Activation-induced cytidine deaminase (AID) is localized to subnuclear domains enriched in splicing factors

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Abstract

Activation-induced cytidine deaminase (AID) is the mutator enzyme in adaptive immunity. AID initiates the antibody diversification processes in activated B cells by deaminating cytosine to uracil in immunoglobulin genes. To some extent other genes are also targeted, which may lead to genome instability and B cell malignancy. Thus, it is crucial to understand its targeting and regulation mechanisms. AID is regulated at several levels including subcellular compartmentalization. However, the complex nuclear distribution and trafficking of AID has not been studied in detail previously. In this work, we examined the subnuclear localization of AID and its interaction partner CTNNBL1 and found that they associate with spliceosome-associated structures including Cajal bodies and nuclear speckles. Moreover, protein kinase A (PKA), which activates AID by phosphorylation at Ser38, is present together with AID in nuclear speckles. Importantly, we demonstrate that AID physically associates with the major spliceosome subunits (small nuclear ribonucleoproteins, snRNPs), as well as other essential splicing components, in addition to the transcription machinery. Based on our findings and the literature, we suggest a transcription-coupled splicing-associated model for AID targeting and activation.

Introduction

Antibody (Ab) diversification in stimulated B cells, by somatic hypermutation (SHM) and class switch recombination (CSR), is dependent on activation-induced cytidine deaminase (AID) [32,47]. SHM generates point mutation in the immunoglobulin (Ig) variable regions while CSR exchanges the Ig heavy chain constant region, giving rise to antibodies with enhanced affinity and new effector functions, respectively [59]. AID initiates these processes by deaminating cytosine to uracil in Ig variable and switch gene regions. The resulting U:G mismatches are usually processed by the uracil-DNA glycosylase UNG2 [10,25,44] and a
pathway that requires the mismatch recognition factors MSH2 and MSH6 [42]. In the next steps, error-prone DNA polymerases and factors involved in non-homologous end-joining (NHEJ) are used to achieve SHM and CSR, respectively [49,59].

AID is a mutator protein and AID off-target activity is responsible for many of the mutations and translocations involved in B cell lymphomagenesis [30,36,46,48]. Thus, understanding its detailed function, targeting and regulation is important. Transcription is required for both SHM and CSR [12,41]. AID physically interacts with the transcription apparatus, likely via the transcription elongation factors, SPT5 [40] and PAF1 [56]. In addition, AID interacts with the spliceosome-associated protein CTNNBL1 (catenin-beta-like protein 1) [7], the splicing regulator PTBP2 (polypyrrolidine tract binding protein 2) [35], as well as several other factors involved in splicing [56] and RNA processing [2]. However, the role of these factors in Ig diversification is still unclear.

AID is a nucleocytoplasmatic shuttling protein that displays a predominantly cytoplasmic localization [43]. The shuttling in and out of the nucleus is driven by an NLS-dependent active nuclear import mechanism [17,39] and exportin1-dependent nuclear export [20], respectively. CTNNBL1 is a NLS-binding spliceosome-associated protein that has been implicated in subcellular trafficking of AID [14], and recently we showed that CTNNBL1 and AID colocalize in nucleoli [17].

In this study, the subnuclear localization of AID and CTNNBL1 and their association with splicing components were examined in more detail. We found that AID and CTNNBL1 accumulate in distinct nuclear domains enriched in spliceosome-associated factors. Furthermore, we identified physical interactions between AID and the major spliceosome subunits. Moreover, our results indicate that activation of AID by PKA phosphorylation [1,6,37] may be linked to the splicing machinery as well. Based on our findings and the literature we suggest a model where a transcription-coupled splicing complex may target AID to its locus and regulate its function.

Materials and methods

Plasmid constructs

Cloning of human AID cDNA (Image clone 4853069) into pECFP/pEYFP-N1 and -C1 vectors (Clontech) and generation of constructs encoding YFP/CFP-tagged AID with C terminal truncations were described previously [17]. The construct encoding the AID splice variant, lacking exon 4, AIDΔ4-YFP (AID1-142-APV-YFP) was made by site-directed mutagenesis of three codons (AID-D143A-Y144P-F145V, 144P-145V generated an internal Agel site) in pAID-YFP followed by Agel digestion and religation. Plasmid encoding untagged AID was generated from the pAID-YFP construct by excision of the YFP gene (Agel-BsrG1 fragment), blunting of ends by T4 DNA polymerase, ligation and reintroduction of AID stop codon. AID-Cherry and AID1-186-Cherry were generated by cloning AID or AID1-186 cDNA (from AID-YFP, or AID1-186-YFP) into the pCherry-N1 vector as Agel-Not fragments. (pCherry-N1 and pCherry-C1 were made by replacing the YFP gene in the pEYFP-N1 and -C1 vectors with the gene encoding Cherry). pEGFP-CTNNBL1 [7] was a generous gift from Dr. Cristina Rada and Dr. Michael Neuberger and the subcloning into pEYFP-C1 and pEYFP-C1 vectors was described previously [17]. pGFP-SMN was kindly provided by Dr. Jianhua Zhou. SMN cDNA was subcloned into the pECFP-C1 and pCherry-C1 as BspE1-BamHI fragments to generate pCFP-SMN and pCherry-SMN. pGFP-U2AF65 was a generous gift from Dr. Maria Carmo-Fonseca (Lisbon, Portugal) to Dr. Marit Otterlei in our laboratory. Mutagenesis was carried out using the Quick-Change™ mutagenesis kit (Stratagene) and confirmed by sequencing.

Cell culture, transfection and confocal microscopy

HeLaS3, Hek-293T and U2OS were cultured in DMEM with 10% fetal calf serum (FCS) and CH12F3 was maintained in RPMI, supplied with 10% heat-inactivated FCS, 50 μM β-mercaptoethanol, 1 mM Na-pyruvate. In addition, all cultures were supplied with 0.03% c-glutamine, 0.1 mg/ml gentamicin or 1 × PenStrep and 2.3 μg/ml fungizone. Cells were transfected with FuGENE HD or X-tremeGENE HP (Roche) according to the manufacturer’s instructions and analyzed 24 h post transfection. When indicated, cells were incubated with 10 ng/ml Leptomycin B (LeptB) (Sigma/ LC-labs) for 2–3 h if not specified otherwise. Permeabilization of cells with digitonin was performed as previously described [17]. Cells were examined in a Zeiss LSM 510 laser scanning microscope (1 μm thickness) with a Plan-Apochromat 63 ×/1.4 oil immersion objective. CFP was excited at 458 nm and detected at 470–500 nm, YFP was excited at 514 nm with detection between 530–550 nm, YFP was excited at 540 nm and detected at 505–550 when co-transfected with Cherry), and Cherry was excited at 543 nm and detected above 615 nm.

Cell fixation and immunofluorescence

Cajal bodies were identified by immunostaining of coilin. Cells were fixed in 2% paraformaldehyde (freshly made in PBS) for 10 min on ice and permeabilized with ice cold methanol (−20 °C, 20 min). Cells where then rinsed in PBS, blocked in PBS containing 2% FCS and labeled with rabbit polyclonal anti-coilin antibody (ab74739, Abcam) (1:300, 1 h at 37 °C) followed by Alexa Fluor 647 nm goat anti-rabbit antibody (#A-21235, Invitrogen).

For detection of nuclear speckles cells were fixed and permeabilized in ice cold methanol (−20 °C, 20 min). Cells with 0.3% TritonX-100 and 5% BSA was used as blocking and staining buffer. Cells were stained with mouse monoclonal anti-SC35 antibody (#S4045, Sigma-Aldrich) (1:2000, overnight at 4 °C), followed by Alexa Fluor 532 nm goat anti-mouse (#A-11002, Invitrogen) or Alexa Fluor 647 nm goat anti-mouse (#A-21235, Invitrogen). Double staining of coilin and SC35 (or AID) were performed on methanol fixed cells using the rabbit polyclonal anti-coilin and mouse monoclonal anti-SC35 (or anti-AID #39-2500, Invitrogen, 1:200) primary antibodies at 4 °C overnight, followed by Alexa Fluor 647 nm goat anti-rabbit and Alexa Fluor 532 nm goat anti-mouse secondary antibodies.

Immunofluorescence staining of PKA was performed by the methanol fixation protocol using PKA catalytic subunit-α rabbit polyclonal antibody (#5C-903, Santa Cruz) (1:100 dilution, overnight at 4 °C) and secondary antibody Alexa Fluor 647 nm goat anti-rabbit. Cells were analyzed by confocal microscopy. Alexa Fluor 532 nm was excited with 543 nm and detected between 560 nm and 615 nm while Alexa Fluor 647 nm was excited with 633 nm and detected above 650 nm.
Preparation of cell extracts, immunoprecipitation and Western blot analysis

Cells expressing GFP- or YFP-tagged proteins were harvested 24 h post transfection and rinsed in PBS. Cell pellets were suspended in HEPES lysis buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 100 mM KCl, 0.5% NP-40) supplemented with 20% glycerol, 1 Complete protease inhibitor (Roche), 1 Phosphatase Inhibitor Cocktail 2 and 3 (Sigma), 200 U/ml OmniCleave™ Endonuclease (Epicentre) and 50 μg/ml RNase A (Sigma). The suspensions were sonicated on ice (3 x 20 s, duty cycle 20% and output 2.5) and cleared by centrifugation. Alternatively, cell pellet was resuspended in 1 packed cell volume of buffer I (10 mM Tris–HCl pH 8.0, 200 mM KCl, 1 mM MgCl₂, 1.5 mM EDTA, 1 Complete protease inhibitor, 1 Phosphatase Inhibitor Cocktail 2/3, 200 U/ml OmniCleave™ and 50 μg/ml RNase A) and lysed by adding 1 packed cell volume of buffer II (buffer I supplemented with 0.5% NP-40 and 40% glycerol). The mixture was rocked for 2 h at 4 °C and cleared by centrifugation. Protein concentrations were measured using the Bradford method (Bio-Rad).

GFP rabbit polyclonal antibodies (made in house) were covalently cross-linked to protein A Dynabeads (Invitrogen) (0.1 μg IgG/μl beads) using dimethyl pimelimidate dihydrochloride (DMP) cross-linker according to the manufacturer’s instructions. Freshly made cell extract (1 mg total protein) was incubated with 30 μl beads at 4 °C and cleared by centrifugation. Protein concentrations were measured using the Bradford method (Bio-Rad).

The primary antibodies used were (dilution/concentration in brackets): rabbit polyclonal anti-GFP (made in-house or #ab290 from Abcam) (0.5 μg/ml or 1:2000); rabbit polyclonal anti-CTNNBL1 (A302-663A, Bethyl) (1:2000); rabbit polyclonal anti-Colin (B-7) (ab74739, Abcam) (1:400); mouse monoclonal anti-AID (#39-2500, Invitrogen) (1:1000); mouse monoclonal anti-AID L7E7 (#4975, Cell Signaling) (1:1000); mouse monoclonal anti-SMN1 2B1 (sc-32313, Santa Cruz) (1:200); rabbit polyclonal anti-Sm B/N (H-9) (sc-374078, Santa Cruz) (1:400); rabbit polyclonal anti-PAKα cat (C20) (sc-903, Santa Cruz) (1:1 μg/ml); mouse monoclonal anti-CDC5L (DB8612362B1) (1:5000); mouse monoclonal anti-POLII (Rp1b-CTD) (#2629, Cell Signaling) (1:1000); rabbit polyclonal anti-SPTS (H300) (sc-28678, Santa Cruz) (1:200); rabbit polyclonal anti-Prp19 (ab27692, Abcam) (1 μg/ml); mouse monoclonal anti-U2AF65 (clone MC3, #U4758, Sigma) (0.1 μg/ml); rabbit polyclonal anti-β-tubulin (#21465, Cell Signaling) (1:1000); mouse monoclonal anti-SRSF1(sc-33652, Santa Cruz) (1:100); rabbit polyclonal anti-RRM5 (#SAB2101960, Sigma) (1:1000). HRP or fluorescent (LI-COR IRDye 680RD)-conjugated swine (or goat) anti-rabbit or HRP-conjugated goat anti-mouse IgG were used as secondary antibodies (1:5000 or 1:25,000, respectively). The HRP membrane signals were developed using SuperSignal West Femto (Pierce), visualized on a Kodak Image station 4000R, and quantified using the Kodak Molecular Imaging NE4 software. Fluorescent Western blot signals were visualized on a LI-COR Odyssey infrared imaging instrument.

Class switching assays

In vitro class switching was measured using flow cytometry. Approximately 2000 CH12F3 cells (10,000 cells/ml) were seeded in flat-bottomed 96-well plates in 200 μl growth medium. Cells were stimulated with 2 ng/ml anti-CD40, and 10 ng/ml murine recombinant IL-4 (Peprotech), and 1 ng/ml human recombinant TGF-β1 (Peprotech) for 4 days. The cells were then stained with LIVE/DEAD violet viability stain (Invitrogen), blocked with Fc receptor antibody (2.4G2) and normal mouse serum (Invitrogen), fixed and permeabilized in CytoFix/Cytoperm™, and washed in PermWash™ containing saponin. Biotin-anti-mouse-IgA (eBioscience, clone 11-44-2) was used for cytoplasmic staining of IgA and Streptavidin-Alexa Fluor 647 (Sigma) was used for detection. Cells were washed twice with PermWash™ and suspended in 200 μl of CellFix™ before analysis. Samples, unstained and single stained controls (including a control with only streptavidin-AF647 for assessment of background staining of endogenous biotin), were analyzed on a FACS Aria. Viable CH12F3 cells were analyzed for YFP expression and IgA expression using FlowJo® version 7.6 for PC software. Reagents were from BD Biosciences if not stated otherwise.

Results

AID is localized in several subnuclear structures

To study subnuclear trafficking of AID, we applied high resolution confocal microscopy to live cells expressing AID with a fluorescent tag (Supplementary Fig. S1A) and inhibited nuclear export by the specific exportin1 (CRM1) inhibitor, leptomycin B (LeptB). Shortly after addition of LeptB, YFP-tagged AID (AID-YFP) entered the nucleus and accumulated in nucleoli where it colocalized with the nucleolar factor, fibrillarin (Fib-Cherry) (Fig. 1A), as expected [17]. However, after prolonged LeptB treatment, AID-YFP was released from nucleoli and accumulated in other nuclear foci (Fig. 1B). To study this observation without adding the nuclear export inhibitor, we used an alternative approach. Digitonin permeabilizes the plasma membrane of cells and enables soluble factors to diffuse out of the cytoplasm and nucleus, while proteins that are structurally bound or are too large for diffusion through the nuclear pores, are retained [58]. In digitonin-treated cells, AID-YFP and the C-terminally truncated nuclear AID variant (YFP-AID1-186) (Supplementary Fig. S1B) associated with nucleoli as well as nucleolar factor, fibrillarin (Fig. 1C). Thus, these results demonstrate that AID associates with several different subnuclear domains.

CTNNBL1 and AID localize to Cajal bodies

The AID-interacting factor CTNNBL1 is part of the Prp19/CDC5L complex [15], which plays a central role during catalytic activation of the spliceosome. Assembly and modification of the major spliceosome subunits occur in distinct nuclear structures such as CBs [34]. This prompted us to investigate whether CTNNBL1 and AID may also be localized to the CBs. In live cells we observed that GFP-tagged CTNNBL1 often accumulated in several small nuclear foci in addition to nucleoli (Fig. 2A). Moreover, CTNNBL1 and nuclear AID (AID1-186)
colocalized at most of these subnuclear domains in both live and fixed cells (Fig. 2B). To identify CBs, we expressed CFP-tagged SMN (survival of motor neuron) protein. The SMN complex accompanies spliceosomal snRNP from cytoplasm to the CBs and constitutes a major component of CBs [38]. Indeed, we found that CTNNBL1 and SMN clearly colocalized in nuclear foci in live HeLa cells (Fig. 2C), which strongly indicates that CTNNBL1 may be targeted to CBs. To exclude the possibility that this was caused by overexpression of SMN protein, we labeled the CBs by immunofluorescence staining of coilin, which is another major component of CBs. Using this approach we demonstrated that CTNNBL1 colocalized with coilin in CBs (Fig. 2D). Next we tested whether CTNNBL1 physically interacted with CB proteins. To this end, GFP-SMN or GFP were expressed in HEK cells and GFP-immunoprecipitation (GFP-IP) was performed from whole cell extracts (WCEs). We used cell extracts that were treated with nucleases to focus on direct protein:protein interactions. In contrast to the GFP control, GFP-SMN clearly pulled down endogenous CTNNBL1 (Fig. 2E). Moreover, applying the same method on GFP-CTNNBL1-expressing cells, we verified that CTNNBL1 also pulls down endogenous coilin (Fig. 2F). Note that the β-tubulin control was neither pulled down by SMN nor CTNNBL1. Taken together, these data show that CTNNBL1 localize to CBs and interacts with major CB associated proteins.

Next, we examined AID with respect to CB localization. Similarly to CTNNBL1, AID colocalized with SMN protein in distinct nuclear foci in live HeLa cells, both at steady state and after inhibition of nuclear export by LeptB (Fig. 3A). Moreover, physical interaction between the SMN protein complex and AID was confirmed by GFP-IP experiments using HEK cells coexpressing GFP-SMN and untagged AID (Fig. 3B). Localization of AID to CBs was also demonstrated in AID-CFP-expressing cells that were immunofluorescence labeled with anti-coilin (Fig. 3C). Likewise, physical association between AID and endogenous coilin and SMN was verified by pull down in extracts from cells expressing either the AID full length protein (YFP-AID) or the C-terminally truncated nuclear AID variant (YFP-AID1-186) (Fig. 3D and E). Importantly, we also demonstrated that endogenous AID accumulates in CBs by double immunofluorescence labeling of AID and coilin in an AID-expressing human B-cell line (Daudi) (Fig. 3F). To map regions on AID required for its accumulation in CBs, AID-YFP variants with C-terminal truncations or mutations were expressed together with CFP-SMN in U2OS and HeLa cells. All C-terminally
truncated/mutated AID variants analyzed, including the physiologically occurring AID splice form lacking exon 4 (AIDΔ4) [57], colocalized with SMN in live cells (Fig. 3G) and with both SMN and coilin in fixed cells (Fig. 3H).

Taken together, these data show that CTNNBL1 and AID localize to CBs and physically interact with CB-associated factors in many cell types including human B cells. Moreover, the C-terminal region of AID is dispensable for its CB localization.

Localization of AID to CBs is independent of CTNNBL1

To further map the AID region essential for CB localization and to study whether targeting of AID to CBs is dependent on interaction with CTNNBL1 we used an extended panel of C-terminally truncated AID mutants (YFP-AID1-n). CTNNBL1 has been implicated in subcellular trafficking of AID [14] and we have previously suggested that CTNNBL1 may target AID to nucleoli [17]. In live cells expressing CFP-SMN protein as CB marker, AID1-29 and AID1-39 were not targeted to Cajal bodies although they were present in the nucleus. However, AID residues 1–84 were sufficient for CB localization (Fig. 4A). Notably, the AID mutants localized to CBs independently of nucleolar targeting (Fig. 4A), and targeting of AID to CBs did not correlate with efficiency of CTNNBL1 pull down (Fig. 4A and B). Neither did the efficiency of CTNNBL1 pull down correlate with pull down of SMN protein (Fig. 4B).

Next, we analyzed AID NLS single-point mutants. AID variants showing disrupted nuclear and nucleolar targeting as well as

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CTNNBL1 binding [17], were still targeted to CBs when coexpressed with SMN (Fig. 4C). Moreover, they pulled down endogenous SMN protein to a relatively higher extent compared to CTNNBL1 (Fig. 4D). In addition, we tested the first characterized CTNNBL1-binding deficient AID mutant, with substitutions of residues 39–42 [7]. This mutant also colocalized with SMN in distinct nuclear bodies (Supplementary Fig. S2).

These results show that the N-terminal part of AID (1–84) is sufficient for CB localization. They further demonstrate that targeting of AID to CBs is disconnected from NLS-driven nuclear localization.

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import and is independent of its binding partner CTNNBL1. However, our findings suggest that CB targeting of AID may be mediated by a mechanism involving the SMN complex.

**AID and CTNNBL1 localize to nuclear speckles and associate with the spliceosomal snRNPs**

Similar to CBs, nuclear speckles are subnuclear structures in the interchromatin compartment. Nuclear speckles are the main sites for maturation and storage of the splicing machinery [54]. To test whether AID may be targeted to these sites, we coexpressed cherry-tagged AID variants (AID-Cherry or AID1-186-Cherry) with the GFP-tagged splicing factor U2AF65 (U2 auxiliary factor 65 kD subunit), which accumulates in nuclear speckles [5,13] (Supplementary Fig. S3). We observed clear colocalization of AID-Cherry and GFP-U2AF65 in nuclear foci in LeptB and digitonin treated cells (Fig. 5A), as well as in methanol fixed cells expressing the truncated nuclear form of AID (Fig. 5B). Supporting this association, we demonstrated that U2AF65 efficiently pulled down AID as well as endogenous CTNNBL1 (Fig. 5C). Likewise, AID pulled down U2AF65 (Fig. 5D). Localization of AID and CTNNBL1 to nuclear speckles was also demonstrated by immunofluorescence staining of the commonly used nuclear speckles marker SC35 (serine/arginine rich splicing factor 2), (Fig. 5E and F). Finally, double immunostaining of coilin and SC35 shows that AID localizes to both CBs and nuclear speckles (Fig. 5G). This suggests that AID may take part in the directional route from CBs to nuclear speckles together with the splicing machinery.

Both CBs and nuclear speckles are enriched in the major spliceosomal building blocks, the snRNPs (U1, U2, U4, U5 and U6) [52,53]. At the core of each snRNP, seven different Smith (Sm) proteins (SmB, SmD1, SmD2, SmD3, SmE, SmF and SmG) form a ring structure around the specific uracil-rich small nuclear RNA (snRNA) molecules (U1-U6 RNA) [38]. GFP-IP from extracts from HEK cells expressing either full length AID (YFP-AID) or the nuclear major domain variant (YFP-AID1-186) [17], showed that AID localizes to both CBs and nuclear speckles (Fig. 5G). This suggests that AID may take part in the directional route from CBs to nuclear speckles together with the splicing machinery.

To get more insight into the association between AID and the splicing machinery in Ig switching B-cells, we used the previously generated CH12F3 mouse B-cell line that stably expresses AID-YFP and the control cell line that expresses the YFP-tag [17] (Fig. 8A). AID with a C-terminal tag (AID-GFP) is biologically active [39,43]. However, to ensure that the stably transfected cell lines were still switch competent, we stimulated the cells with anti-CD40, IL4...
Fig. 4 – Localization of AID to CBs is not dependent of CTNNBL1. (A) Representative confocal microscopy images of live HeLa cells expressing C-terminally truncated AID variants (YFP-AID1-n) together with SMN protein (CFP-SMN). (B) Quantitative WB analysis of CTNNBL1- and SMN-pull down by AID deletion mutants. Proteins were precipitated by GFP-IP from WCE derived from HEK cells expressing C-terminally truncated AID (YFP-AID1-n). YFP-AID1-n (bait), CTNNBL1 and SMN in the IP-fractions were quantified and relative binding were calculated as intensity of CTNNBL1 and SMN, divided by intensity of YFP-AID1-n. The bars represent the mean of 4-6 experiments and standard deviations are indicated on the bars. (C) AID mutants still colocalize with SMN in nuclear spots. Live cell images of U2OS expressing AID NLS mutants together with SMN protein. (D) Quantitative WB analysis of CTNNBL1- and SMN-pull down by AID NLS mutants. Proteins were pulled down by GFP-IP in cell extract derived from HEK cells expressing the YFP-AID NLS mutants. Cells expressing the YFP tag only were used as negative control. Signals in the IP fraction were quantified and relative binding calculated as the mean of 4 experiments.
Fig. 5 – AID and CTNNBL1 localize to nuclear speckles. (A) AID colocalizes with the splice factor U2AF65 in nuclear spots. Representative image of nuclei in permeabilized HeLa cells expressing AID-Cherry together with GFP-U2AF65. Cells were treated with LeptB followed by digitonin directly before analysis in the confocal microscopy. (B) Image of a representative nucleus in methanol fixed cells expressing the C-terminally deleted nuclear form of AID (AID1-186-Cherry) together with GFP-U2AF65. (C) The splicing factor U2AF65 interacts with AID and CTNNBL1. WB showing AID- and CTNNBL1-pull down by GFP-IP in extracts from transfected HEK cells coexpressing GFP-U2AF65 together with AID (untagged). (D) WB showing pull down of endogenous U2AF65 by GFP-IP in extracts from HEK cells expressing YFP-AID. (E) AID colocalizes with the splice factor SC35. Immunofluorescence images of HeLa cell (nucleus) labeled with anti-SC35 antibody (speckles marker). The AID-YFP-expressing cells were permeabilized and fixed with digitonin and methanol, respectively. (F) Nuclear AID and CTNNBL1 both colocalize with SC35. Immunofluorescence images of methanol fixed HeLa cells, coexpressing nuclear AID variants and CTNNBL1, labeled with anti-SC35. (E) AID may associate with CBs and nuclear speckles in the same cell. Immunofluorescence image showing the nucleus of a HeLa cell that express the truncated nuclear AID variant. Cells were methanol fixed and labeled with anti-SC35 and anti-coilin to identify speckles and CBs, respectively.
and TGF-β and measured IgA switch by FACS analysis. Both the YFP-expressing control cells and the AID-YFP-expressing cells switch to IgA with high efficiency after stimulation, 28% and 36%, respectively (Fig. 8A). We then performed GFP-IP experiments and analyzed precipitated proteins by Western blots from unstimulated (−/C0) and CSR-stimulated (+/C0) cells. Notably, stimulation was verified by detection of endogenous AID (25 kD) in the input fractions for all experiments (Fig. 8B). Importantly, quantitation of endogenous and tagged AID from Western blots (mean from six blots) in stimulated cells showed that the total AID level was only 70% higher in the AID-YFP cells than in the YFP control cells (data not shown). This demonstrates that tagged AID is expressed at biologically relevant levels, which makes this a good system to study physical association between AID and its interacting partners.

We identified endogenous AID in the AID-YFP precipitated protein complexes, indicating that we pulled down native AID complexes. Except for AID, the proteins analyzed were generally similarly expressed in stimulated (+) and unstimulated (−) cells (input (+)/input (−) = 1) (Fig. 8B and C). Likewise, association between AID and the transcription machinery (POLII and SPT5), the activating kinase (PKA-α), the major spliceosome snRNP components (Sm proteins), as well as the spliceosome-activating complex (Prp19/CDC5L/CTNNBL1) was not significantly influenced by CSR stimulation (Fig. 8B and C). Neither was the Ser/Arg-rich splice factor 1 (SRSF1) (Supplementary Fig. S4), which was reported to regulate Ig hypermutation in DT40 chicken B cells [24]. In contrast, we found that the association between AID and U2AF65 was significantly reduced in stimulated cells (p < 0.05) (Fig. 8B and C). Interestingly, competition between the constitutive auxiliary splice factor U2AF65 and other polypyrimidine tract-binding proteins is a common strategy to achieve alternative splicing [28]. In fact, the RNA-binding motif protein 5 (RBM5) competes with U2AF65 and promotes exon 4 skipping of AID pre-mRNA [23]. However, we were not able to detect any association between AID and RBM5 in our experiments (Supplementary Fig. S4). Nevertheless, this finding indicates that also components involved in alternative splicing may play a role to regulate the biological activity of AID and that the link between AID and the splicing machinery is central in switching B cells.

Discussion

The nuclear space is highly organized, containing many dynamic nuclear bodies that reflect the diverse functions and activities
Nuclear bodies are not restricted by membranes. Their structural integrity is dependent on interactions between proteins and/or nucleic acids, and exchange of factors between the various bodies and nucleoplasm occurs continuously [27]. Likewise, localization of AID to the various nuclear domains likely reflects its dynamic association with components of these nuclear structures. Previously, we demonstrated that AID and its interaction partner CTNNBL1 are targeted to nucleoli [17]. Here we show that they in addition localize to CBs and nuclear speckles, which are the major spliceosome-associated compartments. Moreover, we demonstrate that AID physically associates with the major spliceosome subunits, the uracil-rich snRNPs (U1, U2, U4, U5 and U6). Biogenesis of the snRNPs is a complex process. Newly synthesized snRNAs are exported to the cytoplasm to undergo various modifications and assembling into snRNPs. The snRNP core complex consists of a heteroheptameric ring of Sm proteins wrapped around the snRNA [45]. The mature cytoplasmic snRNPs are subsequently transported into the nucleus, accompanied by the SMN complex [33,51]. In the nucleus, the snRNPs may be transferred from the SMN complex to coilin in CBs, or accumulate in nucleoli to undergo further modification and assembly, before they enter nuclear speckles [21,50,53,61]. We have identified that AID localizes to all these compartments and interacts with major components (SMN, Coilin, Sm proteins) along this route. This indicates that AID may traffic together with the maturing spliceosome.

The similar localization and interaction pattern observed for AID and CTNNBL1 suggests that they are part of the same complex. The crystal structure of CTNNBL1 was recently solved and supports a direct interaction between AID and CTNNBL1 [19]. The structure reveals a negatively charged protein-binding groove that fits perfectly with the positively charged motif of AID that we identified previously [17]. Although the precise function of CTNNBL1 is unknown, it is part of the Prp19/CDC5L complex, which plays a central role during activation of the spliceosome [14,15]. Our results together with previous reports identifying AID-interacting splice factors such as PTBP2 [35], S3A, SF3B, Prp6 and Prp4 (V), strongly indicate a direct link between AID function and the splicing machinery. Based on this we suggest a cotranscriptional splicing-coupled mechanism for AID targeting and activation (Fig. 8D). Transcription and splicing is intimately linked in vivo [31]. Recently, it was shown that the phosphorylated RNA POLII C-terminal domain promotes splicing activation through recruitment of the U2AF65 and the Prp19 complex [9,16]. Moreover, proteomic analysis of the human RNA POLII complex identified that Ser-Arg-rich splice factors (SR proteins) and all components of the 5′ splice site-binding snRNP (U1), including Sm proteins, associate with the active transcription complex [8]. AID could be linked to this RNA POLII spliceosomal complex both through CTNNBL1 and other splicing- and transcription elongation factors, such as SPT5 [40] and PAF1 [56] (Fig. 8D). In switch regions, the RNA transcripts are extensively paired with the

![Fig. 7 – PKA and AID occur together in nuclear speckles. (A) AID colocalizes with PKA-Cα in nuclear foci. Immunofluorescence image of methanol fixed HeLa cells, expressing YFP-AID1-186 (major domain), labeled with anti-PKA-Cα-Ab. (B) AID colocalizes with PKA-Cα in nuclear speckles. Immunofluorescence image of methanol fixed U2OS cells, expressing CFP-AID1-186, labeled with anti-PKA-Cα and anti-SC35. (C) WB analysis showing pull down of PKA-Cα by AID. GFP-IP was performed in extracts derived from transfected HEK cells expressing YFP-AID full length (AID1-198) or the nuclear YFP-AID1-186 variant. Cells expressing the YFP tag were used as negative control. Live HEK cells expressing negative control (YFP) and the AID variants are shown.](image-url)
transcribed DNA strand [18,60]. Cotranscriptional splicing may bring AID directly in contact with its substrate DNA for targeted activity. Growing evidence for the existence of a tight coupling between DNA methylation status and regulation of pre-RNA processing supports our model linking splicing and DNA editing [4]. Moreover, we find that CSR-defective mutants [17] fail to bind splicing-related factors (this work). This splicing-associated AID targeting model is also consistent with the high mutation rates observed directly downstream of donor splice sites in both variable and switch regions [29].

Interestingly, AID displayed reduced association with the auxiliary splice factor U2AF65 after stimulation. During splicing, U2AF65 binds the polypyrimidine tract element upstream of the 3' splice site and recruits the U2 snRNP to the pre-mRNA branch.
site. This step comprises a major regulatory event in splicing, and competition between the constitutive splice factor U2AF65 and other polypyrimidine tract-binding proteins, such as the AID-interacting factor PTBP2 [35], is a common strategy to mediate alternative splicing [28]. Moreover, U2AF65 displacement is also linked to RNA POLII stalling, which overlaps with the AID target sites [40] and supports the transcription-coupled splicing linked AID targeting model (Fig. 8D).

Phosphorylation of AID by PKA is required for both CSR and SHM in vivo [6]. We found that AID colocalizes with PKA-Cα in nuclear speckles and that nuclear AID also physically associates with PKA. Indeed, this nuclear colocalization may be important to regulate the biological activity of AID. Interestingly, a mechanism for this activation of CSR was recently shown to be mediated by amplification of DNA breaks at switch regions through phosphorylation-dependent interaction of AID with APE1 [55]. Nevertheless, based on the observation that semi-purified AID requires treatment with RNase to remove inhibitory RNA and become catalytically active [3], it is still tempting to speculate that AID may be kept inactive in large RNA-containing snRNP complexes until release mediated by PKA-dependent phosphorylation.

Based on our findings and the literature we suggest a model where both AID targeting and activation is mediated by a cotranscriptional splicing-associated mechanism that is tightly linked to its substrate DNA.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2014.01.004.

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