Repo-Man-PP1

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Coordination of late mitotic events is crucial for the maintenance of genome stability and for the control of gene expression after cell division. Reversible protein phosphorylation regulates this process by de-phosphorylation of mitotic phospho-proteins in a sequential and coordinated manner: this allows an orderly sequence of events to take place during mitotic exit. We have identified Repo-Man/PP1 as a phosphatase complex that regulates temporally and spatially chromatin re-organization and nuclear envelope re-formation during anaphase-telophase.

Following CDK inactivation at anaphase onset, a pool of Repo-Man/PP1 localizes homogeneously to the anaphase chromatin, where it removes the major mitotic phosphorylations on Histone H3 (Thr3, Ser10 and Ser28). We have shown that this de-phosphorylation mediated by Repo-Man/PP1 is essential for the re-establishment of heterochromatin in post-mitotic cells. A second pool of Repo-Man/PP1 targets to the periphery of chromosomes slightly later in anaphase. There, it contributes to the recruitment of Importin β to the chromatin. This fraction of Repo-Man appears to be important in the regulation of nuclear pores and lamina re-assembly since depletion of the complex leads to an abnormal lamina morphology and nuclear shape in G1 cells.

In summary Repo-Man/PP1 represents a molecular coupler between chromatin re-modeling and nuclear envelope re-formation that coordinates these processes during mitotic exit.1
In view of this, it is now of great interest to identify the phosphatases that regulate mitotic exit in space and time and to identify their specific substrates.3,11,12

Chromatin Re-Organization During Mitotic Exit

During early mitosis, chromatin is organized as highly compacted structures known as mitotic chromosomes. At anaphase onset, when the sister chromatids start their journey to the opposite spindle poles, the chromatin undergoes several important changes that are essential for the reformation of a functional interphase nucleus. While several chromosomal proteins (histones and non-histones) are dephosphorylated but remain in place, others leave the chromosomes (e.g., the chromosomal passenger complex (CPC)) and still others are recruited to anaphase chromosomes.

Several studies have suggested that protein phosphatase 1 (PP1) is important for cells to progress from mitosis to G1. For example, PP1 mutants in Drosophila show abnormal sister chromatid segregation and excessive chromosome condensation.13-15 This phosphatase has also been suggested to promote histone dephosphorylation during mitotic exit.16,17 However, the PP1 targeting subunits responsible for these chromatin re-organizations are not known.

Repo-Man was identified as a nuclear protein that is a specific regulatory subunit for PP1,17,18 which targets to the chromatin in anaphase. Repo-Man is mainly localized diffusely in the cytoplasm in early mitosis with a small fraction on mitotic chromosomes. At anaphase onset, Repo-Man is rapidly enriched on the segregating chromatin where it stays until the following entry into mitosis. Repo-Man localization and dynamics are controlled by phosphorylation. CDK1-CyclinB can phosphorylate Repo-Man in vitro. Mitotic Repo-Man (highly phosphorylated) has lower affinity for chromatin. Indeed, the behavior of the small chromosome-associated fraction in early mitosis is very dynamic (Fig 1A, A and C). Chemical inactivation of CDKs by roscovitine causes the re-localization of Repo-Man to chromosomes within a few minutes.19-21 Repo-Man mutants that cannot be fully phosphorylated by CDK localize to the chromatin even during prometaphase-metaphase. From anaphase onset and in interphase, Repo-Man is much less dynamic (Fig 1B, B and C).

Besides regulating Repo-Man localization, CDK phosphorylation has a second important function: it regulates the binding of PP1 to Repo-Man. This phospho-regulation of the complex in early mitosis is extremely important, since it ensures that the Repo-Man/PP1 holoenzyme is not fully activated before anaphase onset. The binding of Repo-Man to PP1 and targeting of the complex to the chromosomes are both very brief and highly dynamic in early mitosis.20 This transient base-line activity is probably necessary and sufficient to maintain the correct level of H3 phosphorylation on the chromosomes.21 This is particularly relevant for the phosphorylation of Histone H3 Thr3, as it represents the docking site for the chromosome passenger complex (CPC) on mitotic chromosomes.22-25 Repo-Man is both a targeting subunit and substrate for PP1. This double regulation of the complex appears to be designed to act as a signal amplifier. In fact, small changes in CDK activity can result in enhanced binding of PP1 to the Repo-Man RVTF motif (Repo-Man docking site for PP1) and in Repo-Man de-phosphorylation. The resulting holoenzyme thus has an increased affinity for the chromatin and can effectively de-phosphorylate histone H3 and possibly other chromosomal substrates (Fig 1C).26

The phosphorylation status of the Repo-Man regulatory subunit rather than

**Figure 1.** Repo-Man/PP1 complex regulation and function in mitosis. (A and B) Analyses of the dynamic behavior of GFP-Repo-Man in prometaphase (A and A) and interphase (B and B) FRAP of HeLa cells transiently expressing GFP-Repo-Man in prometaphase (A and A) and interphase (B and B). Cells were bleached using 488 nm laser line of a confocal microscope. Images were taken before bleaching and at the indicated time points after the end of the bleached pulse (dotted line) at every 2 sec. The bleached area is indicated by a blue circle. Graphs represent corresponding quantitative data for fluorescence recovery kinetics for the bleached area on chromosomes (blue line) and unbleached area on chromosomes (red line) and, for the mitotic cells also the unbleached cytoplasm (green line). Fluorescence intensities of GFP-Repo-Man in the bleached region were measured and expressed as recovery rate. The values represent averages +/- SD from 5 (mitosis) and 5 (interphase) cells. (C) CDK and PP1 control the on/off-rate of Repo-Man onto the chromosomes.
PP1 itself directs the formation and activity of the Repo-Man/PP1 holoenzyme. This appears to contrast with results of a recent study, in which cell-cycle regulation of PP1 was found to occur at the level of the catalytic subunit, through PP1 phosphorylation and inhibitor-1 binding. Our data suggest that there may be different mechanisms for the regulation of PP1 activity in mitosis. It is possible that both are used according to the different substrates targeted.

At anaphase onset, Repo-Man/PP1 stably associates with the chromatin and promotes Histone H3 de-phosphorylation of Thr3, Ser10, and Ser28. The importance of these de-phosphorylations during mitotic exit is not completely understood. Removal of the Thr3 phosphate could be involved in the mechanism of CPC transfer to the spindle midzone, and may be involved in the mechanism of CPC transfer to the spindle midzone, however clear demonstration of such a mechanism is still missing.

The removal of the Ser10 mark on histone H3 seems to be involved in the regulation of the binding of heterochromatin Protein 1 (HP1). HP1 recognizes methylation of H3K9 to direct heterochromatin formation. Aurora B phosphatylates Ser10 on H3 in prophase and this promotes the dissociation of HP1 from pericentric heterochromatin. Association of HP1 with chromatin can occur after dephosphorylation of H3S10. Depletion of HP1 from pericentric heterochromatin. We have shown that the Repo-Man/PP1 complex is responsible for the phospho-switching regulation that mediates the re-establishment of heterochromatin in post-mitotic cells. The coordination of chromatin de-condensation with nuclear reassembly during mitotic exit is not well understood. Following CKD inactivation and the extraction of polyubiquitinated Aurora B by the AAs-ATPase and ubiquitin-dependent chaperone p97, NPC re-assembly starts on the periphery of the segregating chromatin and then proceeds in a step-wise manner. The first re-assembly event is the initiation of nucleoporins as early as anaphase and much earlier than the recruitment of nuclear envelope vesicles. Detailed studies using the Xenopus in vitro system have made great contributions to clarify this process and to identify key players. Moreover, recent studies on the temporal re-association dynamics of nucleoporins (Nups) during mitotic exit have added to our understanding of the post-mitotic assembly of the NPC. The first Nups to accumulate at the chromosome periphery are members of the Nup107-160 complex followed by a small fraction of Nup153 and Nup50. The recruitment of the Nup107-160 complex is mediated by Elys/MEL-28, which can associate to chromatin via its AT-hook. ELYS/MEL-28 seems to represent an essential component in NPC re-assembly both in vitro and in vivo.

NPC reformation is regulated at several levels by importin β. In mitosis, Importin β binds to some of the Nups and prevents their association. During anaphase, RanGTP around chromatin directs the release of the Nups from this inhibitory complex with Importin β, therefore directing the spatial positioning of the NPC. As stated above, it is believed that the first NPC assembly step is conducted by the seeding of single copies of the Nup107-160 complex on the chromatin, mediated by the molecular adaptor ELYS/MEL-28. Importin β negatively regulates the seeding at these sites and subsequent assembly steps are dependent on specific membrane components. Depletion of ELYS/MEL-28 abolishes the assembly of pores at the chromatin periphery and causes the formation of annulate lamellae (membrane stacks of pores in the cytoplasm). Also fundamental to this process are the de-phosphorylation of nucleoporins, chromatin associated factors and NE membrane proteins.

A variety of evidence in the literature supports the involvement of PP1 and PPA2 in nuclear membrane assembly at the M/G1 transition. PP1 appears to be the major mitotic lamin phosphatase responsible for removal of mitotic phosphates from lamin B3 and the A-kinase anchoring protein AKAP149 recruits PP1 at the nuclear envelope (NE) upon somatic nuclear reformation in vitro. PP1 targeting to the NE is also a prerequisite for assembly of B-type lamins. In Drosophila, reassembly of the NPC is blocked by the specific PP1/PP2A inhibitor okadaic acid. Although the identity of the phosphatase responsible for de-phosphorylation of NUPs remains unknown, there are indications that at least NUP153 and NUP 50 can interact with PP1. During our studies aimed at identifying the targets of Repo-Man/PP1 complex on anaphase chromatin we discovered a Nup107-160 complex formation between Repo-Man and importin β. This binding is negatively regulated by Cdk phosphorylation of the N-terminal domain (aa 1–135) of Repo-Man. This interaction appears to be important for targeting at least a fraction of importin β to the periphery of the anaphase chromosomes and it seems to represent a direct structural function of Repo-Man rather than requiring catalytic activity of the Repo-Man/PP1 holoenzyme. At this stage of mitotic exit, Repo-Man also interacts with NUP50 and NUP153. However, more work is required to understand how Repo-Man interacts with this subset of nucleoporins and to determine its biological relevance at the transition from mitosis to G1.

The importin β targeting function of Repo-man may represent an important step in NE re-assembly since it has been shown, at least in vitro, that Importin β levels are critical for proper NE re-assembly. In addition, it has been suggested that the pathway of nuclear pore complex assembly could be regulated at sequential points by transportin and importin β but also that other effectors could exist, particularly for the FG nucleoporins Nup158, Nup153 and Nup50. Our experiments showed that lack of Repo-Man compromises the process of NE reformation: Importin β is not properly recruited to the nuclear rim but form cytoplasmic aggregates that co-localize with NUPs (possibly annulate lamellae) and the nuclear lamina fails to form a smooth structure after cells have completed cytokinesis. Current knowledge leads us to propose the following model. Very early during mitotic exit, the Nup107-160 complex is recruited to regions at the chromosome periphery via the chromatin binding protein ELYS/MEL-28. This is negatively regulated by importin β and transportin.
In the vicinity of these seed regions, Repo-Man docks to the chromatin (directly or indirectly) and brings importin β and an early pool of NUP153/NUP50 (Fig. 2A). In this respect Repo-Man could act either as an assembly factor for these FG NUPS or as a spacer by enriching some regions for importin β and preventing the seeding of more NUP107/160 complex.

Although the binding and recruitment of importin β by Repo-Man seems to be independent of the catalytic activity of the Repo-Man/PP1 holoenzyme, it is plausible to assume that, once the complex is localized to a targeted region of the chromosomal periphery, it could be involved in local de-phosphorylation processes either of NUPS or the lamina during the later steps of the nuclear re-assembly process (Fig. 2C).

Further studies will be required to elucidate the global functions of Repo-Man at this stage of mitosis and to identify substrates critical for nuclear re-assembly. In particular it will be important to clarify the mechanisms and role of the interactions between Repo-Man and NUP153ββ during mitotic exit. It will also be important to determine whether the fraction of Repo-Man bound to the nuclear periphery has a role in the organization and function of the interphase chromatin.

Future Prospective

Contrary to what was believed, the emerging view is that protein phosphorylation processes show stringent and selective substrate specificity. The modifications of chromatin during mitotic exit and the re-formation of a nuclear envelope both require the de-phosphorylation of several proteins in a local and timely manner. To date only three major PP1 targeting subunits have been found to be involved in these chromosome modifications in anaphase: Repo-Man, Nups112, and Aka1. Clearly other targeting subunits must be involved in this complex process and we can hypothesize that the anaphase chromosomes will give us more PP1 targeting subunits that will have specific functions regulating the transitional steps at the G1 boundary.
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