

Spatiotemporal regulation limits the mutagenic potential
of Activation-Induced Deaminase (AID)

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Abstract

Spatiotemporal regulation limits the mutagenic potential
of Activation-Induced Deaminase (AID)

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Activation-induced cytidine deaminase (AID) initiates immunoglobulin (Ig) gene diversification in activated B cells and participates in the erasure of methylation marks that is necessary to genome-wide reprogramming in very early development. AID belongs to the Apobec family of cytidine deaminases. In activated B cells, deamination of C to U by AID triggers repair by error-prone mechanisms, leading to somatic hypermutation, gene conversion and class switch recombination at the Ig genes.

AID has the potential for mutagenesis. Consequently, the genome accumulates off-target deaminations as collateral damage from Ig gene diversification, which are repaired to maintain genomic stability. Pathological activities of AID are evident in many B cell and non-B cell malignancies in which AID is deregulated. AID attacks only single-stranded DNA (ssDNA),

raising the possibility that AID could promote genomic instability during S phase, when DNA becomes transiently single stranded for replication.

The regulation of AID is stringent to minimize pathological outcomes. The role of cell cycle in regulation of AID has not been studied extensively. Here, I sought to address the relationship between cell cycle-dependent nuclear stability of AID and the physiological vs. pathological outcomes of AID activity. I have found that nuclear stability of AID promotes Ig gene diversification in G1 phase, and that nuclear export prevents genomic instability during S/G2/M phase. These results establish the physiological importance of cell cycle-dependent regulation of AID, and show that disruption of normal cell cycle regulation can promote genomic instability and genotoxicity and may contribute to mutagenesis by AID in cancer.

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Dedication

To my family: My dad, Phieu Le; and mom, Ngoc Nhu Dong; and my siblings,
Phuong Le, Nam Le, Tam Le, and Valued Le.

Chapter 1: The biological and pathological ramifications of the mutagenic factor AID

The genome is under constant assault with DNA damage occurring at a rate of 1×10^3 - 1×10^6 lesions/cell/day in human cells [97]. The sources for DNA damage may be either external agents such as UV or ionizing radiation or mutagenic chemicals, or endogenous processes such as reactive oxygen species, collapse of replication forks [1]. Because DNA damage can lead to deleterious mutations that can cause genetic disease and cancer, organisms have evolved highly sophisticated mechanisms devoted to the detection and repair of DNA lesions in order to ensure genomic integrity [2]

Activation-induced deaminase (AID), a member of the Apobec family of cytidine deaminases, deaminates cytosine into uracil in DNA, resulting in somatic hypermutation, gene conversion and class-switch recombination at Ig genes. From an evolutionary standpoint, why would there exist a gene in the genome that can directly mutate the genome itself? AID is indispensable for immunity and early developmental processes [3,4]. And as would be expected for any mutagenic factor, AID is implicated in cancer [5].

1.1. Discovery of the mutagenic factor AID and its role in immunity

The immune system serves as a central line of defense against infections by pathogenic microorganisms. Antibodies are produced by B cells and are essential for the clearance of most pathogens. T cells modulate the antibody response by mediating B cell activation. In order to recognize and respond to many different antigens, both B and T cells express a diverse repertoire of receptors. During development, the rearrangement of V, (D) and J segments creates the primary repertoire for B and T cells, in an antigen-independent manner. Upon encountering antigen, activated B cells undergo additional changes that modify the sequence and structure of

Ig genes, known as Ig gene diversification (Reviewed in [6]).

Three processes of Ig gene diversification

Ig gene diversification consists of three mutagenic processes that alter the sequence and structure of rearranged Ig genes, which already express functional antibody molecules.

1) Somatic hypermutation (SHM) alters the antigen-binding domain of the antibody molecule by introducing point mutations to the rearranged and expressed Ig V regions. Activated B cells migrate to the germinal center, where they proliferate and actively diversify their surface receptor. Clones with high specificity and affinity receptors against a specific antigen are selected and expanded. Hence, SHM when coupled with clonal selection provides a dynamic response against pathogens (Reviewed in [7]).

2) Gene conversion (GC) provides the primary mechanism of altering the sequences at Ig V regions in fowl animals. It involves the transfer of genetic material from the nonfunctional pseudo V regions to the rearranged and expressed Ig V regions [8,9,10].

3) Class-switch recombination (CSR) alters the antibody isotype and effector function. Each constant region at the Ig heavy chain (Igh) locus confers a unique mode of pathogen clearance. CSR occurs at switch regions, located upstream of each constant region. The subsequent deletion of the intervening DNA sequences and the joining of Igh VDJ region to another constant region allows a different antibody isotype to be expressed (Reviewed in [11]).

Discovery and characterization of AID, a key player in Ig gene diversification

Because the processes of Ig gene diversification result in vastly different mutagenic signatures, it was long thought by many that these processes were dependent on distinct

mechanisms. It came as a surprise when AID was found to be responsible for all three processes. In 1999, Honjo and colleagues used a subtraction method to screen for genes that were upregulated in CH12F3-2 B cells stimulated to undergo CSR, and identified a gene encoding a cytidine deaminase [12]. They tested the physiological significance by targeted deletion of the corresponding *Aicda* gene in mice, and found that deletion abolished both CSR and SHM [13]. At the same time, Durandy's group found that patients with hyper-IgM syndrome, characterized by a lack of CSR and SHM activities and an overproduction of IgM, were deficient in AID [14]. Shortly after, AID was shown to be required for gene conversion in chicken B cells [9,10].

The discovery of AID has prompted much effort toward unraveling the molecular details of AID-initiated Ig gene diversification, starting with the characterization of AID (Figure 1-1). DNA sequence analysis revealed that the *Aicda* gene encodes a 28-KDa protein which carries a cytidine deaminase domain and is closely related to the apolipoprotein B mRNA-editing catalytic polypeptide 1 (APOBEC1) [12]. Based on this homology, researchers speculated that the mRNA-editing activity of AID is critical in Ig gene diversification, and suggested that it might act on one or multiple distinct mRNAs that functions in Ig gene diversification. Neuberger and colleagues provided evidence to the contrary by demonstrating that recombinant AID directly acts on DNA in *E. coli*., and that this activity depends upon uracil DNA glycosylase, which recognizes and removes uracil bases in DNA [15]. Biochemical assays of purified AID showed that AID deaminated C to U in DNA and not RNA, with a preference for single-stranded DNA at WRC motifs (W = adenosine or thymidine; R = purine; and C = cytidine) [16,17,18]. Consistent with a DNA mutator activity, in mammalian cells U:G mismatches produced by AID are processed by the same uracil nucleoside glycosylase activity, UNG, that removes uracil bases in DNA in base excision repair (BER), or by MSH2/MSH6, a key factor in the mismatch repair

pathway. Mice in which the *Ung* or *Msh2* genes have been ablated exhibited reduced CSR activity and alteration in the hypermutation spectrum [19], and *Ung*^{-/-}*Msh2*^{-/-} mice exhibit no CSR or SHM [20]. Lastly, AID induces mutations into the genome when ectopically expressed in mammalian cell lines [21,22] and yeasts [23,24], providing further evidence that AID is a DNA mutator.

Targeting AID to transcribed Ig genes

Transcription of Ig genes is a requirement for diversification [25,26,27,28]. Transcription serves two purposes: 1) to provide AID access to ssDNA [17,29] and 2) to recruit AID to sites of transcription pausing by RNA polymerase II [30,31]. The mechanisms by which AID is recruited to Ig loci have to yet to be fully elucidated.

How does AID induce all three processes of Ig gene diversification?

Factors that normally participate in faithful repair pathways are involved in Ig gene diversification. In SHM, CSR and GC, the first step of diversification is production of a DNA nick. This can occur in two ways.

- 1) Uracils created by AID can be excised by UNG, generating an abasic site (AP). The AP lyase activity of the MRE11/RAD50/NBS1 (MRN) complex then cleaves the DNA at the AP site to generate a single-stranded break (SSB) [32].
- 2) Alternatively, the MSH2/MSH6 heterodimer (MutS α) can bind to an U:G lesion and together with Mlh1-Pms2, recruit Exo1. Exo1 then resects DNA starting from the 5' end of the U:G mismatch, creating a gap.

In SHM, DNA synthesis at the break or opposite the AP site by error-prone DNA polymerases could lead to the incorporation of any one of the 4 bases (A, G, C and T) at U:G lesion. Translesion polymerases are thought to carry out mutagenic DNA synthesis to fill in the gap, which results in not only mutation at the C:G base pair but also at neighboring A:T base pairs.

In CSR, DNA deaminated by AID is processed to generate DNA nicks on both DNA strands, by UNG or MSH2/MSH6. Successive excision of nicks on each strand by EXO1 or FEN1 can generate DSBs that can be joined by factors in the non-homologous end-joining pathway.

In GC, factors that normally promote homology-directed repair act at a DNA to transfer information from a donor to the target gene. In chicken B cells deficient in RAD51 paralogues XRCC2, XRCC3, or RAD51B, which function in homology-directed repair, point mutations (non-templated) accumulate at the rearranged Ig V regions instead of templated mutations [33]. This was one of the first observations to suggest that the distinct outcomes of Ig gene diversification are actually branches of a single repair pathway.

1.2. AID in early development

AID was thought to be a B cell-specific factor. However, AID transcript was detected in other cell types, including oocytes, primordial germ cells (PGCs), and embryonic stem (ES) cells in mice [34]; and breast tissue [35] and prostate epithelial cells [22] in humans. In lower vertebrates, AID is expressed in early development [36,37,38], suggesting that AID may have other functions outside of Ig gene diversification. In 2004, Petersen-Mahrt and colleagues provided the first evidence for such a function. Petersen-Mahrt's group showed that AID, like

APOBEC1 can deaminate methylated cytidine into thymidine in ssDNA in vitro [34]. DNA methylation is an important epigenetic mark that is involved in many developmental processes such as differentiation, parental imprinting, and X-chromosome inactivation [4].

The finding that AID deaminates methylated cytidine has prompted investigations to determine the role of AID in early development. Two studies have examined the effect of AID on the methylation state of mouse PGCs and heterokaryons, respectively. In one study, Cokus and colleagues compared genome-wide methylation of PGCs derived from WT and *Aicda*^{-/-} mice, and found that there is a significant increase in methylation in PGCs derived from *Aicda*^{-/-} mice [39]. The other study examined the effects of AID knock down on heterokaryon formation and reprogramming. Blau and colleagues used polyethylene glycol to fuse mouse ES cells to human fibroblasts, creating interspecies heterokaryon, to induce pluripotency in the human nuclei [40]. They found that reprogramming frequency was reduced significantly upon knockdown of AID, and showed that this was due to down-regulated expression of the pluripotency markers Oct-4 and Nanog that could be traced to CpG methylation of the Oct-4 and Nanog promoters.

1.3. AID and cancer

AID deamination is not restricted to Ig genes. Many non-Ig genes have been documented to be targets of AID, including oncogenes *Myc*, *Bcl6*, *Pim1*, *Pax5*, *Fas* and *Rhoh* [41,42,43,44,45]. This observation has led researchers to speculate that AID might be implicated in cancer. Most B lymphoid cancers harbor translocations involving the Ig loci [46]; most notably, the translocation of the proto-oncogene *Myc* to the *IgH* locus in Burkitt lymphoma, which allows *Myc* to be aberrantly expressed under the regulation of the *IgH* promoter [47,48].

AID is required for the *Myc/IgH* translocation, as AID-deficient mice do not exhibit this translocation [49]. In AID transgenic mice, ectopic expression of AID resulted in development of T-cell lymphoma and lung microadenoma, suggesting that AID could promote the transformation and progression of malignancies [50]. Similarly, AID may promote the translocation of the proto-oncogene *Bcl-6*, and it also introduces point mutations into *Bcl6* and other proto-oncogenes that have been documented in diffuse large B-cell lymphoma (DLBCL) [5,42]. In addition, AID expression is correlated with genomic instability in patients with B cell lymphocytic leukemia (B-CLL) [51]. Other non-B cell tumors exhibiting AID-associated genomic instability have also been reported (i.e. T cell lymphomas [50,52]; breast cancer [35,53], colon cancer [54] and gastric cancer [55]).

Most of the studies examining AID expression in cancers have been correlative, and it is still uncertain whether AID expression is the cause or the result of transformation in AID-related malignancies. Nevertheless, the implications of a potent mutagen like AID for cancer are evident, even though understanding the exact role of AID in cancer awaits further investigations.

1.4. The mutagenic potential of AID genomewide

For many years, AID was thought to act almost exclusively on the Ig genes, and considerable effort was devoted to the question of how AID induces mutagenesis at the Ig genes while the rest of the genome remains untouched. It became evident from the ability of AID to drive tumorigenesis in some cancers (see section 1.3) that AID expression poses a threat to the genome. Recently, deep-sequencing has revealed that AID appears to induce mutations genomewide, as shown by evidence that non-Ig genes accumulate mutations at a higher frequency in wild-type activated B cells than in *Aicda*^{-/-} activated B cells [56,57]. Moreover, the

frequency of genomewide mutations increased considerably in *Msh2*^{-/-} *Ung*^{-/-} activated B cells. This suggests that the genome accumulates a considerable mutagenic load as collateral damage from SHM, and that high-fidelity repair protects the genome from AID-induced mutagenesis. Consistent with this, AID induces toxicity upon activation of murine B cells deficient in XRCC2 [58]. XRCC2 is one of the RAD51 paralogs and an important factor in homologous recombination repair and GC. Thus, homologous recombination is required to repair genome-wide DSBs generated by AID.

1.5. Regulation of AID

As a DNA mutator, AID activity is subject to regulation at multiple levels [3].

Transcription and translation of AID

AID transcription is stimulated by signals that induce B cell activation and CSR (lipopolysaccharide, interleukin4, CD40 ligation) in naïve B cells [59], and by estrogen in B cells, and breast and ovarian tissues [35]. Transcriptional regulation of AID involves recruitment of a network of transcription factors (NF-κB, STAT6, HoxC4, Sp1 and Pax5) to four identified regulatory elements at the *Aicda* locus (Reviewed in [3]). Mir-155 and Mir-181 directly bind to the 3' untranslated region of AID mRNA, to regulate AID abundance [60,61,62]. Disruption of the Mir-155 target site increases AID abundance, and also stimulates CSR *in vitro* and *in vivo* [60,61].

Post-translational modifications of AID

AID is subject to post-translational modifications, of which two types have been identified thus far, phosphorylation and ubiquitination. To-date, five phosphorylation sites have been identified: Ser3 [63], Thr27, Ser38, Thr140 and Tyr184 (Reviewed in [3]).

Phosphorylation on anyone or a combination of these sites might impact AID activity, sub-cellular localization and protein stability. Much of the research on post-translational modifications of AID has centered on the phosphorylation of Ser38, carried out by protein kinase A (PKA) [64,65,66,67,68,69]. An AID S38A substitution mutant displayed decrease CSR and SHM activities *in vivo*, and an inability to interact with RPA, a repair factor that is important for AID targeting.

AID is also ubiquitinated. There are eight potential ubiquitylation sites, and mutational analysis suggests that many or all of these may participate in regulating protein stability which limits the half-life of AID [70,71]. Cells are sensitive to AID over-expression [72]. For example, higher level of AID expression is correlated with genomic instability in patients with B-CLL [51].

Sub-cellular localization

AID shuttles between the nucleus and the cytoplasm but is predominately cytoplasmic[73,74,75,76]. AID carries a nuclear export signal (NES) at its C-terminal, which specifies export via the CRM1-dependent pathway. Sub-cellular localization is critical to limiting AID access to DNA substrates in the nucleus and thus, prevent genomic instability. For example, an AID^{F193A} mutant with defective NES resides in the nucleus, and it is toxic to cells [76,77].

Cell cycle regulation

AID only attacks ssDNA [18]. This restricts AID activity to transcribed DNA and regions that become transiently single-stranded during DNA replication in S phase, raising the possibility that AID might be cell cycle regulated. Interestingly, AID-initiated DSBs and point mutations are found mainly in G1 phase cells [59,78,79]. The Maizels laboratory showed that in the chicken DT40 B cell line, AID is evident in the nucleus predominately in G1 phase cells [80].

1.6. Temporal and spatial regulation that limits the mutagenic potential of AID

I became interested in how AID is regulated because it must involve fascinating mechanisms for protecting the genome from a potentially dangerous factor, and deregulation of AID may lead to cancer. I hypothesized that restricting nuclear stability of AID to G1 phase protects the genome from AID-induced mutagenesis.

To test this, I first asked how AID traffics in real time, using live cell imaging. In Chapter 3, I show that AID accumulates in the nucleus in short bursts, in both Ramos B cells and HT1080 fibroblasts.

I then assessed the sub-cellular localization and protein stability of AID throughout the course of the cell cycle. In Chapter 4, I demonstrate that, in the human Ramos B cell line, AID is predominately cytoplasmic in all phases of the cell cycle. By blocking export, I show that nuclear AID is degraded more slowly in G1 phase.

I then asked how nuclear stability relates to function. In Chapter 5 and 6, I establish that AID diversifies Ig genes during G1 phase of cell cycle, and that enforced nuclear localization of AID during S/G2/M phase is toxic.

The work I describe here establishes the physiological importance of cell cycle-dependent regulation of AID, and shows that disruption of normal cell cycle regulation can promote genomic instability. This may contribute to mutagenesis by AID in cancer.

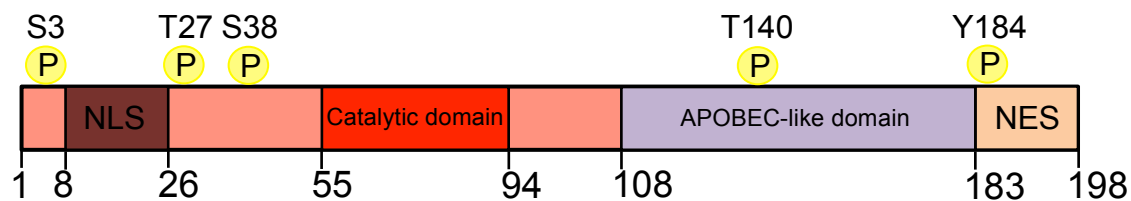


Figure 1-1. Domains of AID.

AID is a small polypeptide composed of 198 amino acids. AID contains a catalytic domain and an APOBEC-like C-terminal domain. AID carries a leucine-rich nuclear export signal (NES) at the C terminus that is recognized by the CRM1-mediated export pathway, and an N-terminal nuclear localization signal (NLS). AID is shown to be phosphorylated at the indicated residues.

Chapter 2: Materials and Methods

Expression constructs

The pEGFP-N3 construct for expression of AID fused at its C-terminus to GFP (AID-GFP) was a gift from Dr. Javier Di Noia (Department of Microbiology and Immunology, University of Montreal, Montreal, Quebec, Canada). I substituted mCherry for a region of GFP flanked by ApaI and BsrGI restriction sites in the pEGFP-N3 construct to generate an AID-mCherry expression construct, pAID-mCh.

Cell cycle reporter constructs p-mKO2-Cdt1 CSII and p-mAG-Gem CSII, in a lentiviral vector, were a gift from Dr. Atsushi Miyawaki (Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan)

pAID-GFP-CSII and pAID-mCh-CSII: I amplified GFP from pEGFP-N3 and mCherry from pAID-mCh (with primers PQL31 5'-ATATCAATTGAGATCCCAA ATGGACAGCC-3' and PQL32 5'-ATATTCTAGATTACTTGTACAG CTCGTCCATGC-3') and inserted these fragments between EcoRI and XbaI sites in p-mAG-Gem CSII, thereby replacing Gem.

pAID-mCh-Cdt1 and pAID-mCh-Gem: I amplified Cdt1 (with primers PQL44 5'-TATATGTACAAGGGATATCCATCACACTGGCGGCC-3' and PQL45 5'-TATATGTACATCTAGATTAGATGGTGTCTCCTGGTCC-3') and Gem (with primers PQL44 5'-TATATGTACAAGGGATATCCATCACACTGGCGGCC-3' and PQL46 5'-TATATGTACATCTAGATTACAGCGCCTTTCTCCG-3') from p-mKO2-Cdt1 CSII or p-

mAG-Gem CSII, and inserted the resulting fragments between BsrGI and XbaI restriction sites of pAID-mCh CSII.

pAID-GFP-Cdt1 CSII and pAID-GFP-Gem CSII: I subcloned Cdt1 and Gem between BsrGI and XbaI restriction sites in pAID-GFP CSII.

AID-mKO2: I amplified mKO2 (with primers mKO2 FOR 5'-ATATGGATCCATCGCCACCATGGTGAGTGTG-3' and mKO2 B REV 5'-ATATTCTAGATTAGCCCTGGGAAGGCAACATTG-3') and inserted the resulting fragment between BamHI and XbaI restriction sites of p-AID-GFP-Cdt1, to replace GFP-Cdt1.

pAID-mKO2-Cdt1 and pAID-mKO2-Gem: I amplified mKO2 (with primers mKO2 FOR and mKO2 REV 5'-ATATGCGGCCGCGCCAGTGTGAT GGATATCCGC-3'), and inserted mKO2 between BamHI and NotI restriction sites in pAID-mCh-Cdt1 and pAID-mCh-Gem CSII, respectively.

Cell Culture and Transfection/Transduction

The human fibrosarcoma cell line, HT1080, was cultured in DMEM media supplemented with 10% FBS, 2 mM L-glutamine, and penicillin/ streptomycin. Lentiviral transductions used 2×10^5 cells grown in medium containing 8 $\mu\text{g/ml}$ of polybrene per well in a 12-well plate. Cells were grown for 3-4 days after transduction and then sorted to enrich for transduced cells expressing the desired fluorescent protein, typically constituting 0.2-10% of the population. For live cell imaging, HT1080 cells were transfected with expression construct carrying AID-GFP,

AID-mCherry or cAID-YFP by LTX transfection as directed in company's protocol (Life Technologies, Cat. # 15338100) or 4-D nucleofector (Lonza).

The human Burkitt lymphoma cell line, Ramos, was cultured in RPM 1640 media supplemented with 10% FBS, 2 mM L-glutamine, penicillin/ streptomycin, 1X non-essential amino acids (Gibco, Cat# 11140-050), 1 mM sodium pyruvate (Gibco, Cat# 11360-070), and 10 mM HEPES (Gibco 15630-080). Ramos B cells were transduced as described for HT1080 cells.

Live-cell imaging

Transfected HT1080 cells were grown in 35mm glass bottom dish (MatTek, sample part # P35G-0-10-C) in 37°C/5% CO₂ incubator at least 24 hr before imaging. Time-lapse microscopy was carried out by DeltaVision microscopy equipped with environmental chamber to control for temperature and CO₂. Cells were imaged every 10 min or 15 min interval for a total duration of 24 hr.

Ramos B cells expressing AID-mCherry were grown in 37°C/5% CO₂ incubator at least 24 hr before imaging. To prevent Ramos B cells from tumbling out of the field during extended period of imaging, LiveCell Array Microscope slide (Nunc, Cat # 130505) was used. Cells were imaged as described for HT1080 cells.

Drug treatment and High Content Screening (HCS) Microscopy and Analysis

Cells expressing AID-mCherry, AID^{F193A}-mCherry, AID-mCherry-Cdt1, AID^{F193A}-mCherry-Cdt1, AID-mCherry-Gem, or AID^{F193A}-mCherry-Gem were plated at a density of 2×10^6 cells/ml and treated with Leptomycin B (50 ng/ml; LC Laboratories; Cat# L6100), MG132 (50 μ M; Z-Leu-Leu-Leu-aldehyde; Sigma-Aldrich Cat# C2211), or both for indicated times.

Cells were then fixed in 3.7% formaldehyde and stained with whole cell stain (HCS CellMask, Invitrogen, Cat# H32714) and DAPI (0.2 $\mu\text{g/ml}$). Fixed cells were then washed, re-suspended in PBS and spun down in a 96-well nuclear microplate (Greiner Bio One, Cat# 655906) for imaging.

Cells were imaged by Thermo Scientific ArrayScan VTI HCS reader. HCS Colocalization BioApplication protocol was used to determine nuclear and whole cell boundaries in individual cells defined by DAPI and HCS CellMask, respectively, and the cytoplasmic region thereby defined as the region between nuclear and whole cell boundaries. The average signal for AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem, and the respective F193A mutants, in the nuclear and cytoplasmic compartments was determined in each cell by measuring the total intensity of mCherry divided by area within each compartment. Nuclear to cytoplasmic ratio (N/C) was calculated for AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem, and the respective F193A mutants, as the ratio of the average signals of nuclear and cytoplasmic mCherry. To ensure that artifacts were not introduced into the analysis, I removed cells that expressed very low levels of mCherry (as these cells exhibit extremely high N/C, and are outliers).

The cytoplasmic signal above or below the nucleus contributes to the apparent “nuclear” signal (see section 4.2). To correct for this bleed-through, a baseline is established by linear regression analysis of nuclear vs. cytoplasmic signals of untreated AID-mCherry transductants (Figure 4-1.B). Each HCS-nuclear reading is adjusted by subtracting the corresponding baseline value from the HCS-nuclear reading. The baseline determined for the untreated AID-mCherry transductants is used to adjust the HCS-nuclear readings in the treated AID-mCherry populations (Chapter 4); AID-mCherry, AID-mCherry-Cdt1, and AID-mCherry-Gem (Chapter 5); and F193A mutants (Chapter 6).

I distinguished G1, S, and G2/M phase cells by DNA content as determined by total DAPI signal. DNA content was ranked and specific fractions of the population assigned to G1, S and G2/M phases in untreated control groups (Figure 4-4.A). Cell cycle profiles of treatment groups were scaled to untreated groups, ranked, and then assigned to respective populations.

Assays of surface IgM loss and cell cycle

To determine fractions of sIgM⁻ cells, at day 3, 7 and 11 post sort, 2 to 5x10⁵ cells were fixed in 3.7% formaldehyde and stained with anti-human IgM (1:500, Southern Biotech Cat# 2022-02), and sIgM⁻ variants quantified by FACS as described [81 Sale, 1998 #848]. To determine cell cycle, cells were stained with DAPI (2 µg/ml) and analyzed by FACS.

Single-cell PCR and Sequencing

Single mCherry⁺ cells expressing AID-mCh, AID-mCh-Cdt1 or AID-mCh-Gem were sorted into 96-well PCR plate containing 20 µl of PCR buffer per well (1 cell/well) at day 7 post sort. Samples were frozen, thawed, and treated with 250 µg/ml proteinase K for 1 hour at 50°C and 5 min at 95°C. The rearranged V_H region was amplified by nested PCR using high-fidelity PfuTurbo DNA polymerase (Stratagene) and first round primers, RV_HFOR QL 5'-TCCCAGGTGCAGCTACAGCAG-3' and JOL48 QL 5'-GTACCTGAGGAGACGGTGACC-3' [81 Sale, 1998 #848] and second round primers, 5'-AGGTGCAGCTACAGCAGTG-3' and 5'-GCCCCAGACGTCCATACC-3'. Predicted sizes of PCR products were confirmed by gel electrophoresis and fragments purified and sequenced.

Chapter 3: AID undergoes nuclear pulses in both Ramos B cells and HT1080 fibroblasts

3.1. Introduction

AID is predominately localized to the cytoplasm and undergoes ubiquitin-dependent proteolysis following entry into the nucleus [70]. This raised the question of whether nuclear levels of AID are constant or oscillate. Oscillation in protein levels represent a compromise between maintenance of a protein at a constant level that is either too low to be useful, or too high to be tolerated, and allow a protein to respond to multiple positive and negative regulatory signals via a feedback loop [82]. Two well-known examples of proteins characterized by oscillating rather than steady state nuclear levels are NF- κ B [83] and p53 [82,84].

To ask if nuclear levels of AID are constant or oscillate, I used live cell imaging to monitor AID in real time in HT1080 human fibroblasts expressing AID-GFP or AID-mCherry. These adherent cells will form stable contacts with a slide and can be imaged for extended periods of time, in contrast to B cells, which tend to tumble during imaging, making it very difficult to track an individual cell for more than 1-2 hr.

3.2. GFP and mCherry tags do not affect AID subcellular localization in HT1080 cells

I first began by using live cell imaging to determine when in the cell cycle AID-GFP or AID-mCherry accumulates in the nucleus, using HT1080 stable transfectants expressing cell cycle-specific reporters mKO2-Cdt1 (G1) or mAG-Gem (S/G2/M), respectively [85] to assign cell cycle phase (Figure 3-1.A). mKO2-Cdt1 and mAG-GEM are fluorescent proteins. I chose to study AID-GFP and AID-mCherry as the GFP and mCherry signals can be readily distinguished from the mKO2 and mAg tags, respectively. I carried out control experiments to verify that AID-

GFP localizes predominately to the cytoplasm and accumulates in the nucleus after treatment with Leptomycin B (LMB), which blocks the CRM-1-mediated nuclear export pathway (Figure 3-2.A). By comparing AID-GFP and AID-mCherry sub-cellular localization, I showed that both localize to the nucleus after brief LMB treatment, (Figure 3-2.B). Lastly, I confirmed that the cell cycle reporters exhibit correct temporal regulation (Figure 3-1.A), and that the mKO2-Cdt1 (G1) and mAG-Gem (S/G2) reporters do not affect normal cell division and cell cycle (Figure 3-1.B).

3.3. Nuclear AID levels oscillate in HT1080 fibroblasts

By live cell imaging of HT1080 fibroblasts expressing AID-GFP/mKO2-Cdt1, I showed that AID-GFP is not continually evident in the nucleus, but accumulates in transient pulses or bursts (Figure 3-3.A, movie in supplemental file), each of which is brief in duration (average 35 ± 2 minutes; N=70). Similar results were observed by analysis of HT1080 expressing AID-mCherry (movie in supplemental file).

I analyzed HT1080 cells expressing the mKO2-Cdt1 reporter (red signal in G1 phase cells) to determine whether oscillation in the AID-GFP signal was evident in G1 phase cells. In an asynchronous culture, approximately half of G1 phase cells exhibited one or more nuclear AID-GFP pulses (Figure 3-3.B).

3.4. Nuclear oscillation does not depend upon Thr-27 or Ser-38 phosphorylation

Mechanisms that confer oscillatory regulation on AID may depend upon recognition of AID by another factor. Since phosphorylation is well known to change interactions with other factors, I tested the possibility that phosphorylation of Thr-27 or Ser-38 might be critical to the

oscillation. Mutation of each of these residues (T27A and S38A) to alanine has been shown to decrease CSR and SHM [65,68]. AID also phosphorylates three additional sites, Ser-3 [63], Thr-140 [86], Tyr-184 [69]. Mutation of Ser-3 and Thr-140 to alanine has shown to affect AID activity [63,86], however the role of phosphorylation of AID at Tyr-184 remains to be determined [69]. I found that the nuclear pulse is not affected by mutation of two of these sites (Thr-27 and Ser-38) to alanine (Figure 3-4).

3.5. Nuclear AID levels oscillate in Ramos B cells

B cells are difficult to document over the course of cell cycle because they are non-adherent and tend to tumble along the slide and out of the field during prolonged imaging. This may be addressed by using slides specially fabricated to contain multiple 20 μ wells, with one B cell/well. By live cell imaging of AID-mCherry-expressing Ramos B cells in such culture wells, I have shown that the AID-mCherry is not continually evident in the nucleus, but accumulates in short pulses, similar to in HT1080 fibroblasts (Figure 3-5, movie in supplemental file).

3.6. Discussion

I have shown that nuclear levels of AID oscillate in both HT1080 fibroblasts and in Ramos B cells. These results suggest that cells may limit the mutagenic potential of AID by restricting the duration of its residence in the nucleus. It is very likely that other factors contribute to regulating the duration and amplitude of these oscillations, by providing either positive or negative feedback. For example, the DNA damage response may measure mutagenesis, and provide feedback when mutation levels are too high or too low. Since oscillatory regulation is not B cell-specific, but also evident in fibroblasts, the regulatory

pathway may apply not only to mutagenesis by AID that promotes Ig gene diversification, but also to AID activity in reprogramming.

Allotment of only a short time for AID to act could both limit mutagenesis and also may allow time for the genome to recover from AID deamination after each pulse. The activity of AID may depend on the number, duration, and nuclear level of the pulses, which may also determine the balance between Ig gene diversification and genomic instability.

An oscillating nuclear signal similar to that of AID has been reported for nuclear factor kappa B (NF- κ B), a transcription factor involved in the regulation of cell division, apoptosis, and inflammation [83,84,87]. In unstimulated cells, inhibitor kappa B (I κ B) binds to NF- κ B and sequester it in the cytoplasm. Upon stimulation, phosphorylation and subsequent proteosomal degradation of I κ B allows NF- κ B to be translocated into the nucleus, and to activate transcription of many genes including I κ B. The transcription of I κ B leads to re-sequestration of NF- κ B, and the delay in the negative feedback loop drives the nuclear pulses [84]. Analogous regulation of AID or any of its interacting partners may control AID nuclear pulses. Future studies will address this by comparing the number, duration, and nuclear level of the pulses between different phases of cell cycle and between HT1080 and Ramos B cells at each stage of cell cycle.

Possible mechanisms regulating AID nuclear localization and its brief nuclear pulses include import, rapid nuclear export, or rapid nuclear degradation. I asked how these mechanisms are regulated during the course of the cell cycle (Chapter 4), and whether the cell cycle-dependence of AID localization and activity relate to function (Chapters 5 & 6).

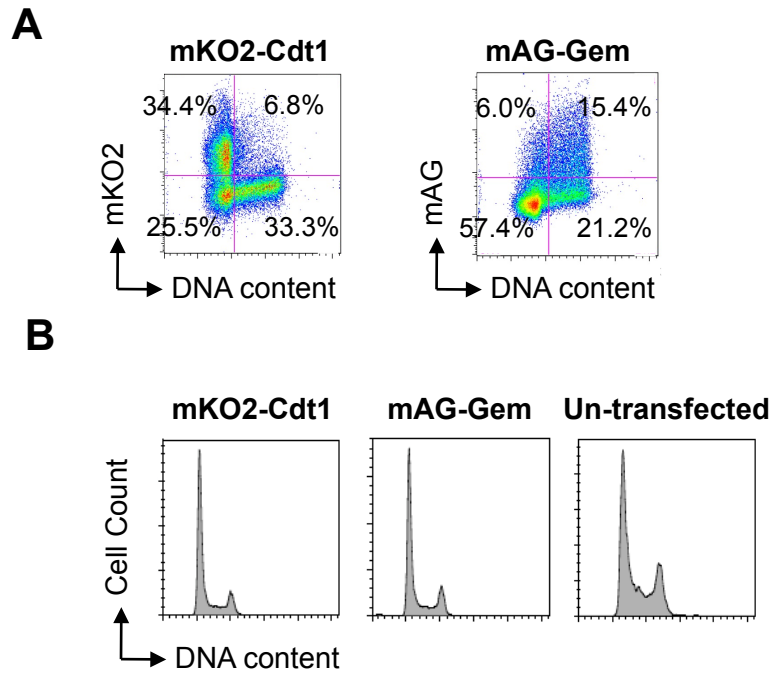


Figure 3-1. Cell cycle reporters to document cell cycle progression of HT1080 fibroblasts.

HT1080 cells were transfected with either the mKO2-Cdt1 or mAG-Gem construct and grown in selection media for stable transfectants. Clonal populations were isolated and expanded. FACS plots of the mKO2-Cdt1 (left) and mAG-Gem (right) transfectants by representative clones are shown, with percentages of G1/mKO+ or G1/mAG+ (top left quadrant); G1/mKO- or G1/mAG- (bottom left quadrant); S/G2/M/mKO+ or S/G2/M/mAG+ (top right quadrant); and S/G2/M/mKO- or S/G2/M/mAG- (bottom right quadrant).

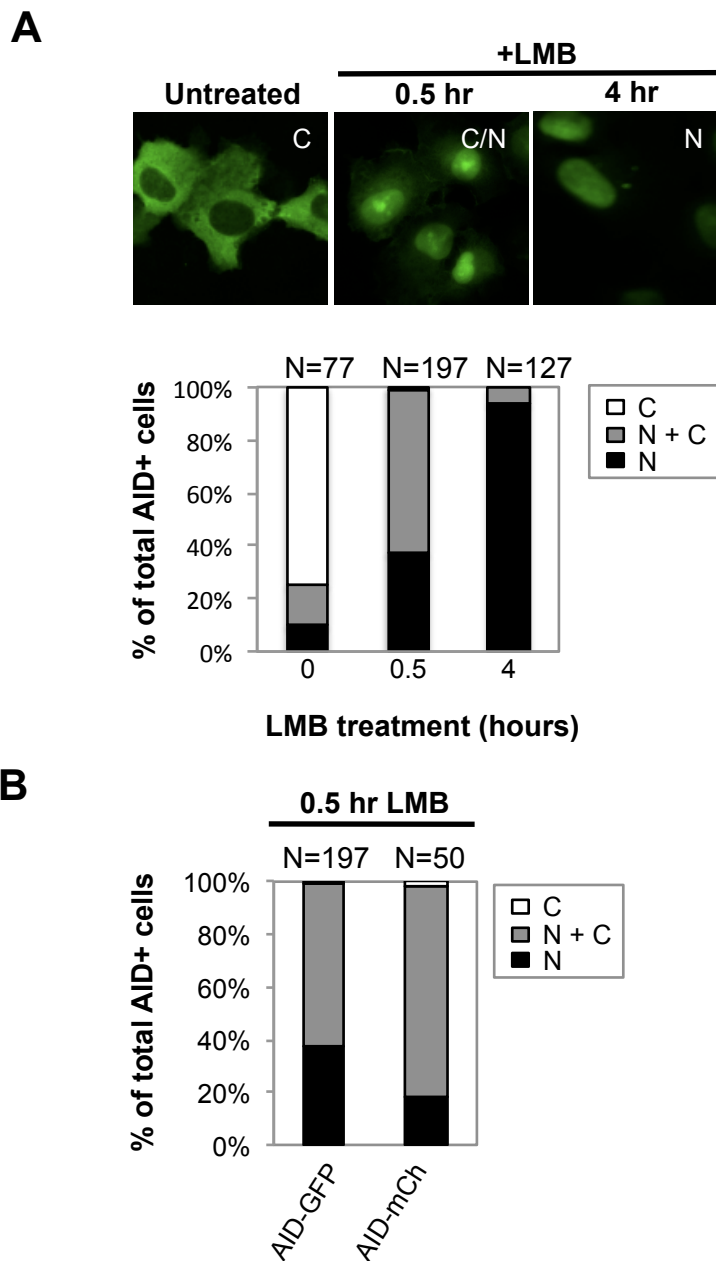


Figure 3-2. Effects of GFP and mCherry tags on AID sub-cellular localization in HT1080 fibroblasts.

A. Immunofluorescence microscopy of HT1080 cells transiently expressing AID-GFP. Top, in untreated cells (left), AID resides in the cytoplasm in majority of the AID-GFP positive cells,

whereas, AID accumulates in the nucleus after 0.5hour and 4hour of LMB treatment (middle and right). Bottom, quantification of AID-GFP subcellular localization in HT1080 cells. Cells expressing AID-GFP were scored for strictly nuclear (black), strictly cytoplasmic (white) or nuclear + cytoplasmic (grey) AID-GFP sub-cellular localization, in the absence of treatment or following treatment with LMB.

B. Comparison between AID-GFP and AID-mCherry sub-cellular localization after 0.5 hour of LMB treatment. Bar graph shows the quantification of cells expressing AID-GFP or AID-mCherry that were scored for strictly nuclear (black), strictly cytoplasmic (white) or nuclear + cytoplasmic (grey) sub-cellular localization

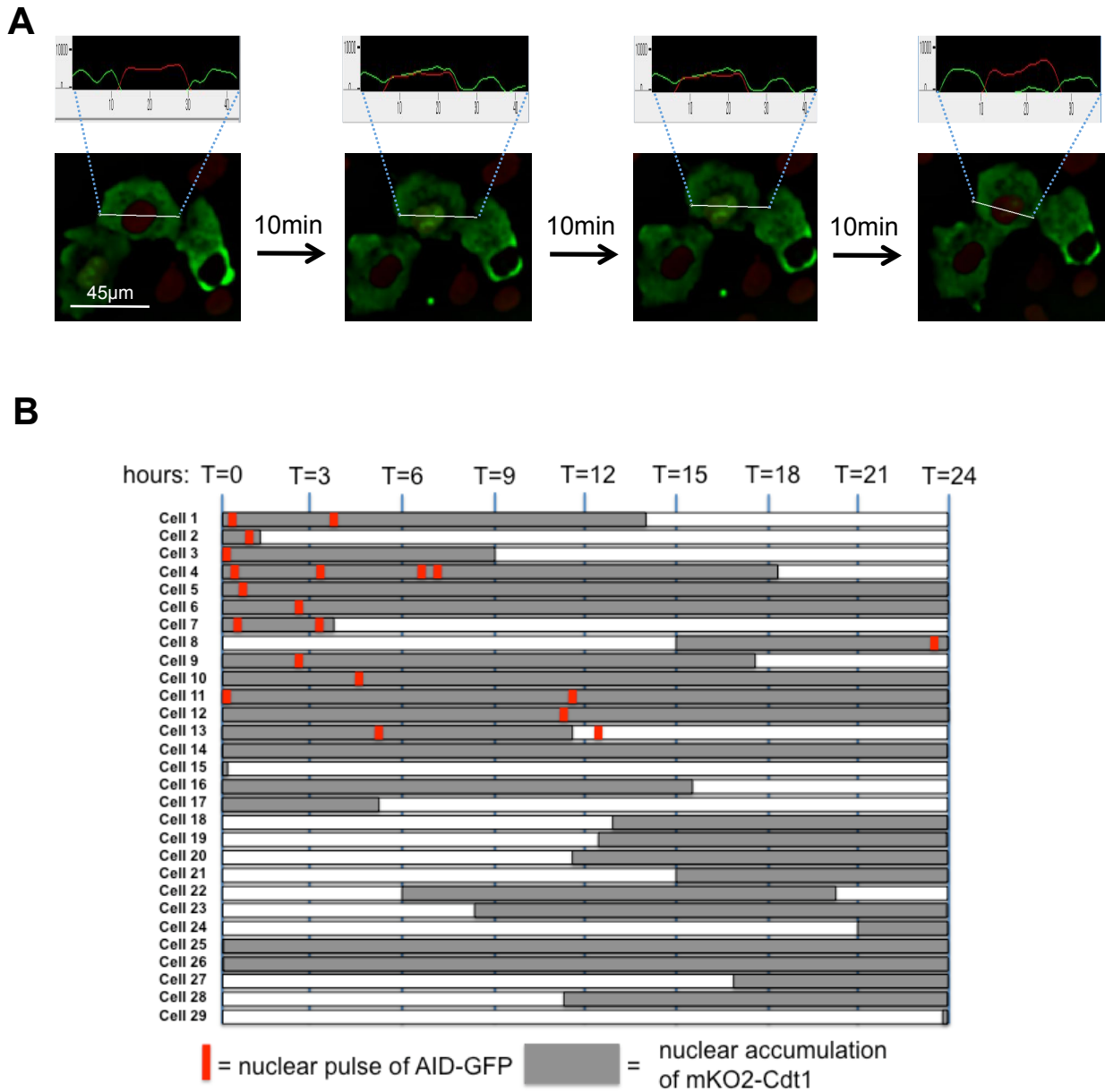


Figure 3-3. Nuclear pulses of AID-GFP in HT1080 cells.

A. Live cell imaging of HT1080 cells expressing both AID-GFP (green) and the mKO2-Cdt1 cell cycle reporter (red). Shown is the nuclear AID-GFP pulse evident in four frames (40 min) from a time-lapse movie of cells imaged every 10 minutes for a total duration of 24 hours. Above each frame, a plot profile shows the fluorescence intensities of AID-GFP (green) and the mKO2-Cdt1 cell cycle reporter (red) along an arbitrary white line.

B. Summary of AID-GFP nuclear pulses in individual HT1080 cells ($n = 29$). Gray bar represents nuclear accumulation of the mKO2-Cdt1 cell cycle reporter; red hatch mark represents each nuclear pulse of AID-GFP.

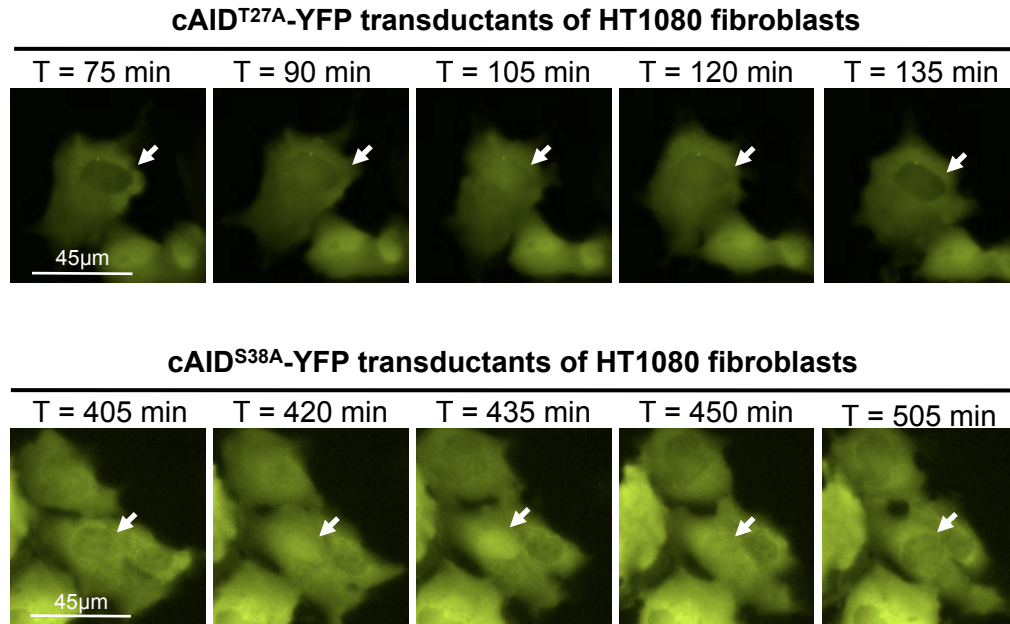


Figure 3-4. Nuclear pulses of AID^{T27A}-YFP and AID^{S38A}-YFP in HT1080 fibroblasts.

Live cell imaging of HT1080 cells expressing either YFP-tagged chicken AID (cAID^{T27A}-YFP or cAID^{S38A}-YFP). Cells were imaged every 15 minutes for a total duration of 24 hours. Top, a nuclear pulse of AID^{T27A}-YFP was documented in the interval 75 min to 135 min of the time lapse movie. Bottom, a nuclear pulse of AID^{S38A}-YFP was documented in the interval 405 min to 505 min of the time lapse movie.

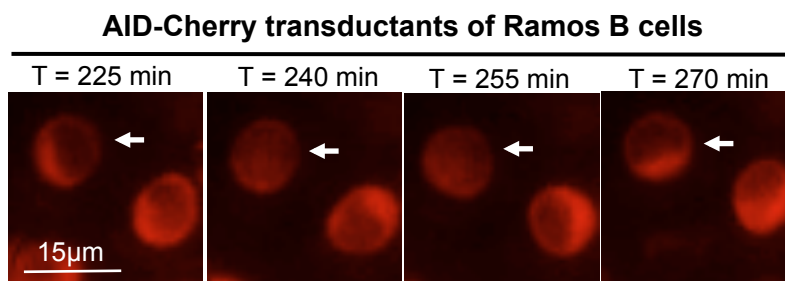


Figure 3-5. Nuclear pulse of AID-mCherry in Ramos B cells.

Live cell imaging of Ramos B cells expressing AID-mCherry. Cells were imaged every 15 minutes for a total duration of 24 hours. A nuclear pulse of AID-mCherry was documented in the interval 225 min to 270 min of the time lapse movie.

Chapter 4: Cell cycle-dependent nuclear stability of AID in Ramos B cells

4.1. Introduction

AID resides in the cytoplasm as part of a large complex [88]. AID retention in the cytoplasm and entry into the nucleus is regulated by phosphorylation [66,67] and by interactions with other proteins, including the initiation factor eEF1A, which actively retains AID in the cytoplasm; the chaperones HSP 40 and HSP 90 [71,88,89,90]; the spliceosome-associated factor CTNBL1 [91]; and the germinal center-associated nuclear protein (GNAP) [92]. The CRM1-dependent nuclear export pathway recognizes a nuclear export signal (NES) in the AID C-terminal and limits the mutator activity of AID by actively exporting it from the nucleus [73]. Proteosomal degradation also limits the half-life of AID [70].

AID may also be subject to cell cycle-dependent regulation. By single-cell imaging of chicken DT40 B cells, our laboratory previously showed that AID is enriched in the nucleus in G1 phase relative to S phase cells [80]. Pol η , the polymerase that carries out the repair step in AID-initiated gene conversion, co-localizes with the rearranged and diversifying Ig λ_R allele predominately in G1 phase [80], suggesting that gene conversion may occur largely within this phase of cell cycle. The point mutations and double-strand breaks (DSBs) characteristic of AID-initiated SHM and CSR are evident in G1 phase cells [59,78,79].

AID acts preferentially on single-stranded DNA [16,17,18,29]. This raises the possibility that restriction of AID activity to G1 phase may prevent its access to regions that become transiently single-stranded during replication in S phase, and thereby limit genomic instability. However, regulation of AID in the course of cell cycle has not been systematically studied. Here

I have asked how AID nuclear stability is regulated in the course of cell cycle in human Ramos B cells.

4.2. HCS-analysis of AID sub-cellular localization in Ramos B cells

To assess AID sub-cellular localization, I chose to study regulation in Ramos B cells. These cells derive from a Burkitt lymphoma, express AID and IgM antibodies (IgM+), and they carry out active somatic hypermutation of their endogenous rearranged IgM gene. Ongoing Ig gene diversification means that the pathways that regulate, respond to and repair AID-initiated damage are maintained in these cells.

I fused the fluorescent protein mCherry to the C-terminus of AID, cloned this into a lentiviral expression vector and delivered the AID-mCherry expression construct into Ramos B cells via transduction. Expression of AID-mCherry affects proliferation only slightly (Figure 4-8.B).

I analyzed cells using high content screening (HCS) microscopy, a high through-put approach to determine protein levels in defined cellular compartments in individual cells [93]. Cells were stained with DAPI and HCS-cell mask (whole cell stain) to define the nucleus and cell perimeter, respectively; and the cytoplasm, which is the region between the nuclear and cell boundaries (Figure 4-1.A). Total intensities of nuclear and cytoplasmic AID-mCherry signals are automatically measured in each cell by HCS-cell analysis software. To ensure that the AID-mCherry level in each compartment is independent of nuclear and cell size, which increases with DNA content (Figure 4-4.B), the average intensity (total intensity/area) in each compartment is quantified. To assess the nuclear accumulation of AID, the ratio of average intensities of nuclear and cytoplasmic AID-mCherry (N/C) is calculated.

AID is exported from the nucleus via a pathway that depends upon CRM1, and treatment with the drug Leptomycin B (LMB) inhibits this pathway and traps AID in the nucleus [74,89]. Representative images fluorescent images of single cells, either untreated or treated with LMB for 0.5 hr (Figure 4-1.A) illustrate nuclear accumulation of AID after LMB treatment. The N/C ratios for the untreated and treated cells shown are 0.80 and 1.34, evidence of nuclear accumulation of AID-mCherry.

AID localizes predominately to the cytoplasm [74,89]. Based on the population analysis of sub-cellular localization, I found that AID-mCherry was predominately cytoplasmic in untreated Ramos B cells (average N/C = 0.81, Figure 4-1.B). However, nuclear AID-mCherry signals with increased linearly with increasing cytoplasmic signals (slope of linear regression = 0.68, Pearson coefficient = 0.87, Figure 4-1.B), suggesting that cytoplasmic signal from above or below the nucleus contributed to an apparent “nuclear” signal. Analysis by confocal microscopy confirmed this, showing that AID-mCherry was mostly absent from the nucleus when out-of-focus signal was eliminated, regardless of the level of cytoplasmic signal (Figure 4-2.A).

To distinguish actual nuclear signal from apparent nuclear signal due to bleed-through from cytoplasm AID-mCherry, I first determined the baseline signal by linear regression analysis of nuclear vs cytoplasmic signals (Figure 4-1.B). The nuclear AID-mCherry signal from each cell was then adjusted by subtracting the corresponding baseline value. Comparison between distributions of adjusted nuclear AID-mCherry levels in an untreated population (average=0) versus LMB-treated population (0.5 hr, average=98.8) confirmed that nuclear AID-mCherry levels increased upon LMB treatment (Figure 4-2.B).

4.3. Nuclear AID, but not cytoplasmic AID, is subject to ubiquitin-dependent proteolysis in Ramos B cells

To ask if AID is subject to ubiquitin-dependent proteolysis, I compared AID-mCherry signals in untreated cells and cells treated with MG132, an inhibitor of the ubiquitin-dependent 26S proteasome [70]. I used HCS microscopy to quantify the nuclear and cytoplasmic AID-mCherry signals in populations of untreated cells and cells treated for 0.5 to 4 hr with MG132. Treatment with MG132 did not affect nuclear and cytoplasmic AID-mCherry signals, as populations at different time points overlapped well with untreated population (Figure 4-3.A, left). Average nuclear and cytoplasmic AID-mCherry signals and the N/C ratio remained constant (Figure 4-3.B).

I then assayed the effect of treatment with LMB (Figure 4-3). Treatment with LMB alone caused a rapid accumulation of AID-mCherry in the nucleus, as evidenced by the ~1.5-fold increase in the average nuclear AID-mCherry signal at 1 hr post-treatment, accompanied by a corresponding decrease in cytoplasmic signal to one half that in untreated cells (Figure 4-3.B). At 1 hr following LMB treatment, the average nuclear AID-mCherry signal rapidly declined, and by 4 hr, the signal was below that in untreated cells, suggesting that nuclear AID is degraded. The average N/C ratio, on the other hand, increased rapidly immediately following treatment with LMB (0-1 hr) and was relatively unchanged at 2 and 4 hr post-treatment (Figure 4-3.B, right). The initial increase in LMB-treated cells reflect nuclear influx of AID-mCherry that is not compensated by normal nuclear export. The observation that the AID-mCherry signal was unchanged from 2-4 hr suggested that nuclear AID-mCherry may be destabilized.

I compared stability of nuclear and cytoplasmic AID-mCherry by treating cells with both LMB + MG132 (Figure 4-3). This resulted in an ~1.7-fold increase in the average nuclear AID-

mCherry signal at 1 hr post-treatment. The signal was unchanged thereafter (2-4 hr). This contrasts with the decrease in nuclear AID-mCherry signal in cells treated with LMB alone (Figure 4-3.B, left), and shows that the ubiquitin-dependent proteolysis destabilizes nuclear AID.

Treatment with LMB + MG132 caused a rapid decrease in cytoplasmic AID-mCherry level within 1 hr of treatment, similar to treatment with LMB alone. At 2 and 4 hr post-treatment, the cytoplasmic signal in cells treated LMB + MG132 was only slightly higher than that in cells treated with LMB alone (Figure 4-3.B, middle). Thus, cytoplasmic AID-mCherry is not affected by MG132.

These results show that nuclear AID, but not cytoplasmic AID, is rapidly degraded by ubiquitin-dependent proteolysis. Evidence of nuclear destabilization of AID has also been provided by comparison of nuclear and cytoplasmic fractions of tagged AID from cell extracts from the human Burkitt lymphoma line, BL2, and activated primary murine B cells [70]. The average N/C ratios in cells treated with LMB + MG132 or LMB alone were 3.1 and 2.4, respectively at 2 hr post-treatment, suggesting nuclear AID is destabilized by ubiquitin-dependent proteolysis (Figure 4-3.B, right).

As described in Section 4.2, cytoplasmic signal can contribute to apparent nuclear signal as determined by HCS. I therefore also determined the adjusted nuclear AID-mCherry level as described in section 4.2, for cells treated with MG132, LMB, or LMB + MG132 (Figure 4-3.C). Treatment with MG132 did not affect the average adjusted nuclear AID-mCherry level. In cells treated with LMB, the average adjusted level increased rapidly within 1 hr of treatment, followed by a rapid decrease through 4 hr post-treatment to approximately 50%, as similarly observed without the adjustment for bleed-through (Figure 4-3.C). However, the signal from LMB-treated cells remained above the baseline of the signal from untreated cells, suggesting that AID-

mCherry was not completely degraded during the 4 hr period in which it accumulated in the nucleus and was unable to exit. Treatment with LMB + MG132 caused an increase in signal by 230 units above baseline to a level that remained constant at 2 and 4 hr post-treatment, as was observed without adjustment for bleed-through (Figure 4-3.C).

4.4. AID is predominately cytoplasmic in all phases of cell cycle in Ramos B cells

To evaluate the sub-cellular localization of AID throughout the course of the cell cycle in Ramos B cells, I first assigned G1, S, and G2/M phase cells by DNA content (total DAPI signal). DNA content was ranked and assigned to G1, S and G2/M phases (see Material and Methods, Figure 4-4.A).

Nuclear, cytoplasmic and whole cell area increase in the course of the cell cycle, as did total and average AID-mCherry signals in each compartment (Figure 4-4.B & 4-4.C). However, the average N/C ratios were nearly identical in G1, S and G2/M phase cells (0.80, 0.80 and 0.84, respectively, Figure 4-4.D). This is consistent with previous observations that AID mainly resides in the cytoplasm in almost all cells [74,89].

Cytoplasmic localization reflects the dynamic nuclear-cytoplasmic shuttling of AID with a net balance in favor of nuclear export [74,89]. Nonetheless, the rates of nuclear entry and export may differ during the course of the cell cycle. I determined the rate of nuclear entry in cells treated with LMB + MG132, to ensure that the nuclear level was unaffected by protein degradation. Relative rates were calculated by determining the slope of the best-fit line of the average nuclear signals within 0 - 1 hr of LMB + MG132 treatment for G1, S and G2/M phase, and expressed as relative to G1 phase (Figure 4-5.C). I found that the rates of entry were similar

in G1 phase and S phase cells, and slightly lower in G2/M compared to in G1 phase. Future studies will assess the rate of export in each phase of cell cycle.

4.5. Nuclear AID is degraded more slowly in G1 phase than S/G2/M phase in Ramos B cells

To ascertain whether destabilization of AID is cell cycle-dependent, I utilized HCS to quantify nuclear and cytoplasmic levels of AID-mCherry in each phase of cell cycle in untreated Ramos B cells and cells treated with MG132, LMB or MG132 + LMB. Control experiments verified that treatments with MG132, LMB or MG132 + LMB extending up to 4 hr did not perturb the cell cycle (Figure 4-6). Treatment with MG132 did not significantly change the nuclear or cytoplasmic AID-mCherry levels in G1, S or G2/M phase cells (Figure 4-5.A, left). Treatment with LMB caused an increase in nuclear AID-mCherry level within 0-1 hr in each phase of the cell cycle, followed by a decrease from 1-4 hr of treatment (Figure 4-5.A, middle). At 4 hr post-treatment, the nuclear AID-mCherry signal had dropped to 89% (G1), 73% (S), and 66% (G2/M) of that in G1, S and G2/M untreated cells, respectively. Treatment with LMB caused the cytoplasmic AID-mCherry signal to decrease rapidly by 1 hr post-treatment, and continue to decrease more slowly thereafter, in all phases of cell cycle. The average cytoplasmic AID-mCherry signals in G1 phase cells appeared to be slightly higher than in S and G2/M phase cells at 2-4 hr post-treatment. LMB+MG132 treatment caused the nuclear AID-mCherry signal to increase rapidly and then plateau, accompanied by a rapid decrease and then plateau in cytoplasmic AID-mCherry signal, in all phases of cell cycle (Figure 4-5.A, right). Thus, degradation followed nuclear import. In cells treated either with LMB alone or with LMB + MG132, both nuclear and cytoplasmic AID-mCherry levels were higher in G1 phase compared to S and G2/M phase cells in the period from 2-4 hr post-treatment.

Comparison of the slopes of the LMB response curves at 1-2 hr post-treatment suggested that the rate at which nuclear AID is initially degraded differs in different phases of cell cycle. I calculated the relative rates of nuclear degradation by determining the rate at which the average nuclear AID-mCherry signal decreased between 1-2 hr of treatment, as defined by the slope of the line. Population average rates were determined for 4 independent experiments, and expressed relative to rates in G1 phase. This showed that rates of initial degradation were 1.56-fold and 1.54-fold higher in S and G2/M phase, respectively, than in G1 phase ($p=0.02$ and $p=0.03$, Figure 4-5.B). Thus nuclear degradation of AID-mCherry occurs significantly more slowly in G1 phase than in S or G2/M phase cells.

4.6. Ubiquitin-dependent proteolysis does not depend on Ser-3, Thr-27, Ser-38, or Thr-140 phosphorylation in Ramos B cells

Phosphorylation is known to regulate ubiquitination and subsequent degradation. To ask if phosphorylation regulates nuclear degradation of AID, I assessed the effect of mutation of each of the known phosphorylation sites of AID (Ser-3, Thr-27, Ser-38, Thr-140 or Tyr-184) to alanine on the kinetic of response of nuclear AID following LMB treatment.

I transduced Ramos B cells with lentivirus bearing AID-mCherry, AID^{S3A}-mCherry, AID^{T27A}-mCherry, AID^{S38A}-mCherry, AID^{T140A}-mCherry, or AID^{Y184A}-mCherry expression constructs, and sorted mCherry(+) cells at day 3 post-transduction. Cells were treated with LMB for 0-5 hr; and nuclear signals were quantified by HCS-microscopy.

HCS analysis showed that most of the phosphorylation mutants respond to LMB similarly as WT. Nuclear signals of AID^{S3A}-mCherry and AID^{S38A}-mCherry were nearly identical to that of AID-mCherry 0-5 hr post-treatment (Figure 4-7). In contrast, nuclear

AID^{T27A}-mCherry and AID^{T140A}-mCherry signals were slightly below that of nuclear AID-mCherry signal at 1-4 hr post treatment. Nuclear signal of AID^{Y184A}-mCherry increased within 0-1 hr of treatment to a level that is higher than nuclear AID-mCherry signal (1.5-fold vs 1.4-fold of untreated, respectively; Figure 4-7). Interestingly, nuclear AID^{Y184A}-mCherry signal decreased more rapidly within 1-2 hr of treatment than the nuclear signal AID-mCherry, indicating that phosphorylation of AID on Y184 might increase AID protein stability in the nucleus.

4.7. The AID^{F193A} mutation prevents nuclear export, resulting in altered subcellular localization accompanied by decreased protein levels in Ramos B cells

AID carries a nuclear export signal (NES) and is exported from the nucleus by the CRM1-mediated pathway. To ask how nuclear export contributes to the regulation of AID subcellular localization, I took advantage of the ability of a point mutation in the NES to block export [76,77]. AID bearing the F193A mutation is not transported by the CRM1-dependent export pathway and localizes spontaneously to the nucleus. I compared mCherry signals in the nucleus and cytoplasm of AID^{F193A}-mCherry and control AID-mCherry transductants. Cells were transduced with a retroviral expression construct, mCherry+ transductants sorted 3 days later, and mCherry signal analyzed by flow cytometry at 7 days after sorting. Cell cycle profiling by flow cytometry (Figure 4-8.A) showed that the fraction of cells exhibiting an mCherry signal was reduced in AID^{F193A}-mCherry relative to control AID-mCherry transductants in G1 phase (29.4% vs. 43.7%) and S/G2/M phases (28.7% vs. 47.8%).

Fluorescence microscopy showed that AID^{F193A}-mCherry was localized to the nucleus. HCS analysis illustrated that the NES mutation not only conferred altered subcellular localization,

as predicted, but also caused a decrease in mCherry signal in both the nucleus and cytoplasm. In AID^{F193A}-mCherry transductants, the average total, apparent nuclear and cytoplasmic signals were 111, 142 and 63 units respectively, and the N/C ratio was 2.3 (Table 4-3). In contrast, in AID-mCherry transductants, the average total, apparent nuclear and cytoplasmic signals were 444, 424 and 474 units respectively, and the N/C ratio was 0.9 (Table 4-3).

4.8. Nuclear AID is subjected to proteosomal degradation during the course of cell cycle in HT1080 fibroblasts

To address whether HT1080 cells exhibited spatiotemporal regulation of AID comparable to that demonstrated for Ramos B cells, I transduced HT1080 cells with lenti virus carrying AID-mCherry construct and sorted mCherry + cells 3 days post transduction. I used HCS to quantify the nuclear and cytoplasmic AID-mCherry levels in populations of untreated cells and cells treated with MG132, LMB or LMB + MG132 for 0-5 hr. HCS analysis showed that the nuclear and cytoplasmic signals were both unaffected by MG132 treatment as populations treated with MG132 for 0.5 to 5 hr overlapped well with untreated population (Figure 4-9.A, left). Treatment with LMB alone resulted in a rapid 1.7-fold increase in nuclear signal at early times post-treatment (up to 2 hr), followed by a rapid decline (Figure 4-9.B, left; blue line). The cytoplasmic signal was reduced to one-third the level in untreated cells in response to treatment with LMB alone (Figure 4-9.B, middle; blue line). Treatment with both LMB + MG132 resulted in a 2.6-fold increase in nuclear signal (Figure 4-9.B, left; green line), and reduced the cytoplasmic signal, although somewhat less (Figure 4-9.B, middle, green line).

To quantify nuclear accumulation, we calculated the ratio of average nuclear and cytoplasmic AID-mCherry signals (N/C). This ratio was 0.9 for untreated cells, and was

unaffected by treatment with MG132 (Figure 4-9.B, right; orange line). Treatment with LMB resulted in a rapid increase in this ratio, to 1.9; and treatment with LMB + MG132 resulted in an even more dramatic increase, to 2.5 (Figure 4-9.B, right). These results show that nuclear AID is destabilized by ubiquitin-dependent proteolysis in HT1080 cells, as it is in Ramos B cells.

I then determined AID-mCherry signals in G1, S and G2/M phase cells. The N/C ratio was essentially identical in G1, S and G2/M phase cells (0.89, 0.88 and 0.90, respectively, data not shown). MG132 treatment has no effect on nuclear and cytoplasmic AID-mCherry signals during the course of the cell cycle (Figure 4-10.A, left). LMB + MG132 treatment caused an increase in nuclear signal to a level approximately 2.6-fold of untreated at 5 hr post-treatment in G1 phase, 2.5-fold in S, and 2.6-fold in G2/M phase cells (Figure 4-10.A, right). LMB + MG132 treatment caused a slight decrease in cytoplasmic level in all phases of the cell cycle (Figure 4-10.A, right).

In G1 phase cells, a rapid increase in AID-mCherry signal was evident within 1 hr post-treatment with LMB, followed by a slight decrease (1-2 hr) and then a rapid decrease (2-5 hr). A similar pattern was evident in G2/M phase cells. In S phase cells, an initial rapid increase in AID-mCherry signal was evident within 1 hr post-treatment with LMB, and degradation did not ensue immediately, but only at 2 hr post-treatment. Comparison of the slopes of the LMB response curves between the 2 and 3 hour times points suggested that the initial rates of loss of nuclear AID-mCherry signal were similar, independent of cell cycle stage (Figure 4-10.A, middle). The relative rate of nuclear degradation was approximately 1.15-fold higher in G2/M than in G1 and S phase (Relative rates: 1.00 (G1), 0.96 (S), 1.15(G2/M); Figure 4-10.B).

4.9. Discussion

The experiments described in this Chapter confirm that AID predominately resides in the cytoplasm and accumulates in the nucleus upon inhibition of the CRM1-dependent nuclear export pathway resulted. They provide new evidence that nuclear but not cytoplasmic AID undergoes ubiquitin-dependent proteolysis, and that this is cell cycle-dependent .

Previously cytoplasmic AID was found to be subject to destabilization by proteolysis, but with longer half-life than nuclear AID [70,71,90]. However, our results clearly show that cytoplasmic AID is unaffected by MG132 treatment (Figure 4-3.A, left; Figure 4-3.B, left). Thus, if AID does undergo cytoplasmic degradation, this may occur by a pathway that does not involve the ubiquitin-dependent proteasome that is inhibited by MG132. HSP90 and HSP40 stabilize and retain AID in the cytoplasm have been shown to be important in regulating AID abundance and activity [71,89,90]. Definition of the specific E3 ligases and other factors that regulate AID stability in both cellular compartments should be fertile ground for future studies.

I showed that AID is predominately cytoplasmic in all phases of cell cycle, as predicted given that AID is predominately cytoplasmic in almost all cells in asynchronous cultures. This suggests that the rate of nuclear export exceeds the rate of nuclear entry in each phase of the cell cycle. I further showed that the rates of nuclear entry differ during the course of the cell cycle, with comparable rates evident in G1 and S phases and a slightly slower rate evident in G2/M phase (Figure 4-5.C). It will be of interest to determine whether the rate of nuclear export is modulated during the course of the cell cycle.

I also found that ubiquitin-dependent proteolysis destabilizes nuclear AID in every stage of the cell cycle, albeit more slowly in G1 phase. AID is polyubiquitinated, with 8 lysine residues of AID and an uncharacterized N-terminal motif that are targets of E3 ubiquitin ligases (possibly NEDD4 and RRNF126), which confer the de-stabilization of nuclear AID [70,94,95].

It would be interesting to see whether mutation to anyone or a combination of these lysine residues affect the rate of AID degradation during specific phases of cell cycle. Additionally, REG-gamma, a proteasomal activator, associates with nuclear AID and modulates AID nuclear abundance and activity [77]. This factor may itself be cell cycle regulated or contribute to the cell cycle-dependent regulation of AID nuclear stability.

In addition, I found that AID^{Y184A}-mCherry is degraded faster in response to LMB treatment compare to AID-mCherry. It would be interesting to determine whether phosphorylation of AID at Tyr-184 regulates the cell cycle-dependent nuclear stability of AID.

Cells are sensitive to cytotoxic DNA damage caused by off-target activity of nuclear AID. This is evident from the reduced cell proliferation observed in cells that express the AID^{F193A}-mCherry, which is sequestered to the nucleus, as shown here and by others [76,77]. Hence, nuclear export is important in limiting AID-initiated genomic instability.

The nuclear pool of AID is continually depleted by nuclear export and protein degradation in the nucleus, and replenished by import of protein from the cytoplasm. The AID^{F193A} NES mutation caused a 2-fold reduction in total signal, and a modest but detectable reduction in nuclear AID. The reduction in total and nuclear signals are likely to reflect degradation of AID^{F193A}-mCherry that has been sequestered in the nucleus, where proteolysis occurs. The AID^{F193A} NES mutation caused a 4-fold reduction in cytoplasmic AID. This reduction in cytoplasmic signal exceeds the reduction in total signal, suggesting that nuclear export of AID normally contributes to replenishing the cytoplasmic pool of protein.

I have demonstrated that both nuclear export and proteasomal degradation limit the nuclear stability of AID. It is interesting that even though the AID^{F193A}-mCherry is expressed at a much lower level than AID-mCherry (almost certainly due to ubiquitin-dependent proteolysis,

based on the stabilizing effect of MG132 treatment on nuclear AID), the fact that it cannot be exported out of the nucleus results in toxicity. This suggests that nuclear export not only protects AID from proteosomal degradation in the nucleus, but also protects the genome from mutagenesis, and that proteosomal degradation alone cannot prevent mutagenesis. It would be interesting to ask if a specific loss of AID degradation (by mutating the ubiquitin-ligase targets of AID) further exacerbates the effect of mutation of the NES.

It is interesting that in HT1080 cells, the rate of degradation of nuclear AID is similar between the phases of cell cycle. In Ramos B cells, rate of degradation is slower in G1 phase, which suggests that some component of cell cycle-dependent of nuclear AID is B cell-specific. However, because both HT1080 and Ramos B cells exhibit nuclear pulses of AID, this difference may not be a large contributing factor in oscillatory regulation (see Chapter 3).

The evidence for cell cycle-dependent regulation of nuclear stability of AID that I have presented in this Chapter almost certainly has implications for determining the balance between immunoglobulin diversification and genomic instability. It may also affect AID activity in cancer cells, where cell cycle and damage signaling may be deregulated.

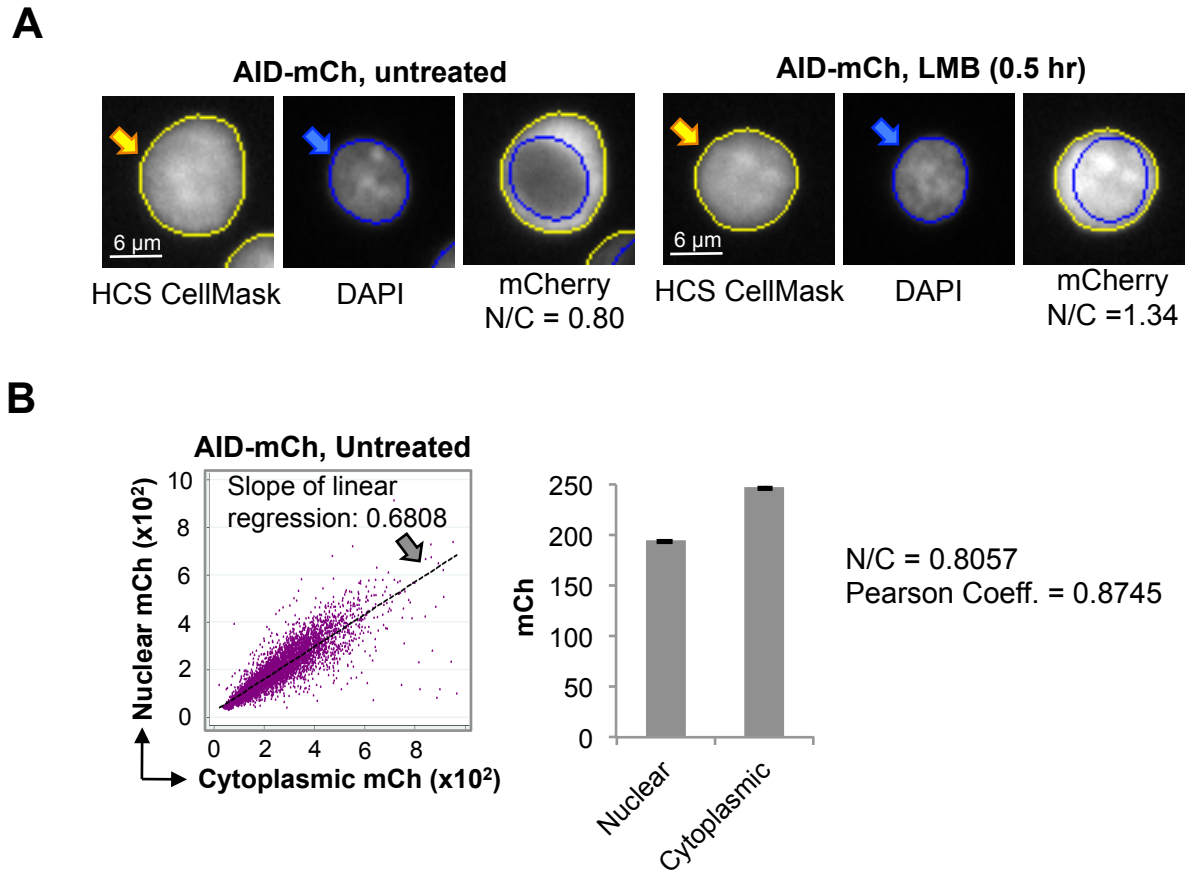


Figure 4-1. Analysis of sub-cellular localization of AID-mCherry in individual Ramos B cells by high content screening (HCS) microscopy.

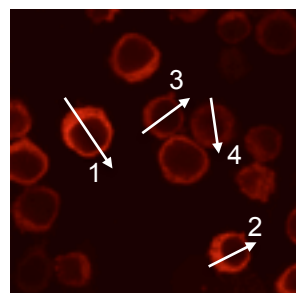
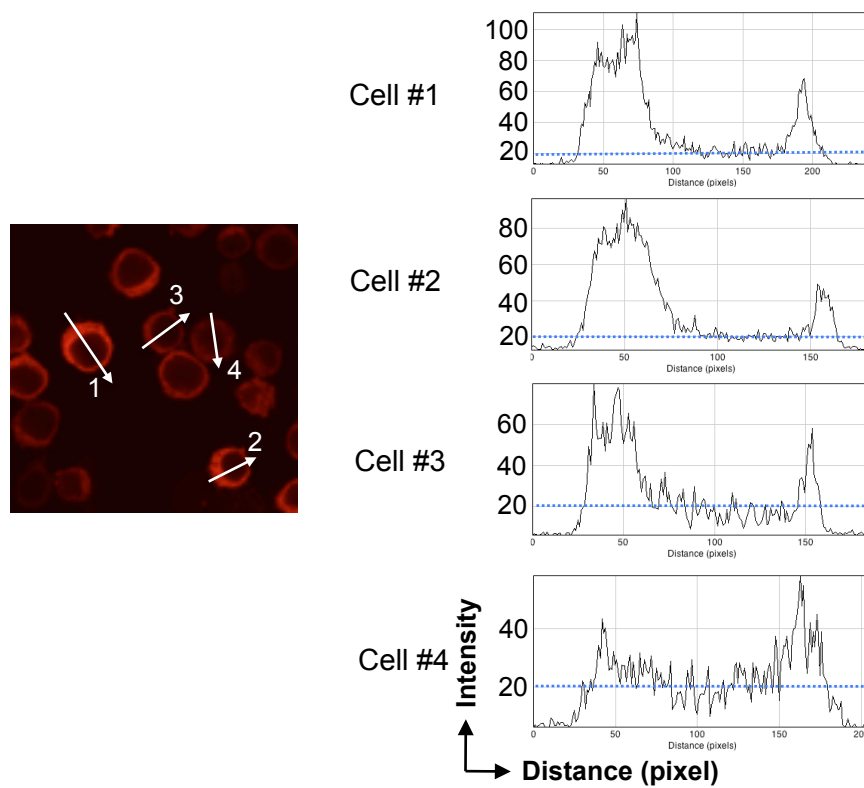
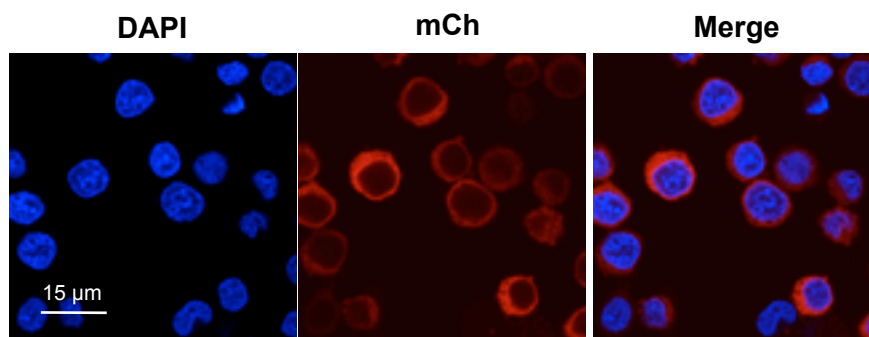
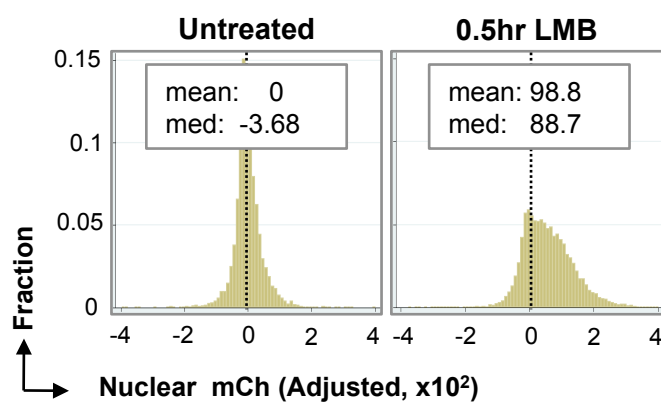
A. Representative examples of AID-mCherry sub-cellular localization quantified by HCS in untreated cells and LMB-treated (0.5 hr) cells. AID-mCherry signal is determined by whole cell boundary, defined by HCS CellMask, yellow line; nuclear boundary, defined by staining with DAPI, blue line. Cytoplasmic AID-mCherry signal is determined by measuring the average mCherry signal intensity (total intensity/area of cytoplasm) within the space between the cell and nuclear boundaries; and nuclear AID-mCherry signal is determined by measuring the average mCherry signal intensity (total intensity/area of nucleus) within the nuclear boundary. The ratio of the nuclear to cytoplasmic signals (N/C) quantifies nuclear accumulation. Typically, AID is cytoplasmic and $N/C < 1$, as in the untreated cell shown on the left; but treatment with LMB inhibits nuclear export of AID, resulting in $N/C > 1$, as in the cell on the right.

B. Population-based assessment of AID sub-cellular localization. Right, scatter plot of nuclear vs. cytoplasmic mCherry signals of AID-mCherry transductants. Dash line represents the slope of linear regression analysis. Left, average nuclear and cytoplasmic signals are quantified and graphed with SEM as black bars. Average nuclear-to-cytoplasmic ratio and the Pearson coefficient are shown.

Figure 4-2. Assessment of nuclear AID-mCherry levels after adjusting for the contribution of cytoplasmic signal in Ramos B cells.

A. Fluorescent images of AID-mCherry transductants acquired by confocal fluorescent microscopy. DAPI (left), mCherry (middle) and merge (right) are shown. Plot files of fluorescence intensities of mCherry along arbitrary lines as indicated for 4 representative AID-mCherry transductants exhibiting a range of maximum fluorescence intensities.

B. Histograms of nuclear mCherry relative to baseline for untreated (Left) and 0.5 hour of LMB (Right). Nuclear signal was adjusted by subtracting corresponding baseline value (as determined by linear regression analysis (Figure 1.B) from each HCS reading (see material and methods). Mean and median are tabulated as shown.

A**B**

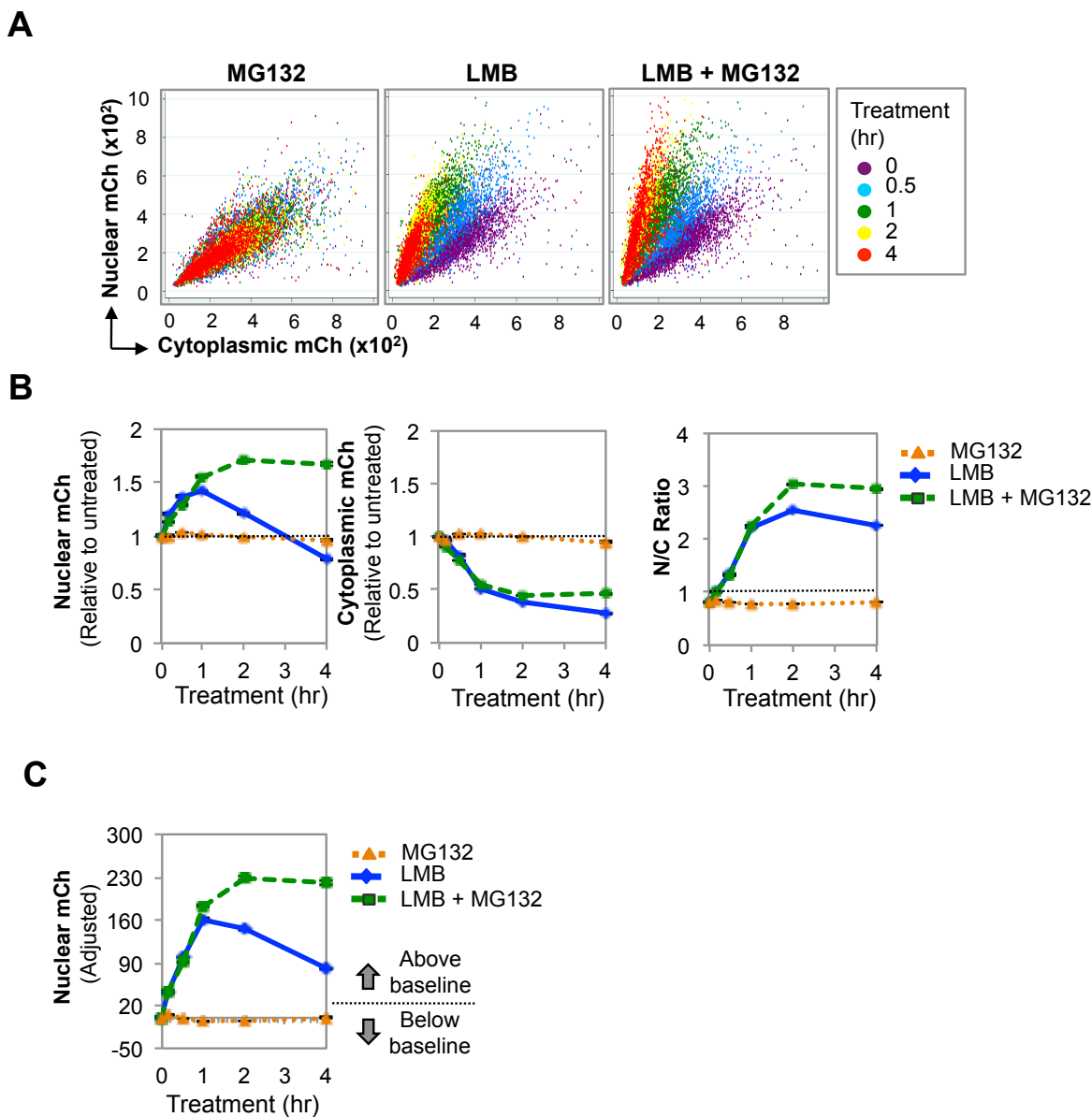


Figure 4-3. HCS assessment of AID nuclear stability in Ramos B cells.

A. Scatter plots of nuclear vs. cytoplasmic AID-mCherry signals for populations of individual cells, untreated (purple) or treated with MG132, LMB, LMB + MG132 for 0.5 hour (blue), 1 hour (green), 2 hour (yellow), or 4 hour (red).

B. Quantification of nuclear and cytoplasmic AID-mCherry signal and N/C ratio in treated relative to untreated cell populations at indicated times post-treatment with LMB (diamond/solid), MG132 (triangle/dotted), or both (square/dash). This experiment was repeated 3 times for LMB treatment, and once for MG132 and LMB + MG132. Each point on the graph represents the population average, and black bars represent SEM of the population, which are minuscule and cannot be seen. Dotted line represents no change (fold change of 1). For statistical tests, see Table 4-1.

C. Assessment of nuclear mCherry relative to baseline (dotted line) at indicated times post-treatment with LMB (diamond/solid), MG132 (triangle/dotted), or both (square/dash). Each point on the graph represents the population average, and black bars are SEM of the population.

Compared to Untreated

	0 hr		0.167 hr		0.5 hr		1 hr		2 hr		4 hr	
	N		N	p	N	p	N	p	N	p	N	p
LMB												
Nuclear AID-mCh	6141	3394	1.59E-59		4752	8.55E-185	6393	1.07E-289	3327	3.49E-61	5023	1.17E-130
Cytoplasmic AID-mCh	6141	3394	2.30E-02		4752	1.07E-53	6393	0.00E+00	3327	0.00E+00	5023	0.00E+00
N/C	6141	3394	0.00E+00		4752	0.00E+00	6393	0.00E+00	3327	0.00E+00	5023	0.00E+00
LMB + MG132												
Nuclear AID-mCh	6141	5009	9.40E-37		4682	1.09E-132	6029	0.00E+00	1988	1.01E-207	1918	6.78E-168
Cytoplasmic AID-mCh	6141	5009	2.08E-16		4682	2.61E-93	6029	0.00E+00	1988	0.00E+00	1918	0.00E+00
N/C	6141	5009	0.00E+00		4682	0.00E+00	6029	0.00E+00	1988	0.00E+00	1918	0.00E+00
MG132												
Nuclear AID-mCh	6141	5569	6.80E-01		5822	4.54E-04	3063	5.41E-01	2862	5.14E-01	5569	9.91E-04
Cytoplasmic AID-mCh	6141	5569	2.56E-03		5822	1.62E-02	3063	1.27E-02	2862	7.77E-01	5569	7.92E-05
N/C	6141	5569	3.08E-14		5822	2.60E-02	3063	9.75E-16	2862	1.99E-06	5569	6.55E-05

LMB vs. LMB + MG132 treatment

	0.167 hr	0.5 hr	1 hr	2 hr	4 hr
Nuclear AID-mCh	5.37E-08	6.21E-08	8.52E-25	6.89E-99	3.54E-265
Cytoplasmic AID-mCh	1.75E-07	1.84E-05	4.49E-10	8.17E-22	1.59E-118
N/C	3.25E-01	2.41E-03	3.67E-07	1.48E-101	2.60E-135

LMB vs. MG132 treatment

	0.167 hr	0.5 hr	1 hr	2 hr	4 hr
Nuclear AID-mCh	1.34E-51	7.10E-141	1.38E-275	5.13E-53	2.13E-59
Cytoplasmic AID-mCh	6.01E-01	3.89E-80	0.00E+00	0.00E+00	0.00E+00
N/C	2.58E-243	0.00E+00	0.00E+00	0.00E+00	0.00E+00

MG132 vs. LMB + MG132 treatment

	0.167 hr	0.5 hr	1 hr	2 hr	4 hr
Nuclear AID-mCh	7.23E-30	1.59E-94	0.00E+00	8.91E-201	1.39E-175
Cytoplasmic AID-mCh	3.54E-07	2.52E-135	0.00E+00	0.00E+00	6.44E-256
N/C	2.11E-267	0.00E+00	0.00E+00	0.00E+00	0.00E+00

Table 4-1. Probability test for Figure 4-3.B.

Statistical tests were performed using two-tailed, unpaired Student's t-test, assuming unequal variances, for comparison of nuclear and cytoplasmic AID-mCherry signal and the N/C ratio between different treatment groups and between different times post treatment and untreated control in each treatment group.

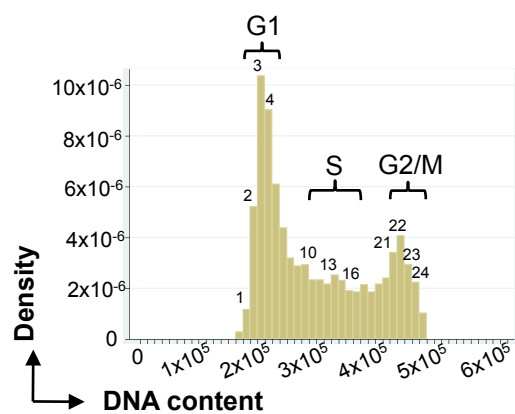
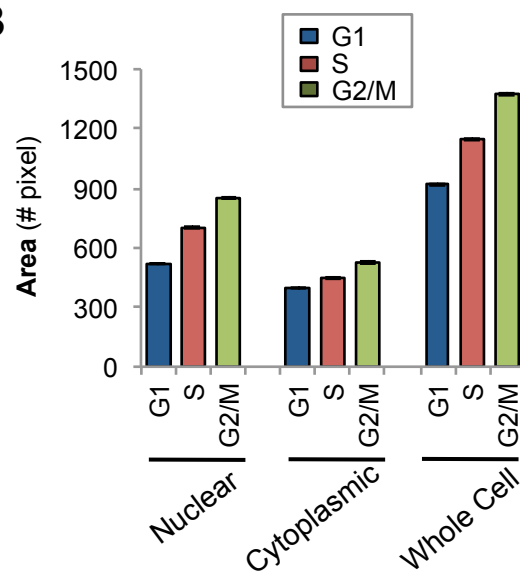
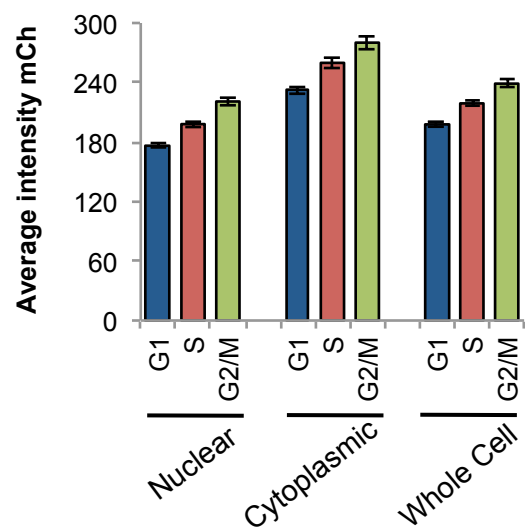
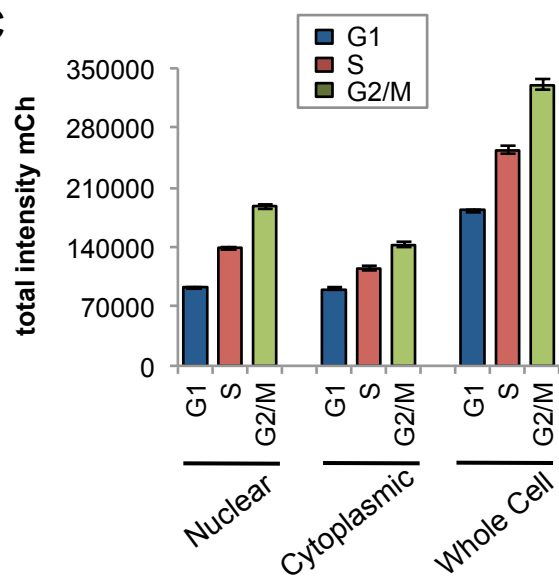
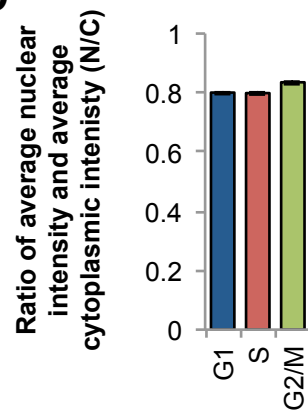
A**B****C****D**

Figure 4-4. HCS assessment of DNA content, nuclear/cell size, and AID-mCherry signals during the course of the cell cycle in Ramos B cells.

A. G1, S, and G2/M phase Ramos cells are distinguished by DNA content, which is measured by total intensity of DAPI. DNA content is ranked, and ranks 1-4 are assigned to G1, ranks 10-16 to S and ranks 21-24 to G2/M. Example of cell cycle profile and G1, S, and G2/M populations is shown for untreated.

B. Bar graph of average nuclear, cytoplasmic, and whole cell area for G1 (blue), S (red), and G2/M (green) phase B cell populations. Error bars denote SEM of the population and are too small to see clearly.

C. The population average of total intensity (left) and average intensity (right) of AID-mCherry expressed in Ramos B cells in the nuclear and cytoplasmic compartments and the whole cell are shown for G1, S and G2/M phase cells.

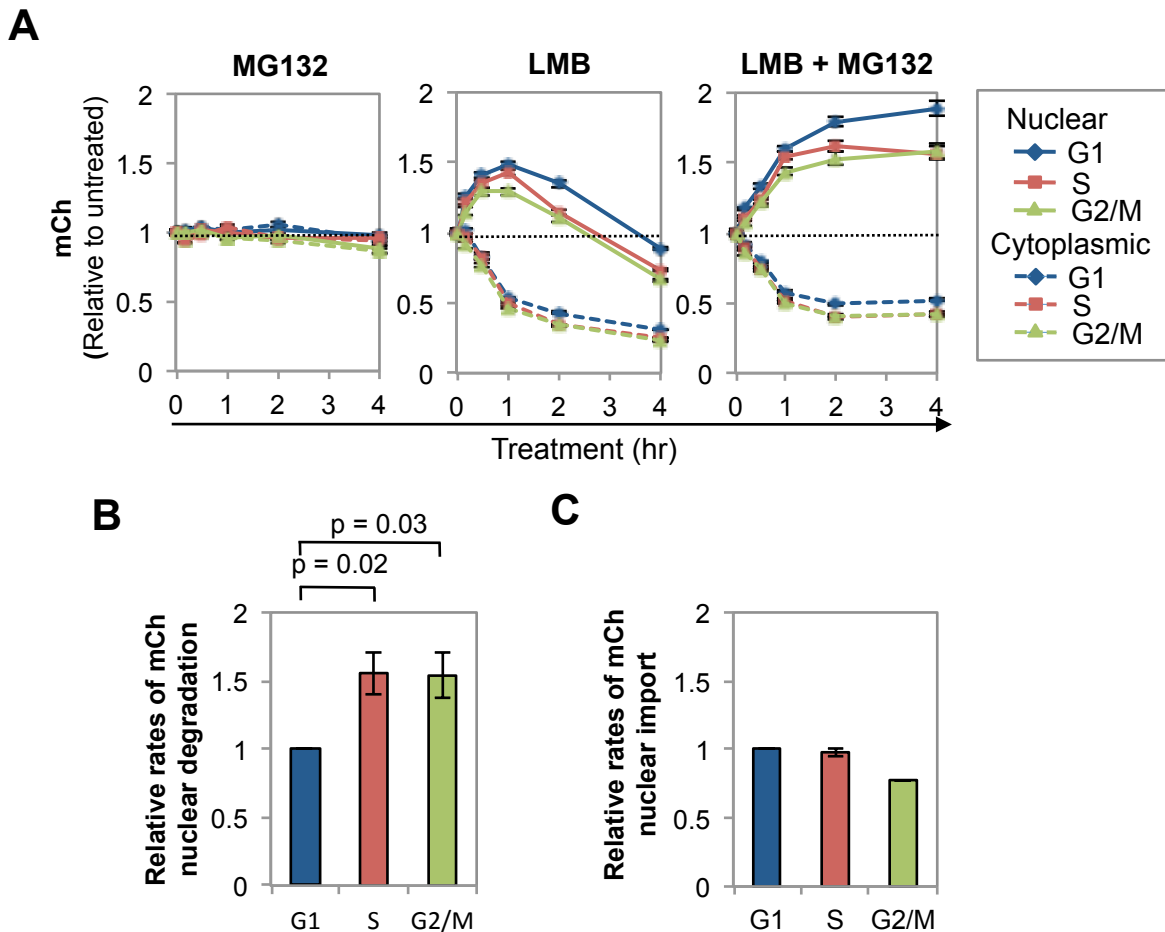


Figure 4-5. Nuclear AID is more stable in G1 phase than S/G2/M phase in Ramos B cells.

A. Kinetics of response of AID-mCherry nuclear (solid lines) and cytoplasmic (dashed lines) signals at indicated times following treatment with MG132, LMB or LMB + MG132 in G1 (blue), S (red) and G2/M (green) phase cells. Each point on the graph represents the population average, and black bars are SEM of the population. Dotted line represents no change (fold change of 1). For statistical tests, see Table 4-2.

B. Relative rates of nuclear degradation of AID-mCherry in LMB-treated cells in G1, S and G2/M phases. Rates were estimated as the slope of the line defined by the population averages at 1 hour and 2 hour treatment time points in 4 independent experiments, and values are presented relative to the slope in G1 phase. This experiment was repeated 3 times for cells treated with LMB, and once for cells treated with MG132 and LMB + MG132. SEM is shown as black bars. Significance (p values) shown above graph was determined by two-tailed, unpaired Student's t-test, assuming unequal variances.

	Nuclear AID-mCherry			Cytoplasmic AID-mCherry			N/C		
	G1 vs. S	G1 vs. G2/M	S vs. G2/M	G1 vs. S	G1 vs. G2/M	S vs. G2/M	G1 vs. S	G1 vs. G2/M	S vs. G2/M
LMB									
0 hr	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	8.08E-01	4.17E-05	1.15E-05
0.167 hr	1.58E-01	4.44E-04	5.08E-02	2.68E-01	2.01E-03	6.89E-02	4.84E-01	3.67E-03	1.87E-03
0.5 hr	4.35E-02	4.37E-04	1.37E-01	6.37E-01	1.87E-02	2.10E-02	6.44E-05	1.59E-01	4.68E-06
1 hr	1.08E-02	7.08E-13	8.72E-06	2.12E-03	3.01E-11	5.81E-04	9.53E-06	5.64E-09	3.61E-02
2 hr	6.57E-10	5.86E-12	2.48E-01	4.02E-16	2.24E-14	8.65E-01	3.60E-09	1.21E-09	3.74E-01
4 hr	2.50E-21	4.02E-32	1.75E-04	2.26E-20	3.80E-21	8.03E-03	6.48E-03	3.09E-07	1.38E-02
LMB + MG132									
0.167 hr	2.56E-03	2.76E-04	2.91E-01	1.98E-01	4.68E-03	1.30E-01	9.83E-02	1.37E-02	9.21E-04
0.5 hr	1.29E-04	1.99E-04	6.60E-01	8.76E-03	3.78E-03	5.58E-01	9.19E-02	1.87E-02	3.85E-04
1 hr	1.22E-01	8.08E-07	1.66E-03	2.95E-06	2.40E-09	1.33E-01	6.66E-26	2.32E-18	8.29E-01
2 hr	1.42E-03	1.83E-05	1.49E-01	2.24E-11	1.39E-06	4.50E-01	1.24E-13	1.39E-05	2.87E-01
4 hr	1.68E-06	1.77E-05	8.02E-01	3.19E-10	2.87E-04	8.70E-01	1.17E-04	4.05E-11	5.50E-04
MG132									
0.167 hr	8.74E-02	2.40E-01	7.76E-01	1.04E-01	1.53E-01	9.09E-01	6.85E-01	1.42E-06	9.86E-05
0.5 hr	6.03E-01	6.03E-01	9.82E-01	3.42E-02	3.04E-01	3.95E-01	4.85E-03	9.52E-06	3.53E-02
1 hr	8.91E-01	4.59E-02	8.50E-02	4.82E-01	1.68E-02	8.20E-03	4.66E-01	1.01E-10	1.43E-07
2 hr	5.11E-02	1.56E-01	7.65E-01	4.90E-03	3.95E-04	4.10E-01	2.45E-02	1.05E-12	9.98E-08
4 hr	6.17E-01	1.01E-03	4.88E-03	8.63E-01	5.71E-03	5.40E-02	8.18E-02	2.12E-01	8.03E-01

Compared to Untreated

LMB (G1) Nuclear AID-mCherry Cytoplasmic AID-mCherry N/C	0 hr	0.167 hr		0.5 hr		1 hr		2 hr		4 hr	
	N	N	p	N	p	N	p	N	p	N	p
	1899	1112	9.08E-30	1532	9.26E-75	2100	9.51E-125	1022	2.09E-41	1415	1.89E-11
	1899	1112	8.66E-01	1532	1.75E-18	2100	1.25E-131	1022	3.02E-181	1415	5.84E-266
LMB (S) Nuclear AID-mCherry Cytoplasmic AID-mCherry N/C	1899	1112	7.42E-134	1532	0.00E+00	2100	0.00E+00	1022	0.00E+00	1415	0.00E+00
	0 hr	0.167 hr		0.5 hr		1 hr		2 hr		4 hr	
	N	N	p	N	p	N	p	N	p	N	p
	1141	594	3.21E-13	844	7.38E-33	1218	7.89E-60	634	3.08E-07	1045	2.52E-43
LMB (G2/M) Nuclear AID-mCherry Cytoplasmic AID-mCherry N/C	1141	594	3.57E-01	844	1.67E-09	1218	7.27E-99	634	4.58E-159	1045	4.62E-199
	1141	594	2.11E-76	844	3.67E-280	1218	0.00E+00	634	0.00E+00	1045	0.00E+00
	0 hr	0.167 hr		0.5 hr		1 hr		2 hr		4 hr	
	N	N	p	N	p	N	p	N	p	N	p
LMBMG132 (G1) Nuclear AID-mCherry Cytoplasmic AID-mCherry N/C	934	493	5.50E-06	719	1.00E-19	949	6.39E-24	457	1.23E-03	684	1.06E-47
	934	493	8.79E-03	719	9.48E-15	949	1.00E-84	457	2.01E-118	684	1.75E-153
	934	493	6.40E-51	719	4.05E-267	949	0.00E+00	457	7.84E-228	684	0.00E+00
	0 hr	0.167 hr		0.5 hr		1 hr		2 hr		4 hr	
LMBMG132 (S) Nuclear AID-mCherry Cytoplasmic AID-mCherry N/C	N	N	p	N	p	N	p	N	p	N	p
	1899	1558	4.38E-20	1420	8.23E-56	1880	1.54E-145	567	1.11E-70	422	6.70E-54
	1899	1558	3.70E-05	1420	4.32E-24	1880	3.57E-109	567	2.12E-130	422	1.89E-102
	1899	1558	2.19E-166	1420	0.00E+00	1880	0.00E+00	567	2.08E-266	422	1.56E-181
LMBMG132 (G2/M) Nuclear AID-mCherry Cytoplasmic AID-mCherry N/C	0 hr	0.167 hr		0.5 hr		1 hr		2 hr		4 hr	
	N	N	p	N	p	N	p	N	p	N	p
	1141	954	7.85E-06	895	5.11E-19	1107	2.45E-76	393	2.14E-39	411	2.31E-29
	1141	954	1.69E-05	895	1.28E-24	1107	8.92E-92	393	4.16E-130	411	1.98E-116
MG132 (G1) Nuclear AID-mCherry Cytoplasmic AID-mCherry N/C	1141	954	6.86E-102	895	0.00E+00	1107	0.00E+00	393	7.71E-180	411	1.32E-154
	0 hr	0.167 hr		0.5 hr		1 hr		2 hr		4 hr	
	N	N	p	N	p	N	p	N	p	N	p
	934	580	1.03E-02	570	1.03E-11	811	1.04E-42	263	7.47E-21	293	1.50E-24
MG132 (S) Nuclear AID-mCherry Cytoplasmic AID-mCherry N/C	934	580	9.48E-07	570	3.39E-19	811	2.54E-73	263	3.30E-86	293	1.19E-52
	934	580	3.00E-47	570	5.54E-211	811	0.00E+00	263	2.87E-96	293	6.83E-104
	0 hr	0.167 hr		0.5 hr		1 hr		2 hr		4 hr	
	N	N	P	N	p	N	p	N	p	N	p
MG132 (G2/M) Nuclear AID-mCherry Cytoplasmic AID-mCherry N/C	1899	1413	1.90E-01	1701	1.07E-01	1860	6.52E-01	949	3.28E-01	605	2.51E-01
	1899	1413	5.23E-01	1701	1.52E-01	1860	2.41E-01	949	4.36E-02	605	5.27E-02
	1899	1413	1.30E-03	1701	3.08E-03	1860	2.21E-08	949	8.03E-07	605	1.21E-01
	0h	0.167h		0.5h		1h		2h		4h	
MG132 (S) Nuclear AID-mCherry Cytoplasmic AID-mCherry N/C	N	N	p	N	p	N	p	N	p	N	p
	1141	782	5.19E-01	1033	4.77E-01	1042	8.22E-01	583	2.14E-01	616	1.06E-01
	1141	782	5.08E-02	1033	5.66E-01	1042	1.44E-01	583	2.98E-01	616	1.34E-01
	1141	782	3.29E-03	1033	8.26E-01	1042	3.84E-05	583	5.62E-02	616	2.40E-04
MG132 (G2/M) Nuclear AID-mCherry Cytoplasmic AID-mCherry N/C	0 hr	0.167 hr		0.5 hr		1 hr		2 hr		4 hr	
	N	N	p	N	p	N	p	N	p	N	p
	934	591	8.00E-01	791	5.32E-01	828	1.48E-01	431	4.54E-01	436	8.76E-06
	934	591	1.03E-01	791	8.28E-01	828	3.15E-01	431	8.62E-02	436	3.11E-05
	934	591	3.15E-04	791	1.78E-01	828	1.16E-02	431	2.33E-01	436	8.60E-01

Table 4-2. Probability test for Figure 4-5.A.

Statistical tests were performed using two-tailed, unpaired Student's t-test, assuming unequal variances, for comparison of nuclear and cytoplasmic AID-mCherry signal and the N/C ratio between G1 and S; G1 and G2/M; and S and G2/M at different hours post-treatment in each treatment group.

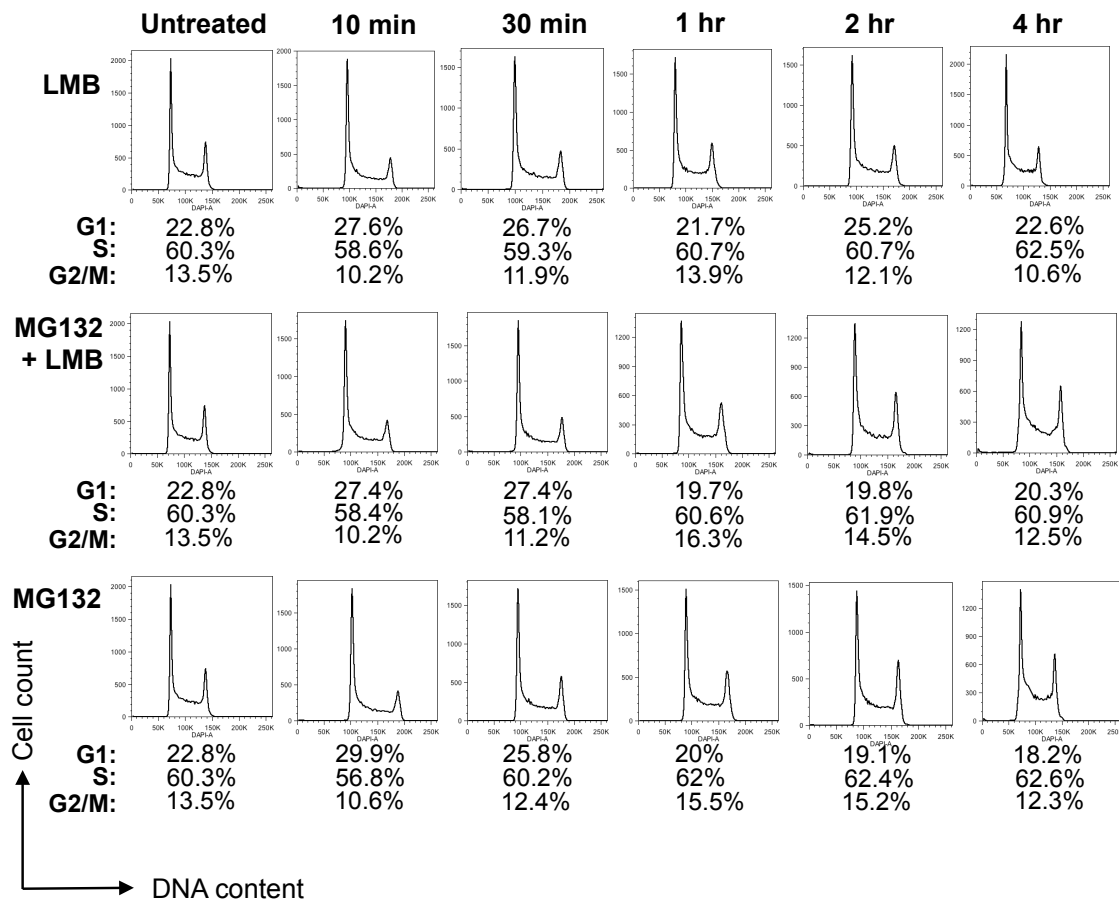


Figure 4-6. Cell cycle profile is unaltered with LMB and/or MG132 treatment in Ramos B cells.

Cell cycle profile following treatment of Ramos B cells with LMB, LMB + MG132, and MG132 for indicated time. Estimated percentage of cells in G1, S, and G2/M (as determined by the Watson Pragmatic computational model in FlowJo) are tabulated below each cell cycle profile.

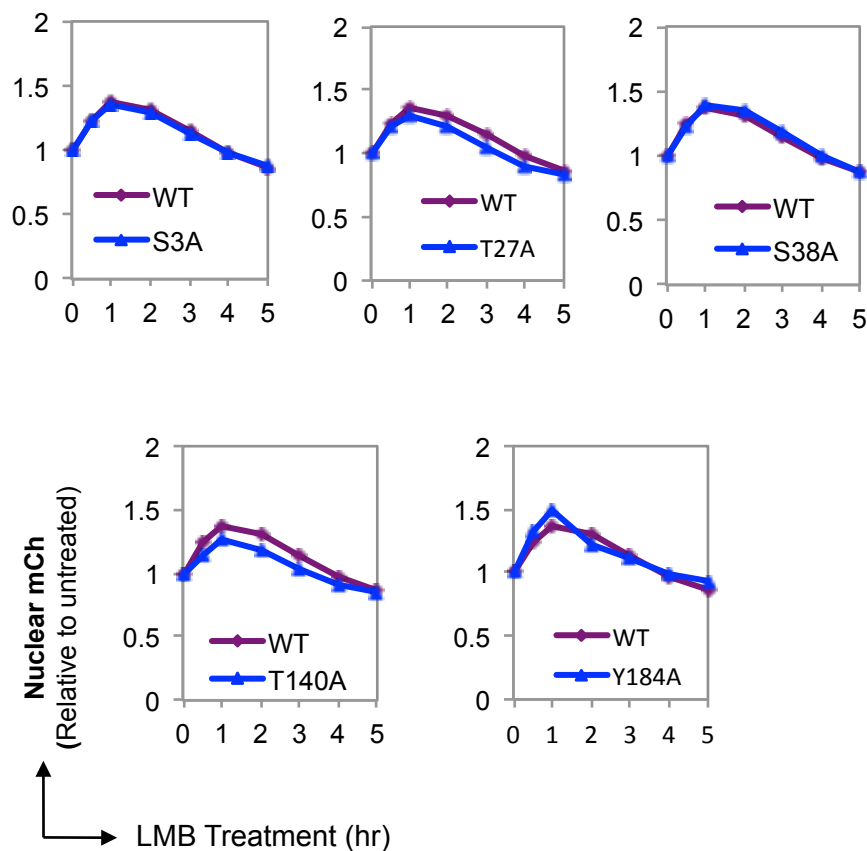


Figure 4-7. Comparison of nuclear degradation of AID between WT and individual phosphorylation mutants in Ramos B cells.

Kinetics of response of AID^{WT}-mCherry (black line) and AID^{S3A, T27A, S38A, T140A, or Y184A}-mCherry (blue lines) nuclear signals at indicated times following treatment with LMB. Each point on the graph represents the population average, and black bars are SEM of the population.

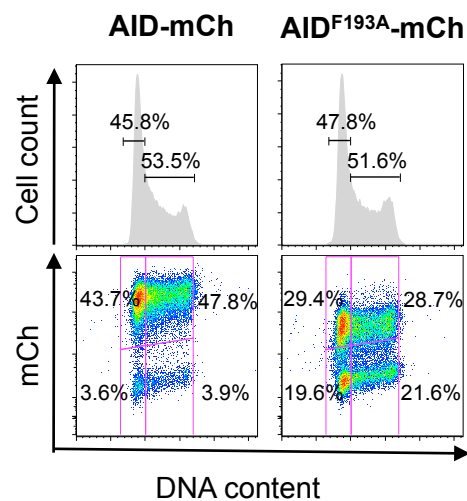
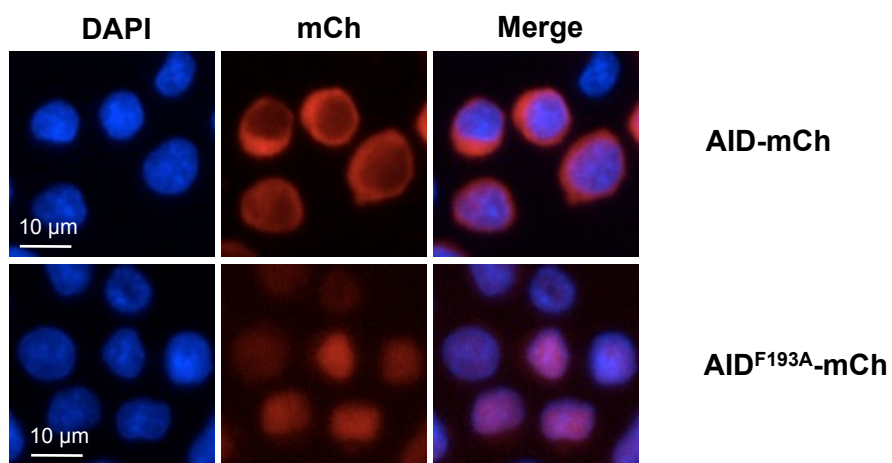
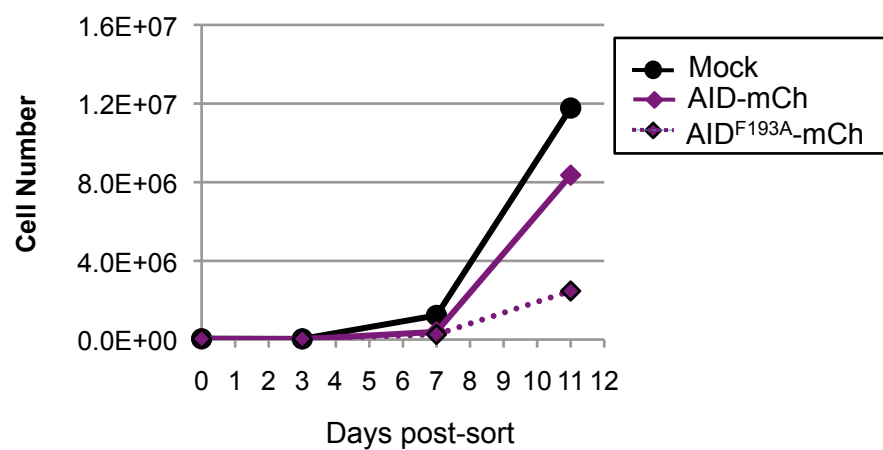
A**B****C**

Figure 4-8. AID^{F193A}-mCherry exhibits nuclear localization in Ramos B cells.

A. Representative FACS profiles of AID-mCherry and AID^{F193A}-mCherry transductants. Top, cell number relative to cell cycle (DNA content), with fractions of G1 and S/G2/M phase cells indicated. Bottom, mCherry+ signal relative to DNA content, with percentage of G1/mCherry+ (top left quadrant), G1/mCherry- (bottom left quadrant), S/G2/M/mCherry+ (top right quadrant) and S/G2/M/mCherry- (bottom right quadrant).

B. Representative images of AID-mCherry and AID^{F193A}-mCherry transductants. DAPI (left), mCherry (middle), and merge (right) are shown.

C. Quantification of cell viability over days in culture for Ramos B cells expressing AID-mCherry, and AID^{F193A}-mCherry, or mock-transduced. Number of viable cells was determined at day 3, 7, and 11 post sorting mCherry+ transductants for mock (circle, black solid line), AID-mCherry (diamond, purple solid line) transductants, and AID^{F193A}-mCherry (diamond, purple dotted line).

	AID-mCh		AID ^{F193A} -mCh		p-value
	N	mean	N	mean	
Total mCh	14666	444.2	13485	111.4	0
Cytoplasmic mCh	14666	473.6	13485	62.9	0
Apparent nuclear mCh	14666	423.7	13485	142.0	0
N/C	14666	0.9	13485	2.3	0
Adjusted nuclear mCh	14666	0.9	13485	67.5	0

Table 4-3. Comparison of cellular distribution of AID-mCherry and AID^{F193A}-mCherry.

The number of cells (N) and the average total, cytoplasmic, apparent nuclear and adjusted nuclear mCherry signals and the N/C ratio are tabulated for AID-mCherry and AID^{F193A}-mCherry transductants. Statistical test using two-tailed, unpaired Student's t-test, assuming unequal variances for comparison between AID-mCherry and AID^{F193A}-mCherry.

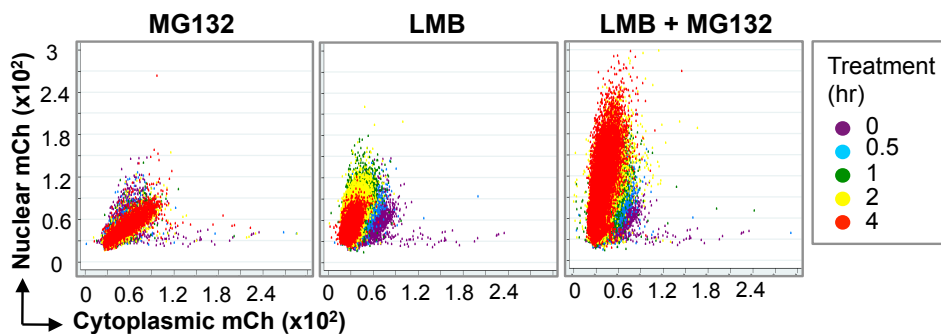
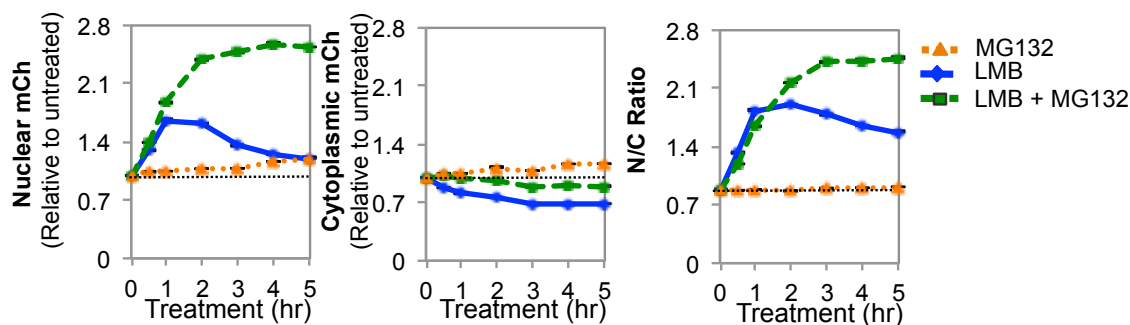
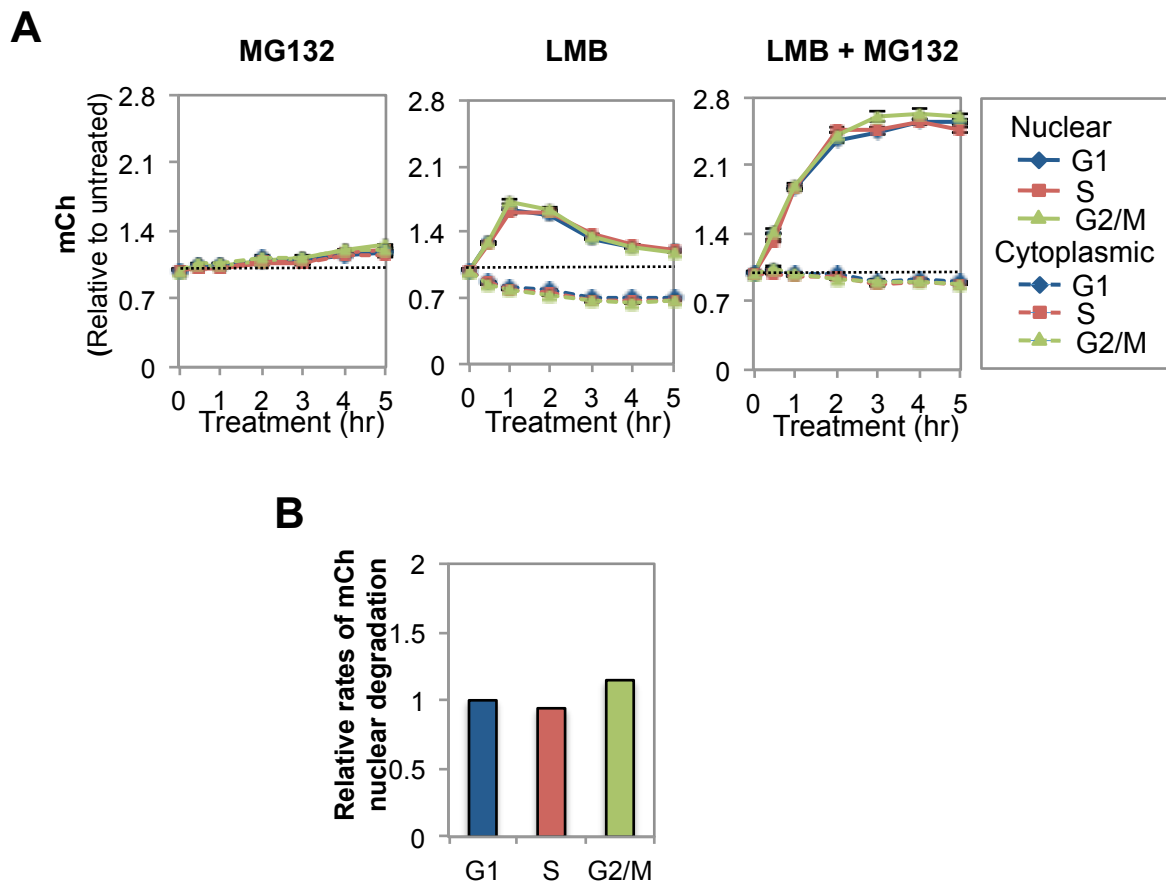
A**B**

Figure 4-9. HCS assessment of AID nuclear stability in HT1080 fibroblasts.

A. Scatter plots of nuclear vs. cytoplasmic AID-mCherry signals for populations of individual cells, untreated (purple) or treated with MG132, LMB, LMB + MG132 for 0.5 hour (blue), 1 hour (green), 2 hour (yellow), or 4 hour (red).

B. Quantification of nuclear and cytoplasmic AID-mCherry signal and N/C ratio in treated relative to untreated cell populations at indicated times post-treatment with LMB (diamond/solid), MG132 (triangle/dotted), or both (square/dash). Dotted line represents no change (fold change of 1).



Chapter 5: Cell cycle-dependent nuclear stability of AID regulates Ig gene diversification in Ramos B cells

5.1. Introduction

AID must be in the nucleus to gain access to its DNA substrates. I have shown that both proteasomal degradation and nuclear export limit nuclear AID (Chapter 4). I next asked how cell cycle might regulate AID. The Cdt1 and Gem tags, which derive from cell cycle regulators, can direct a fused fluorescent protein into the nucleus and target the protein for destruction at specific stages of cell cycle [85]. I used these tags to restrict nuclear AID to G1 and S/G2/M phase, respectively.

5.2. Cdt1 and Gem tags restrict AID abundance to G1 and S/G2/M phase, respectively

In control experiments, I verified that the Cdt1 and Gem tags confer predicted G1 or S/G2M phase-restricted stability to the fused fluorescent proteins, by cell cycle profiling of Ramos B cells expressing mKO2-Cdt1 or mAG-Gem fluorescent fusion proteins (Figure 5-1.A and Figure 5-1.B, respectively). By fluorescence microscopy, I showed that these tagged reporter proteins localized to the nucleus (Figure 5-1.C).

I then assessed the effect of cell cycle restriction on AID. I generated cells for this analysis by transducing Ramos B cells with lentivirus bearing AID-mCherry, AID-mCherry-Cdt1 or AID-mCherry-Gem expression constructs. I sorted mCherry⁺ cells 3-4 days after transduction, and at 11 days after sorting I collected cells for analysis.

I analyzed cell cycle distribution and the mCherry signal of the cell populations. I found that cell cycle profiles were similar in all transductants at 11 days post-sort (Figure 5-2.A, top).

Thus, expression of Cdt1- or Gem-tagged AID-mCherry does not alter cell cycle distribution. The tags did affect cell cycle dependence of AID-mCherry expression, as predicted. In AID-mCherry transductants, the mCherry signal was evident throughout cell cycle, with similar fractions of G1 and S/G2/M phase cells exhibiting a clear mCherry signal (43.7% and 47.8%, Figure 5-2.A, left). In contrast, among AID-mCherry-Cdt1 transductants, more G1 phase than S/G2/M phase cells exhibited an mCherry signal (42.0% and 23.7%; Figure 5-2.A, middle). Among AID-mCherry-Gem transductants, fewer G1 phase than S/G2/M phase cells exhibited an mCherry signal (25.0% and 53.4%; Figure 5-2.A, Right).

Moreover, considerable loss of signal intensity occurred during the 11 days between initial sorting for mCherry⁺ expression and cell cycle profiling in both AID-mCherry-Cdt1 and AID-mCherry-Gem transductants. In those populations, 33.5% and 19.5% of cells respectively exhibited reduction in signal intensity to below the gate used for initial sorting, compared to only 7.5% of AID-mCherry cells (Figure 5-2.A). Profiles of mCherry signal as percent of maximum signal show the reduction in signal intensity among the mCherry(+) cells in AID-mCherry-Cdt1 and AID-mCherry-Gem transductants (Figure 5-2.B). These results suggested that cells in which expression of the Cdt1- or Gem-tagged protein is downregulated may have a proliferative advantage, as would occur if expression of those fusion proteins is toxic. Analysis of cell number following the initial sort confirmed this. AID-mCherry-Cdt1 transductants exhibited considerable mortality, evident as an 8-fold decrease in cell number compared to AID-mCherry transductants at 11 days post-sort (Figure 5-3). Mortality was also evident among AID-mCherry-Gem transductants.

5.3. The Cdt1 tag confers nuclear localization on AID-mCherry

Fluorescence microscopy immediately showed a clear difference between the AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem transductants: the mCherry⁺ signal from AID-mCherry-Cdt1 transductants was nuclear, even in the absence of LMB treatment (Figure 5-4).

5.4. G1-restricted AID is predominately nuclear whereas S/G2/M-restricted AID is predominately cytoplasmic

I quantified nuclear and cytoplasmic distributions and the ratio of nuclear to cytoplasmic mCherry signal (N/C) by high content screening (HCS) microscopy. Quantification of average mCherry signal intensity per pixel showed that total signal in AID-mCherry transductants was approximately 3-fold higher than in either AID-mCherry-Cdt1 or AID-mCherry-Gem transductants, and that AID-mCherry-Cdt1 transductants exhibited the lowest signal (Figure 5-5.A, left; Table 5-1), consistent with differences in signal documented above (Figure 5-2.A). Comparisons of apparent nuclear and cytoplasmic signals documented similar differences, and revealed a strikingly lower cytoplasmic signal evident among AID-mCherry-Cdt1 transductants (Figure 5-5.A and Table 5-1). This difference was especially evident upon graphing the N/C ratio, which was 1.6 for AID-mCherry-Cdt1 transductants, and slightly below 1.0 for the other transductants (Figure 5-5.A and Table 5-1). This is consistent with the predominantly nuclear localization of AID-mCherry-Cdt1 evident by immunofluorescence microscopy (Figure 5-4).

To quantify the significance of the preferential nuclear localization of AID-mCherry-Cdt1, I first used a linear model of nuclear vs. cytoplasmic signal (Figure 5-5.B, top) to correct for the contribution of cytoplasmic signal to apparent nuclear signal (see Materials and Methods). The adjusted nuclear mCherry signal in AID-mCherry-Cdt1 transductants was much higher than

in either AID-mCherry ((54.5 compared to 0.9; $p=0$) or AID-mCherry-Gem (54.5 compared to - 0.5; $p=0$) transductants (Figure 5-5.B, bottom; Table 5-1). There was a more modest but nonetheless significant difference between the adjusted nuclear mCherry signal of AID-mCherry and AID-mCherry-Gem transductants ($p=0.0565$). Thus, the Cdt1 tag promotes nuclear localization and/or nuclear stability of AID-mCherry.

5.5. Cell cycle dependence of subcellular distributions of AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem

To determine if nuclear localization of AID-mCherry was dependent on cell cycle, I analyzed the total, apparent nuclear, and cytoplasmic mCherry signals, as above, for cells in G1, S, and G2/M phases. AID-mCherry exhibited relatively constant signal across all phases of the cell cycle (Figure 5-6.A and Table 5-2). AID-mCherry-Cdt1 total, apparent nuclear, and cytoplasmic signals were highest in G1 phase, intermediate in S phase, and lowest in G2/M phase. This is consistent with the premise that the Cdt1 tag will target AID-mCherry-Cdt1 for degradation during S/G2/M phases. AID-mCherry-Gem exhibited the opposite trend, as signal was lowest in G1 phase and dramatically increased from S to G2/M phase (Figure 5-6.A and Table 5-2). The N/C ratios of AID-mCherry and AID-mCherry-Gem transductants showed little variation in the course of cell cycle, and were very slightly below unity in all phases of the cell cycle (Figure 5-6.A and Table 5-2). The N/C ratio of AID-mCherry-Cdt1 transductants was > 1 in all phases of the cell cycle, and highest in G1 phase (Figure 5-6.A and Table 5-2).

I corrected for the contribution of cytoplasmic signal to apparent nuclear signal as above. The adjusted nuclear AID-mCherry-Cdt1 was clearly greatest during G1 phase (88.8). Thus, the

Cdt1 tag enhanced the nuclear signal in G1 phase cells. The AID-mCherry-Gem signal was predominantly cytoplasmic in G1 phase, but nuclear in G2/M phase (Figure 5-6.B and Table 5-2).

To show that cytoplasmic vs. nuclear localization did not reflect impaired nuclear entry, I treated cells with LMB to inhibit nuclear export. Treatment with LMB caused nuclear accumulation of AID-mCherry and AID-mCherry-Gem, with similar kinetics, and greatly increased the apparent N/C ratio of AID-mCherry-Gem (Figure 5-7). Thus nuclear entry was not impaired. Notably, the AID-mCherry-Cdt1 apparent nuclear signal increased very transiently following LMB treatment, then diminished, suggesting that AID-mCherry-Cdt1 undergoes rapid degradation if confined to the nucleus. Consistent with this, MG132 treatment increased the apparent nuclear signal of AID-mCherry-Cdt1 but not AID-mCherry or AID-mCherry-Gem.

5.6. Enhanced nuclear localization of AID in G1 phase accelerates Ig gene diversification

AID has a well-established role in Ig gene diversification. If AID functions in the nucleus in G1 phase, then enhanced nuclear localization in G1 phase conferred by the Cdt1 tag (Figure 5-7.B) is predicted to accelerate Ig gene diversification. I tested this hypothesis by examining the sIgM loss rate of AID-mCherry, AID-mCherry-Cdt1 or AID-mCherry-Gem transductants [81,96]. mCherry⁺ cells were selected at 3-4 days post-transduction, then cultured for 7 additional days before analysis of the fraction of sIgM⁻ cells. This fraction was 3.6% in mock-transduced Ramos B cells (not shown), evidence that the cells retained activity of AID and the hypermutation pathway. This fraction was 7.9% in AID-mCherry transductants, increased to 46.5% in AID-mCherry-Cdt1 transductants ($p=0.007$ relative to AID-mCherry); and 7.1% in AID-mCherry-Gem transductants (Figure 5-8). This is a powerful indication that nuclear localization of AID-mCherry during G1 phase is a driving force in Ig gene diversification.

Similar results were evident using reporters in which the mCherry cassette was substituted by mKO2, a fluorescent tag reported to respond more rapidly to cell cycle-dependent degradation [85]. The sIgM loss frequencies were nearly identical in AID-mKO2-Cdt1 and AID-mCherry-Cdt1 transductants (47.1% vs. 46.5%); and AID-mKO2-Gem relative to AID-mCherry-Gem transductants (5.4% vs. 7.1%; Figure 5-9.B).

5.7. Enhanced nuclear localization of AID in G1 phase accelerates accumulation of point mutations in Ig V_H regions

I sorted single mCherry⁺ cells from cultures of AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem transductants, carried out single-cell PCR and sequenced amplified IgV_H regions. Sequence analysis revealed that the mutation frequency in IgV_H regions was 2.66 per kb in AID-mCherry transductants, 3.84/kb in AID-mCherry-Cdt1 transductants ($p=2.4 \times 10^{-9}$) and 2.40 per kb in AID-mCherry-Gem transductants ($p=6.3 \times 10^{-3}$) (Figure 5-10.A). Most mutations were point mutations, however a few deletions and insertions were detected, most frequently in AID-mCherry-Cdt1 transductants (Figure 5-10.B). Thus, the enhanced nuclear localization of AID in G1 phase that is conferred by the Cdt1 tag results in acceleration of somatic hypermutation.

Accelerated hypermutation was especially evident in diagrams of mutant lineages, which document more changes per generation in the AID-mCherry-Cdt1 transductants (Figure 5-10.C).

As is characteristic of AID-initiated mutagenesis, 82% of point mutations in AID-mCherry transductants were at G or C, and only 18% at A or T (Figure 5-10.D and Table 5-3). This is very similar to previously published analyses of IgV_H hypermutation by endogenous AID in Ramos B cells [81,96]. A notable difference in AID-mCherry-Cdt1 or AID-mCherry-Gem

transductants was an increase in the fraction of mutations at G, accompanied by an apparent decrease in the fraction of mutations at A and T (Figure 5-11 and Table 5-3).

5.8. Discussion

Here, I have investigated the impact of cell cycle-dependent nuclear stability of AID on Ig gene diversification and cell survival, using Cdt1 and Gem tags to restrict nuclear stability to G1 or S/G2/M phases, respectively.

I found that AID-mCherry-Cdt1 localized almost exclusively to the nucleus in Ramos B cells. This is consistent with the effect of this tag on the mKO2 fluorescent reporter in Ramos B cells, as mKO2-Cdt1 resides in the nucleus, and the fluorescent signal is restricted to G1 phase cells (Figure 5-1.C, top). The Cdt1 tag did not compromise nuclear export, as LMB treatment, which blocks export via the CRM1-mediated pathway, caused an increase in nuclear AID-mCherry-Cdt1 accompanied by a corresponding decrease in cytoplasmic protein.

While the Cdt1 tag override normal mechanisms for cell cycle-dependent regulation of AID, the Gem tag did not have a comparably robust or independent effect. Total, nuclear and cytoplasmic levels of AID-mCherry-Gem were significantly lower in G1 phase compared to S/G2/M (Figure 5-5). Thus, AID-mCherry-Gem is subject to cell-cycle dependent proteosomal degradation, directed by the Gem tag, as predicted. However, it was initially surprising that AID-mCherry-Gem expression produced a strong cytoplasmic signal but no nuclear signal. The Gem tag contains two NLS's, and in control experiments I showed that it confers nuclear localization to the AG fluorescent protein in Ramos B cells (Figure 5-1.C, bottom) along with predominate expression in S/G2/M phase. The cytoplasmic localization of AID-mCherry-Gem suggests that the mechanisms that prevent nuclear localization of AID during S/G2/M phase

override the effect of the Gem tag. Nuclear import was not impaired, because treatment with LMB caused AID-mCherry-Gem to accumulate in the nucleus. Nuclear destabilization did not eliminate the protein, as treatment with MG132 had very little effect. The remaining possibility is that nuclear export promotes accumulation of AID-mCherry-Gem in the cytoplasm rather than the nucleus during S/G2/M phases. I confirm this in Chapter 6, by showing that the export defective derivative AID^{F193A}-mCherry-Gem can localize to the nucleus in S/G2/M phases. Thus nuclear export appears to contribute to cell cycle regulation of nuclear AID.

I showed that Ig gene hypermutation was accelerated in Ramos B cells expressing AID-mCherry-Cdt1, which localizes to the nucleus primarily in G1 phase. AID-mCherry-Cdt1 transductants exhibited a significant increase in the rate of sIgM loss and in mutation frequency at the rearranged and expressed IgV_H region compared to AID-mCherry transductants and AID-mCherry-Gem transductants. Accelerated hypermutation in these transductants is consistent with previous reports that detected AID-initiated damage and enrichment of repair factors at Ig genes predominately in G1 phase [59,78,79]; Ordinario, 2009 #120; Sharbeen, #129}.

Notably, AID-mCherry-Cdt1 transductants exhibited high levels of cytotoxicity, as compared with AID-mCherry or AID-mCherry-Gem transductants. The reduced viability associated with AID-mCherry-Cdt1 expression could mean that the presence of AID in the nucleus in G1 phase is toxic. However, cell cycle restriction of AID was not absolute. Some AID-mCherry-Cdt1 transductants exhibited a nuclear mCherry+ signal in S/G2/M phases, which may account for toxicity. It is possible that AID-mCherry-Cdt1 may not be subject to all forms of regulation necessary to ensure that diversification occurs while protecting the genome from instability, as would occur if the Cdt1 tagged protein was not effectively destabilized in S/G2/M phase. Experiments described in Chapter 6 address this possibility.

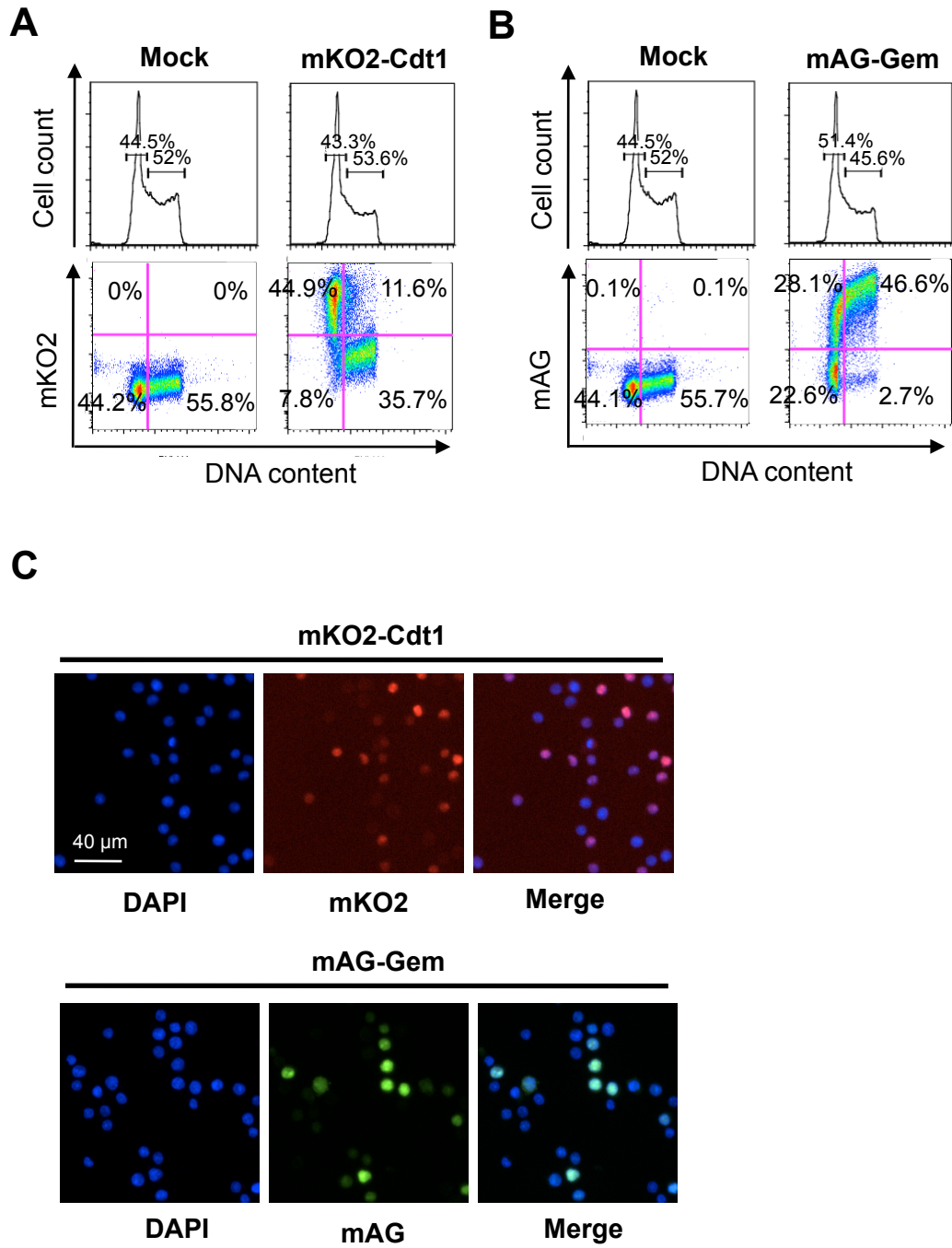


Figure 5-1. Cdt1 and Gem tags limit stability of fluorescent proteins mKO2 and mAG, respectively.

A. FACS profiles of cell cycle (above) and mKO2 signal vs. DNA content (below) for mock-transduced Ramos cells and cells transduced with an mKO2-Cdt1 expression construct. Gates: G1, mKO2⁺ (top left); S/G2M, mKO2⁺ (top right); G1, mKO2⁻ (bottom left); S/G2M, mKO2⁻ (bottom right).

B. FACS plots of cell cycle (above) and mAG signal vs. DNA content (below) for mock-transduced Ramos B cells and cells transduced with a mAG-Gem construct. Gates: G1, mAG (top left); S/G2M, mAG (top right); G1, mAG (bottom left); S/G2M, mAG (bottom right).

C. Fluorescent images of mKO2-Cdt1 and mAG-Gem transductants. Top, DAPI (left), mKO2 (middle) and merge (right) are shown for mKO2-Cdt1 transductants. Bottom, DAPI (left), mAG (middle) and merge (right) are shown for mAG-Gem transductants.

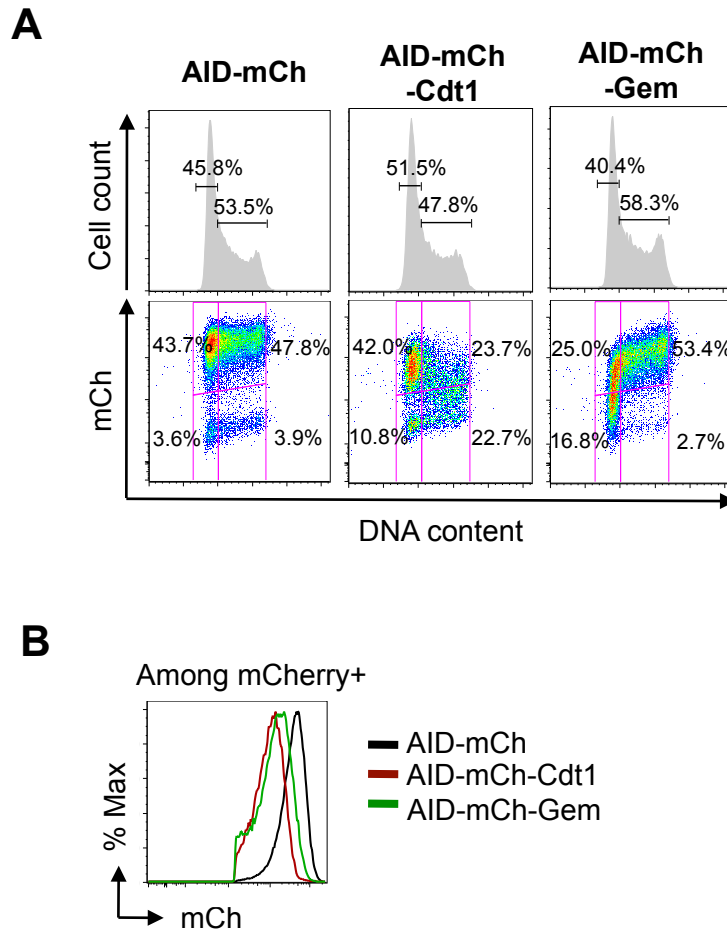


Figure 5-2. Abundance of Cdt1- and Gem-tagged AID-mCherry.

A. Representative FACS profiles of AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem transductants. Top, cell number relative to cell cycle (DNA content), with fractions of G1 and S/G2/M phase cells indicated. Bottom, mCherry⁺ signal relative to DNA content, with percentage of G1/mCherry⁺ (top left quadrant), G1/mCherry⁻ (bottom left quadrant), S/G2/M/mCherry⁺ (top right quadrant) and S/G2/M/mCherry⁻ (bottom right quadrant).

B. Profiles of mCherry signal in the mCherry⁺ gate as percent of maximum for AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem transductants.

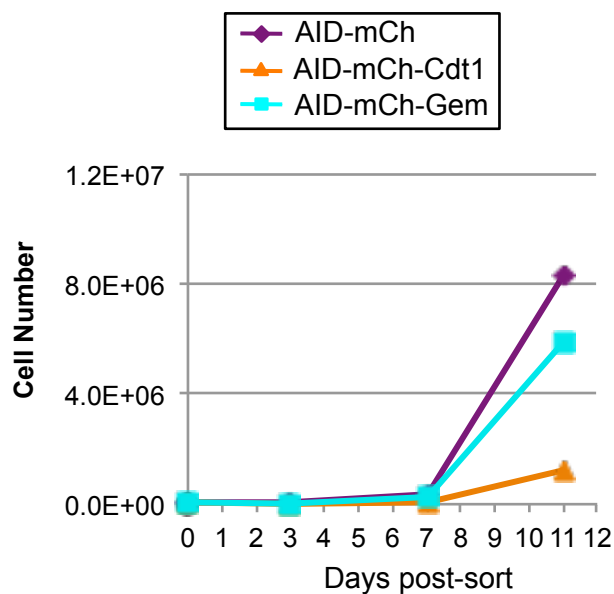


Figure 5-3. Expression of AID-mCherry-Cdt1 is toxic.

Quantification of cell viability over days in culture for Ramos B cells expressing AID-mCherry, AID-mCherry-Cdt1, and AID-mCherry-Gem. Number of viable cells was determined at days 3, 7, and 11 post sorting mCherry+ transductants.

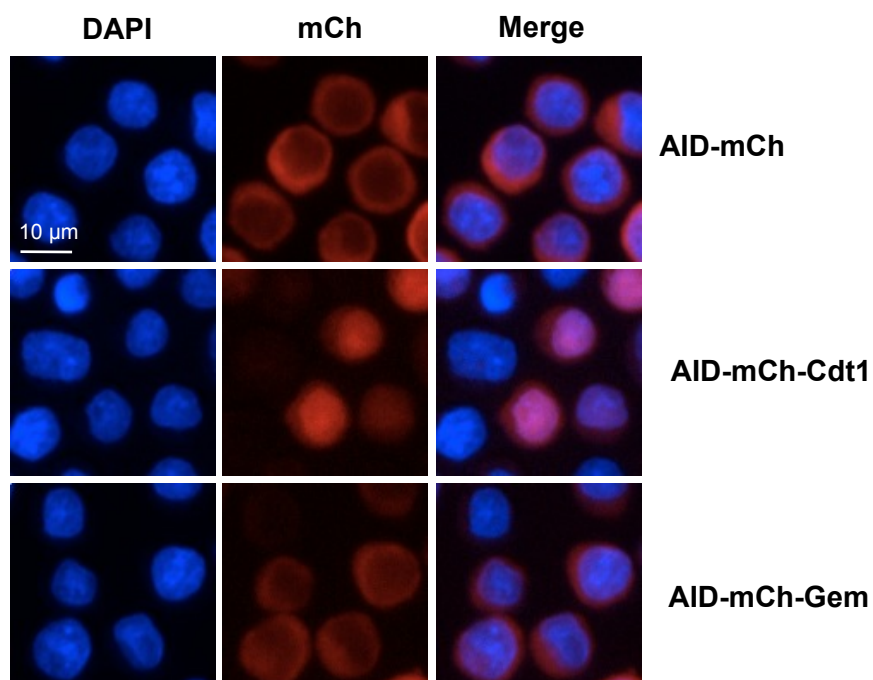


Figure 5-4. AID-mCherry-Cdt1 exhibits nuclear localization.

Representative images of AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem transductants. DAPI (left), mCherry (middle), and merge (right) are shown for AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem transductants.

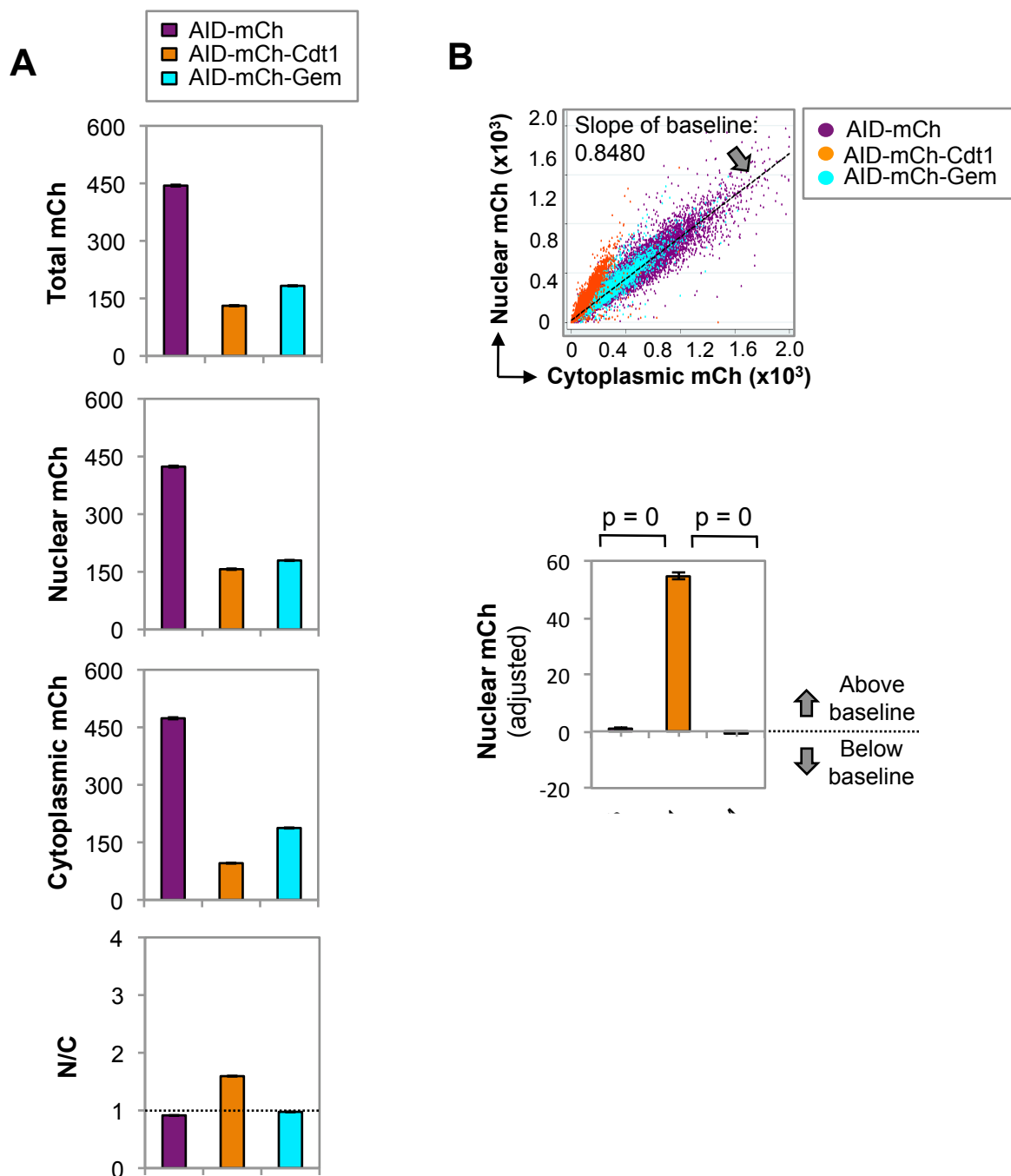


Figure 5-5. HCS-analysis AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem sub-cellular localization.

B. Total, nuclear and cytoplasmic mCherry levels and N/C quantified by HCS for AID-mCherry, AID-mCherry-Cdt1 or AID-mCherry-Gem transductants are shown. Each point on the graph represents the population average, and black bars are SEM of the population.

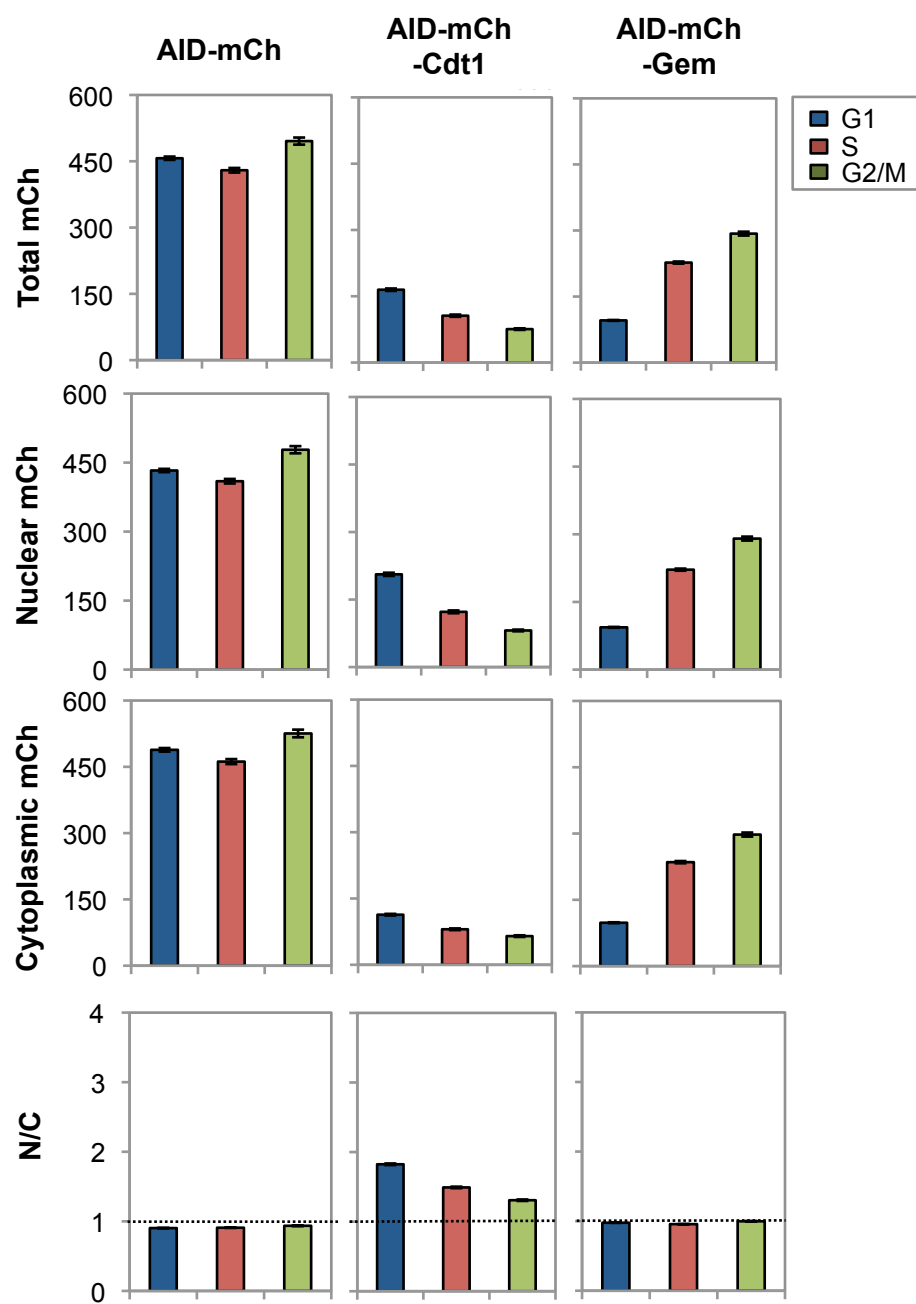
C. Left, scatter plot of nuclear vs cytoplasmic mCherry signals for populations expressing AID-mCherry (purple), AID-mCherry-Cdt1 (orange) or AID-mCherry-Gem (cyan). Dash line represents the baseline defined by linear regression analysis of AID-mCherry population. Right, distribution of nuclear AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem relative to baseline (dotted line). Each point on the graph represents the population average, and black bars are SEM of the population.

	AID-mCh		AID-mCh-CDT1		AID-mCh-GEM	
	N	mean	N	mean	N	mean
Total mCh	14666	444.2	6854	130.9	17309	182.9
Cytoplasmic mCh	14666	473.6	6854	95.8	17309	187.5
Apparent nuclear mCh	14666	423.7	6854	156.9	17309	179.7
N/C	14666	0.9	6854	1.6	17309	1.0
Adjusted nuclear mCh	14666	0.9	6854	54.6	17309	-0.5

	AID-mCh vs. AID-mCh-CDT1	AID-mCh vs. AID-mCh-GEM	AID-mCh-CDT1 vs. AID-mCh-GEM
	p-value	p-value	p-value
Total mCh	0	0	0
Cytoplasmic mCh	0	0	0
Apparent nuclear mCh	0	0	7.67E-30
N/C	0	9.30E-239	0
Adjusted nuclear mCh	0	0.06	0

Table 5-1. Comparison of cellular distribution of AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem.

The number of cells (N) and the average total, cytoplasmic, apparent nuclear and adjusted nuclear mCherry signals and the N/C ratio are tabulated for AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem transductants. Statistical test using two-tailed, unpaired Student's t-test, assuming unequal variances for comparisons among AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem transductants.

A

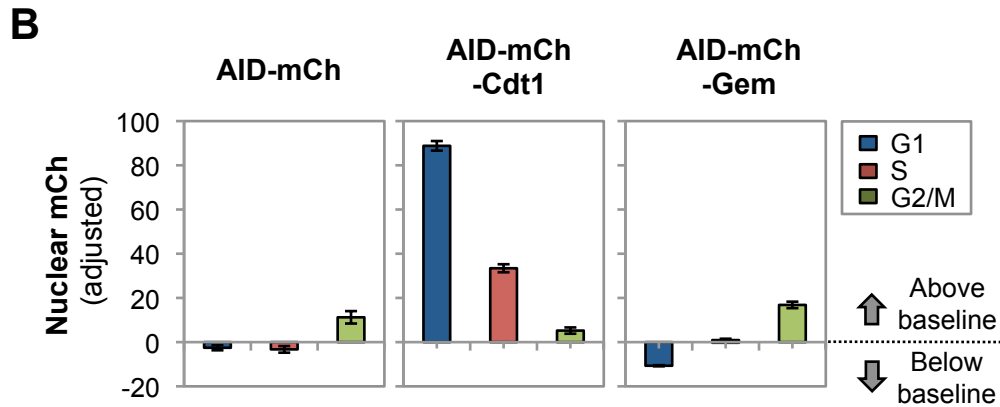


Figure 5-6. Cellular distribution of AID-mCherry, AID-mCherry-Cdt1, and AID-mCherry-Gem during the course of the cell cycle.

A. HCS quantification of nuclear and cytoplasmic mCherry levels and N/C for AID-mCherry, AID-mCherry-Cdt1 or AID-mCherry-Gem transductants are shown for G1 (blue), S (red) and G2/M (green) phase. Each point on the graph represents the population average, and black bars are SEM of the population.

B. Distribution of nuclear AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem relative to baseline (dotted line) for cells in G1, S or G2/M phase. Each point on the graph represents the population average, and black bars are SEM of the population.

	AID-mCh						G1 vs. S	G1 vs. G2/M	S vs. G2/M
	G1		S		G2/M				
	N	mean	N	mean	N	mean			
Total mCh	5282	456.9	2730	429.7	1269	495.7	2.3E-05	1.3E-05	6.88E-12
Cytoplasmic mCh	5282	488.5	2730	461.8	1269	525.5	0.0002	0.0001	1.06E-09
Apparent nuclear mCh	5282	432.8	2730	409.5	1269	478.0	0.0001	1.80E-07	2.32E-13
N/C	5282	0.9	2730	0.9	1269	0.9	0.1863	1.02E-06	0.0001
Adjusted nuclear mCh	5282	-2.5	2730	-3.3	1269	11.2	0.6984	5.90E-06	5.43E-06

	AID-mCh-Cdt1						G1 vs. S	G1 vs. G2/M	S vs. G2/M
	G1		S		G2/M				
	N	mean	N	mean	N	mean	p-value	p-value	p-value
Total mCh	1785	164.8	1285	106.0	899	75.7	6.27E-53	3.43E-142	1.52E-21
Cytoplasmic mCh	1785	113.4	1285	80.4	899	64.8	1.54E-33	3.87E-69	1.05E-08
Apparent nuclear mCh	1785	206.1	1285	122.7	899	81.4	6.27E-66	1.23E-177	1.64E-28
N/C	1785	1.8	1285	1.5	899	1.3	5.73E-89	1.04E-196	2.67E-32
Adjusted nuclear mCh	1785	88.8	1285	33.4	899	5.2	3.43E-80	2.49E-192	3.31E-33

	AID-mCh-Gem						G1 vs. S	G1 vs. G2/M	S vs. G2/M
	G1		S		G2/M				
	N	mean	N	mean	N	mean	p-value	p-value	p-value
Total mCh	5680	95.9	3305	227.1	2011	292.9	0	1.18E-299	6.96E-38
Cytoplasmic mCh	5680	98.2	3305	235.2	2011	297.7	0	5.21E-286	1.43E-31
Apparent nuclear mCh	5680	93.7	3305	221.6	2011	290.4	0	8.84E-301	6.21E-42
N/C	5680	1.0	3305	1.0	2011	1.0	1.46E-12	1E-05	4.78E-16
Adjusted nuclear mCh	5680	-10.7	3305	0.9	2011	16.8	1.23E-65	6.37E-71	3.61E-23

Table 5-2. Comparison of cellular distribution of AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem during the course of the cell cycle.

The number of cells (N) and the average total, cytoplasmic, apparent nuclear and adjusted nuclear mCherry signals and the N/C ratio are tabulated for G1, S and G2/M cells in AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem transductant populations. Statistical test using two-tailed, unpaired Student's t-test, assuming unequal variances for comparisons among G1, S and G2/M phase cells in AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem transductant populations.

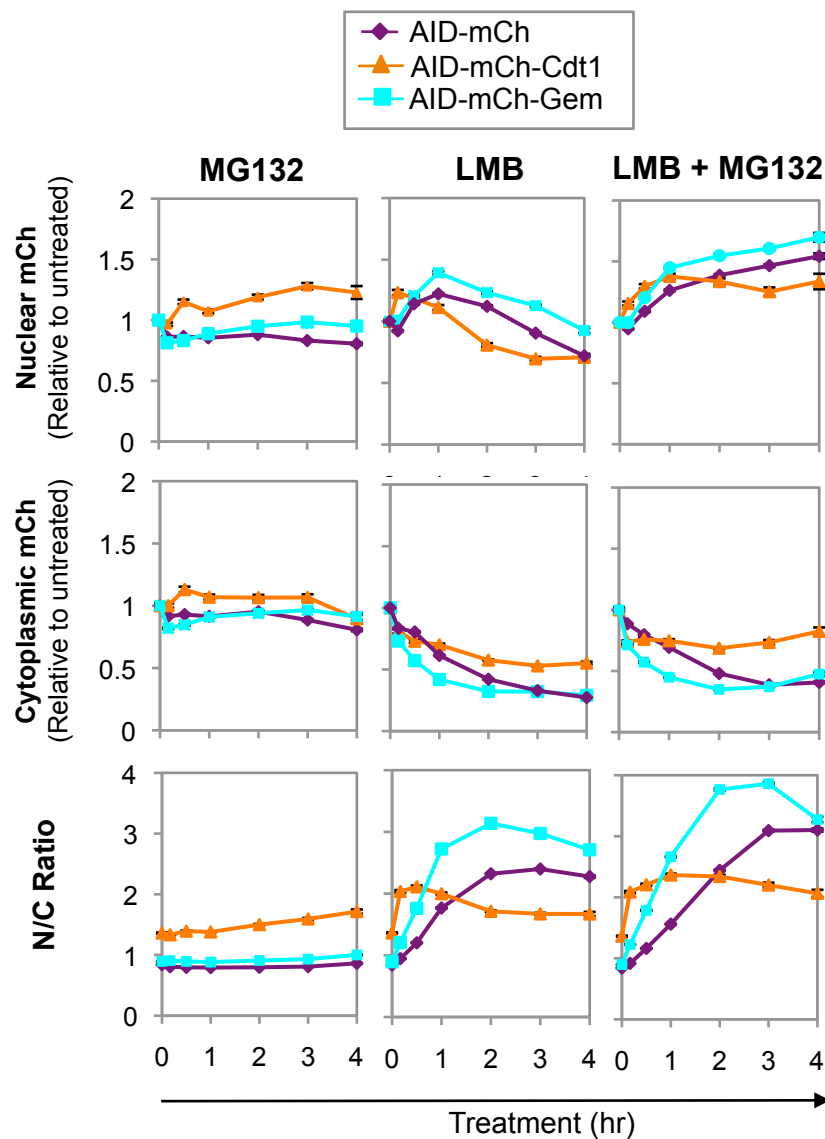


Figure 5-7. Destabilization and redistribution of AID-mCherry, AID-mCherry-Cdt1, and AID-mCherry-Gem upon treatment with MG132, LMB, or both.

Relative nuclear and cytoplasmic mCherry levels and N/C after treatment with MG132, LMB, or both in Ramos B cells expressing AID-mCherry (purple), AID-mCherry-Cdt1 (orange), or AID-mCherry-Gem (cyan). Each point on the graph represents the population average, and black bars are SEM of the population.

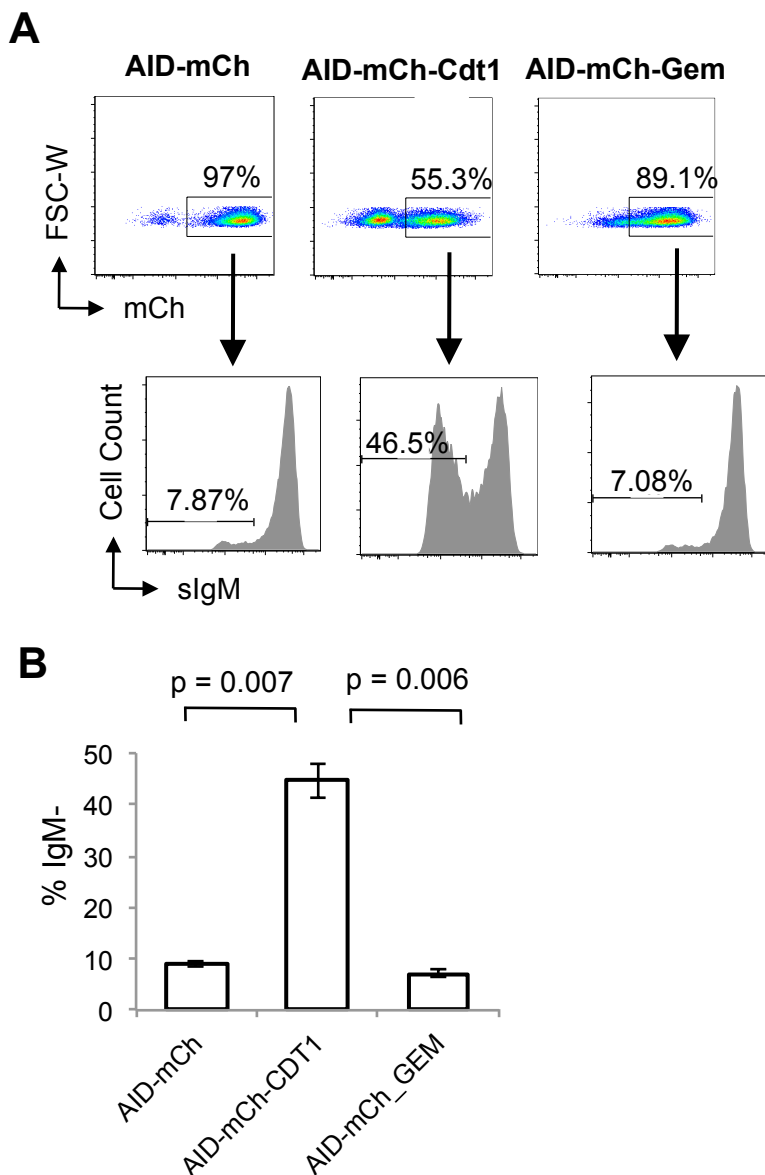


Figure 5-8. Accelerated sIgM loss in AID-mCherry-Cdt1 transductants.

A. Representative FACS profiles of AID-mCherry, AID-mCherry-Cdt1, AID-mCherry-Gem transductants. Top, mCherry⁺ signal gated relative to mock transductants (not shown). Percentage of mCherry⁺ cells indicated. Bottom, sIgM staining profile of mCherry⁺ cells from gate above, with percentage of sIgM⁻ shown above bar indicating gate for sIgM⁻.

B. Quantification of average percentage of sIgM⁻ cells and SEM, based on 3 independent experiments. Two-tailed, unpaired Student's t-test, assuming unequal variances, was used to determine statistical significance.

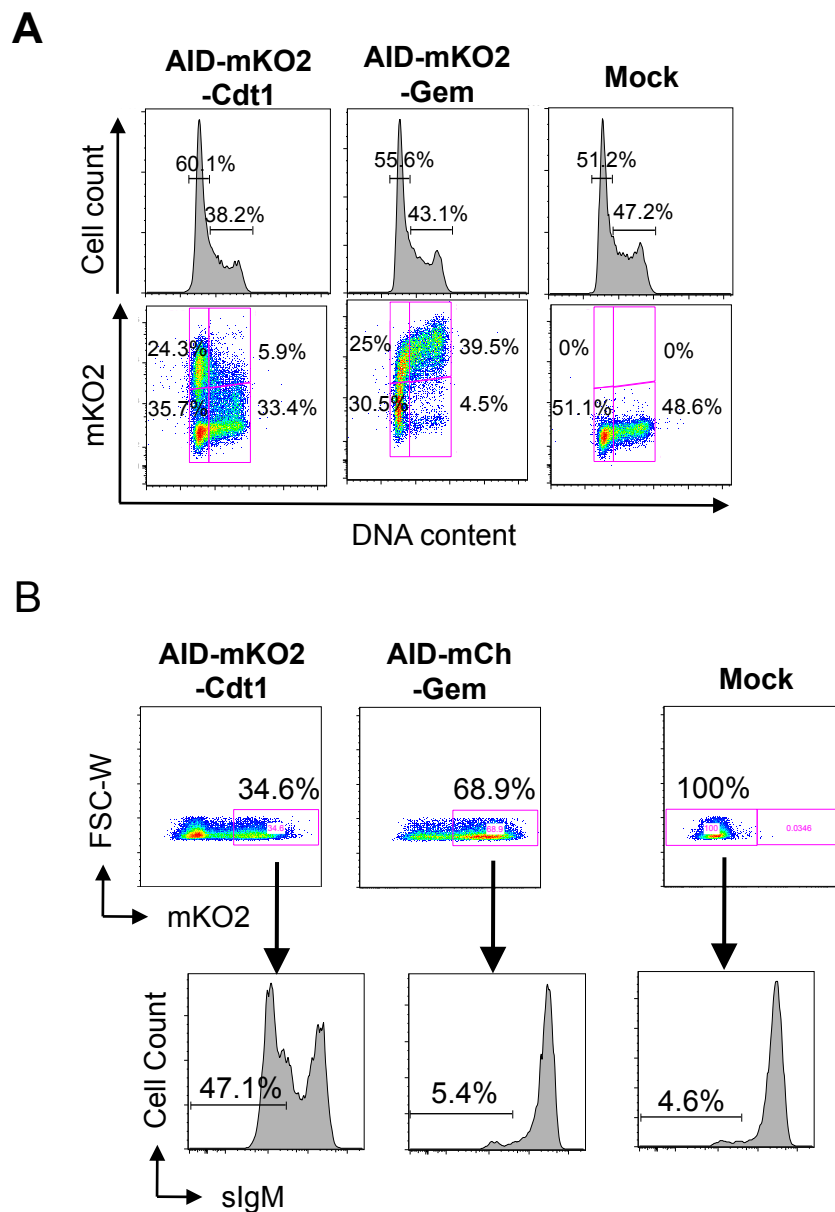


Figure 5-9. Mutagenic activity of AID-mKO2-Cdt1 is similar to AID-mCherry-Cdt1.

A. FACS profiles of AID-mKO2-Cdt1 and AID-mKO2-Gem and mock-transductants. Top, cell number relative to cell cycle (DNA content), with fractions of G1 and S/G2/M phase cells indicated. Bottom, mKO2 signal relative to DNA content, with percentage of G1/mKO2+ (top left quadrant), G1/mKO2- (bottom left quadrant), S/G2/M/mKO2+ (top right quadrant) and S/G2/M/mKO2- (bottom right quadrant).

B. FACS profiles of AID-mKO2-Cdt1, AID-mKO2-Gem and Mock. Top, mKO2+ expression gated relative to mock transductants. Bottom, sIgM staining profile of mKO2+ cells from gate above, bar indicating gate for sIgM- as described in [81 Sale, 1998 #848].

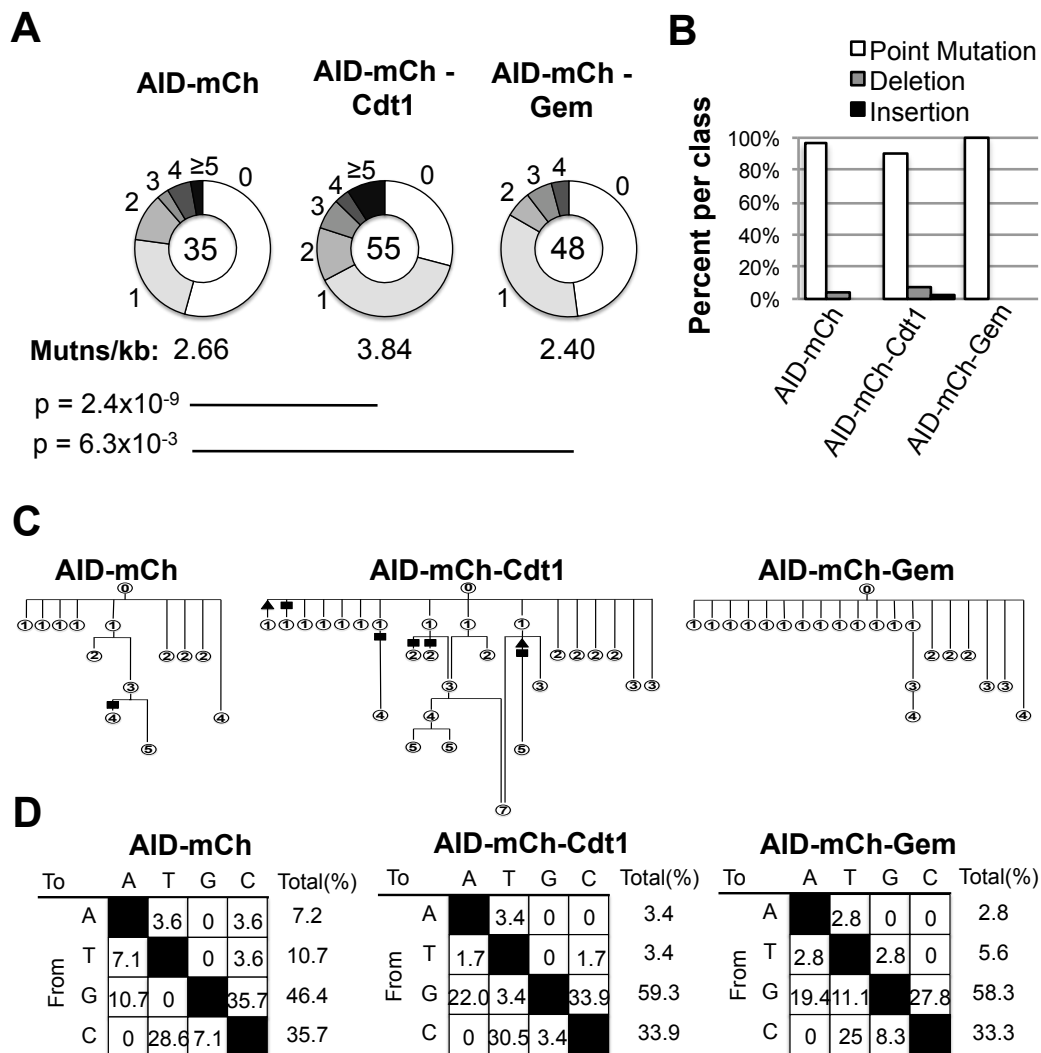


Figure 5-10. Increased mutation frequency at rearranged IgV_H regions in cells expressing AID-mCherry-Cdt1.

A. Distribution of mutations at rearranged IgV_H regions in single cells expressing AID-mCherry, AID-mCherry-Cdt1, or AID-mCherry-Gem. Proportions of sequences exhibiting 0, 1, 2, 3, 4, 5, and 6 mutations are shown. Each segment of the pie chart represents the proportion of sequences with the indicated number of mutations, with the total number of sequences analyzed shown in the middle and frequency of mutations (mutns/kb) at the bottom. Statistical significance was determined by chi square test using WT data as expected values.

B. Percent of point mutations (white), deletions (gray), and insertions black for AID-mCherry, AID-mCherry-Cdt1, or AID-mCherry-Gem transductants.

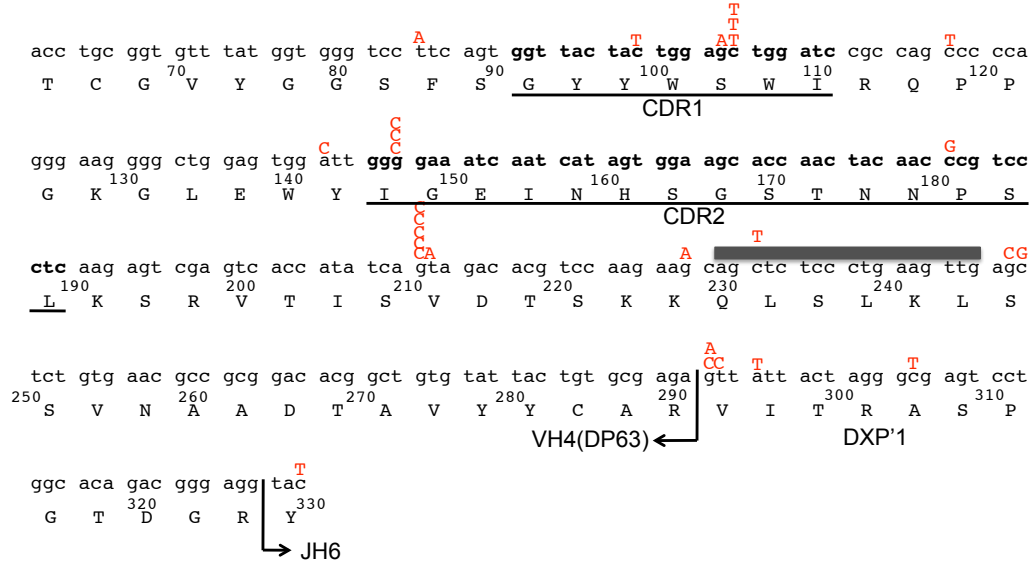
- C. Genealogy of mutation spectra in AID-mCherry, AID-mCherry-Cdt1, or AID-mCherry-Gem transductants. Only sequences with distinct mutation spectra are included. Numbers in circles tally point mutations; filled squares represent deletions; and filled triangles represent insertions.
- D. Percentage of each possible single nucleotide substitution among point mutations, with percentage of all point mutations that occur at each nucleotide shown on the right.

Mutations at	AID-mCh	AID-mCh-Cdt1	AID-mCh-Gem	Ramos*
A	7%	3%	3%	10%
T	11%	3%	6%	8%
G	46%	59%	58%	47%
C	36%	34%	33%	35%
A + T	18%	7%	8%	18%
G + C	82%	93%	92%	82%
Among mutations at G+C				
Mutations at	AID-mCh	AID-mCh-Cdt1	AID-mCh-Gem	Ramos*
G	57%	64%	64%	58%
C	43%	36%	36%	43%
Transition and transversion mutations				
Mutations	AID-mCh	AID-mCh-Cdt1	AID-mCh-Gem	Ramos*
Transitions	43%	54%	44%	51%
Transversions	57%	46%	56%	49%
* previously published (Ref. 96)				

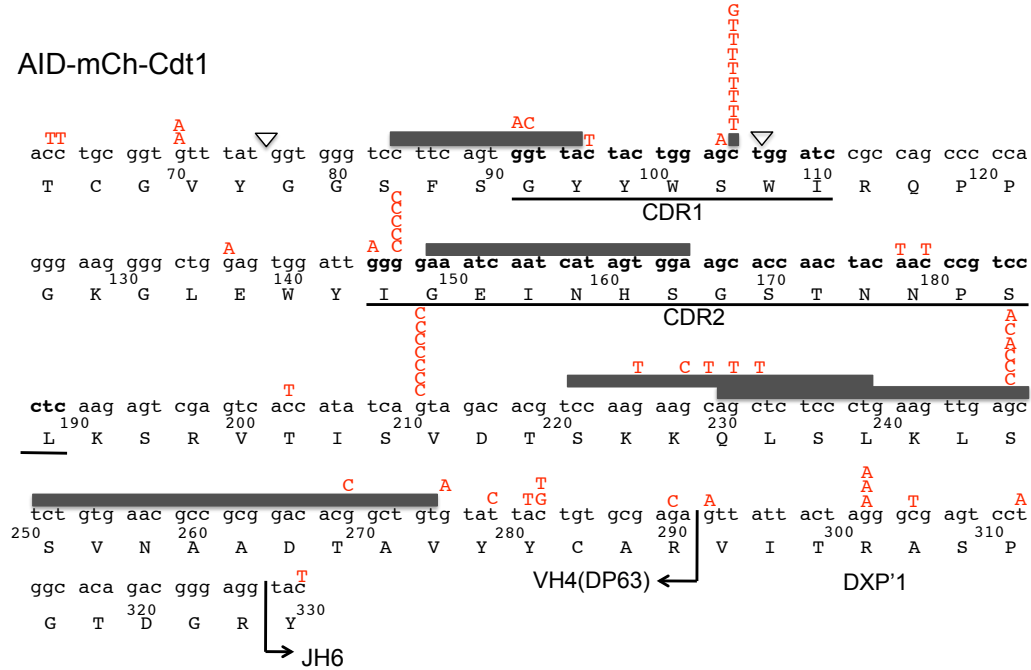
Table 5-3. Comparison of point mutations in AID-mCherry, AID-mCherry-CDT1, and AID-mCherry-GEM transductants.

Percentage of point mutations that occur at each nucleotide is shown for AID-mCherry, AID-mCherry-CDT1, and AID-mCherry-GEM, as well as percentage that are transitions and transversions.

AID-mCh



AID-mCh-Cdt1



AID-mCh-Gem

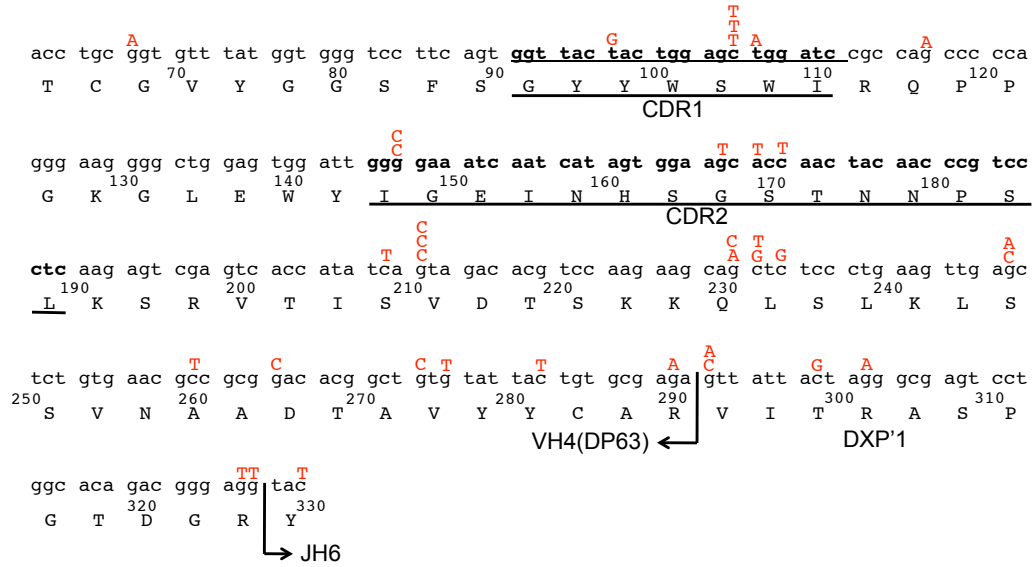


Figure 5-11. Distribution of mutations along IgV_H in cells expressing AID-mCherry, AID-mCherry-Cdt1, or AID-mCherry-Gem.

Point mutations are displayed above the parental sequence in red. Closed bars and open triangles represent deletions and insertions, respectively. Only sequences, pooled from two independent experiments, with a unique mutation spectrum are documented. Locations of CDR1 and CDR2 are indicated. Positions of nucleotides are numbered starting from the first base of first codon as +1. Corresponding amino acids are shown below each codon and numbered starting from the first codon as +1.

Chapter 6: The role of nuclear export in limiting cell cycle-dependent mutagenesis by AID in Ramos B cells

6.1 Introduction

AID-mCherry-Cdt1 expression not only accelerated Ig gene hypermutation but was also cytotoxic (Chapter 5). Among AID-mCherry-Cdt1 transductants, most mCherry⁺ cells were in G1 phase (42.0%), and a minority were in S/G2/M phases (23.7%; Table 6-1). This raised the possibility that deamination in S/G2/M phase and not G1 phase was the source of cytotoxicity. To more cleanly assign toxicity to events initiated in either G1 or S phase, I tested the effect of additional mutations. My evidence that AID undergoes proteolysis in the nucleus (Chapter 4) suggested that it might be possible to confer more rigorous cell cycle-dependent regulation by using the F193A mutation to trap AID in the nucleus (Chapter 4). I analyzed Ramos B cells expressing AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem to test this hypothesis. I found that AID^{F193A}-mCherry-Cdt1 transductants exhibit accelerated Ig gene hypermutation without accompanying cytotoxicity.

6.2. AID^{F193A}-mCherry-Cdt1 is present in the nucleus only in G1 phase

Cell cycle profiling by flow cytometry showed that the cell cycle distribution of the AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 or AID^{F193A}-mCherry-Gem transductant populations was indistinguishable (Figure 6.1). However, among AID^{F193A}-mCherry-Cdt1 transductants, a robust mCherry signal was evident in only a minority of cells (28%) at 11 days following the initial sort of mCherry⁺ transductants (Figure 6.1). This is well below the fraction of mCherry⁺ cells observed among AID-mCherry (91.5%), AID-mCherry-Cdt1 (65.7%) or AID^{F193A}-mCherry

(58%) transductants at comparable times after transduction and initial sorting (Figure 6.1 and Table 6-1). Strikingly, essentially all mCherry+ cells among AID^{F193A}-mCherry-Cdt1 transductants were in G1 phase (27%) and very few were in S/G2/M phase (1.7%; Figure 6.1 and Table 6-1). In contrast, more than half of all AID^{F193A}-mCherry-Gem transductants (55.5%) exhibited a robust mCherry+ signal; and while most mCherry+ cells in that population were in S/G2/M (36%), a considerable fraction was in the G1 phase gate (19%) (Figure 6.1, right; Table 6-1).

Thus, cell cycle restriction was complete in AID^{F193A}-mCherry-Cdt1 but not AID^{F193A}-mCherry-Gem transductants (Figure 6-1 and Table 6-1).

6.3. Enforced nuclear localization in G1 phase correlates with increased cell viability

I generated AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 or AID^{F193A}-mCherry-Gem transductants of Ramos B cells, sorted mCherry+ cells at day 3 after transduction, and analyzed cell number on days 3, 7 and 11 post-sort. It was immediately obvious that the AID^{F193A}-mCherry and AID^{F193A}-mCherry-Gem transductants exhibited considerably reduced growth relative to the AID^{F193A}-mCherry-Cdt1 transductants (Figure 6-2). By day 11, the numbers of cells in AID^{F193A}-mCherry and AID^{F193A}-mCherry-Gem transductant populations were 28% and 38% of those in AID^{F193A}-mCherry-Cdt1 transductant populations. Cell numbers were similar between AID-mCherry and AID^{F193A}-mCherry-CDT1 transductants (Figure 6-2).

6.4. AID^{F193A}-mCherry-Cdt1 nuclear signal is restricted to G1 phase

Fluorescence microscopy showed that AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 or AID^{F193A}-mCherry-Gem transductants exhibit nuclear localization (Figure 6-3). To determine

the nuclear mCherry signal during the course of the cell cycle, I used HCS microscopy. HCS analysis showed that the unadjusted nuclear mCherry signal in AID^{F193A}-mCherry-CDT transductants was 2-fold reduced in G1 phase and 10-fold reduced in S/G2/M phase relative to AID^{F193A}-mCherry transductants (Figure 6-4.A and Table 6-3). AID^{F193A}-mCherry-Gem transductants exhibited greatly reduced G1 phase signal, and 2-fold reduced S/G2/M phase relative to AID^{F193A}-mCherry transductants (Figure 6-4.A and Table 6-3).

The importance of cycle regulation was highlighted by comparison of the adjusted nuclear levels of AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem after correcting for cytoplasmic bleed through, as described in Chapter 4 above (section 4.2) (Figure 6-4.B and Table 6-3). In AID^{F193A}-mCherry transductants, the average adjusted nuclear signal was highest in G1 phase, and diminished somewhat during S phase and increased moderately in G2/M phase (Figure 6-4.B, left; Table 6-3). The decrease between G1 and S phases is consistent with my evidence that AID is degraded more slowly in G1 phase than in S and G2/M phases of cell cycle (Chapter 4). In contrast, nuclear mCherry signal was restricted exclusively to G1 phase in AID^{F193A}-mCherry-Cdt1 transductants (Figure 6-4.B and Table 6-3). Thus, AID^{F193A}-mCherry-Cdt1 is trapped in the nucleus and destabilized in S/G2/M phases by the Cdt1 tag. Among AID^{F193A}-mCherry-Gem transductants, a nuclear mCherry signal was evident in G1 phase, and increased 3-fold or more during S and G2/M phases of cell cycle, when the Gem tag confers stability (Figure 6-4.B and Table 6-3).

6.5. Forced nuclear localization of AID causes increased somatic hypermutation

To test how enforced nuclear localization of AID during G1 or S/G2/M phase affected Ig gene diversification, I compared the sIgM loss rates of Ramos AID^{F193A}-mCherry, AID^{F193A}-

mCherry-Cdt1 and AID^{F193A}-mCherry-Gem transductants. mCherry⁺ cells were sorted at 3 days post-transduction, then cultured for 3, 7 and 11 days before quantifying the fraction of sIgM-cells. At day 7, this fraction was 83.5% in AID^{F193A}-mCherry transductants, 67.0% in AID^{F193A}-mCherry-Cdt1 transductants, and 73.2% in the AID^{F193A}-mCherry-Gem transductants (Figure 6-5.A). Comparing the fractions of sIgM-cells at different days post sort, the fraction of sIgM-cells increased by 2-fold from day 3 to day 7 for AID^{F193A}-mCherry (34.2% to 83.5%), AID^{F193A}-mCherry-Cdt1 (27.6% to 67.0%) and AID^{F193A}-mCherry-Gem (24.1% to 73.2%) transductants (Figure 6-5.B). At day 11, the fraction of sIgM-cells was 65.9% in AID^{F193A}-mCherry transductants, 68.2% in AID^{F193A}-mCherry-Cdt1 transductants, and 72.2% in the AID^{F193A}-mCherry-Gem transductants (Figure 6-5.B).

6.6 Discussion

The effect of AID^{F193A}-mCherry-Cdt1 on cell proliferation contrasts with that of AID-mCherry-Cdt1, which caused clear reduced cell growth (Figure 5.3). I conclude that AID must be in the nucleus to undergo degradation, and AID that escapes via export in G1 phase is able to re-enter and damage DNA later in the cell cycle.

Limiting AID activity depends largely on nuclear export, as AID^{F193A}-mCherry transductants, AID^{F193A}-mCherry-Cdt1 transductants, and AID^{F193A}-mCherry-Gem transductants all exhibited comparable rates of sIgM loss that are substantially higher than the WT AID derivatives. Because AID deamination is restricted to S/G2/M phase in AID^{F193A}-mCherry-Gem transductants, mutagenic repair of AID-induced DNA damage may occur either during S/G2/M phase, or AID deamination introduced in S/G2/M may persist through cell division and be repaired in G1 phase in the subsequent cell cycle, or both. Elucidating the timing and

mechanisms of repair at AID-induced lesions in the context of cell cycle will be of interest for future studies. In either case, misregulated AID enzymatic activity has consequences for the genome due to off-target activity [41,42,43,44,45].

Interestingly, cells were affected similarly by the expression of AID^{F193A}-mCherry and AID AID^{F193A}-mCherry-Gem, which are nuclear through out cell cycle and in S/G2/M, respectively. In both of these cases, cells expressing AID during S/G2/M had vastly impaired growth rates, indicating that the cells had a large AID-induced cytotoxic burden. The toxicity observed may be attributed to the presence of AID in the nucleus during S or G2/M phase, because cells that express AID^{F193A}-mCherry-Cdt1, which resides in the nucleus exclusively in G1 phase, did not exhibit substantial cell death. Consistent with this, cells that express AID-mCherry-Cdt1 experienced similar toxicity to AID^{F193A}-mCherry and AID^{F193A}-mCherry-Gem transductants, perhaps due to persistent expression of AID-mCherry-Cdt1 evident in a fraction of S phase cells (although the level was much less in S than in G1 phase).

These experiments demonstrated that nuclear stability of AID promotes Ig gene diversification, and that nuclear export protects the genome from AID-induced mutagenesis during S/G2/M phase. In G1 phase, AID deamination would be restricted to transcribed genes; whereas in S phase, DNA replication yields ssDNA that would also be a target for AID deamination. It is plausible that nuclear AID is especially deleterious to cells during S phase because AID could promote mutagenesis throughout the genome, not just at transcribed regions that are surveyed for DNA damage by transcription-coupled repair processes. It will be of interest to assess the frequency and identity of AID-dependent mutations genome-wide at different stage of cell cycle to clarify the role of AID in genomic instability.

	mCh+	mCh+ G1	mCh+ S/G2/M
AID-mCh	91.5%	43.7%	47.8%
AID-mCh-CDT1	65.7%	42.0%	23.7%
AID-mCh-GEM	78.4%	25.0%	53.4%
AID ^{F193A} -mCh	58%	29.4%	28.7%
AID ^{F193A} -mCh-CDT1	28%	26.7%	1.7%
AID ^{F193A} -mCh-GEM	78.4%	19.4%	36.1%

Table 6-1. Cell cycle distribution of mCherry + cells for AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem and their F193A mutant derivatives.

Percentage of total cells that is mCherry + cells, mCherry + G1 phase cells and mCherry + S/G2/M phase cells in AID-mCherry, AID-mCherry-Cdt1, AID-mCherry-Gem, AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem transductant populations.

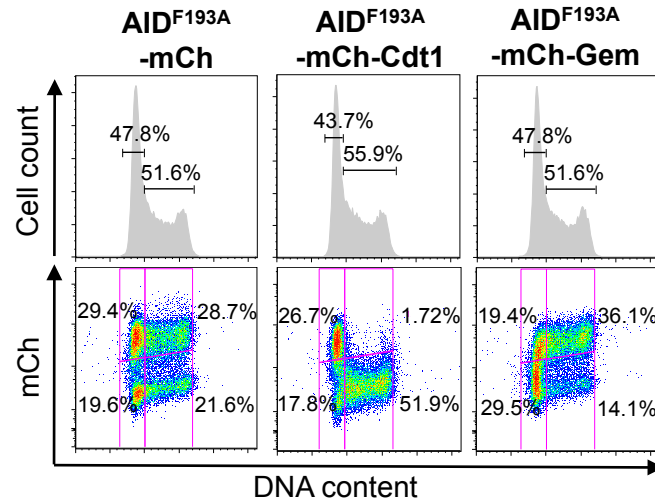


Figure 6-1. Abundance of AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem.

Representative FACS profiles of AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem. Top, cell number relative to cell cycle (DNA content), with fractions of G1 and S/G2/M phase cells indicated. Bottom, mCherry+ signal relative to DNA content, with percentage of G1/mCherry+ (top left quadrant), G1/mCherry- (bottom left quadrant), S/G2/M/mCherry+ (top right quadrant) and S/G2/M/mCherry- (bottom right quadrant).

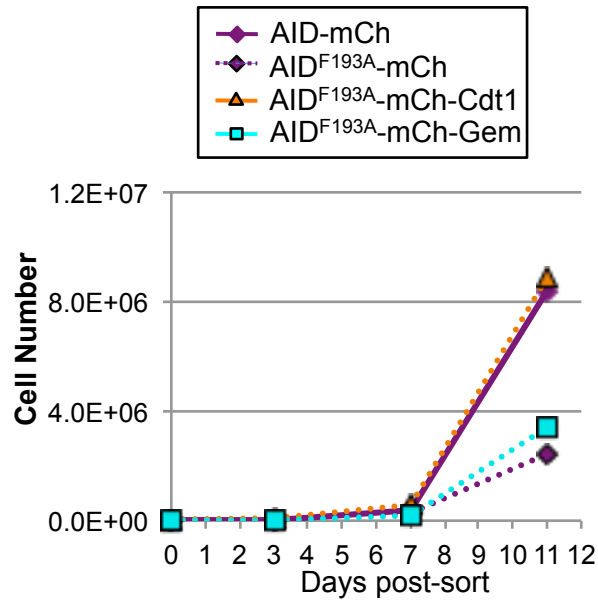


Figure 6-2. AID^{F193A}-mCherry and AID^{F193A}-mCherry-Gem transductants exhibited considerably reduced growth relative to the AID^{F193A}-mCherry-Cdt1 transductants. Quantification of cell viability over days in culture for Ramos B cells expressing of AID-mCherry (purple solid line, diamond), AID^{F193A}-mCherry (purple dotted line, diamond), AID^{F193A}-mCherry-Cdt1 (orange dotted line, triangle), and AID^{F193A}-mCherry-Gem (cyan dotted line, square). Number of viable cells was determined at days 3, 7, and 11 post sorting mCherry+ transductants.

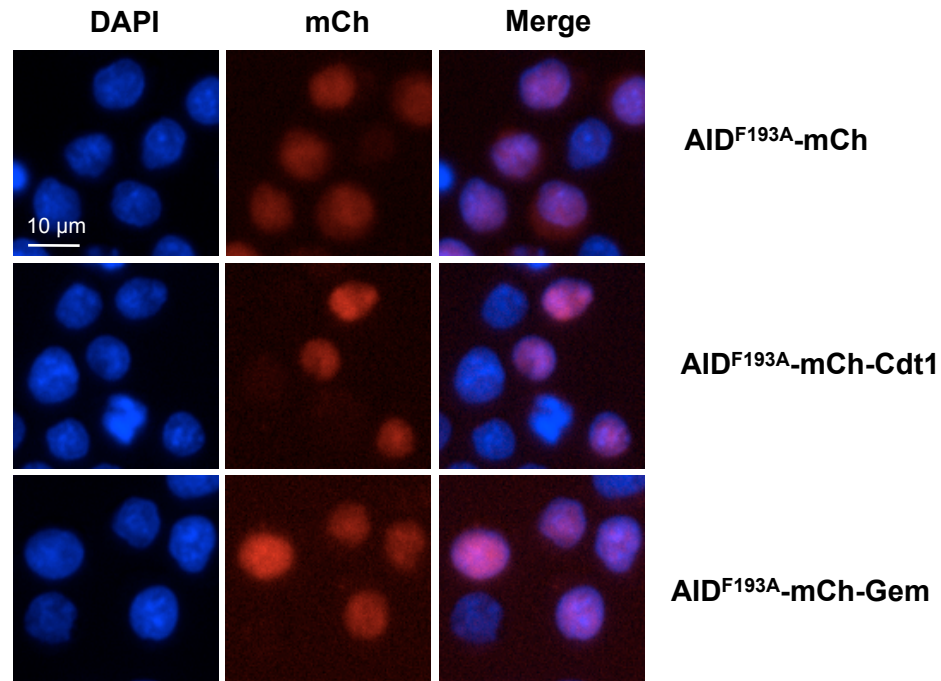
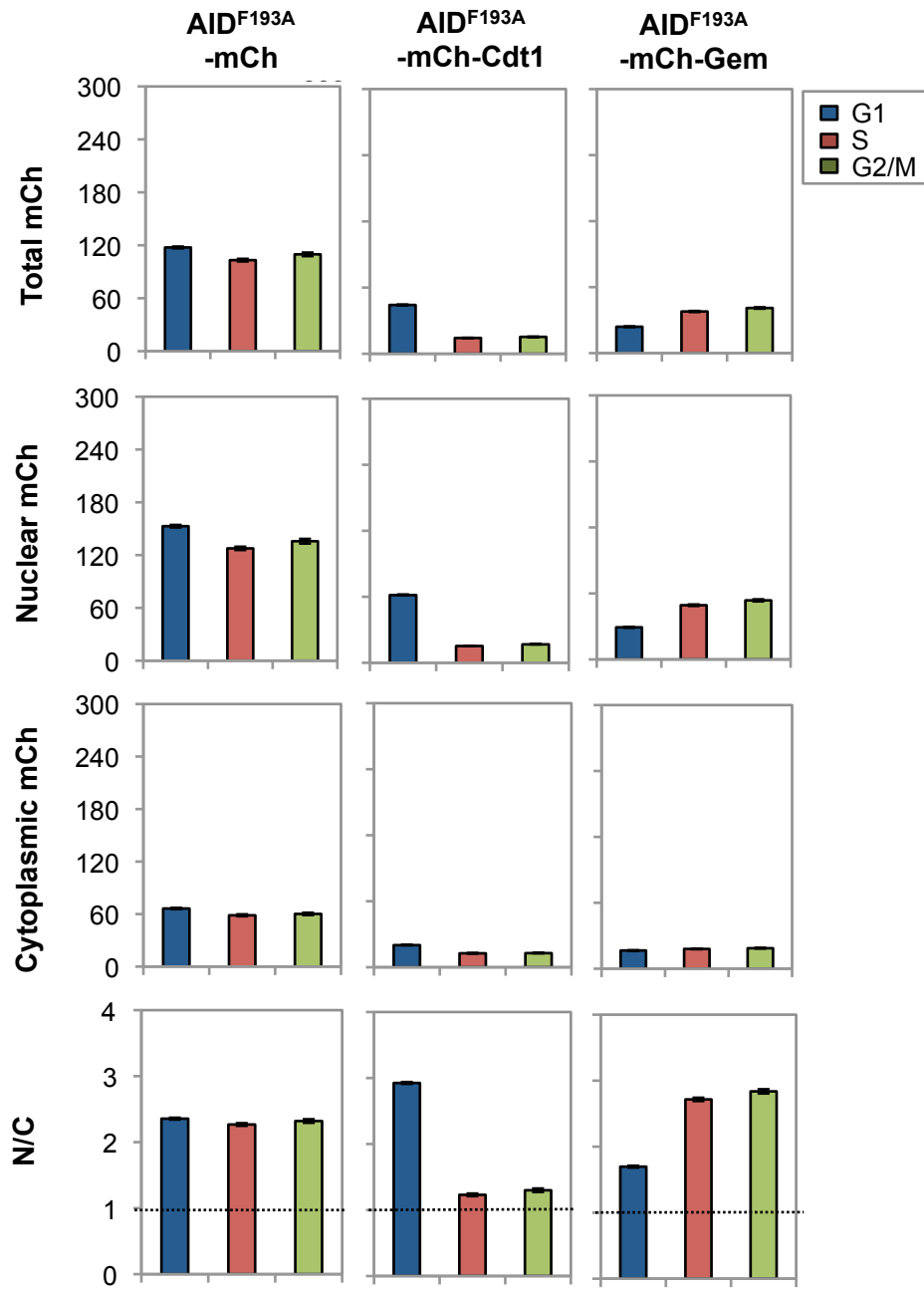


Figure 6-3. AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem exhibit nuclear localization.

Representative images of AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem transductants. DAPI (left), mCherry (middle), and merge (right) are shown for AID-mCherry, AID-mCherry-Cdt1 and AID-mCherry-Gem transductants.

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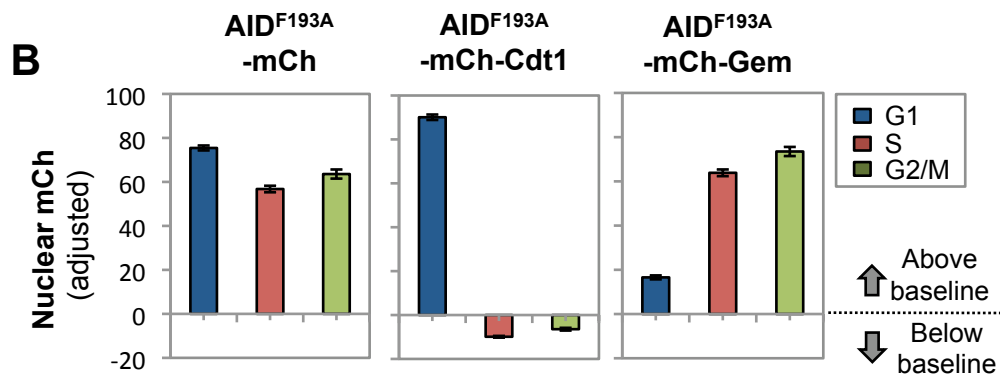


Figure 6-4. Cellular distribution of AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem during the course of the cell cycle.

A. HCS quantification of average nuclear and cytoplasmic mCherry levels and N/C are shown for G1 (blue), S (red) and G2/M (green) phase in each AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem transductants. Each point on the graph represents the population average, and black bars are SEM of the population.

B. Distribution of nuclear AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem relative to baseline (dotted line) for cells in G1, S or G2/M phase. Each point on the graph represents the population average, and black bars are SEM of the population.

	AID^{F193A}-mCh		AID^{F193A}-mCh-Cdt1		AID^{F193A}-mCh-Gem	
	N	mean	N	mean	N	mean
Total mCh	13485	111.4	11790	76.5	9223	83.4
Cytoplasmic mCh	13485	62.9	11790	41.6	9223	44.7
Apparent nuclear mCh	13485	142.0	11790	101.2	9223	107.0
N/C	13485	2.3	11790	2.2	9223	2.4
Adjusted nuclear mCh	13485	67.5	11790	44.8	9223	47.9

	AID^{F193A}-mCh vs. AID^{F193A}-mCh- CDT1	AID^{F193A}-mCh vs. AID^{F193A}-mCh- GEM	AID^{F193A}-mCh- CDT1 vs. AID^{F193A}- mCh-GEM
	p-value	p-value	p-value
Total mCh	0	5.09E-260	4.0E-19
Cytoplasmic mCh	0	1.52E-254	6.6E-17
Apparent nuclear mCh	1.17E-250	2.86E-210	5.8E-07
N/C	8.21E-25	1.21E-02	3.8E-29
Adjusted nuclear mCh	5.35E-110	0	2.3E-03

Table 6-2. Comparison of cellular distribution of AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem.

The number of cells (N) and the average total, cytoplasmic, apparent nuclear and adjusted nuclear mCherry signals and the N/C ratio are tabulated for AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem transductants. Statistical test using two-tailed, unpaired Student's t-test, assuming unequal variances for comparisons among AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem transductants.

	AID ^{F193A} -mCh						G1 vs. S	G1 vs. G2/M	S vs. G2/M
	G1		S		G2/M				
	N	mean	N	mean	N	mean	p-value	p-value	p-value
Total mCh	5047	117.5	2281	103.2	1396	109.7	1.08E-15	5.48E-04	8.65E-03
Cytoplasmic mCh	5047	66.4	2281	58.6	1396	60.2	3.22E-09	4.89E-05	3.65E-01
Apparent nuclear mCh	5047	152.9	2281	127.6	1396	135.8	2.65E-27	9.87E-09	1.16E-02
N/C	5047	2.4	2281	2.3	1396	2.3	2.56E-04	2.35E-01	1.14E-01
Adjusted nuclear mCh	5047	75.4	2281	56.8	1396	63.6	2.24E-23	5.55E-07	7.67E-03

	AID ^{F193A} -mCh-Cdt1						G1 vs. S	G1 vs. G2/M	S vs. G2/M
	G1		S		G2/M				
	N	mean	N	mean	N	mean	p-value	p-value	p-value
Total mCh	5192	110.6	1649	35.9	951	38.3	0	0	7.58E-06
Cytoplasmic mCh	5192	50.2	1649	31.7	951	32.1	3.56E-224	5.72E-261	4.39E-01
Apparent nuclear mCh	5192	153.9	1649	38.1	951	41.9	0	0	4.00E-07
N/C	5192	2.9	1649	1.2	951	1.3	0	0	2.33E-06
Adjusted nuclear mCh	5192	90.1	1649	-9.9	951	-6.5	0	0	3.45E-06

	AID ^{F193A} -mCh-Gem						G1 vs. S	G1 vs. G2/M	S vs. G2/M
	G1		S		G2/M				
	N	mean	N	mean	N	mean	p-value	p-value	p-value
Total mCh	3049	60.0	1689	94.6	1222	102.6	2.43E-127	9.42E-117	4.82E-05
Cytoplasmic mCh	3049	41.5	1689	45.0	1222	46.6	3.26E-06	4.18E-11	6.13E-02
Apparent nuclear mCh	3049	73.0	1689	123.2	1222	134.2	9.73E-131	1.29E-111	1.15E-04
N/C	3049	1.7	1689	2.7	1222	2.8	1.62E-228	2.27E-181	2.87E-03
Adjusted nuclear mCh	3049	16.6	1689	63.9	1222	73.5	4.47E-142	8.24E-120	1.73E-04

Table 6-3. Comparison of cellular distribution of AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem during the course of the cell cycle.

The number of cells (N) and the average total, cytoplasmic, apparent nuclear and adjusted nuclear mCherry signals and the N/C ratio are tabulated for G1, S and G2/M cells in AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem transductant populations. Statistical test using two-tailed, unpaired Student's t-test, assuming unequal variances for comparisons among G1, S and G2/M phase cells in AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem transductant populations.

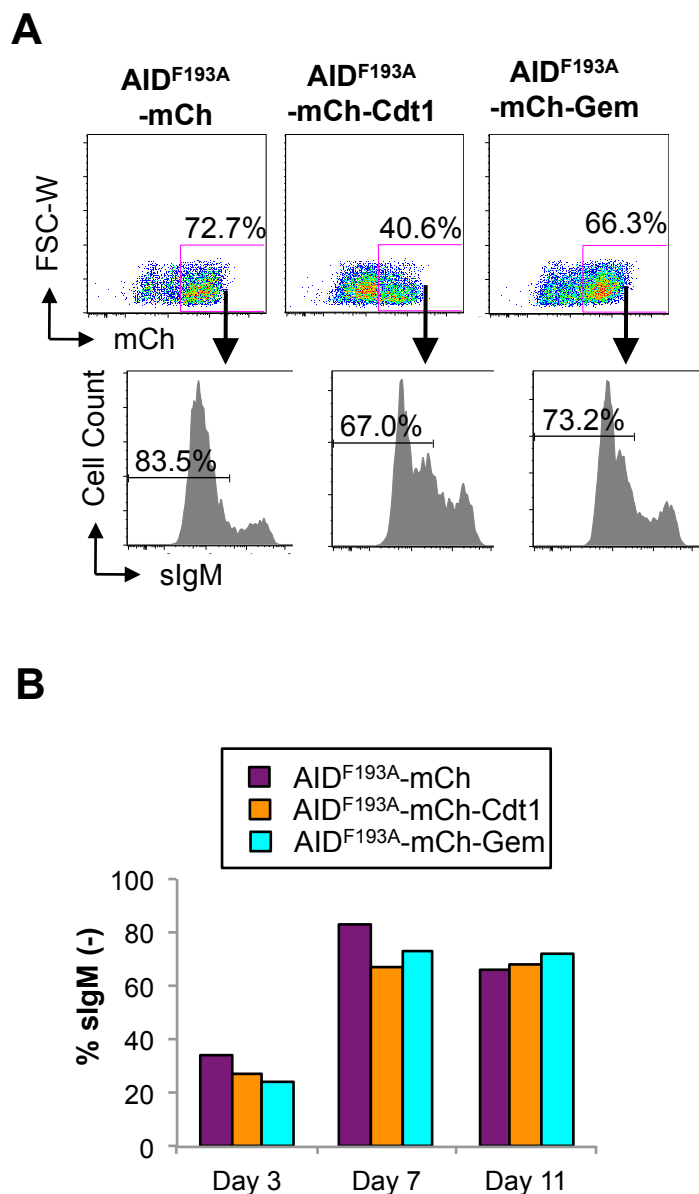


Figure 6-5. Mutagenic activity of AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem.

A. Representative FACS profiles of AID^{F193A}-mCherry, AID^{F193A}-mCherry-Cdt1 and AID^{F193A}-mCherry-Gem transductants. Top, mCherry⁺ signal gated relative to mock transductants (not shown). Percentage of mCherry⁺ cells indicated. Bottom, sIgM staining profile of mCherry⁺ cells from gate above, with percentage of sIgM⁻ shown above bar indicating gate for sIgM⁻.

B. Percentage of sIgM⁻ cells at day 3, 7 and 11 post-sort for AID^{F193A}-mCherry (purple bars), AID^{F193A}-mCherry-Cdt1 (orange bars) and AID^{F193A}-mCherry-Gem (cyan bars) transductants.

Chapter 7: Conclusions and future directions

AID is a required factor for Ig gene diversification. As a DNA mutator, it has the potential to induce genome-wide mutagenesis. Consequently, the genome can accumulate off-target mutations as collateral damage from Ig gene diversification, which are repaired by high-fidelity repair mechanisms to restore genomic stability. The regulation of AID is stringent to prevent AID pathological activities, as evident in both B cell and non-B cell malignancies which exhibit deregulation of AID. Because AID functions in the nucleus, some regulation is spatial, and involves limiting active AID in the nucleus. The role of cell cycle in AID regulation has not been studied extensively.

7.1 Summary

In Chapter 3, I examined the possibility that nuclear AID levels oscillate, which would enable feedback regulation, by live cell imaging. I show that AID exhibits pulses of nuclear localization that are very similar to the nuclear pulses displayed by NF- κ B. This is an exciting discovery because it suggests that AID activity is limited to a very small window of time, which may allow the genome to recover from AID-induced deaminase after each pulse. Future studies will characterize the nuclear pulse in the course of cell cycle, as AID physiological vs pathological activities may depend on the number, duration, and nuclear level of the pulse.

I have identified new mechanisms that confer spatiotemporal regulation to AID. Population-based analysis of AID in nuclear and cytoplasmic compartments revealed that AID is predominately cytoplasmic and is subjected to proteosomal degradation in the nucleus, as I have shown in Chapter 4. AID is cytoplasmic in all phases of cell cycle, suggesting that nuclear-

cytoplasmic shuttling of AID favors nuclear export. Degradation of nuclear AID occurs more slowly in G1 phase compared to S and G2/M phase, consistent with the view that AID functions in G1 phase. Nuclear entry does not appear to be critical to cell cycle regulation, as the rate of nuclear entry was essentially constant throughout cell cycle.

To test whether nuclear stability of AID in G1 phase is important to Ig gene diversification, I utilized Cdt1 and Gem cell cycle tags to restrict nuclear stability of AID to G1 and S/G2/M phase, respectively, as shown in Chapter 5. I found that the Cdt1 tag, but not the Gem tag, is able to confer nuclear localization of AID, suggesting that AID may be stable in the nucleus only in G1 phase cells. Stabilizing AID in G1 phase by means of the Cdt1 tag significantly increased the rate of hypermutation, as measured both by the rate of sIgM loss (7.9% sIgM⁻ in AID-mCherry transductants vs. 46.5% sIgM⁻ in AID-mCherry-Cdt1 transductants; $p=0.007$) and by the mutation frequency at rearranged and expressed IgH V region (mutns/kb = 2.66 in AID-mCherry transductants and 3.84/kb in AID-mCherry-Cdt1 transductants ($p=2.4 \times 10^{-9}$)).

Sub-cellular localization of AID depends largely on nuclear export, which protects AID from degradation and prevents AID-induced mutagenesis. To ask if nuclear export is cell cycle regulated, I created F193A mutants to inhibit nuclear export (Chapter 6). AID^{F193A} resides predominately in the nucleus, and cells that express this protein exhibit massive cell death. Similarly, cells that express AID^{F193A}-mCherry-Gem, which stabilizes nuclear AID during S/G2/M phase, exhibit massive cell death. In contrast, cells that express AID^{F193A}-mCherry-Cdt1, which restricts nuclear AID to G1 phase, exhibited no cell death beyond that characteristic of cells expressing WT AID. Together these results support the hypothesis that nuclear stability of

AID promotes Ig gene diversification in G1 phase, and that nuclear export protects the genome from AID-induced mutagenesis during S/G2/M phase.

7.2. Future directions.

The results that I report in this thesis have implications for how the mutagenic activity of AID is balanced with the need to protect genomic sequence.

There is a great deal known about AID interactions with other proteins, but cell cycle-specificity has not been studied. Determining whether AID interacts with specific partners at specific stages of cell cycle may reveal how oscillatory regulation is conferred upon AID.

Similarly, AID post-translational modification, especially by phosphorylation, has been extensively studied, but to date the cell cycle-dependence of these modifications has not been examined. My results suggest that this will be a fruitful area for new research.

I have shown directly that the presence of AID in the nucleus during S phase but not G1 phase is toxic to cells. To show that this is due to AID-induced mutagenesis, deep sequencing should be carried out on DNA from cells expressing AID^{F193A}-mCherry-Gem and AID^{F193A}-mCherry-Cdt1. My results predict a much higher mutational burden in the former. Because AID is known to have off-target activity, particularly at oncogenes, it will be interesting to learn whether enforced mutagenesis in S phase targets mutations throughout the genome, or if the mutation frequency is elevated at the genes targeted by AID in diversifying B cells.

Balancing Ig gene diversification and genomic instability is an important research focus for studying B cell lymphomas. It is known that AID can promote genomic instability in many B cell lymphomas and other malignancies, but the relationship between AID and tumorigenesis is often blurred. It is possible that AID deregulation arises from the transformation of cells. If so,

then what events during the transformation process would result in AID deregulation. It is also possible that deregulation of AID initiates transformation, possibly by unleashing its mutagenic activity. If so, then how does AID-induced mutagenesis lead to the transformation and progression of malignancies? New answers to these questions may emerge by asking whether the regulatory mechanisms I have discovered govern AID and AID-induced mutagenesis are deregulated in tumors that express AID, or if one or more of these levels of regulation is deregulated. Careful assessment of spatiotemporal regulation of AID in malignant cells will provide new insights into the roles of AID in cancer.

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