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The impact of heterochromatin on DSB repair.

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Abstract:

DNA non-homologous end-joining (NHEJ) is the major DNA double strand break (DSB) repair pathway in mammalian cells. Although NHEJ-defective cell lines show marked DSB repair defects, cells defective in Ataxia Telangiectasia mutated (ATM) repair most DSBs normally. Thus, NHEJ functions independently of ATM signalling. However, ~ 15 % of radiation-induced DSBs are repaired with slow kinetics and require ATM and the nuclease, Artemis. DSBs persisting in the presence of an ATM inhibitor, ATMi, localise to heterochromatin suggesting that ATM is required for repairing DSBs arising within or close to heterochromatin. Consistent with this, we show that siRNA of key heterochromatic proteins, including KAP-1, HP1, HDAC1/2, relieves the requirement for ATM for DSB repair. Further, ATMi addition to cell lines with genetic alterations that impact upon heterochromatin, including Suv39H1/2-knockout, ICFa-syndrome and Hutchinson-Guilford Progeria cell lines, fails to impact on DSB repair. KAP-1 is a highly dose dependent, transient and ATM-specific substrate and mutation of the ATM phosphorylation site on KAP-1 influences DSB repair. Collectively, the findings show that ATM functions to overcome the barrier to DSB repair posed by heterochromatin. However, even in the presence of ATM, γ-H2AX foci form on the periphery rather than within heterochromatic centres. Finally, we show that KAP-1's association with heterochromatin is diminished as cells progress through mitosis. We propose that KAP-1 is a critical heterochromatic factor that undergoes specific modifications to promote DSB repair and mitotic progression in a manner that allows localised and transient chromatin relaxation but precludes significant dismantling of the heterochromatic superstructure.

Introduction

Ataxia Telangiectasia (A-T) mutated (ATM) is a phosphoinositol 3-kinase like kinase (PIKK) that lies at the heart of a signal transduction response to DNA double strand breaks (DSBs) [1]. The very existence of a highly complex DSB signalling 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 serves further to demonstrate the importance of an appropriate response to DSB formation [2]. ATM signalling activated by DSB formation promotes cell cycle checkpoint arrest, apoptosis and also influences DSB repair. Whilst apoptosis functions to remove damaged cells from the cycling population, cell cycle checkpoint arrest can both enhance the opportunity for repair and serve as an alternative to apoptosis to limit the proliferative capacity of damaged cells. DNA non-homologous end-joining (NHEJ) functions as the major DSB rejoining pathway in mammalian cells [3]. A-T cell lines rejoin the majority of DSBs with normal kinetics demonstrating that most NHEJ occurs independently of ATM signalling [4, 5]. However, the rejoining of ~ 15 % of irradiation (IR) induced DSBs require ATM and additional proteins that function in the ATM signal transduction process, including γ-H2AX and 53BP1 [5]. Previous studies have demonstrated that whilst the majority of DSBs are repaired with fast kinetics in mammalian cells, a subset of breaks is rejoined with slower kinetics [6]. Strikingly, ATM is required for the slow component of DSB repair, which represents an ~ 15 % subset of X- or γ-ray induced DSBs [5]. ATM-dependent DSB repair also requires the nuclease, Artemis [5]. Artemis nuclease activity has the capacity to cleave hairpin ended DSBs and to remove 3' or 5' single stranded overhangs following remodelling of the DNA ends by the DNA-dependent protein kinase (DNA-PK) [7, 8]. Furthermore, Artemis is an ATM-dependent substrate after radiation [5, 9-11]. For this, and additional reasons, it was suggested that ATM may function to promote Artemis dependent end processing prior to rejoining by NHEJ [5]. However, in a more recent study, a closer look at the ATM dependency for DSB repair following exposure to a range of agents producing DNA ends of differing complexity suggested that there was only a weak correlation between end complexity and ATM dependent DSB repair [12]. Whilst the impact of ATM/Artemis in the repair of complex DSBs requires further examination and should not be discounted,

we sought additional or alternative explanations for the role of ATM in the repair of X or γ-ray induced DSBs. Here, we discuss the impact of heterochromatin on DSB repair and the role of ATM in promoting DSBs that are located within or close to heterochromatic DNA.

DSBs that require ATM for their repair are localised to the periphery of heterochromatic DNA regions.

Depending upon cell type, around 10-25 % of mammalian DNA is transcriptionally inactive or "silenced" and is highly compacted by heterochromatinisation. Driven by mounting evidence that chromatin architecture strongly influences repair processes and that ATM signalling modifies chromatin structure, we considered the possibility that ATM may be specifically required for DSB repair within heterochromatic DNA [13, 14]. To examine this, we exploited the mouse NIH 3T3 cell line in which pericentric and centromeric heterochromatic regions can be readily visualised as densely staining DAPI regions, termed chromocentres [12, 15]. Initially, we verified that regions visualised by DAPI staining in mouse NIH3T3 cells were enriched for heterochromatic factors including trimethylated K9 of histone H3, Kruppel-associated box (KRAB)-associated protein 1 (KAP-1), heterochromatin protein 1 (HP1) and centromeric protein A, (CENP-A), and depleted for the known euchromatic markers, HMGB1 and the transcription factor, E2F1 (See Fig 2 in [12]). Following exposure of NIH 3T3 cells to 3 Gy IR in the presence of a specific ATM inhibitor (ATMi; KU55933), we examined the localisation of γ-H2AX foci present at early and late times. At 0.5 hr post IR, γ-H2AX foci were stochastically distributed throughout the nucleus (Fig 1). Using high resolution imaging, we estimated that ~ 20% of the γ-H2AX foci present at 0.5 hr co-localised (i.e. overlapped or bordered) with chromocentres. Strikingly, the γ-H2AX foci that remained at 24 hr post IR in the presence of ATMi were predominantly (~ 70%) localised to the periphery of the chromocentres (Fig 1). We also enumerated the rate of loss of all γ-H2AX foci regardless of localisation and those that localised to the periphery of densely DAPI staining chromocentres (classified as DSBs located within or close to heterochromatic DSBs (HC-DSBs) and, by subtraction, estimated the rate of loss of DSBs considered to be located within euchromatic DNA (EC-DSBs). Strikingly, we

observed that in control cells the EC and HC-DSBs were repaired with fast and slow kinetics, respectively, and that addition of ATMi specifically impacted upon the repair of the HC-DSBs (Fig 1B). These findings suggest that the slow component of DSB repair may represent the repair of DSBs located close to or within heterochromatic DNA and that ATM is specifically required for the repair of such DSBs.

Knock down or loss of heterochromatic building factors relieves the requirement for ATM for DSB repair.

The findings above predicted that reducing the heterochromatic DNA content might diminish the requirement for ATM for DSB repair. A range of DNA and histone modifications as well as the recruitment of specific heterochromatin building factors function co-ordinately to create heterochromatic DNA [16, 17]. DNA methylation by DNA methyl transferases is the most significant DNA modification, whilst histone H3 acetylation promotes a more open chromatin conformation and histone H3 methylation (H3 K9 me) confers a more closed conformation. The histone deaceytylases HDAC1, HDAC2 and HDAC3, are important in removing heterochromatin-inhibiting histone H3 acetylation whilst Suv39H1/2 are important SET-domain histone methyltransferases. Additionally, HP1 and the co-repressor, KAP-1, are critical heterochromatic building proteins [18]. Heterochromatin building occurs in an interwoven manner with loss of any component adversely affecting compaction similar to the demise of a carefully stacked pack of cards following the removal of any single card. We, therefore, examined the impact of a range of critical heterochromatic components on the ATM dependency for DSB repair. Strikingly, we observed that knockdown or loss of any one of several heterochromatic factors relieved or reduced the DSB repair defect imparted by addition of ATMi [12] (summarised in Table 2). Thus, following KAP-1 siRNA, DSBs were repaired at a similar rate to that observed in control cells with or without addition of ATMi. Similar results were obtained following HDAC1/2 siRNA or HP1 siRNA. Further, mouse embryo fibroblasts (MEFs) knocked out for the Suv39H1/2 histone methyltransferases, which show reduced HP1 localisation within pericentric heterochromatin, also showed a diminished DSB repair defect following ATMi addition, reflecting a reduced dependency upon ATM [19]. We also examined cells from

Immunodeficiency Centromere-instability Facial-anomalies syndrome [type a] (ICFa) Syndrome and Hutchinson-Gilford Progeria Syndrome (HGPS), both of which show progressive heterochromatin disorganisation, and, in both cases, observed a diminished DSB repair defect at 24 hr following ATMi addition compared to normal human cell lines. Collectively, these results suggest that the manipulation of essential heterochromatic building factors whilst not affecting the normal rate of DSB repair *per se*, impacts upon the requirement for ATM for DSB repair consolidating the notion that ATM is specifically needed for repair of DSBs located within or close to heterochromatic DNA.

KAP-1 is a highly DSB dose-dependent and specific ATM substrate.

KAP-1 harbours the motif, LSSQE, which encompasses the consensus ATM phosphorylation motif (S/TQ). We raised antibodies that recognise KAP-1 phosphorylated at the predicted ATM phosphorylation site (S824). Western blotting using these α-pS824-KAP-1 (pKAP-1) antibodies confirmed that, whilst there was no detectable signal in undamaged cells, they recognise an IR-inducible substrate that has the mobility of KAP-1 (Fig 2). Moreover, KAP-1 phosphorylation was almost entirely ATM dependent with no detectable signal observed in an $ATM^{-/-}$ lymphoblastoid cell line (LBL) and no significant reduction being found in an ATR-deficient LBL (ATR-SS cells) following treatment with IR or etoposide, an agent that induced DSBs following topoisomerase II inhibition (Fig 2). Further, we did not detect any evidence of pKAP-1 following exposure to HU or UV, agents which do not directly induced DSBs. These findings strongly suggest that KAP-1 is an unusually specific ATM substrate that is only induced by direct DSB induction. Additionally, pKAP-1 was observed at early times post IR (10 to 30 min, depending on dose), its magnitude was highly dose dependent and was relatively transient with its rate of decay being dose dependent, largely disappearing by 6 h post physiological exposure levels.

Importantly, ectopic expression of S824A KAP-1 in cells that were knocked down for endogenous KAP-1 showed a similar DSB repair defect to that observed in A-T cell lines whilst expression of S824D KAP-1 conferred repair kinetics that were similar to wildtype and unaffected by ATMi addition [12]. Collectively, these findings argue that

S824 is a critical phosphorylation site on KAP-1, which is regulated by ATM-dependent phosphorylation and is required for ATM-dependent DSB repair.

Heterochromatin is a barrier to DSB repair and signalling which ATM partially relieves.

The findings above, taken together with additional published observations [12] strongly suggest that ATM is specifically required to repair DSBs that arise within or proximal to heterochromatin, which is achieved by phosphorylation of KAP-1, a co-repressor that interacts with HP1. The finding that ATM is specifically required for the slow component of DSB repair and that DSBs associated with heterochromatin are repaired with slower kinetics compared to DSBs located within euchromatin [12], suggest that heterochromatin poses a barrier to the DSB repair that is relieved, at least in part, by ATM. We aimed to evaluate how KAP-1 phosphorylation impacts upon heterochromatin. In Fig 1, we showed that, in the presence of ATMi, the persisting γ-H2AX foci at 24 hr post 3 Gy are located on the periphery of the densely staining DAPI chromocentres. It is striking that, although the γ-H2AX foci partially encroach into the densely DAPI staining regions, complete overlap is rarely observed. These findings raise the possibility that ATM-dependent KAP-1 phosphorylation might overcome the barrier to γ-H2AX foci expansion posed by heterochromatin. We, therefore, examined the relationship between γ-H2AX foci formation and chromocentre location in wildtype cells, where ATM is functional. Since HC-DSBs are repaired more slowly that EC-DSBs, we anticipated that the DSBs remaining unrepaired at later times in a wild type cell line would be enriched for HC-DSBs. Consistent with this, we observed that the γ-H2AX foci persisting at 24 hr post 3 Gy were, like those remaining in the presence of ATMi, predominantly located around the periphery of the chromocentres (Fig 4). Using high resolution imaging, we delineated the chromocentre regions (shown in blue), the γ-H2AX foci (green) and the regions of overlap (red). The regions of overlap are predominantly on the edge of the chromocentres consistent with the notion that heterochromatin poses a barrier to signal expansion. Strikingly, there was only limited encroachment of γ-H2AX formation into the chromocentric mass (Fig 4), similar to our previous observations in cells treated with ATMi [12]. These findings, therefore, argue that although ATM phosphorylation events

may serve to relax the heterochromatin to an extent that allows DSB repair, they do not result in overt dismantling of the heterochromatic super-structure and do not fully relieve the barrier to foci expansion posed by heterochromatin. In this context, it is also important to note that the densely DAPI staining chromocentres in KAP-1 siRNA treated NIH3T3 cells are clearly detectable albeit with moderately diminished levels of H3 K9 trimethylation (see supplementary material in Ref [12].

The distribution of KAP-1 on chromatin is re-organized during mitosis.

The results described above and published findings suggest that ATM's impact on heterochromatin occurs via phosphorylation of KAP-1 on S824, which modifies KAP-1 binding to heterochromatin and triggers heterochromatin relaxation [12, 14]. The interaction of HP1 with chromatin during interphase is promoted by H3K9me3. Notably, although H3K9me3 does not alter during mitosis, the binding of HP1 to chromatin has been reported to diminish [20]. Rather than affecting the histone methylation code, evidence suggests that mitotic cells regulate HP1 chromatin binding via H3S10 phosphorylation by Aurora B kinase [21]. Driven in part by our goal to examine ATM's role in DSB repair in G2 phase, we examined the localisation of KAP-1 during the cell cycle. Immunofluorescence analysis of G1 phase cells using α-KAP-1 antibodies showed a pronounced enrichment of KAP-1 at the densely DAPI staining chromocentres, consistent with the substantial evidence that these represent regions of heterochromatin, to which KAP-1 is preferentially localised (Fig 4A). In G1 phase, these densely staining chromocentres were also enriched for H3K9me3 but were depleted for the euchromatic marker, H3K9acetyl (Fig 4BC). In contrast, in G2-phase, the DAPI regions were more diffuse compared to G phase and contained KAP-1, TriMeK9 and AcetylK9 H3, suggesting that these regions no longer uniquely represent heterochromatic chromocentres (Fig 4BC). We monitored KAP1 localisation through G2/M using phospho-H3 staining to delineate cell cycle positions. Cells with minimal (but detectable) phospho-H3 signal, determined to be at the very beginning of G2-phase, have similar KAP-1 enriched chromocenters to G1/S cells. As levels of phospho-H3 increase and cells progress through G2-phase towards the mitotic boundary, some (but not all) KAP-1 is redistributed from dense DAPI regions to give pan nuclear staining, indicating the active

re-organization of heterochromatin in preparation for mitotic chromosome hyper-condensation (Fig 4D). It was notable that pan nuclear KAP-1 localisation observed during mid/late G2 was unaffected by detergent extraction (Fig 4E), suggesting that KAP-1 remains bound to chromatin but that the densely staining DAPI regions may less stringently represent ‘pure’ heterochromatic chromocentres. In further contrast, as cells progressed through mitosis, the localisation of KAP-1 was observed to be completely distinct to that of the DAPI staining, indicating the release of KAP-1 from chromatin during progression through mitosis (Fig 4). Thus, although KAP-1 partly co-localised with DAPI staining in very late G2, in prophase, metaphase and anaphase there was almost no KAP-1 co-localising with DAPI staining. Strikingly, in late telophase, KAP-1 rapidly relocalised to chromatin. These findings closely parallel studies of HP1 localisation during progression through mitosis [21].

Collectively, these findings suggest that in G2 phase, heterochromatic DNA becomes more diffusely organised than in G1 phase and during transition through mitosis both KAP-1 and HP1 are released from the chromatin.

Discussion.

A-T cell lines display dramatic radiosensitivity, which, more than twenty years ago, was proposed to be due to a defect in the repair of chromosomal DSBs [22]. Despite this early observation, consolidation of this notion was slow in surfacing primarily because the majority of DSBs are repaired normally in A-T cells when assessed by physical methods such as pulsed field gel electrophoresis (PFGE) [4]. Although DSB repair defects were detected by PFGE, the modest impact and high doses necessitated by the technique limited the significance of the findings [4]. Later, A-T cells were demonstrated to display cell cycle checkpoint defects and the biological impact of ATM loss was attributed to defective checkpoint function [23]. Only more recently with the advent of γ-H2AX foci analysis as a sensitive monitor of DSB repair following physiologically relevant doses of IR, was a repair defect in A-T cell lines confirmed and its contribution to A-T radiosensitivity appreciated [5]. However, the mechanistic role that ATM plays in DSB repair has, until recently, remained unclear.

In a recent study and consolidated further here, we show that ATM is specifically required for the repair of DSBs located close to or within heterochromatic regions [12]. The organisation of heterochromatin in mouse and human cells is distinct due, in part, to the fact that mouse cells have acrocentric chromosomes. Of relevance to our recent work, is the exploitation of the mouse NIH3T3 cell line, in which heterochromatin is organised into readily visualised chromocentres that show dense DAPI staining. Recently, we and others have observed that γ-H2AX foci formation does not occur within chromocentres but rather on their periphery (see Fig 1, 4) [24]. Strikingly, although the γ-H2AX foci that form in A-T cells are smaller than those in control cells, we observed a similar magnitude of γ-H2AX encroachment into the chromocentres in either the presence or absence of ATM kinase activity. Further, the γ-H2AX foci that remain at later times in a control or an A-T cell line are preferentially located at the periphery of chromocentres and show a similar magnitude of overlap with them. This is consistent with our finding that DSBs associated with chromocentres (HC DSBs) are repaired with slower kinetics than DSBs located within euchromatic DNA and that A-T is specifically defective in the slow component of DSB repair, i.e. in the repair of HC DSBs. We also show that knockdown or loss of multiple factors that contribute to the heterochromatic super-structure including

KAP-1, HDAC1/2, Suv39H1/2 and HP1 can relieve the requirement for ATM for DSB repair. Finally, KAP-1 is a highly sensitive ATM substrate with its phosphorylation being rapidly lost as DSB repair proceeds. We also previously showed that KAP-1 phosphorylation at S824 is required for its role in ATM-dependent DSB repair [12]. Collectively, these findings strongly suggest that following the introduction of DSBs, ATM phosphorylates KAP-1, which promotes repair of DSBs located within or close to heterochromatic DNA. This raises the question of how KAP-1 phosphorylation impacts upon the heterochromatic super-structure. Ziv et al previously showed that exposure to neocarzinostatin, a radiomimetic DNA damaging agent, increased accessibility of micrococcal nuclease to DNA in an ATM-dependent manner [14]. Critically, they showed that KAP-1 siRNA also increased accessibility and that expression of S824A or S824D KAP-1 prevented or enhanced accessibility, respectively. Additionally, we showed previously that the presence of KAP-1 in a chromatin fraction enriched for heterochromatic proteins diminished after IR in an ATM-dependent manner. Taken together, these findings suggest that ATM-dependent KAP-1 phosphorylation modifies its chromatin binding, which, in turn, influences heterochromatic structure. However, the precise impact of KAP1 phosphorylation is unclear and clearly the higher order chromatin structure is not dismantled following IR-induced KAP-1 phosphorylation. Therefore, we suggest that KAP-1 phosphorylation confers sufficient localised heterochromatic relaxation to allow DSB repair without necessitating significant disassembly of the higher order chromatin structure. Possibly consistent with this, we observed that the duration and extent of KAP1 phosphorylation is short and highly dose dependent. Thus, we propose that the DSB response restricts the duration, location and extent of KAP-1 phosphorylation to limit the overall impact on higher order structure but promote localised DSB repair. In contrast, we show that KAP-1's distribution and chromatin association is dramatically altered as cells progress through mitosis, similar to the release previously observed for HP1. Global chromatin alterations are required to mediate the dramatic DNA condensation needed for cell division, which coincides with a significant decrease in many nuclear processes (eg transcription). In this context, it is perhaps not surprising that the mechanisms of chromatin alteration following DSB formation and mitotic entry are distinct. Finally, we demonstrate that KAP-1 is

phosphorylated predominantly by ATM with minimal phosphorylation contributed by ATR following, for example, replication-fork stalling or UV irradiation. It is likely that since higher order chromatin structure is dismantled during replication, KAP-1 phosphorylation might be less important for DSB repair during S-Phase or at collapsed replication forks, thus eliminating the need for ATR to phosphoryate this substrate. Given the fact that the majority of DSB response substrates are phosphorylated redundantly by ATM and ATR, the unusual specificity of KAP-1 phosphorylation is noteworthy and potentially important.

In summary, KAP-1 is a strong and functionally critical ATM substrate, whose phosphorylation is highly dose dependent and transient. Collectively, the emerging data suggest that KAP-1 is a “keystone” heterochromatic building factor that can become modified after DSB formation as well as during the cell cycle to allow critical but highly defined changes to the higher order structure necessary for DSB repair and potentially for events during mitosis.

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Figure 1. DSBs that persist following IR treatment in the absence of ATM kinase activity are localised around the periphery of chromocentres. DMSO or 10 μM KU55933 ATM inhibitor (ATMi) was added to confluent NIH 3T3 cells. 30 minutes later, cells were irradiated with 2 Gy IR and harvested 0.5 or 24 hours post-IR, as indicated. Cells were fixed and immunostained for γH2AX (red) and DAPI (green). Images shown are representative of the total cell population. At 0.5 hr post IR, ~ 20 % of DSBs overlap or touch chromocentres. In contrast, by 24 hr, ~ 70 % of DSBs are in contact with the periphery of chromocentres.

Figure 2. KAP-1 is an efficient and specific ATM phosphorylation substrate.
Panel A: GM02188 (WT), GM03189D (A-T) and DK0064 (ATR-SS) were either irradiated with 0, 2, 5, or 10 Gy gamma radiation (IR) and harvested 30 minutes later, irradiated with 0, 10, 20 or 40 J/m2 ultraviolet-C (UV) radiation and harvested 120 minutes later, treated with 1, 2 or 5 mM hydroxyurea (HU) and harvested 120 minutes later or treated with 5, 10 or 20 μM etoposide (ETP) and harvested 120 minutes later. KAP-1 was immunoblotted as in Figure 1. Forty micrograms of whole cell extract (prepared as described previously, was immunoblotted for either pS824 KAP1 or total KAP1 [12].

Figure 3. The restriction of heterochromatin on γ-H2AX foci formation is not relieved by the presence of ATM. Panels A-C: Confluent NIH3T3 cells were irradiated with 1 Gy IR and harvested 0.5 hr later. Cells were fixed and immunostained for γH2AX (green) and DAPI (blue). High resolution 3D images of deconvolved Z-stacks were obtained, showing DAPI-chromocenters (A, blue), γH2AX foci (B, green) and overlayed images (C). Panel D: Using softWoRx® Suite software, quantitative measurements of densely-stained DAPI and γH2AX foci were made and a 3D wireframe model was generated showing regions of blue-green overlap in red (indicated by arrows). The 3D model was rotated along the Z-axis (i-iv). Panel E: An enlarged image of the region of the z-axis rotated image shown in Panel D (iii), outlined by the white dashed lines.

Figure 4. KAP-1 is removed from chromatin during progression through mitosis. Panel A: Confluent G0/G1 phase NIH 3T3 cells were fixed and immunostained with KAP-1 (green), tri-methylated K9 histone H3 (TriMeK9 H3, red) and DAPI (blue). Panel B: Logarithmically growing NIH 3T3 cells were fixed and immunostained with acetylated K9 histone H3 (AcetylK9 H3, green), TriMeK9 H3 (red) and DAPI (blue). Panel C: Logarithmically growing NIH 3T3 cells were fixed and immunostained with AcetylK9 H3 (green), KAP-1 (red) and DAPI (blue). Panel D: Logarithmically growing NIH 3T3 cells were fixed and immunostained with KAP-1 (green), histone H3 phosphoserine 10 (phospho-H3, red) and DAPI (blue). Progression through G2/M was assessed by phospho-H3 staining and DAPI morphology. Panel E: Logarithmically growing NIH 3T3 cells were first extracted with 0.1% (v/v) Triton X100 in PBS for 30 seconds before being fixed and immunostained as in (D). N.B. The specific morphologies of KAP-1, TriMeK9 H3 and Acetyl K9 H3 during G2-phase shown in panels B and C were confirmed by co-staining each with a G2/M-phase marker such as phosphoserine 10 of histone H3 or Cyclin B1.

Table 1:

Treatment or cell line	DMSO treated cells (No. H2AX foci at 24 h)	ATMi treated cells (No. H2AX foci at 24 h)
Scrambled siRNA	**2.1 ± 0.4**	**10.7 ± 0.8**
KAP-1 siRNA	**3.2 ± 1.5**	**3.6 ± 1.0**
HP1αβγ siRNA	**2.8 ± 1.5**	**4.4 ± 0.4**
HDAC1/2 siRNA	**5.2 ± 1.1**	**5.8 ± 1.3**
SUV39H1/2$^{+/+}$ MEFs	**0.8 ± 0.4**	**8.6 ± 0.4**
SUV39H1/2$^{-/-}$ MEFs	**1.9 ± 0.2**	**5.0 ± 0.4**
Normal fibroblasts	**0.9 ± 0.2**	**9.3 ± 0.6**
HGPS fibroblasts	**1.1 ± 0.3**	**5.0 ± 0.6**
ICFa fibroblasts	**5.2 ± 2.6**	**7.5 ± 2.6**

Legend: The results in this table show the number of γ-H2AX foci present at 24 h post irradiation with 3 Gy in the absence (first column) or presence (second column) to ATMi addition. ATMi was added 30 min prior to irradiation. Data is only show for the 24 h time point. Full analysis is given in [12]. In some instance, there was a high background number of γ-H2AX foci. We assessed whether the number of foci was elevated relative to this background. Thus, for example, following HDAC1/2 siRNA, there were elevated γ-H2AX foci in the absence of ATMi but this was not further increased by ATMi addition, and the foci remaining was less than that observed in cells treated with scrambled siRNA and ATMi. Thus, we consider that HDAC1/2 siRNA at least partially alleviates the requirement for ATM for DSB repair.

FIGURE 1: 3T3 cells with ATM-dependent foci on chromocentres

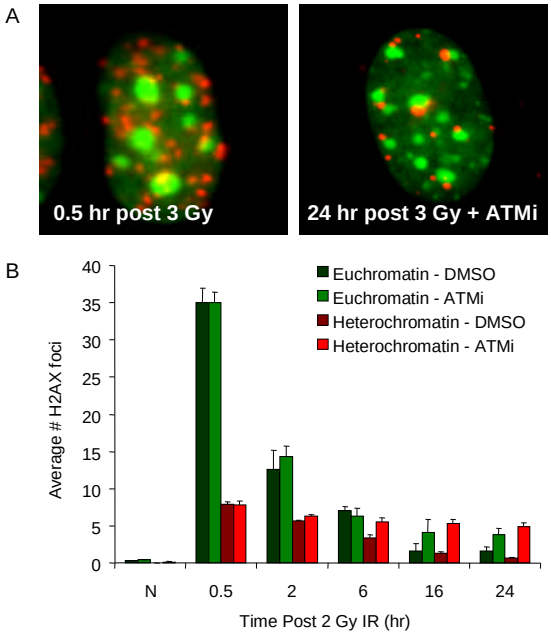

FIGURE 2: pKAP-1 DSB/ATM dependence and linear induction

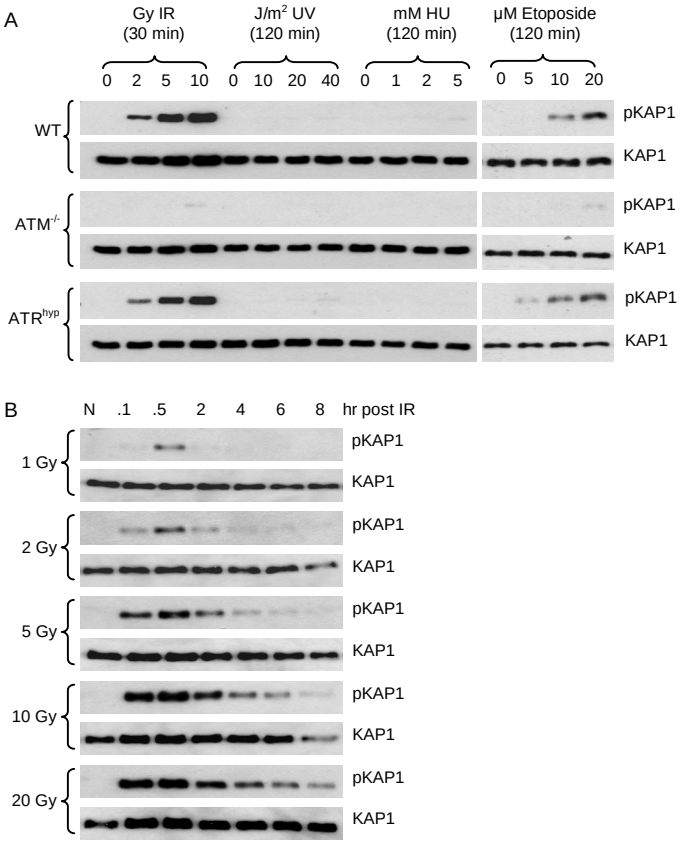

FIGURE 3: WT cells showing foci on periphery

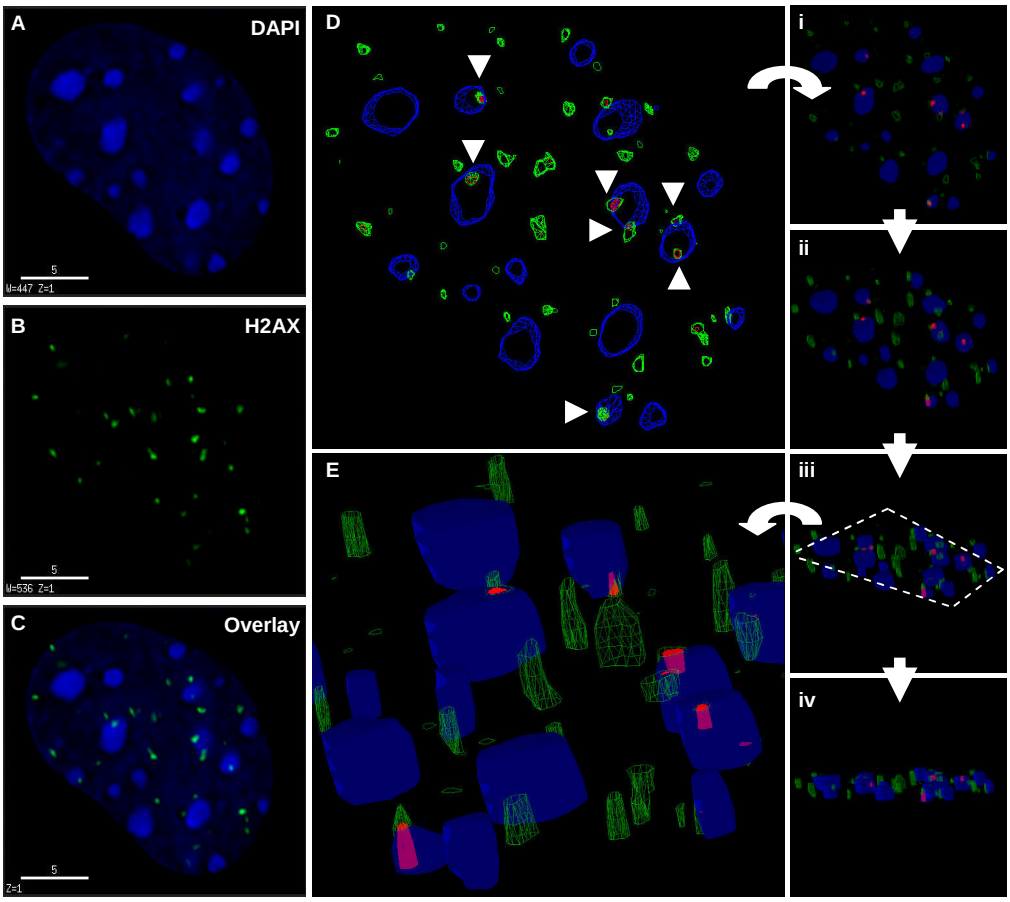

FIGURE 5: KAP-1 localisation in G2/M