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Etoposide Induces Nuclear Re-Localisation of AID

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Abstract

During B cell activation, the DNA lesions that initiate somatic hypermutation and class switch recombination are introduced by activation-induced cytidine deaminase (AID). AID is a highly mutagenic protein that is maintained in the cytoplasm at steady state, however AID is shuttled across the nuclear membrane and the protein transiently present in the nucleus appears sufficient for targeted alteration of immunoglobulin loci. AID has been implicated in epigenetic reprogramming in primordial germ cells and cell fusions and in induced pluripotent stem cells (iPS cells), however AID expression in non-B cells is very low. We hypothesised that epigenetic reprogramming would require a pathway that instigates prolonged nuclear residence of AID. Here we show that AID is completely re-localised to the nucleus during drug withdrawal following etoposide treatment, in the period in which double strand breaks (DSBs) are repaired. Re-localisation occurs 2-6 hours after etoposide treatment, and AID remains in the nucleus for 10 or more hours, during which time cells remain live and motile. Re-localisation is cell-cycle dependent and is only observed in G2. Analysis of DSB dynamics shows that AID is re-localised in response to etoposide treatment, however re-localisation occurs substantially after DSB formation and the levels of re-localisation do not correlate with H2AX levels. We conclude that DSB formation initiates a slow-acting pathway which allows stable long-term nuclear localisation of AID, and that such a pathway may enable AID-induced DNA demethylation during epigenetic reprogramming.

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Introduction

Genomes are protected from damage and mutation by a plethora of enzymes, however certain cell types perform carefully orchestrated DNA rearrangements and mutational programs that create or enhance population diversity. In B cells, VDJ recombination generates a naïve population of cells expressing different immunoglobulins (Ig). B cells are activated after encountering

proliferation. Replication through an abasic site requires translesion synthesis with random replacement of the missing nucleotide, resulting in dC-dN mutations [14]. Mutations at dA:dT base pairs also occur in SHM although these cannot be directly introduced by AID/UNG. Instead, dU:dG mispairs produced by AID are recognised by the Msh2/Msh6 heterodimer [15-17], instigating a non-classical mismatch repair pathway that results in the re-synthesis of surrounding DNA by the error prone polymerase [18].

AID has emerged as a candidate for epigenetic reprogramming as it has the potential to demethylate 5-methylcytosine (5mC). Direct deamination of 5mC by AID has been demonstrated *in vitro* [19], forming a dT:dG mismatch that could be repaired by thymine DNA glycosylase [20] and further processing to yield a demethylated dC:dG pair. Evidence also exists for the deamination of 5-hydroxymethylcytosine (5hmC) by AID [21]. However, recent studies have questioned this mechanism as AID prefers C to 5mC or 5hmC as a substrate *in vitro* [22-24], but AID could still demethylate 5mC indirectly by initiating homologous recombination or long patch repair at neighbouring residues [25,26]. Whatever the mechanism, compelling *in vivo* data links AID with epigenetic reprogramming: Aid$^{-/-}$ mice show defects in the removal of DNA methylation during primordial germ cell (PGC) formation [27], and AID is required for the expression of key reprogramming factors during cell fusion reprogramming and iPS cell generation [28-30] and for the mesenchymal-epithelial transition in mammary epithelial cells [31]. AID can also demethylate DNA in early zebrafish embryos [32].

DNA deamination occurs in the nucleus, but though AID is technically small enough to diffuse through nuclear pores it is restricted to the cytoplasm and carries a specific nuclear import signal [33-35]. This import signal is offset by a strong Crm1-dependent nuclear export signal [34,35] and a cytoplasmic interaction with eEF1A that inhibits import [33,36]; as a result AID shuttles rapidly across the nuclear membrane with the vast majority remaining in the cytoplasm at steady state. To further limit activity, the stability of AID is low in the nucleus [37]; REG- targets AID for proteasomal degradation through an N-terminal motif [38] and a further destabilising motif is present at the C-terminal [39], although these negative regulators are partially offset by a stabilising interaction with YY1 [40].

Over-expressed AID is rigorously excluded from the nucleus, but enough protein transiently shuttles through the nucleus to cause detectable SHM [17], and SHM and CSR can be reconstituted by ectopic AID expression in NIH/3T3 cells showing that no B cell specific factors are required [41,42]. Furthermore, on-going SHM occurs in Ramos cells, a B cell lymphoma cell line that constitutively expresses AID without any sign of AID re-localisation to the nucleus [17,43,44]. These data suggest that no change in steady state AID localisation is required for function in B cells. Nonetheless, cells with nuclear AID are observed in germinal centres where B cell activation occurs [45] and also in sperm [46], suggesting that a dedicated pathway exists for AID nuclear re-localisation. Because other cell types express little if any AID relative to B cells, we speculated that any realistic role for AID in genome-wide demethylation would require a pathway to provide stable, long-term nuclear residence. Here we demonstrate that AID re-localises to cell nuclei for extended periods following DNA damage.

Results

Etoposide treatment causes nuclear AID re-localisation

Nuclear accumulation of ectopically-expressed AID has been reported in HEK293 cells treated with -rays, hydrogen peroxide or bleomycin [47]. e ı oside tre

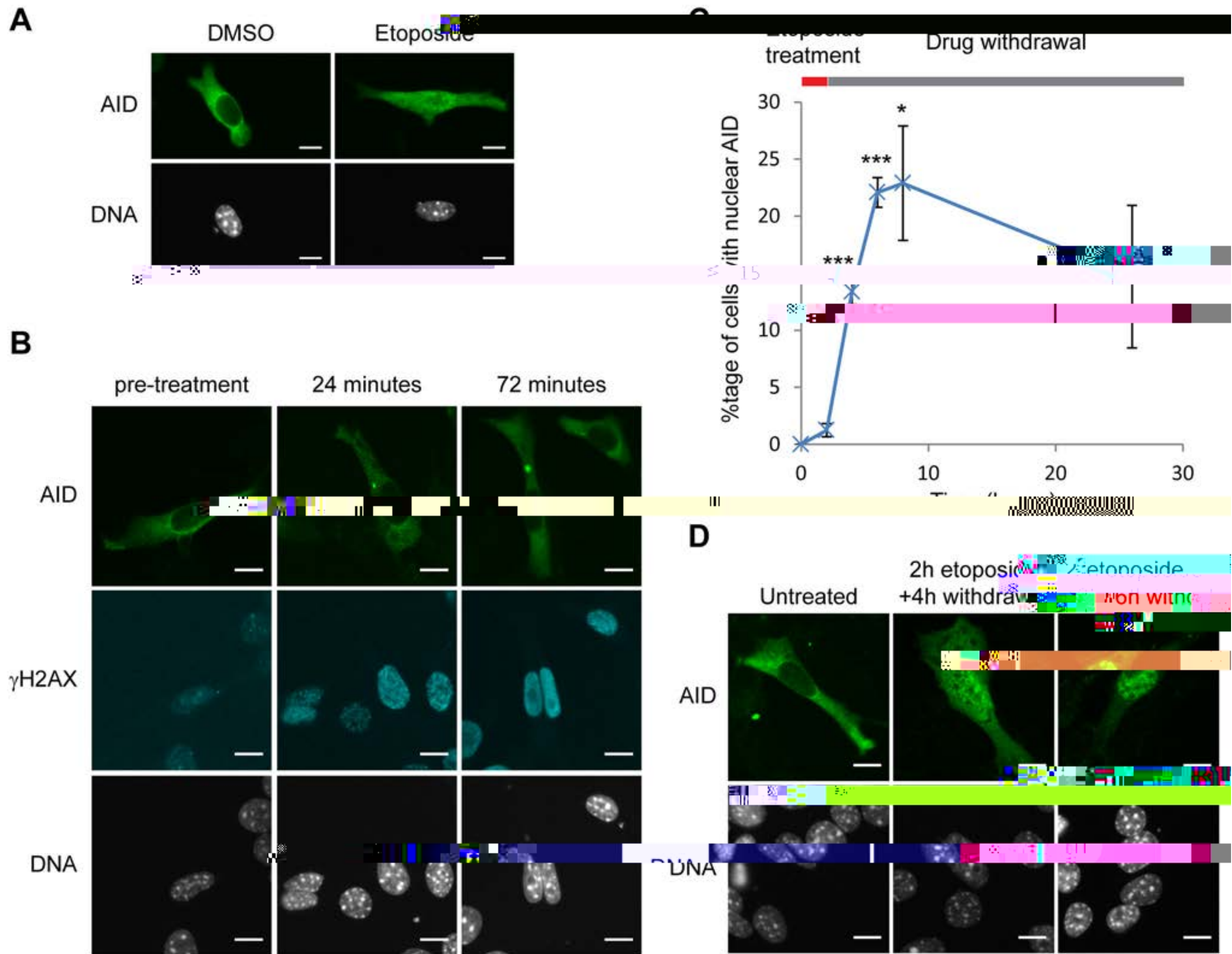

Figure 1. Etoposide induces AID re-localisation. A) AID localisation in NIH/3T3 cells transiently transfected with FLAG-AID and treated for 2 hours with 200μM etoposide or vehicle control (DMSO). Most etoposide-treated cells were indistinguishable from controls, however, the rare cells shown with nuclear AID (<1% transfected cells) in the drug treated sample were never observed in the controls. B) AID distribution after 24 and 72 minutes treatment with 200μM etoposide compared to H2AX formation. Images were captured at equivalent exposure times to allow visual comparison of signal intensity. C) Percentage of transfected cells showing nuclear AID localisation after 2 hours treatment with 200μM etoposide followed by drug withdrawal for 0, 2, 4, 6 and 24 hours. Data is from three independent experiments, two using FLAG-AID and one using GFP-AID constructs to ensure that the specific tag was not the source of the effect. Error bars represent ±1 s.e., *** $p<0.01$, * $p<0.05$ for Student's *t*-test comparing given time point to pre-treatment (time=0). D) AID localisation after 2 hours etoposide treatment followed by 4 and 6 hours drug withdrawal. Scale bars at bottom right of each image indicate 10μm.

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showing AID re-localisation, out of 42 cells imaged), measured as the time at which the nuclear signal exceeded the cytoplasmic signal. After re-localisation AID remained in the nucleus for an extended time (>10 hours), in most cases until the end of the experiment, although in three out of sixteen cases the protein returned to the cytoplasm (Figure 2A, Movie S1). Co-transfection with a nuclear-localised RFP construct confirmed the continuous nuclear localisation (Movie S5). The re-localisation of AID into the nucleus occurred over ~110 minutes (standard deviation ±25 minutes, n=16) (Figure 2B), and showed no further fluctuations across the extended nuclear residence. Of the 26 cells that did not undergo complete AID re-localisation, 24 showed no evidence of nuclear AID during the experiment suggesting that AID re-localisation is generally an all or nothing event. The two exceptions showed a very transient nuclear AID entry lasting 20-30 minutes (Figure S1B). These data show that after etoposide treatment, cells can stably maintain AID in the nucleus for extended periods (up to 19 hours) without undergoing cell death at least in the short term.

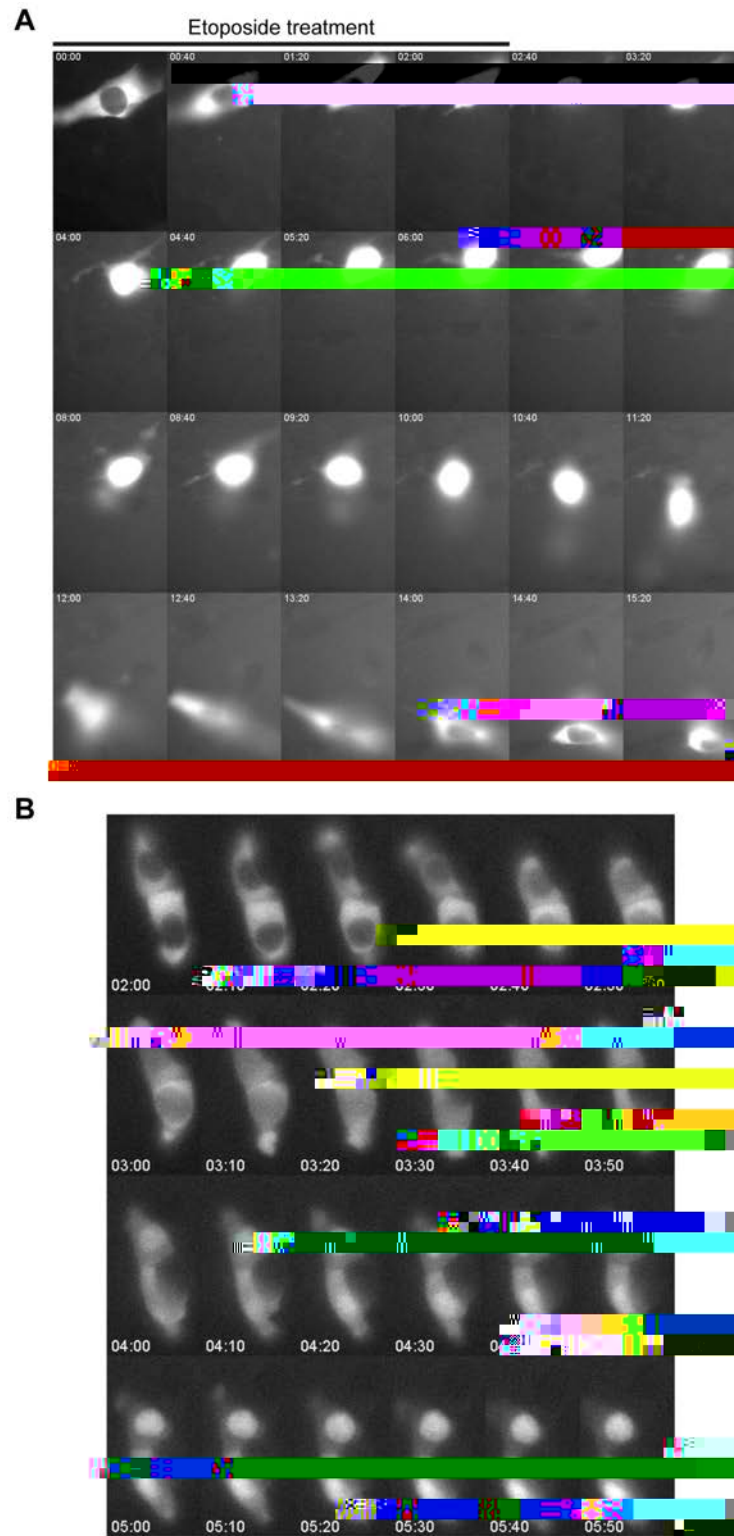

Figure 2. Dynamics of AID re-localisation. A) Montage of frames from live-cell imaging of an NIH/3T3 cell expressing GFP-AID, treated for 2 hours with 200μM etoposide and then monitored after drug withdrawal. Each frame represents 40 minutes. This video can be seen in full in Movie S1. B) Montage of two cells undergoing cytoplasmic to nuclear re-localisation of AID after etoposide withdrawal. Frames are shown at 10 minute intervals, t=0 at the addition of etoposide, first frame shown here is at etoposide removal. This video can be seen in full in Movie S3.

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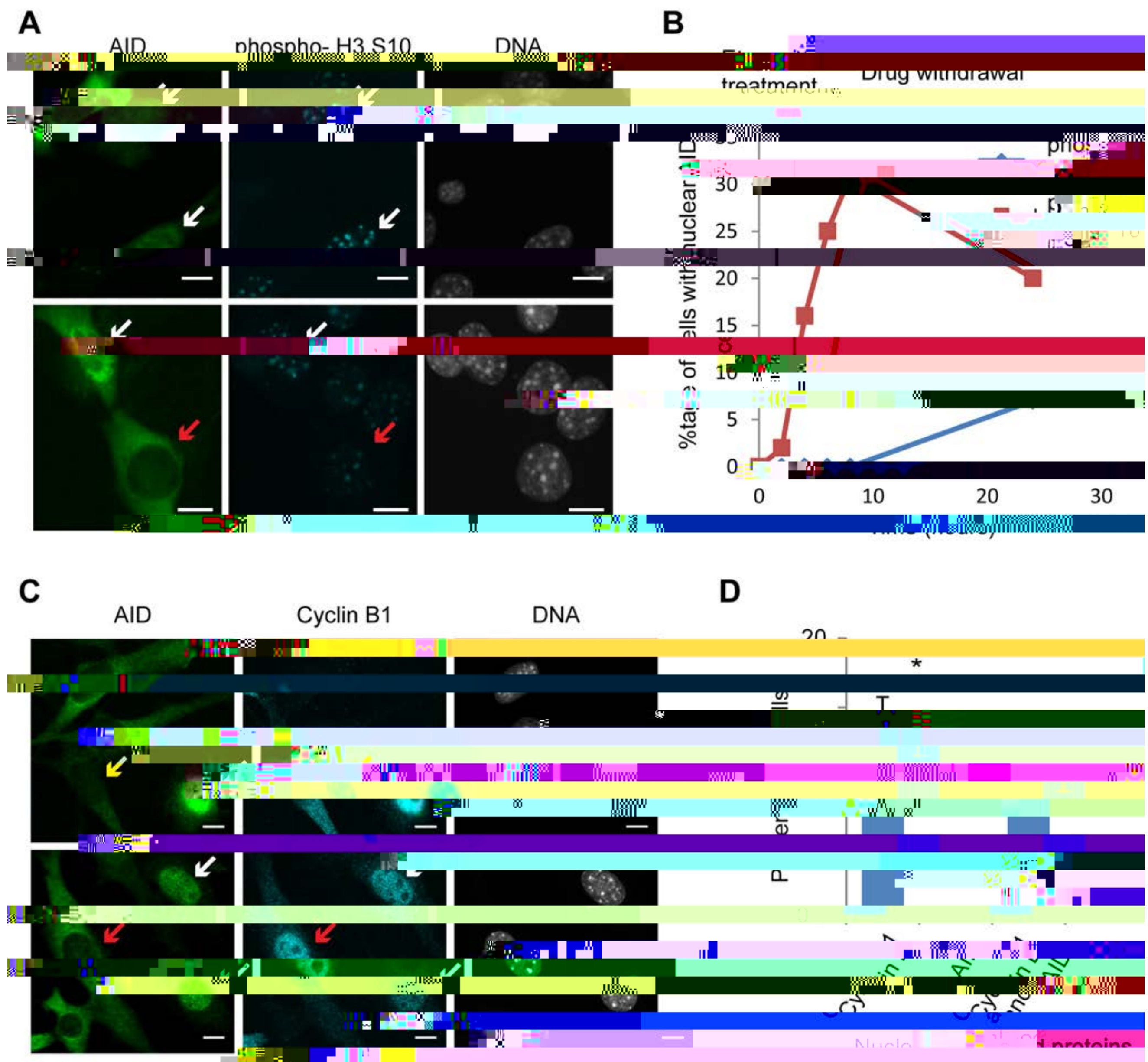

Figure 3. Cell cycle association of AID re-localisation.

The proportion of cells showing AID nuclear re-localisation was highly reproducible between replicate experiments, but we noted that transient transfections show ~25% re-localisation whereas the stable cell line shows only ~10% for the same dose of etoposide. This may reflect a requirement for high levels of AID which would not be physiologically relevant, but may also reflect the physiology of the stable cell line; stable AID expressing lines are hard to derive, presumably due to

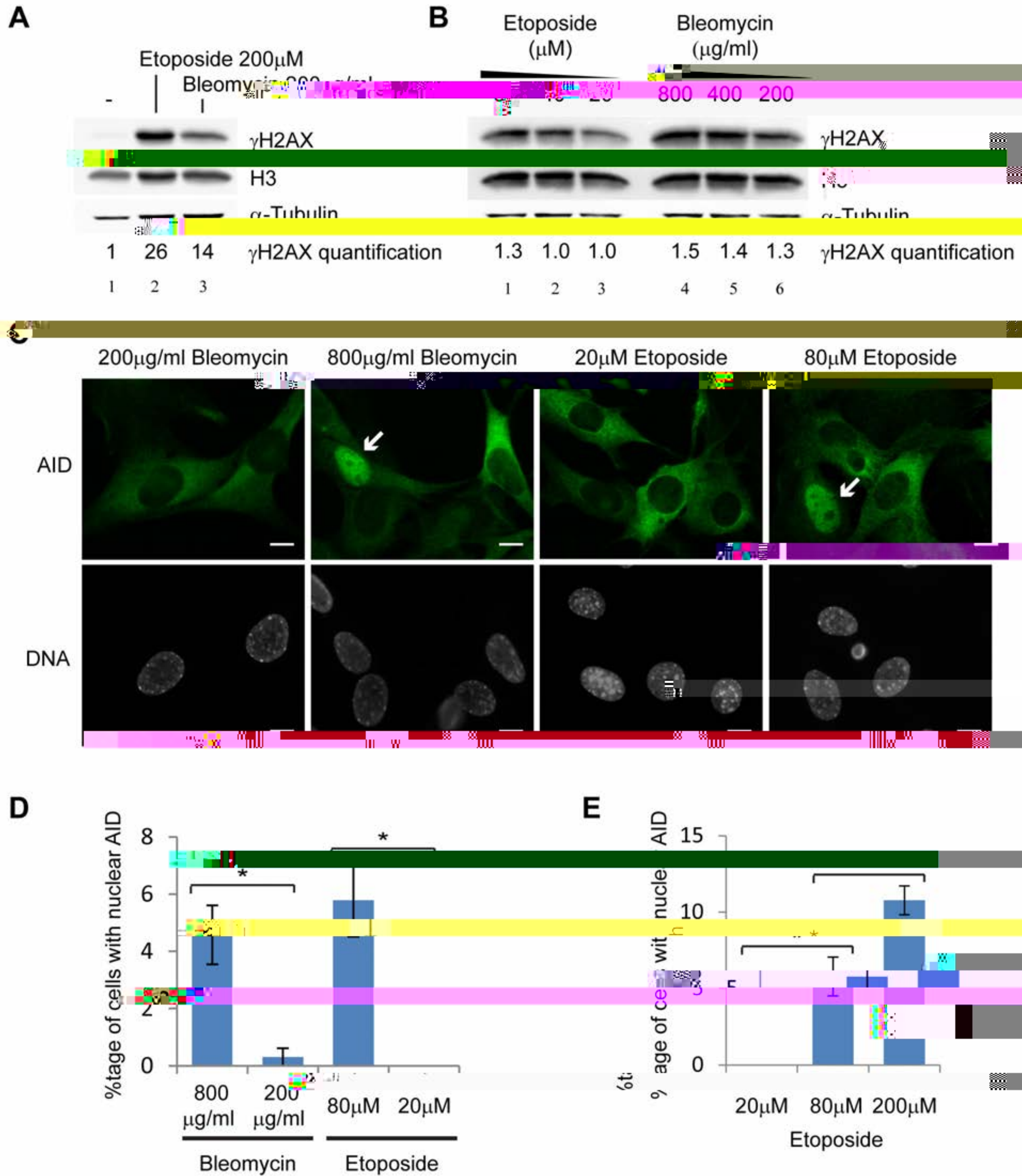

Figure 4. DSB requirement for AID nuclear localisation. A) Western blot showing H2AX levels in NIH/3T3 cells treated for 2 hours with 200μM etoposide or 200μg/ml bleomycin, the concentration used in [47]. Histone H3 and -tubulin are shown as loading controls, quantification of H2AX is relative to tubulin. B) Western blot of H2AX levels in NIH/3T3 cells after 2 hours treatment with given concentrations of etoposide and bleomycin. Controls as in A, quantification of H2AX is relative to tubulin. C) Localisation of AID in a stable NIH/3T3 cell line expressing FLAG-AID, treated with bleomycin and etoposide at different doses for 2 hours and then allowed to recover for 5 hours. White arrows indicate cells with nuclear AID. Scale bars at bottom right of each image indicate 10μm. D) Quantification of cells shown in C with nuclear AID under different drug treatments. E) AID nuclear localisation with increasing etoposide concentration, cells treated as in C. Error bars represent ±1 s.e., * $p<0.05$ for Student's *t*-test, n=3 samples per drug treatment except n=2 for 20μM etoposide and n=5 for 200μM etoposide, >100 cells were counted in each sample.

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AID-mediated mutagenesis, and may therefore survive by down-regulating AID import.

The mechanism that maintains cytoplasmic AID has been well-characterised, and strong AID nuclear accumulation phenotypes have been observed after removal of the Crm1-

methods for fluorescence detection (see protocols at www.cellsignal.com) and imaged using a LI-COR Odyssey system. Antibodies were rabbit - H2AX (CST 9718) at 1:1,000, rabbit -H3 (Millipore 07-690) at 1:5,000 and mouse - tubulin (CST 3873) at 1:10,000, DyLight-conjugated secondary antibodies (CST) were used at 1:15,000.

Supporting Information

Figure S1. Nuclear re-localisation of AID. A) Nuclear re-localisation of AID in NIH/3T3 cells transiently transfected with an AID-HA construct. Cells were treated with 200μM etoposide followed by 6 hours of drug withdrawal before staining. No nuclear localised AID was observed amongst untreated control cells. B) Montage of frames from live-cell imaging of an NIH/3T3 cell expressing GFP-AID, treated for 2 hours with etoposide followed by drug withdrawal. Each frame represents 10 minutes. This is one of only two cells observed (out of 42) that underwent transient nuclear re-localisation of AID. This video can be seen in full in Movie S4.
(TIF)

Figure S2. Dynamics of H2AX compared to AID localisation. A) Western blots showing H2AX accumulation in NIH/3T3 cells treated with no drug, etoposide (200μM), bleomycin (200μg/ml) or ICRF-193 (100μM), samples were taken across a 2 hour drug treatment and 6 hours of drug withdrawal. H3 is shown as a loading control. B) Localisation of AID in a stable NIH/3T3 cell line expressing FLAG-AID, treated with etoposide, bleomycin or ICRF-193 doses given in A for 2 hours followed by 6 hours of drug withdrawal. Nuclear AID was only observed in etoposide-treated cells.
(TIF)

Figure S3. AID re-localisation at low etoposide concentration. Cells from Figure 4C treated with 20μM etoposide showing clear nuclear AID re-localisation. These cells were extremely rare (only a few were seen per cover slip), and were not encountered while performa

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