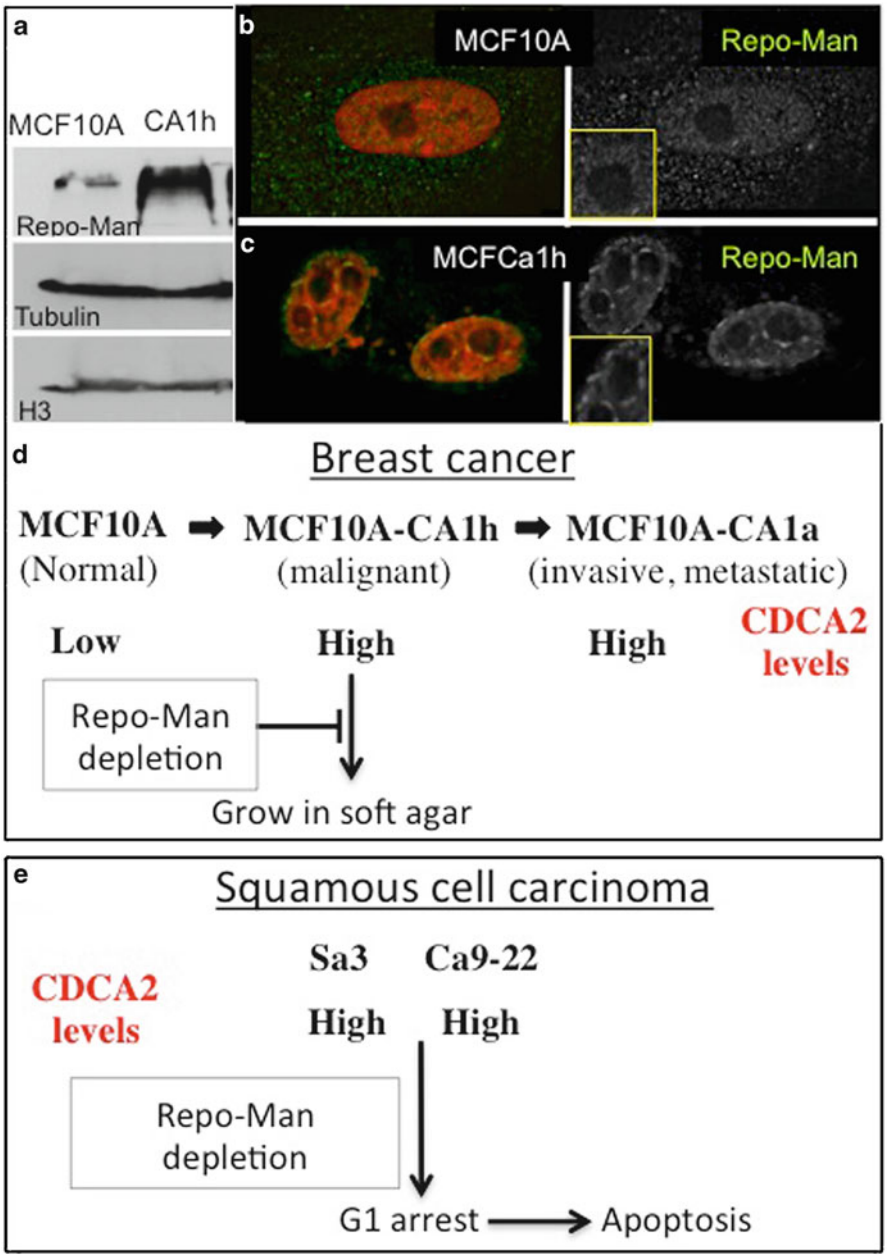

Fig. 3 Repo-Man functions in cancer progression. (**a**) Repo-Man is overexpressed in malignant breast cancer cell lines (CA1h) compared to normal breast cancer cells (MCF10A) in whole cell lysates. (**b**, **c**) In the malignant breast cancer cells, Repo-Man accumulates at the periphery of the nucleoli. (**d**) Repo-Man (*CDCA2*) levels are upregulated in breast cancer cells. Depletion of Repo-Man in malignant breast cancer cells restores the normal DNA damage response and blocks the ability of these cells to grow in soft agar [13]. (**e**) Depletion of Repo-Man in squamous cell carcinoma cell lines causes a G1 arrest and sensitizes the cells to DNA damaging agents [25]

(together with *KIF14*) for the metastatic prognosis of this cancer type. Kif14 belongs to the large family of kinesin proteins and appears to be a well-known oncogene that is overexpressed and associated with metastatic outcomes in lung [35], breast [36], ovary [37], and liver [38] carcinomas.

From the work presented above, it appears that increased levels of Repo-Man may represent a relatively common signature in human cancer biology. Analyses of Repo-Man (*CDCA2*) expression in different cancer types compared to their corresponding normal tissues clearly show this to be the case for many cancer types (Fig. 3a–e), though, interestingly, not all types show elevated expression of Repo-Man. These initial studies highlight Repo-Man as an important player in cancer, though much work is needed to test for a correlation with the prognosis for most of these cancer types, what is the molecular mechanism, and, most importantly, is the Repo-Man/PP1 complex a potential drug target for therapy?

Is Repo-Man (CDCA2) a Cancer Driver Gene?

Based on the data available so far it is quite clear that Repo-Man represents a biomarker for poor prognosis in cancer progression for at least some tumor types. What is less clear is why this is the case and which one of the functions so far revealed for this protein is the most relevant in cancer biology. The understanding of this aspect is extremely important if Repo-Man represents a drug target: we need to understand if the catalytic or the structural function of the protein is required for cancer progression.

Clearly all these cancer types and in particular their metastases are characterized by a high level of genome instability. However, it has been reported in several papers that Repo-Man can act at different levels that all promote genome instability. First, there are data supporting its role in modulating the DDR, therefore providing a platform for mutations and chromosome rearrangements. Second, it controls the regulation of chromosome segregation and Aurora B function. Impairing Aurora B leads to an increase of aneuploidy and lagging chromosomes. Lagging chromosomes are not lost in the cells but give rise to the formation of micronuclei. These phenomena, although very well known and widely used as a diagnostic signature for chromosome instability, have been recently shown to be the source of even greater genome instability [39, 40]. In this respect, increased Repo-Man levels could represent one of the means for generating the instability that characterizes late-stage cancers. Third, it has been clearly shown that Repo-Man is the phosphatase for histone H3 and that compromising its function causes problems in chromatin organization after cell division. Fourth, a change in the chromatin landscape for methylation and acetylation could well be another source of instability due to drastic changes in gene expression profiles. We do not know at the present time which type of chromatin Repo-Man binds to and how its expression levels alters the gene expression landscape of cells. It is clearly a question that needs to be addressed in the future.

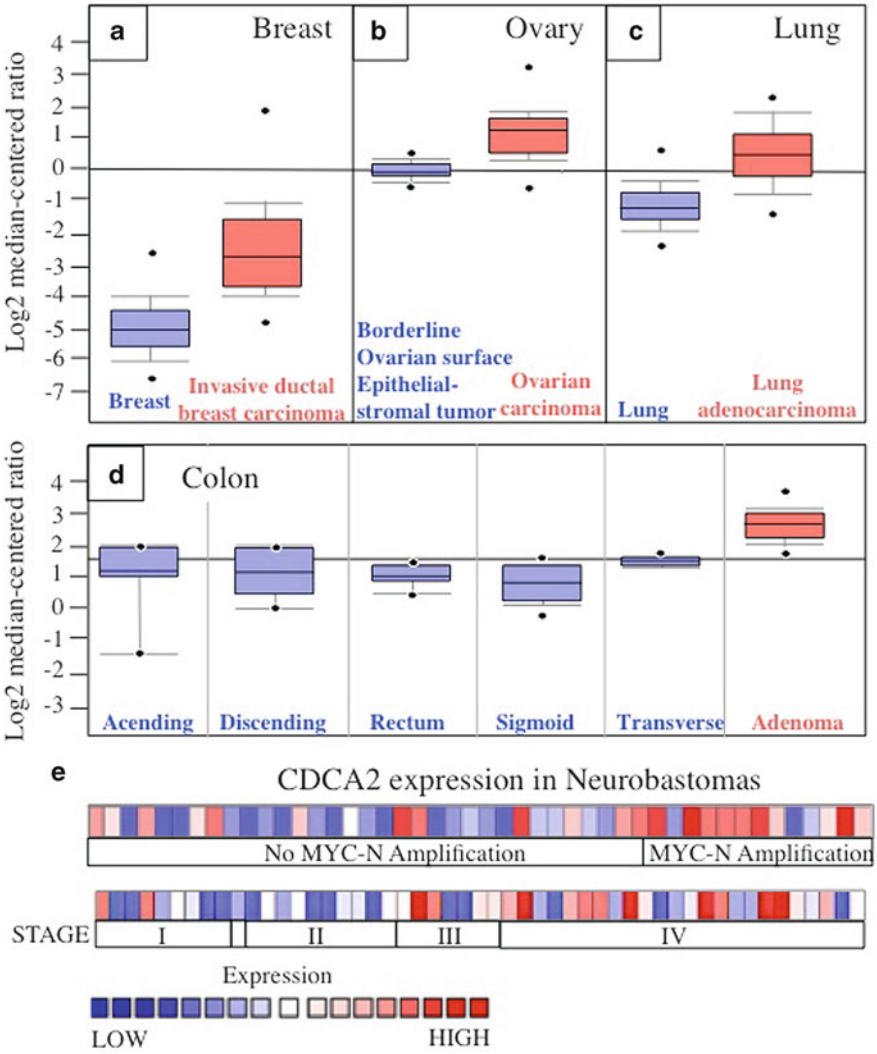

Fig. 4 Repo-Man overexpression in cancer. (**a**–**d**). Repo-Man is overexpressed in breast, ovary, lung, and colon cancers as shown by microarray data obtained in Oncomine. (**e**) Repo-Man is overexpressed in Neuroblastomas [32] and the expression levels correlate with Myc-N expression (Oncomine analyses)

Moreover, if we take a look at the localization of the protein in cancer cells where it is overexpressed, we can clearly see not just a general enhanced staining, but also a distinct pattern with accumulation to the nuclear periphery and at the periphery of the nucleoli (Fig. 4c). This is in contrast to normal interphase nuclei where it is evenly distributed in the nucleoplasm (Fig. 4b). Because it has been shown that Repo-Man binds Nup153 and Importinβ and is involved in aspects of nuclear

envelope reformation, we could also contemplate a scenario where this protein plays a role in the abnormal dynamics of the nuclear lamina that have been reported in cancer cells [40–42]. Addressing the role of Repo-Man in several cancer types and analyzing all of these aspects will help to clarify how Repo-Man is important in cancer progression and why. The answers to these questions are essential to develop adequate strategies to block the important aspect of Repo-Man function in cancer.

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