Heterochromatic DNA Double Strand Break Repair

Aaron A. Goodarzi, Angela T. Noon, Penny A. Jeggo

Genome Damage and Stability Centre, University of Sussex, Brighton BN1 9RQ, UK

Eukaryotic chromatin is segregated into highly condensed heterochromatin and comparably relaxed euchromatin. Although heterochromatic gene expression is either transiently or permanently impeded, the integrity of heterochromatic DNA is critical for cell survival as it contributes to the regulation of nuclear architecture, gene expression, ribosome biogenesis, chromosome stability and mitosis. Formed by a plethora of proteins, structurally complex heterochromatin is generally inaccessible to DNA processing enzymes, including those repair factors required to re-join DNA double strand breaks (DSBs). To be repaired, heterochromatic lesions require the Ataxia Telangiectasia Mutated (ATM) pathway to transiently modify heterochromatic factors surrounding the DSB, relaxing its structure and thereby allowing DNA non-homologous end-joining (NHEJ) to function. Cells deficient for ATM or proteins involved in its signalling cascade repair euchromatic DSBs normally but are unable to resolve lesions within heterochromatin. Depletion of key heterochromatic proteins, including the KAP-1 transcriptional co-repressor, Heterochromatin Protein 1 (HP1) or histone deacetylases 1&2 (HDAC1&2), relieves the requirement for ATM signalling in DSB repair. Importantly, KAP-1 is a highly dose dependent, transient and specific substrate of ATM and the manipulation of KAP-1 phosphorylation regulates heterochromatic DSB repair. We propose that KAP-1 is a critical heterochromatic factor that undergoes specific modifications following DSB formation to promote repair in a manner that allows localised and transient chromatin relaxation but precludes widespread dismantling of the heterochromatic superstructure.

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Heterochromatic Structure and Function

Heterochromatin is literally defined as chromatin that is different (*hetero-*) from "true" (*eu-*) chromatin. Indeed, heterochromatin is functionally distinct and is often spatially segregated from the bulk chromatin of eukaryotic nuclei.[1] Subdivided into constitutive and facultative components, heterochromatin is highly condensed and generally comprises between 10 and 25% of total chromatin, depending on age, cell-type and species.[2,3] Broadly defined, constitutive heterochromatin is transcriptionally inert and remains so throughout life, while facultative heterochromatin originates as euchromatin and forms as genes are silenced during development and ageing. Constitutive heterochromatin includes the centromeric and pericentromeric satellite DNA as well as telomeric DNA at the ends of chromosomes. Facultative heterochromatin includes those promoters, genes and chromosomes that are silenced during development or senescence. A classic example of facultative heterochromatin is the mammalian female inactive X chromosome, which is randomly silenced at an early stage of embryogenesis to prevent functional aneuploidy of X-linked genes.[2,3] The presence and function of heterochromatin is essential for eukaryotic life, contrib-uting to the regulation of mitosis (centromeres and pericentromeric DNA), gene expression (regulating promoter accessibility), ribosome biogenesis (controlling nucleolar integrity and ribosomal DNA expression), chromosome stability (telomere-mediated chromosomal end protection) and the overall stability of the nucleus.[4]

Although the precise function, location and timing of the myriad types of heterochromatin differ, they share many common molecular components that are required to implement transcriptional silencing and chromatin compaction. By varying the parts and the order of their assembly, functionally distinct loci can be established from a basic set of heterochromatic building blocks.[4] The major components of heterochromatin are summarised in Figure 1. These include primary silencing factors, heterochromatic foundation factors, histone methylation building enzymes, chromatin architecture complexes and DNA methylation proteins. Unique to given DNA sequences, the primary silencing factors include transcriptional repressors, functional siRNA and histone variants that specify regions of programmed silencing. The heterochromatin foundation factors include the transcriptional co-repressors (such as KRAB-domain Associated Protein 1 (KAP-1)) and chromatin compaction 'adaptors' (such as Heterochromatin

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Address correspondence: Aaron A. Goodarzi, M.D., Ph.D., Genome Damage and Stability Centre, University of Sussex, Brighton BN1 9RQ UK
TEL: +44-1273-678482, FAX: +44-1273-678121, E-mail: a.goodarzi@sussex.ac.uk, p.a.jeggo@sussex.ac.uk
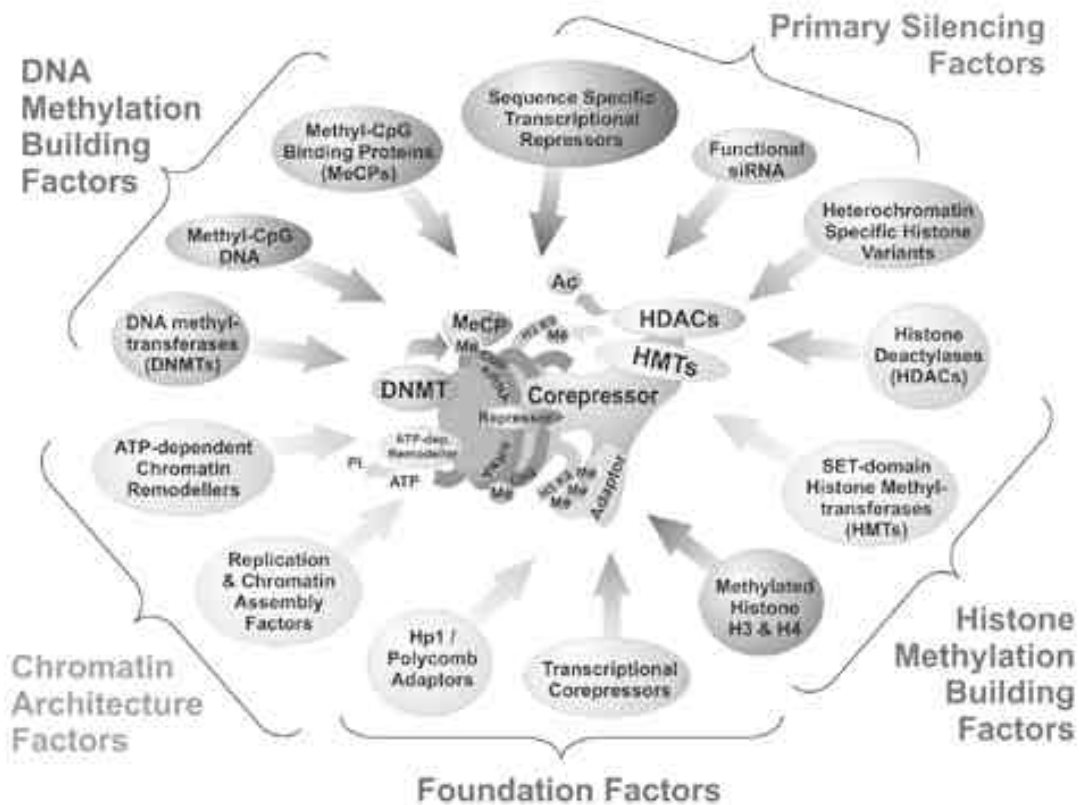
Figure 1. Heterochromatin Building Factors. Primary silencing factors include sequence-specific transcriptional repressors, functional siRNA and histone variants. The heterochromatin foundation factors include the transcriptional co-repressors and chromatin compaction 'adaptors' that are recruited by the primary silencing factors. Foundation factors recruit histone methylation building factors to catalyse the removal of pro-transcriptional acetyl-groups from histones (via histone deacetylases (HDACs)), replacing them with anti-transcriptional methylation (via histone methyl-transferases (HMTs)). This, in turn, serves to recruit and retain more compaction adaptors. Mediating the assembly of heterochromatin, chromatin architecture factors provide the energy to enable the necessary histone re-arrangements (ATP-dependent chromatin remodellers) and/or re-assembly (replication & chromatin assembly factors). Finally, DNA methylation building factors (DNA methylases (DNMTs)) methylate the underlying DNA (at CpG islands), branding target DNA with a 'silencing' signal that is bound and protected by Methyl-CpG binding proteins (MeCPs).

Protein 1 (HP1) or polycomb) that are universally recruited by the primary silencing factors to promote heterochromatinization. Drafted in by the foundation factors, histone methylation building factors catalyse the removal of pro-transcriptional acetyl-groups from histones (via histone deacetylases (HDACs)), replacing them with a profusion of anti-transcriptional methylation (via histone methyl-transferases (HMTs)). This, in turn, serves to recruit and retain more foundation factors (specifically HP1). The increasing abundance of HP1 forms inter- and intra-nucleosomal interactions, coalescing into complexes that collapse and condense nearby chromatin. Mediating the assembly of the heterochromatic superstructure, chromatin architecture complexes provide the energy to enable the necessary histone re-arrangements (ATP-dependent chromatin remodellers) and/or re-assembly (replication-associated histone assembly factors). Finally, DNA methylation building factors (DNA methylases (DNMTs)) paint the underlying DNA (at CpG islands) with methyl groups, branding target DNA with a 'silencing' signal that is bound and protected by Methyl-CpG binding proteins (MeCPs). Cooperating together, these factors establish a self-reinforcing heterochromatic superstructure around basal chromatin that compacts, silences and

spatially segregates target DNA, effectively isolating it from the robust metabolic activity occurring on euchromatic DNA.

Disassembling Heterochromatin

Once assembled, heterochromatin is highly stable and successfully excludes the majority of euchromatic proteins, maintaining gene silencing and preserving the integrity of its structure. Generally speaking, the heterochromatinization of a given genomic region in non-dividing cells is sustained perpetually once achieved. However, it is essential that heterochromatic DNA participates in such nuclear processes as DNA replication or mitosis. In both cases, the heterochromatic superstructure is removed from the underlying chromatin only for the time required for each event and is rapidly re-assembled upon completion. During S-phase, the vast majority of heterochromatin remains unperturbed until all other sequences have been replicated, whereupon heterochromatic DNA is duplicated rapidly and accompanied by immediate heterochromatin re-assembly before entry into G2-phase.[1] During very late G2 and early M-phase, the integrity of

heterochromatin (with the notable exception of the centromeres) gives way to the ultra-condensation of chromosomes required for cell division.[5] Regions marked for silencing (indicated by DNA and histone methylation) are re-assembled into heterochromatin without delay once the nuclear envelope re-forms and mitotic chromatin de-condenses. Indeed, only under conditions of extraordinary nuclear re-arrangement is the heterochromatic barrier removed in its entirety. Under ordinary circumstances, the general integrity of heterochromatin is not perturbed at all in quiescent or post-mitotic differentiated cells. However, under conditions of genomic trauma, such as the formation of DNA double strand breaks following oxidative stress or ionizing radiation exposure, emerging evidence suggests that dynamic and localized changes to heterochromatin are important for DNA repair and cellular survival.

Heterochromatin and the DNA double strand break response

DNA double strand breaks (DSBs) elicit genomic rearrangements and chromosomal fragmentation if not repaired in an accurate and timely manner.[6] Mounting evidence suggests that both DNA damage induced signalling and repair processes influence and are influenced by higher-order chromatin. Laser-induced damage results in chromatin relaxation in the area surrounding the DSB.[7] Studies in yeast have shown that histone eviction is required for the proper recruitment of DSB repair factors.[8] Chromatin modifiers such as NuA4 histone-acetyltransferase complexes are also needed for efficient DSB repair.[9] Moreover, both yeast and mammalian systems indicate that higher-order chromatin serves as a barrier to the expansion of damage response signalling and that reduced chromatin compaction (by linker histone H1 depletion) enhances cell cycle checkpoint arrest and radioresistance.[10-12]

In mammalian cells, Ataxia Telangiectasia (A-T) mutated (ATM) lies at the centre of a signal transduction cascade that responds to the presence of DSBs, leading to cell cycle checkpoint activation, while DNA non-homologous end joining (NHEJ) operates as the primary mechanism of repair.[6] Within the ATM-signalling pathway, a hierarchy of so-called 'mediator' proteins, including the Mre11/Rad50/NBS1 (MRN) complex, histone H2AX, MDC1, RNF8, RNF168 and 53BP1 are required to concentrate ATM activity around the DSB. The major components of this hierarchy are summarized in Figure 2 and involve DSB recognition by the MRN complex, MRN-dependent ATM activation, ATM-dependent H2AX phosphorylation, γH2AX-dependent MDC1 retention, MDC1-dependent further MRN recruitment (leading to additional ATM-activation / γH2AX-expansion) and MDC1-dependent RNF8 and RNF168 recruitment, which promotes 53BP1 retention. Recent data suggests that an important role of 53BP1, following recruitment to γH2AX foci, is to mediate the localization and concentration of ATM activity in the vicinity of the DSB (Noon et al., manuscript under consideration). The very existence of a highly complex DNA damage response attests to the significance of DSBs as lesions promoting genomic instability and/or cell death. The dramatic

clinical features caused by loss of ATM in A-T patients also serve to demonstrate the importance of the ATM-signalling.[6] Indeed, cells deficient or dysfunctional (i.e. mutated for any one component) for the hierarchical ATM-signalling response are generally highly radiation sensitive, having both cell cycle checkpoint arrest and DSB repair defects.

ATM signalling controls cell cycle checkpoint arrest and cells lacking ATM are unable to impede the progress of DNA replication or mitotic entry following DSB formation.[6] By contrast, the majority of DSB repair is ATM-independent and only ~10-20% of initially incurred DSBs remain un-repaired in the absence of ATM, histone H2AX or 53BP1.[13] Recently, we demonstrated that the ATM-dependent sub-component of DSB repair corresponds to lesions located within regions of heterochromatin.[14] Importantly, knockdown of the heterochromatic building factors KAP-1, HP1 or HDAC1/2 alleviated the requirement for ATM in DSB-repair, suggesting (i) that the heterochromatic superstructure is a barrier to DSB repair and (ii) ATM-signalling functions to bypass this barrier. Previous to these observations, a direct connection between heterochromatin and ATM-signalling was made when KAP-1 (also called TIF1ß, TRIM28 or KRIP-1) was identified as an ATM substrate, being robustly phosphorylated at S824.[15] The effect of KAP-1 phosphorylation on chromatin was described as pan-nuclear, resulting in the universal relaxation of nucleosomes. We later showed that DSB repair within heterochromatin cannot proceed in the absence of KAP-1 phosphorylation (pKAP-1) and, importantly, that expression of a KAP-1 phospho-mimic (S824D) bypasses the requirement for ATM in DSB repair.[14]

## The induction, regulation and impact of KAP-1 phosphorylation

The extreme C-terminus of KAP-1 harbours the LSS[824]QE sequence, which is a near-perfect consensus motif for ATM-mediated phosphorylation at serine 824. KAP-1 phosphorylation is rapid (within several minutes of DSB induction) and highly dose-dependent, linearly increasing as the number of DSBs increases and decreasing as they are repaired (Noon et al. manuscript under consideration).[15] Predominantly DSB and ATM dependent, no detectable pKAP-1 signal is observed in ATM$^{-/-}$ cells and no significant induction was found following exposure to DNA damaging agents that do not directly cause DSBs. This makes KAP-1 unique among DSB response substrates examined to date, most of which are redundantly phosphorylated by either DNA-PK or ATR (protein kinases closely related to ATM) or inducible by other forms of damage than a DSB (such as replication fork collapse, base damage or DNA single strand breaks). Importantly, ectopic expression of the KAP-1 phosphorylation mutant (S824A) in cells depleted for endogenous KAP-1 showed an indistinguishable and epistatic DSB repair defect to ATM deficient or inhibited cells.[14] Expression of the phosphorylation mimic (S824D) conferred normal repair kinetics that were unaffected by ATM inhibition. Collectively, these findings argue that KAP-1 S824

phosphorylation is critically required for ATM-dependent DSB repair.

Once KAP-1 is phosphorylated, it is tightly regulated to minimize the duration and location of heterochromatic perturbation to only what is absolutely necessary for DSB repair. Initially, pKAP-1 forms at both DSBs demarcated by γH2AX foci and throughout the nucleus in apparently undamaged chromatin (Noon et al. manuscript under consideration).[15] Pan-nuclear pKAP-1 generally dissipates within a few hours of DSB induction and, at later times, we have observed that slow-repairing γH2AX foci are associated with intense foci of pKAP-1 (Noon et al. manuscript under consideration). pKAP-1 foci associated DSBs correspond to those which are repaired with slow kinetics in normal cells and require ATM-signalling for repair. The changing pattern of pKAP-1 over time is likely due to the nature of ATM activation, which first spreads rapidly throughout the nucleus and later is thought to be retained within the DSB vicinity (Noon et al., manuscript under consideration).[6] Thus, pKAP-1 forms where ATM localizes, rather than by any change in its own distribution or mobility (indeed, the distribution of KAP-1 in vivo does not change following DSB induction). Importantly, the retention and concentration of ATM within the DSB vicinity is entirely dependent on functional 53BP1. Thus, cells deficient for MDC1, RNF8, RNF168 or 53BP1 (which manifest an inability to localize 53BP1 to γH2AX foci) all display identical DSB repair defects to ATM loss or inhibition, as they are unable to maintain pKAP-1 within the DSB vicinity where it is functionally relevant to the repair process (Figure 2). pKAP-1 is also subject to very robust turnover by protein phosphatase activity (Goodarzi et al, unpublished observations). This, presumably, serves to rapidly return heterochromatin to 'normal' following DSB

repair completion. However, rapid pKAP-1 turnover also necessitates the continual KAP-1 rephosphorylation in order for DSB repair to proceed and, given the abundance of KAP-1 in heterochromatic regions, a very high local concentration of active ATM (thus necessitating 53BP1 function). The identity and regulation of this protein phosphatase activity is unknown.

The defined impact of phosphorylation on the KAP-1 dependent heterochromatic superstructure is unclear. No changes in the intrinsic mobility of GFP-labelled KAP-1 or the physical association of KAP-1 with other heterochromatic proteins (HP1 or HDAC1/2) following DSB formation have been detected to date.[14,15] Previously, pKAP-1 has been described as resulting in the universal relaxation of nucleosomes and the broad alteration of KAP-1's co-purification with nuclease-resistant chromatin, indicating that KAP-1 phosphorylation does measurably affect the state of nucleosome condensation.[14,15] The mechanism by which this occurs is uncertain, although there is evidence that S824 phosphorylation interferes with the SUMO-ligase activity of KAP-1's carboxyl-terminal tandem PHD/Bromo-domain.[16] The prevention of KAP-1 auto-sumoylation is predicted to interfere with the ability of KAP-1 to functionally interact with components of the NuRD complex, a Nucleosome Remodelling and histone Deactylase activity required to trigger heterochromatinization.[16] Although alterations to histone acetylation or methylation have been reported to occur following DSB induction, few have been shown to be induced rapidly (i.e. with comparable kinetics to KAP-1 phosphorylation) or to be specific to heterochromatin; thus, the precise impact of pKAP-1 on NuRD activity is unclear.[17] It is possible that the ATP-dependent histone remodelling activity (versus the histone deacetylase activity) of the NuRD complex is sufficiently
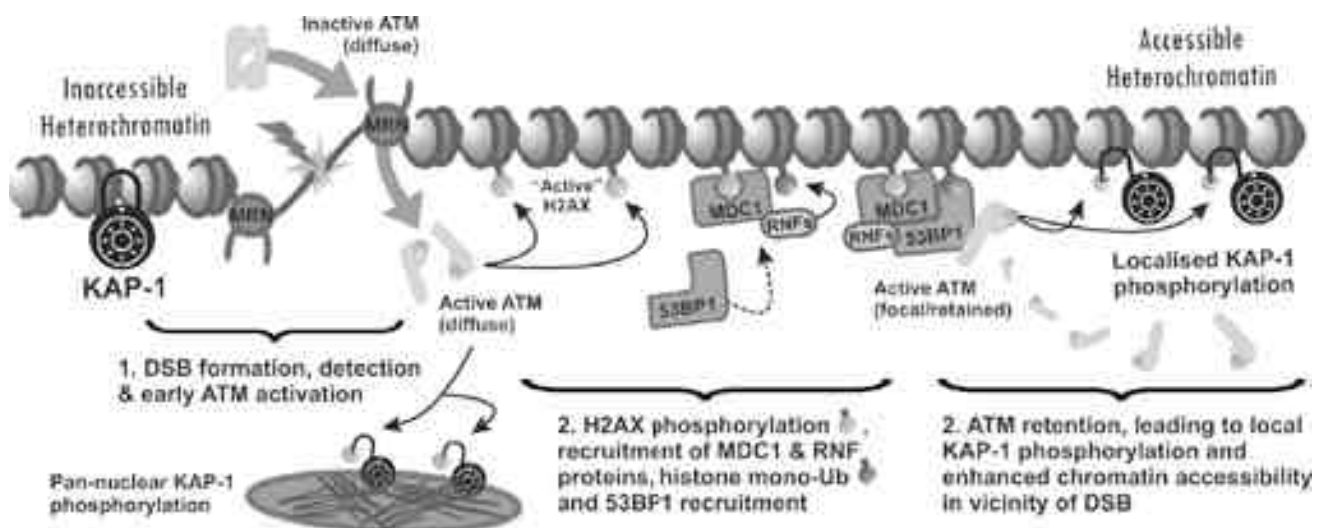
Figure 2. The Heterochromatic DNA double strand break response. DNA double strand breaks (DSBs) induced by ionizing radiation or radiomimetic chemical exposure are recognized by the Mre11/Rad50/Nbs1 (MRN) complex. The MRN-complex enables the monomerization and autophosphorylation of ATM, activating it. Active ATM then phosphorylates histone H2AX in the vicinity of the DSB, which serves to recruit MDC1. MDC1 recruits further MRN which leads to additional ATM-activation and γH2AX-expansion. MDC1 also recruits RNF8 and RNF168, which promote histone mono-ubiquitnation. This, in turn, promotes 53BP1 retention. Recent data suggests that an important role of 53BP1 is to mediate the localization and concentration of ATM activity in the vicinity of the DSB. The concentrated ATM activity is then able to phosphorylate KAP-1 within regions of heterochromatin, rendering DSBs in these areas amenable to DSB repair.

inhibitory to the repair process that it requires transient inactivation for DSB ligation to occur. Further research is required to test this possibility and determine the precise mechanism by which S824 phosphorylation impacts upon the function of KAP-1.

The bigger picture:

Interestingly, lower eukaryotes, which have minimal heterochromatin, have a diminished role for ATM-signalling in their DSB response.[18] By contrast, ATM-signalling is of major importance to the DSB response of higher eukaryotes, where a larger genome and more complex developmental program has resulted in increased constitutive and facultative heterochromatin. It is likely that the increased prominence of ATM-signalling in the DSB response (compared to TEL1-signalling in yeast) correlates with the increasing complexity of chromatin architecture observed through eukaryotic evolution.

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