E3-ubiquitin ligase Nedd4 determines the fate of AID-associated RNA polymerase II in B cells

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Programmed mutagenesis of the immunoglobulin locus of B lymphocytes during class switch recombination (CSR) and somatic hypermutation requires RNA polymerase II (polII) transcription complex-dependent targeting of the DNA mutator activation-induced cytidine deaminase (AID). AID deaminates cytidine residues on substrate sequences in the immunoglobulin (Ig) locus via a transcription-dependent mechanism, and this activity is stimulated by the RNA polII stalling cofactor Spt5 and the 11-subunit cellular noncoding RNA 3′–5′ exonucleolytic processing complex RNA exosome. The mechanism by which the RNA exosome recognizes immunoglobulin locus RNA substrates to stimulate AID DNA deamination activity on its in vivo substrate sequences is an important question. Here we report that E3-ubiquitin ligase Nedd4 destabilizes AID-associated RNA polII by a ubiquitination event, leading to generation of 3′ end free RNA exosome RNA substrates at the Ig locus and other AID target sequences genome-wide. We found that lack of Nedd4 activity in B cells leads to accumulation of RNA exosome substrates at AID target genes and defective CSR. Taken together, our study links noncoding RNA processing following RNA polII pausing with regulation of the mutator AID protein. Our study also identifies Nedd4 as a regulator of noncoding RNAs that are generated by stalled RNA polII genome-wide.

[*Keywords*: activation-induced deaminase; immunoglobulin locus transcription; Nedd4; RNA polymerase II stalling; RNA polymerase II ubiquitination; noncoding RNA]

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Prior to the discovery of noncoding RNA (ncRNA) as a major subclass of eukaryotic genome regulators (Ebert and Sharp 2012; Rinn and Chang 2012), the presence of noncoding germline transcripts in the immunoglobulin (Ig) locus had attracted the attention of many molecular biologists and immunologists (Alt et al. 1982). Accumulating over the last four decades, ample evidence has unequivocably established that the synthesis of long noncoding germline transcripts in the Ig locus plays a pivotal role in recruiting B-cell-specific DNA mutator factors recombination activation genes (RAG-1 and RAG-2) and activation-induced cytidine deaminase (AID) to their target DNA sequences (for review, see Schatz et al. 1992; Keim et al. 2013). AID is a ssDNA cytidine deaminase; AID's activity depends on transcription, cofactors, and transcription-driven secondary DNA structures to identify substrate DNA, which it subsequently mutates to promote class switch recombination (CSR) and somatic hypermutation (SHM) (Chaudhuri et al. 2007; Keim et al. 2013). CSR is an AID-dependent chromosomal deletion–recombination event that alters the IgH locus in such a way that the host B cell is now capable of expressing antibodies that have an isotype different from IgM. One important, unanswered question relates to how a genome-wide process like transcription regulates AID in such a fashion that specific DNA single-strand mutations and DNA double-strand breaks at variable (V) genes and switch (S) sequences are generated in a controlled manner in the Ig locus. Recent advances in the understanding of RNA polymerase II (polII) regulation during transcription initiation, elongation, and termination at various DNA sequences provided insights that have helped to elucidate RNA polII's role in regulating AID targeting and mutagenic activity (Besmer et al. 2006; Wang et al. 2006; Rajagopal et al. 2009; Pavri et al. 2010; Basu et al. 2011). Work in multiple laboratories has focused on the state of the eukaryotic transcription complex with which AID is

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associated. Following transcription initiation at transcription start sites (TSSs), RNA polII undertakes "promoter escape," a process that is regulated stringently by many regulatory mechanisms. These regulatory mechanisms include the action of various DNA helicases that catalyze melting of supercoiled promoters (a TFIIH-dependent mechanism) and recruitment of various RNA polII-associated cofactors signaled by RNA polII C-terminal phosphorylation events at the Ser-5 (S5) residue. Following promoter escape, another step that regulates the entry of RNA polII into elongation mode is "RNA polII pausing," also referred to as "promoter-proximal transcription pausing (PPTP)." Paused RNA polII molecules are poised to undergo rapid entry into transcription elongation mode if provided with adequate signaling cues. The paused RNA polII complex is associated with additional cofactors NELF and DSIF (containing the proteins Spt4 and Spt5). Following phosphorylation of NELF, DSIF, and the C-terminal tail of RNA polII at Ser-2 (S2) by the kinase P-TEFb, NELF is released from the RNA polII stalled complex, and this event signals that the RNA polII can now enter the elongation phase. Capping of the nascent transcript associated with the paused RNA polII promotes RNA polII entry into the elongation phase (Cramer et al. 2008; Cheung and Cramer 2012). It is believed that AID associates with one or many states of the RNA polII following "promoter escape" (Pavri and Nussenzweig 2011; Kenter 2012; Keim et al. 2013).

Recent studies using a combination of genome sequencing of AID-expressing B cells devoid of repair pathways (thus a lack of repair of AID-generated DNA lesions genome-wide) and AID-DNA chromatin immunoprecipitation (ChIP) studies have revealed that AID can mutate various parts of the B-cell genome (Liu et al. 2008; Pavri et al. 2010; Yamane et al. 2011). Based on these studies, it has been proposed that AID identifies its potential target sequences genome-wide by binding to the Spt5-containing transcriptional "pausing" complex and promoting DNA deamination due to the stimulatory role of its cofactor, the ncRNA processing complex RNA exosome (Pavri et al. 2010; Basu et al. 2011; Stavnezer 2011; Kenter 2012). In a previous study, we proposed that the stalled RNA polII-bound AID complex is first identified by the 3′–5′ RNA exonuclease RNA exosome (Liu et al. 2006; Lykke-Andersen et al. 2009) that then displaces the RNA from the DNA/RNA hybrid formed in the transcription complex bubble. The displacement provides AID access to the ssDNA on template and nontemplate strands of the RNA polII-associated transcription bubble at AID target sequences in the Ig locus (Basu et al. 2011). We reported that in in vitro reactions, AID can deaminate cytosines on template and nontemplate strands of transcribed dsDNA in the presence of the purified RNA exosome complex (Basu et al. 2011). How the 3′–5′ RNA exonucleolytic RNA exosome identifies a transcriptionally stalled RNA polymerase complex and displaces the nascent RNA from the transcription complex to generate ssDNA substrates on which AID can act is an important question. Paused RNA polII can undergo two fates. First, it can backtrack and realign with the nascent transcript to continue transcription with the help of its cofactor TFIIS (which cleaves the 3′ end of the nascent transcript to effectively realign the transcript with the template DNA), allowing transcription elongation (Fig. 1A). Alternatively, the RNA polII can be destabilized by its ubiquitination to effect transcription termination and thereby prevent accumulation of stalled RNA polII on the DNA (Anindya et al. 2007, 2010; Svejstrup 2010). It has been reported that degradation-inducing polyubiquitination of RNA polII is initiated by a monoubiquitination event signaled by the HECT domain-containing E3 ligase Nedd4 (Fig. 1A; Rotin and Kumar 2009). Following Nedd4-mediated K63 monoubiquitination, RNA polII is polyubiquitinated by another enzyme at a K48 site to promote proteasome-mediated degradation (Harreman et al. 2009). Here we investigate the state of RNA polII that is complexed with AID and its target sequences at the Ig locus and in the remainder of the genome. We observed that Nedd4 ubiquitinates the AID-associated transcription complex to regulate its CSR-catalyzing activity at the Ig locus. We provide evidence that Nedd4 activity is required for generation of RNA exosome target sequences at potential oncogenic "off-targets" of AID, such as c-myc. Taken together, these observations generate compelling evidence for the mechanism by which AID bound with stalled RNA polII uses RNA exosome to mutate its DNA substrates without allowing the initiation of catastrophic genomic instability.

Results

Nedd4 binds the AID complex in B cells

Following transcriptional pausing, RNA polII can either (1) backtrack and reinitiate its transcriptional attempts, (2) terminate its transcriptional attempts, or (3) overide the whole pausing process and enter the elongation phase. Possibilities 1 and 2 are outlined in Figure 1A. To determine which of these states is attained by the RNA polII during transcription of switch sequence IgSμ, we used ChIP to monitor enrichment of IgSμ sequences from B cells stimulated for IgG1 CSR with all of the following markers: TFIIH (a marker for the RNA polII complex in cells that have undergone "promoter escape"), TFIIS (RNA polII backtracking and nascent RNA cleavage), Nedd4 (RNA polII ubiquitination), and known IgSμ-binding proteins Spt5, AID, and Exosc3. In these experiments, we isolated naïve B cells from the spleen and stimulated them to undergo CSR to IgG1 using a cocktail of LPS and IL4. After 2 d of CSR stimulation, we performed ChIP reactions with the above-mentioned proteins and identified the binding efficiencies to switch sequence IgSμ with the help of quantitative PCR (qPCR) (for details, see the Materials and Methods). We found that Nedd4, TFIIS, and TFIIH bind to the nonrepetitive assayable DNA regions of IgSμ (5′IgSμ), indicating that RNA polII (as identified by TFIIH) is present on 5′IgSμ poised to undergo backtracking and elongation (as marked by the presence of TFIIS) and/or to undergo ubiquitination-mediated destabilization (as marked by the binding of Nedd4) (Fig. 1B).

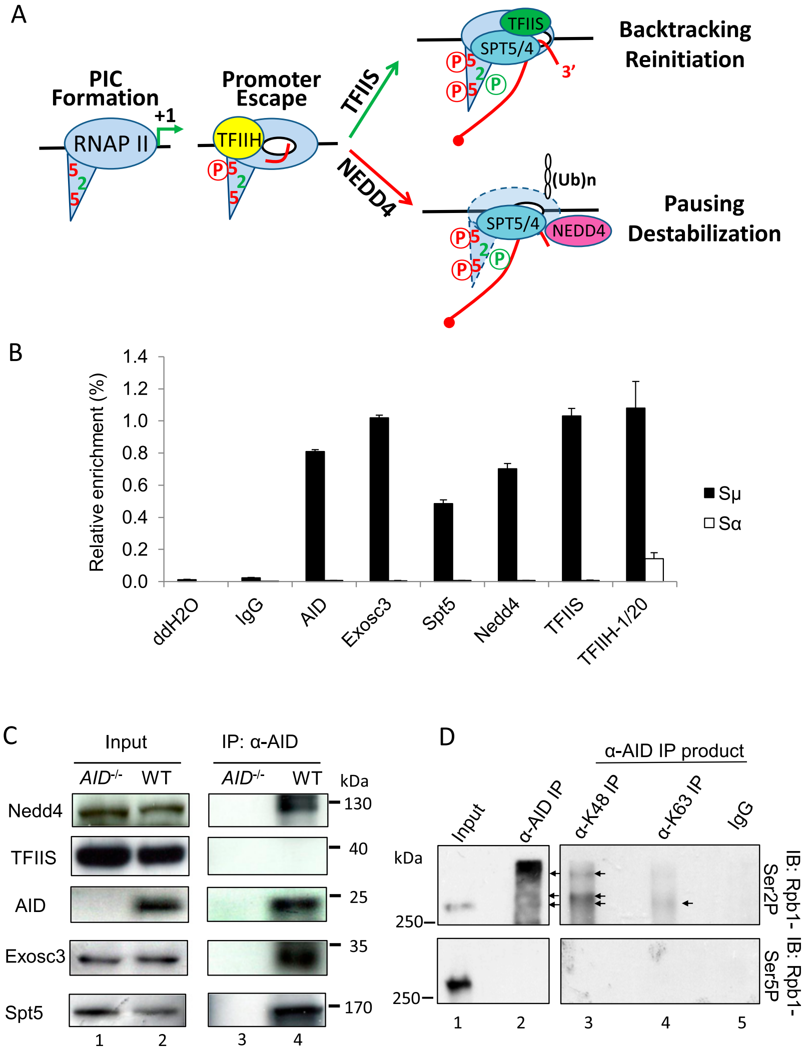

Figure 1. AID-associated transcription pausing complex in B cells contains E3-ubiquitin ligase Nedd4. (*A*) Schematic of possible "transition states" of transcriptionally paused Spt5-associated RNA polII complex in transcribed Ig switch (IgS) sequences in B cells. Following TFIIH-driven promoter escape, transcriptionally stalled RNA polII may enter the transcription elongation phase through a combination of backtracking and reinitiation (a TFIIS-dependent process) or undergo pausing and destabilization followed by ubiquitination-mediated degradation (a Nedd4-dependent process). (*B*) 5′IgSμ bound by AID, ExoSc3, Nedd4, TFIIS, or TFIIH in mouse primary B cells stimulated for CSR to IgG1, determined with ChIP assay. IgSα is a switch sequence that is not transcriptionally activated and acts as a negative control. (*C*) Interaction of AID with Nedd4, TFIIS, ExoSc3, and Spt5 observed by AID coimmunoprecipitation followed by Western blotting in CSR-stimulated B cells. (*D*) Two rounds of immunoprecipitation with extracts of CH12-F3 cells stimulated for IgA CSR and treated with MG132. The first immunoprecipitates with AID antibody (lane *2*) were boiled and then subjected to immunoprecipitation with K48 or K63 linkage-specific poly-Ub antibodies (lanes *3*,*4*) along with IgG control (lane *5*).

However, we realize that not all 5′IgSμ-bound RNA polII isolated from a heterogeneous population of CSR-stimulated B cells will be in the same configuration simultaneously. Some 5′IgSμ bound by RNA polII will be in the elongation phase, some will be in the backtracking phase, and a small subset will be undergoing ubiquitination-mediated degradation. Next, we wanted to know which of these RNA polII complexes is associated with AID (schematized in Fig. 1A). To determine the state of AID-bound RNA polII in B cells, we immunoprecipitated the AID complex from nuclear extracts obtained from AID$^{-/-}$ and AID$^{+/+}$ mouse splenic B cells that were stimulated for CSR (to IgG1) and evaluated the presence of various components of the transcription complex (for details, see the Materials and Methods). We observed that AID immunoprecipitates RNA polII stalling factor Spt5, RNA exosome subunit Exosc3, and RNA polII-destabilizing E3-ubiquitin ligase Nedd4 (Fig. 1C, lane 4). We did not find the marker for backtracking RNA polII complex (TFIIS) in our AID immunoprecipitates (Fig. 1C). In support of some of these observations, interaction-mapping analysis based on published literature provides evidence that AID family member protein APOBEC3G (Conticello et al. 2005) forms a functionally relevant complex with RNA polII and Nedd4 (Supplemental Fig. S1A; Dussart et al. 2005). In Supplemental Figure S1A, we show the interaction network of APOBEC3G with Nedd4 and RNA polII. We also performed immunoprecipitation reactions in mouse B cells with TFIIS and detected the presence of TFIIS and Spt5 in the immunoprecipitate but not AID (Supplemental Fig. S1B). We conclude that AID interacts with stalled RNA polII component Spt5, RNA exosome, and Nedd4. The Spt5-associated, AID-associated, and RNA exosome-associated RNA polII is enriched with Nedd4 but does not contain detectable levels of TFIIS (Fig. 1C).

To determine whether AID-associated RNA polII is ubiquitinated in B cells and probe the nature of this ubiquitination linkage, we immunoprecipitated AID-associated RNA polII (first immunoprecipitation) from a B-cell line (CH12F3) that can undergo CSR to IgA following stimulation with LPS, IL-4, and TGFβ cytokines. We boiled this sample to remove protein–protein interactions and then immunoprecipitated RNA polII with anti-

K48Ub (Fig. 1D, lane 3) or anti-K63Ub (Fig. 1D, lane 4) antibodies to determine the nature of the specific ubiquitination linkage. We probed the K48-modified or K63-modified RNA polII immunoprecipitate with specific antibodies against Ser-5 phosphorylated RNA polII or Ser-2 phosphorylated RNA polII. We observed in repeated experiments that AID is bound to Ser-5 phosphorylated RNA polII (higher exposures reveal the Ser-5 phosphorylated RNA polII signal on the Western blot) (see Supplemental Fig. S1C) and also to Ser-2 phosphorylated RNA polII (Fig. 1D; Supplemental Fig. S1C). Moreover, we observed that Ser-2 phosphorylated and ubiquitinated RNA polII immunoprecipitates with AID and is enriched at a higher molecular weight due to ubiquitination (Fig. 1D). We note that in addition to K48 linkage, RNA polII may be modestly modified by a K63-Ub linkage (Fig. 1D). Taken together, these experiments show that in B cells, AID associates with RNA polII ubiquitinated with a K48 linkage; this RNA polII has undergone promoter escape and is poised to enter elongation, since it is marked by RNA polII C-terminal domain (CTD) Ser-2 phosphorylation. Consistent with reports using yeast as a model system, we found that Ser-2 phosphorylated RNA polII is a target of ubiquitination in B cells (Harreman et al. 2009).

Nedd4 promotes AID activity in B cells

To check whether Nedd4 is involved in AID function, we generated Nedd4 knockdown CH12F3 cells by lentiviral transduction of shRNA specifically targeting Nedd4 mRNA (we refer to these cells as shNedd4). A nonmammalian shRNA control (SHC002) was prepared as well. CH12F3 cells can be stimulated for IgA CSR in ex vivo conditions following incubation with LPS, IL4, and TGFβ. We observed a clear diminution in CSR efficiency to IgA in shNedd4 cells after 72 h of stimulation (Fig. 2A, left panel). The reduction in CSR in shNedd4 cells compared with SHC002 control cells was confirmed in five separate experiments (Fig. 2A, right panel; three individual experiments shown in Supplemental Fig. S2A). To check whether the CSR deficit in shNedd4 cells results from the change of expression level of important proteins for CSR such as AID and RNA exosome, we

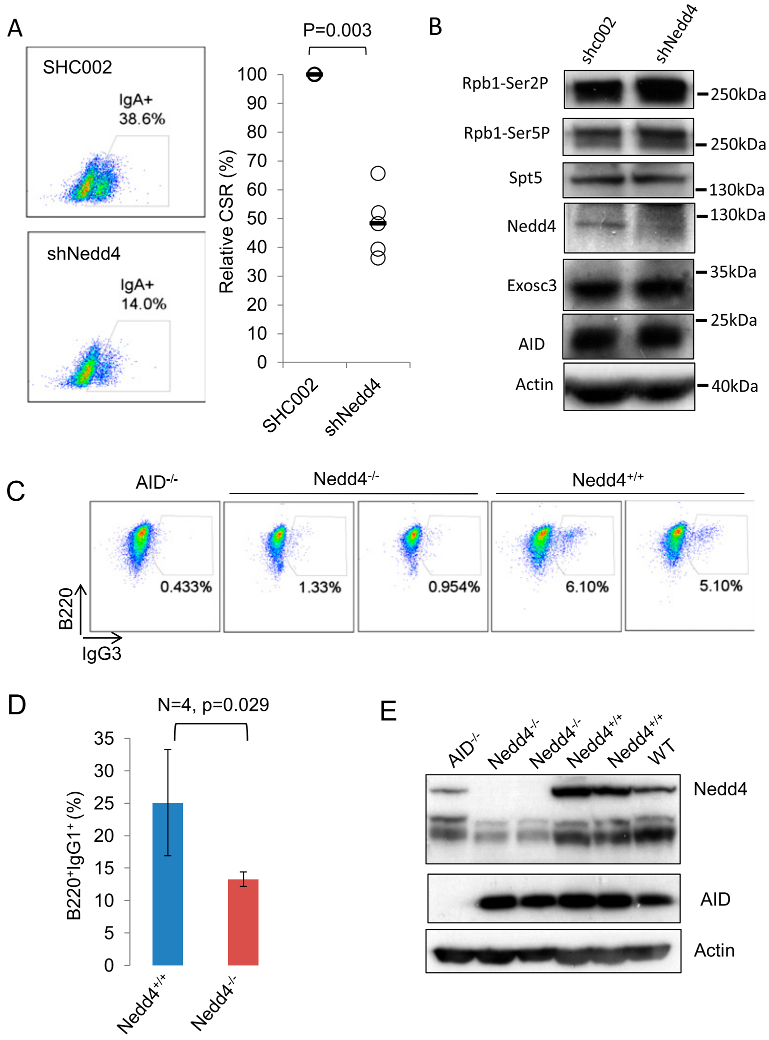

Figure 2. Nedd4-deficient B cells have impaired CSR. (*A*) Representation of CSR efficiency of shNedd4 and SHC002 CH12F3 cells stimulated for IgA CSR (*left*) from five independently performed experiments (*right*). (—) The mean. (*B*) Protein expression in shNedd4 and SHC002 cells illustrated with Western blotting. IgG3 CSR (*C*), IgG1 CSR (*D*), and protein expression (*E*) of B cells obtained from Nedd4$^{-/-}$ and Nedd4$^{+/+}$ mice. AID$^{-/-}$ is a negative control for CSR; different chimeric mice were used in each figure for the data of each Nedd4 genotype.

examined the protein levels of shNedd4 and SHC002 cells by Western blot. Using actin as a loading control, it was immediately evident that shNedd4 cells express reduced levels of Nedd4 protein, while the expression of the other proposed components of the AID/RNA polII complex in these cells, such as phosphorylated RNA polII (Rpb1: Ser-2 and Ser-5 phosphorylated), Spt5, AID, or Exosc3, did not show any appreciable decrease in their expression levels (Fig. 2B). Normal cell proliferation is required for optimal CSR. To exclude the possibility that the CSR deficit in shNedd4 cells is due to defective proliferation of shNedd4 cells, we examined cell proliferation of shNedd4 and SHC002 cells via cell number counting and observed no variation in proliferation between shNedd4 cells and control SHC002 cells after 72 h in culture (Supplemental Fig. S2B). We confirmed that observation by using the VPD450 dye dilution technique (Supplemental Fig. S2C). Another requirement during CSR is the robust transcription of switch sequences. We found that shNedd4 cells do not have a deficiency in switch sequence transcripts (Iμ or Iα) (discussed later; see Fig. 4; Supplemental Fig. S5). Since Nedd4 shares some protein homology with another potential E3-ligase, Nedd4L (Nedd4-2) (Kamadurai et al. 2009), we also generated a knockdown line of Nedd4L protein in CH12F3 cells. We confirmed knockdown of Nedd4 and Nedd4L transcripts by qPCR and found that the transcript levels of both genes were approximately half of those expressed in CH12F3 cells (Supplemental Fig. S3A). We found that shNedd4 cells are defective in CSR, but the shNedd4L cells undergo IgA CSR similar to that of the control SHC002 (wild-type) cells (Supplemental Fig. S3B).

Since the cell line CH12F3 might override certain epigenetic programs by virtue of its transformed status, we evaluated the role of Nedd4 in stimulating CSR in primary B cells. For this purpose, we obtained fetal liver chimeric mice that express a C-terminally deleted form of Nedd4 (consistent with previous published literature, we refer to these B cells as Nedd4$^{-/-}$ [Yang et al. 2008]) (for details of the generation of these chimeric mice, see the Materials and Methods). As a control, we also obtained chimeric mice that expressed wild-type Nedd4 (Nedd4$^{+/+}$). Using LPS, we stimulated splenic B cells from these mice to undergo CSR to IgG3 and with anti-CD40 antibody together with IL4 for CSR to IgG1. We found that CSR to IgG3 (Fig. 2C) and IgG1 (Fig. 2D) is reduced in Nedd4$^{-/-}$ B cells in comparison with Nedd4$^{+/+}$ controls. We show the defect in IgG3 CSR levels using a FACS plot, since efficiency of IgG3 CSR in ex vivo cultures is low, and FACS plots are widely accepted as the proper representation of the actual phenotype. The efficiency of IgG1 CSR is high, and thus we represent the data using a quantitative plot. We used these IgG1CSR-stimulated B cells to prepare protein extracts from the Nedd4$^{+/+}$ and Nedd4$^{-/-}$ B cells and observed the loss of full-length Nedd4 expression in the Nedd4$^{-/-}$ B cells (Fig. 2E, top panel). However, Nedd4 deficiency does not affect AID expression levels in these cells (Fig. 2E, middle panel). We performed growth curves using these B-cell cultures and concluded that Nedd4$^{-/-}$ and Nedd4$^{+/+}$ B cells proliferated similarly during the 72-h assay (Supplemental Fig. S2D). To determine whether AID activity is indeed decreased during IgG1 CSR, we evaluated the level of mutation at the 5′ end of the IgSμ switch regions (donor switch sequence and direct target of AID activity) and found reduced AID-induced mutations in Nedd4$^{-/-}$ B cells (~50% reduction of overall mutation frequency in Nedd4$^{-/-}$ B cells) (Supplemental Table SI). We would have liked to measure the mutation frequency at the core IgSμ, a region where we expect complexation of AID/Spt5/RNA exosome/Nedd4 (discussed in our proposed model in Fig. 6, below), but these regions are difficult to clone due to their G-richness and other DNA sequence properties.

Next, we examined whether Nedd4 deficiency may induce genomic instability in B cells and lead to inhibition of CSR. For that purpose, we assayed for genomic stability in Nedd4$^{+/+}$ and Nedd4$^{-/-}$ B cells using telomeric FISH (T-FISH) assays (Franco et al. 2006). In these T-FISH assays, chromosome instability can be detected easily by observing loss of labeled telomeres. We did not find any observable genomic instability in Nedd4$^{-/-}$ B cells in comparison with Nedd4$^{+/+}$ cells (Supplemental Fig. S4). Taken together, we conclude that Nedd4 functions to promote CSR for both IgG3 and IgG1 isotypes in primary B cells (Fig. 2C,D). Nedd4 deficiency also leads to decreased CSR to IgA in CH12F3 cells (Fig. 2A). Thus, Nedd4 is an important component of the CSR machinery in B cells at multiple isotypes in the IgH locus. Based on these observations, we proceeded to determine the mechanism by which Nedd4's E3 ubiquitination activity promotes AID function during CSR.

Nedd4 promotes AID interaction with its cofactors, Spt5 and RNA exosome, on transcribed Ig switch sequences in B cells

As Nedd4 binds the AID complex and promotes AID function in B cells, we wanted to evaluate whether the level of RNA exosome subunit Exosc3 and Spt5, two marker proteins of the active AID/RNA polII complex, associates with AID in a Nedd4-dependent fashion. We noted that Nedd4 deficiency does not affect the overall expression of AID, Spt5, or Exosc3, as the total content of these proteins in the input samples is comparable in SHC002 and shNedd4 cells (Fig. 3A). We then immunoprecipitated AID from SHC002 and shNedd4 cells and found that in the shNedd4 cells, the steady-state level of the AID/RNApolII/RNA exosome complex is significantly reduced, as observed by the lack of Spt5 and Exosc3 in AID immunoprecipitates obtained from shNedd4 cells (Fig. 3A). However, at this moment, it is not possible to conclude whether Nedd4 influences the order of recuitment of RNA exosome and AID to the stalled RNA polII complex.

To determine the effect of Nedd4 deficiency on AID and RNA exosome binding to IgSμ, we used ChIP assays in SHC002-CH12F3 or shNedd4-CH12F3 cells. We found that AID and Exosc3 bind to IgSμ following CSR stimulation in CH12F3 (SHC002) cells, but this interaction is reduced in Nedd4-deficient (shNedd4) cells (Fig. 3B).

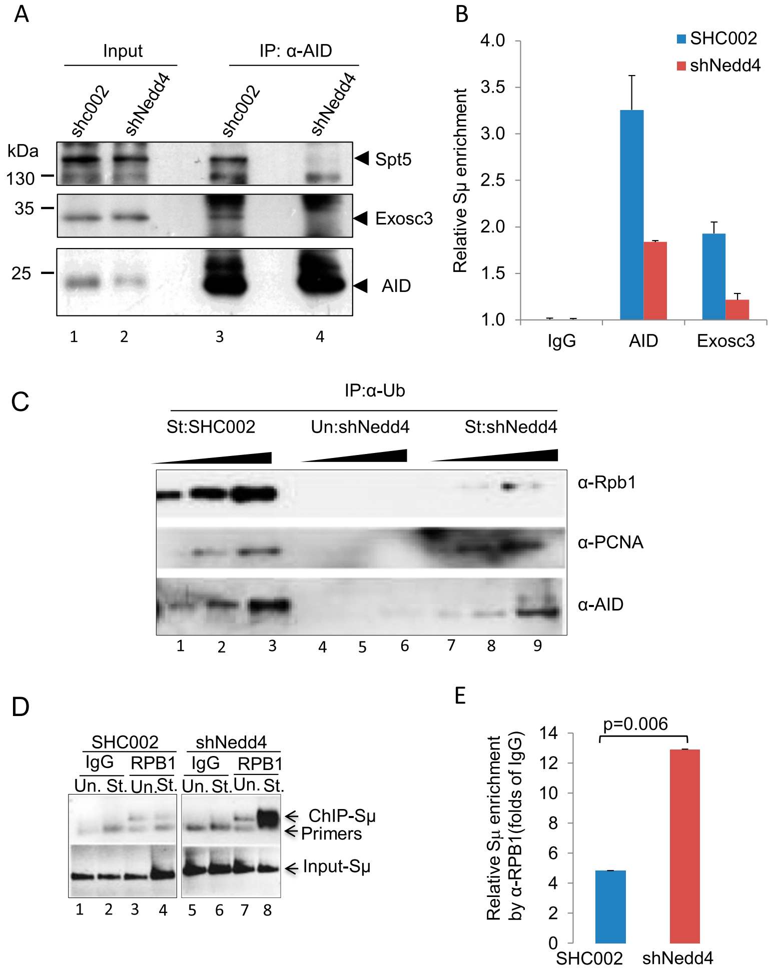

Figure 3. Nedd4 promotes AID interaction with its cofactors, Spt5 and RNA exosome, on transcribed Ig switch (IgS) sequences while decreasing steady-state levels of RNA polII bound to IgS. (*A*) Coimmunoprecipitation assays to evaluate interaction of AID with Spt5 and Exosc3 in SHC002 or shNedd4 cells. (*B*) Association with IgSμ switch region DNA by AID and Exosc3 in SHC002 and shNedd4 cells, determined with ChIP. Ubiquitination of RNA polII (*C*) and steady-state binding of RNA polII at IgSμ (*D*,*E*) in SHC002 and shNedd4 cells treated with MG132. (St) Stimulated for CSR; (Un) without CSR stimulation. The ChIP products were either assayed by DNA gel electrophoresis (*D*) or quantitated by qPCR (*E*).

Thus, we conclude that in B cells, Nedd4 facilitates the complexation of AID and its cofactors, RNA exosome and Spt5, on its physiological DNA substrates. We proceeded to seek a mechanistic interpretation of Nedd4-dependent AID interaction with the RNA polII-associated Spt5 and RNA exosome complex during CSR.

Nedd4 controls the steady-state level of IgS-associated RNA polII by its ubiquitination activity

We focused on determining the regulation of IgSμ-bound RNA polII by Nedd4. From previously published literature, we were aware that monoubiquitination of RNA polII by Nedd4 at sites of DNA damage marks the RNA polII complex for polyubiquitination-mediated degradation (Anindya et al. 2007; Harreman et al. 2009). Given that Nedd4 is a component of the AID-bound transcription complex and binds to transcription-activated IgSμ (Fig. 1B,C), we investigated whether RNA polII ubiquitination is Nedd4-dependent and determined its accumulation levels in transcribed IgSμ. We assayed for RNA polII binding to IgSμ in CH12F3 cells proficient and deficient in Nedd4. We chose to analyze only IgSμ, since it is the most robustly transcribed switch sequence and thus provides the best opportunity to perform biochemical assays that require protein complex detection. We performed ubiquitinated protein immunoprecipitation experiments with IgA CSR-stimulated cells and observed that unlike in the SHC002 cells, shNedd4 cells have reduced ubiquitinated RNA polII (Fig. 3C, cf. lanes 1–3 and 7–9). In contrast, we did not see a significant decrease in the levels of ubiquitinated PCNA in these cells (a known monoubiquitinated protein in B cells) (Langerak et al. 2007) and saw only a slight change of AID ubiquitination (Fig. 3C). A fraction of AID is ubiquitinated in B cells, as has been reported previously by other groups (Aoufouchi et al. 2008; Delker et al. 2013). These results demonstrate that in CSR-stimulated B cells, a portion of cellular RNA polII is ubiquitinated by Nedd4.

We subsequently wanted to determine whether the steady-state level of IgSμ-bound RNA polII is dependent on the Nedd4 activity in these B cells. We chromatin-immunoprecipitated RNA polII from SHC002 and shNedd4 cells and assayed for RNA polII association with IgSμ by conventional PCR (Fig. 3D) and qPCR (Fig. 3E). By both methods, we observed that there is increased accumulation of RNA polII on IgSμ in shNedd4 cells in comparison with SHC002 controls. Taken together, these experiments demonstrate a role of Nedd4 in the turnover of RNA polII resident at IgS sequences during CSR in activated B cells, potentially by a polyubiquitination-mediated degradation event.

Because there is more RNA polII associated with IgS in Nedd4-deficient B cells, we checked whether there are more IgS transcripts in Nedd4-deficient B cells. We observed that in shNedd4 cells, IgSμ and IgSα germline transcripts levels are stabilized at levels above those seen in control SHC002 cells (Supplemental Fig. S5A,B). Consistently, we also found that in Nedd4$^{-/-}$ primary B cells, the steady-state level of IgSγ1 switch sequence transcript is higher than that seen in Nedd4$^{+/+}$ B cells upon CSR stimulation (Supplemental Fig. S5C). The stabilizing effect of germline transcripts in Nedd4-depleted CH12F3 cells (Supplemental Fig. S5A,B) is much higher than that seen in Nedd4 mutant primary B cells (Supplemental Fig. S5C). This may be due to the fact that CH12F3 cell lines survive better than primary B cells in culture and accumulate more RNA, thus providing robust means to evaluate increased accumulation of germline transcripts. Based on these observations, we conclude that Nedd4 determines CSR efficiency in B cells, potentially by cotranscriptionally regulating the steady-state levels of RNA polII associated with IgS switch regions.

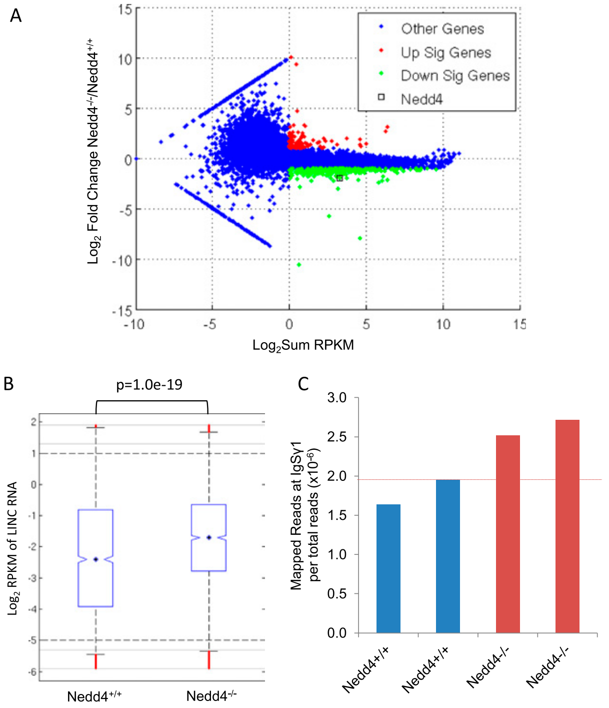

Figure 4. Nedd4-deficient B cells have increased levels of long ncRNA genome-wide. Whole-genome RNA sequencing from Nedd4$^{-/-}$ and Nedd4$^{+/+}$ B cells grown under conditions of IgG1 CSR for 3 d. (*A*) Gene expression of coding genes in Nedd4$^{+/+}$ and Nedd4$^{-/-}$ B cells was evaluated from RNA sequencing data sets for genes that are diffentially expressed in the two sets. Fold change in gene expression of individual genes for Nedd4$^{-/-}$ B cells over that of Nedd4$^{+/+}$ B cells is presented. Coding genes expressed at the same levels in Nedd4$^{-/-}$ and Nedd$^{+/+}$ B cells are represented by blue dots, genes expressed higher in Nedd4$^{-/-}$ B cells are represented by red dots ($\log_2$ sum reads per kilobase per million mapped reads [RPKM] > 0; $\log_2$ fold change Nedd4$^{-/-}$/Nedd4$^{+/+}$ > 1), and genes that have decreased expression in Nedd4$^{-/-}$ B cells are represented by green dots ($\log_2$ sum RPKM > 0; $\log_2$ fold change Nedd4$^{-/-}$/Nedd4$^{+/+}$ < −1). (*B*) Large ncRNA subtype lincRNA expression levels in Nedd4$^{+/+}$ and Nedd4$^{-/-}$ B cells. lincRNAs identified by Guttman et al. (2009) were assayed for expression levels in this plot. (*C*) Summation of total transcript reads of the IgSγ1 switch sequence in Nedd4$^{+/+}$ and Nedd4$^{-/-}$ B cells from two separate analyses performed on RNA isolated from two different mice of each genotype.

Nedd4 regulates ncRNA biogenesis at AID target sequences

If Nedd4 mediates destabilization of RNA polII at AID target sequences and activates RNA exosome-mediated degradation of nascent ncRNAs, we would expect that there would be an accumulation of RNA exosome substrate transcripts at AID target loci in Nedd4$^{-/-}$ B cells. To determine whether this is indeed the case, we decided to perform RNA sequencing of the whole genome of Nedd4$^{+/+}$ and Nedd4$^{-/-}$ B cells. We isolated ribosomal RNA (rRNA)-depleted total RNA from Nedd4$^{+/+}$ and Nedd4$^{-/-}$ B cells following CSR activation to IgG1. We performed high-throughput RNA sequencing and analyzed the levels of various coding RNAs and ncRNAs in these B cells (for details of total mapped reads, see Supplemental Fig. S6A). We quantitated the genome-wide exome expression level in Nedd4$^{+/+}$ and Nedd4$^{-/-}$ cells and found slight but not statistically significant differences in mRNA levels genome-wide over the total genome expression. We listed the read counts per million bases analyzed of the Nedd4$^{+/+}$ and Nedd4$^{-/-}$ coding gene transcriptomes (Supplemental Table S2) and identified pathways that could vary between these two genotypes (see Supplemental Fig. S6B,C for pathways that could be up-regulated or down-regulated in the Nedd4$^{-/-}$ B cells, respectively). We did not observe significant changes in the subset of genes that are expressed in the Nedd4$^{+/+}$ and Nedd4$^{-/-}$ samples, although there are a few genes that are up-regulated or down-regulated as shown in Figure 4A. As determined using STRING pathway analysis software (Supplemental Fig. S6B,C), we did not observe any changes in pathways that directly affect transcription, cell cycle progression, or DNA repair pathways that indirectly could have affected CSR. We then assayed for various types of RNAs that are noncoding in nature to determine whether transcription of the noncoding genome by RNA polII is affected in Nedd4$^{-/-}$ B cells. A family of ncRNAs that is expressed in B cells is large intergenic ncRNAs (lincRNAs). We are interested in this subgroup, since IgS germline transcripts share a number of properties with lincRNAs, including the observations that germline transcripts do not code for proteins, are spliced, and are polyadenylated, all three properties having also been observed in recently characterized lincRNAs (Rinn and Chang 2012). Indeed, we found that lincRNA levels are increased in Nedd4$^{-/-}$ B cells in comparison with those present in Nedd4$^{+/+}$ cells (Fig. 4B). (A detail of the TSS and transcription end site of each of the lincRNAs that we analyzed is presented in Supplemental Table S3.) Based on this finding, we specifically inspected the steady-state expression level of germline transcript IgSγ1 in Nedd4$^{-/-}$ B cells. We chose IgSγ1 transcript levels due to the fact that they are expressed in primary B cells only following CSR stimulation, are known to form a secondary RNA structure, and are polyadenylated and thus represent closely the canonical description of lincRNAs. We observed that the percentage of mapped reads (normalized to the remainder of the RNA genome) at the IgSγ1 region is increased in Nedd4$^{-/-}$ B cells over that seen in Nedd4$^{+/+}$ cells (Fig. 4C) in two separate RNA sequencing experiments. The increase in the IgSγ1 mapped reads per million reads of the total Nedd4$^{-/-}$ transcriptome is ~25%–40% compared with that found in the Nedd4$^{+/+}$ control.

Another type of ncRNA that could be a target of the RNA exosome complex is TSS-associated ncRNA (TSS-ncRNA). TSS-ncRNAs have been implicated in recruitment and regulation of RNA polII during the transcription initiation phase, although the exact role in this process is not understood. We evaluated the expression levels of TSS-RNAs genome-wide for various loci and found that there is no significant alteration in the spectrum of genes that express TSS-ncRNAs in Nedd4$^{+/+}$ and Nedd4$^{-/-}$ B cells (Fig. 5A). We evaluated and found no differences in the start site positioning or length of TSS-ncRNAs in the Nedd4$^{-/-}$ and Nedd4$^{+/+}$ B cells compared with TSSs genome-wide (Fig. 5B). These findings indicate that Nedd4 activity deficiency does not alter the distribution or location of TSS-ncRNAs genome-wide. Since we found that Nedd4 interacts with RNA polII associated with AID (Fig. 1C), we decided to investigate the levels of TSS-associated transcripts in Nedd4$^{-/-}$ cells specifically at genes that have been previously reported to be mutated by AID or bound with AID in B cells based on data obtained from AID ChIP assays (Fig. 5C). Recently published literature indicates that on a genome-wide basis, AID binds to many genes where RNA polII stalling occurs. We found that AID-bound genes have higher amounts of TSS-RNAs in Nedd4$^{-/-}$ B cells compared with those observed in Nedd4$^{+/+}$ B cells (Fig. 5C). We wanted to

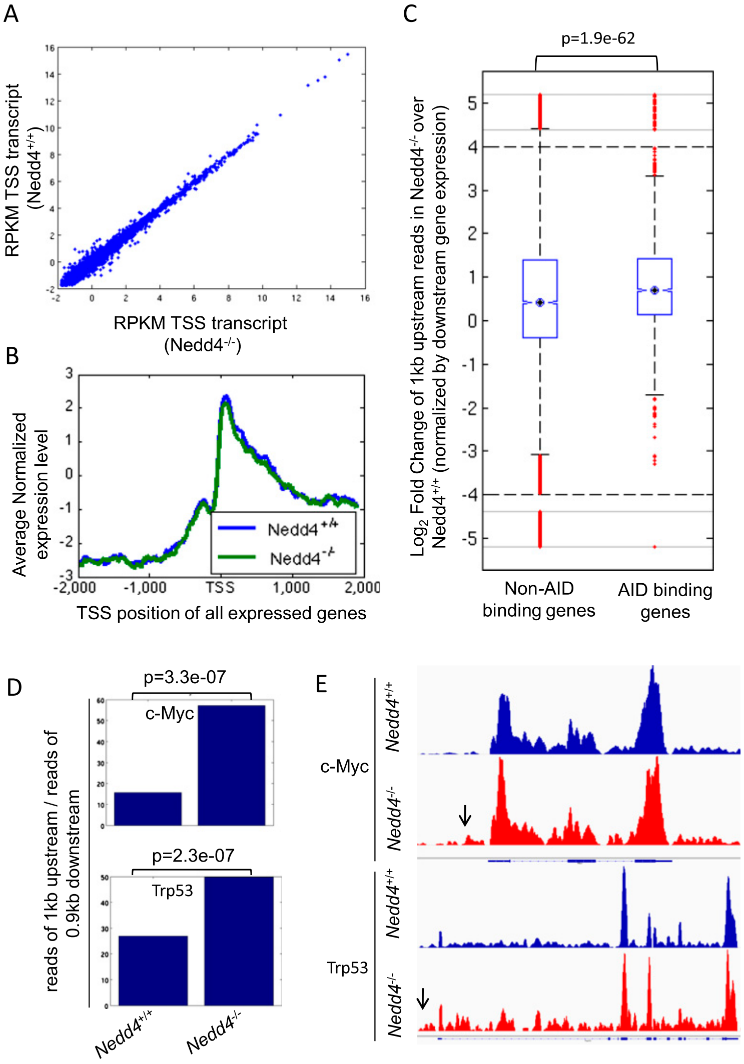

Figure 5. Nedd4 is required for processing of TSS transcripts at AID target sequences in B cells. TSS-ncRNA in Nedd4$^{+/+}$ and Nedd4$^{-/-}$ B cells. (*A*) Comparison of expressed TSS-ncRNAs in Nedd4$^{+/+}$ and Nedd4$^{-/-}$ B cells depicted by their RPKM levels. (*B*) Evaluation of the TSS position and expression level of TSS-ncRNA transcripts and their cognate coding transcripts genome-wide in Nedd4$^{+/+}$ or Nedd4$^{-/-}$ B cells. (*C*) Evaluation of TSS-ncRNA levels (evaluated for the 1000 base pairs [bp] upstream of the genic TSS) at nontargets and targets of AID (Yamane et al. 2011) in Nedd4$^{-/-}$ B cells relative to that seen in Nedd4$^{+/+}$ B cells. (*D*) A quantitative estimate of the level of TSS-associated upstream transcripts (≤1 kb in length) at the cMyc locus (*top* panel) and the p53 locus (*bottom* panel) in Nedd4$^{+/+}$ and Nedd4$^{-/-}$ B cells. (*E*) The expression profile of TSS-RNA at the c-myc locus (*top* panel) and the p53 locus (*bottom* panel) in Nedd4$^{+/+}$ and Nedd4$^{-/-}$ B cells. The region of TSS-RNA is shown with an arrow; the RNA sequencing data plots were derived using Investigative Genomics Viewer software.

directly visualize and evaluate the presence of TSS-RNA at AID "off-target" genes. We are aware that c-myc has been reported in various studies to be a robust "off-target" of AID (Ramiro et al. 2006; Liu et al. 2008; Pasqualucci et al. 2008). More importantly, AID-mediated mutations in c-myc and p53 have now been established to initiate Burkitt's lymphoma (Ramiro et al. 2007) and gastric epithelial cancer (Matsumoto et al. 2007; Shimizu et al. 2012), respectively. As seen in Figure 5, D and E, the level of upstream TSS-associated transcripts at c-myc is increased in Nedd4$^{-/-}$ B cells. In quantitative terms, the level of upstream transcripts at the c-myc TSS is approximately fourfold higher in Nedd4-deficient B cells based on the level found in Nedd4$^{+/+}$ B cells (P-value = 3.2700×10^{-7}) when normalized to downstream reads initiating from the TSS of c-Myc (Fig. 5D). Similarly, when we assayed for the presence of TSS-associated upstream transcripts at the Trp53 locus, we found increased stabilization of these transcripts in conditions of Nedd4 deficiency (Fig. 5D,E). These experiments provide further evidence that Nedd4 regulates nascent noncoding transcript levels at certain loci that are mutatable by AID in B cells by initiating their processing and/or degradation by the AID cofactor complex RNA exosome.

Discussion

In this study, we evaluated the fate of the RNA polII complex that stimulates AID's ability to deaminate DNA target sequences genome-wide. We propose two fates of RNA polII that it could encounter following promoter escape (a TFIIH-dependent mechanism) and after attaining transcriptional pausing: (1) cleavage of the nascent RNA in the transcription elongation complex followed by reinitiation of the polII (a TFIIS-dependent mechanism) or (2) destabilization of RNA polII via the ubiquitination pathway that leads to exposure of the nascent RNA in the collapsing transcription bubble (a Nedd4-dependent mechanism) (Anindya et al. 2007; Cheung and Cramer 2012). We found that Nedd4 and TFIIH complex with AID; these observations suggest that a combination of RNA polII backtracking and RNA polII destabilization functions as a possible mechanism that supports AID mutagenesis activity. Based on these observations and published work from other laboratories, we updated our model of how AID identifies its target sequences in the IgH locus and genome-wide and incorporates mutations. Following transcription initiation at various IgH- and non-IgH-localized DNA sequences, RNA polII undergoes promoter-proximal stalling or may undergo stalling in its elongation phase (Fig. 6B). The conditions that promote promoter-proximal stalling are in the process of being unraveled, although it is quite possible that environmental cues and promoter-proximal DNA sequences may promote RNA polII stalling (Saunders et al. 2006). RNA polII stalling at regions significantly downstream from TSSs could be caused by pretermination events of RNA polII induced by various conditions, including the pres-

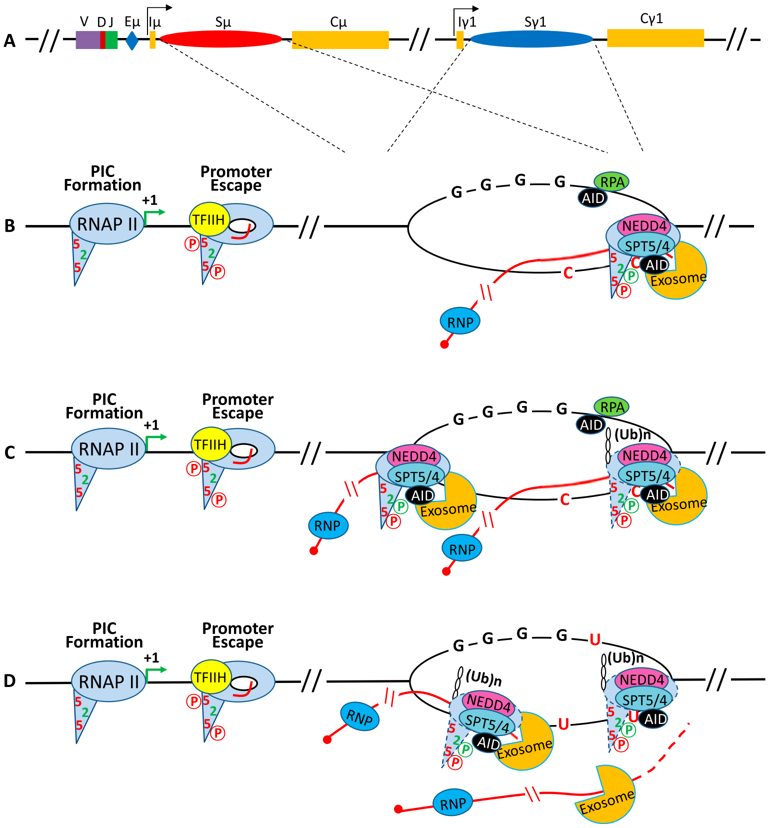

Figure 6. A schematic representation of AID regulation via the transcription complex in the Ig switch sequence. (*A*) A simplified representation of an Ig switch region structure that contains a G-rich core sequence (blue oval) and the promoter preceding Iγ1. (*B*) Transcription at switch sequences can cause secondary DNA structures (R-loops) that impede and stall RNA polII (RNAP II). Moreover, stalled RNA polII (bound with Spt5 and RNA exosome) recruits AID and Nedd4. (*C*) Nedd4 present in the RNA polII/RNA exosome/AID complex promotes the ubiquitination of RNA polII and disengages it from the nascent RNA transcript. (*D*) The RNA exosome is now able to degrade the transcript in the transcription bubble to allow AID access to the template and nontemplate strands of its target sequence. In this model (adapted from Sun et al. 2013), multiple sets of the stalled AID–RNA polII–RNA exosome–Nedd4 complex are present in the R-loop ssDNA structure in the switch sequence; for the purpose of simplicity, we show two representative complexes.

ence of secondary structures on the template DNA (Li and Manley 2006; Richard and Manley 2009). In either case, the stalled RNA polII will be required to relinquish the associated nascent transcripts as an RNA exosome substrate in order to resolve the paused state. Indeed, AID-mediated mutations can occur within the first 100–500 base pairs (bp) from the TSS (associated with the promoter-proximal stalled complex), as seen during SHM in variable genes or certain other AID "off-target" genes. Mutations also occur at >2 kb downstream from TSSs (associated with the pretermination RNA polII complex), as seen in switch sequences. For the RNA exosome to be able to identify and degrade stalled RNA polII-associated transcripts at DNA sequences where AID mutates its targets, it has to be able to find a 3′ end free transcript. In this study, we provide evidence that Nedd4 induces RNA polII ubiquitination, which consequently promotes the generation of the 3′ end free nascent RNA transcript by displacing the associated RNA polII transcription complex (Fig. 6B,C). We propose that this RNA polII ubiquitination event occurs in a complex that contains Spt5, RNA exosome, AID, and Nedd4 (Fig. 6B,C); the absence of any of the components destabilizes the complex (Fig. 3A; Basu et al. 2011). We propose that once the RNA exosome degrades the nascent germline transcript associated with the AID-associated transcription complex, the template and nontemplate strands of the IgS sequence are exposed for AID-mediated mutagenesis (Fig. 6D). This model is in line with the known activity of RNA exosome. Transcriptional complex-associated ncRNAs are substrates of the RNA exosome complex. For example, in *Saccharomyces pombe*, RNA exosome-mediated cotranscriptional degradation of centromeric RNA has been implicated in maintenance of transcriptional silencing and genomic integrity (Buhler et al. 2007; Reyes-Turcu and Grewal 2012; Yamanaka et al. 2013). RNA exosome substrate TSS-associated ncRNA promoter upstream transcripts (PROMPTs) have also been identified in mammalian cells (Preker et al. 2008; Seila et al. 2008). In addition, other RNAs—such as viral RNAs (Zhu et al. 2011), intergenic cryptic unstable transcripts (Wyers et al. 2005), rRNA, small nucleolar RNAs (snoRNAs) (Allmang et al. 1999), etc.—are reported to be RNA exosome substrates in vivo (Houseley et al. 2006). Understanding of the transcriptional properties (length, TSS information, and directionality) of these substrate RNAs and the mechanism of their recognition by the RNA exosome complex will provide further insight into how RNA exosome identifies and processes transcripts in the IgH locus, as proposed in Figure 6.

We do not exclude the possibility that other mechanisms can also provide the RNA exosome access to the AID/RNA polII-associated nascent RNA. Residual CSR in Nedd4$^{-/-}$ B cells indicates that there could be other pathways that provide AID access to transcribed switch sequences. Interaction of AID with the replication protein complex RPA due to phosphorylation of AID at residue Ser-38 has been implicated in providing AID the ability to mutate nontemplate strand switch sequences. A high density of nontemplate strand DNA breaks can be sufficient to induce some DNA double-strand breaks and cause inefficient CSR. It is also possible that "scrunching" of RNA polII may displace the template DNA strand from the RNA and override the role of the RNA exosome and/or Nedd4 in providing AID with a suitable substrate. However, until now, "scrunching" has only been demonstrated with bacterial RNA polII (Revyakin et al. 2006), and thus its feasibility as a mechanism for generation of ssDNA with mammalian RNA polII requires further work. Negative supercoiling preceding the transcribing RNA polII can also generate ssDNA that is the target of AID protein (Shen and Storb 2004; Longerich et al. 2006). Finally, we do realize that transcription-coupled DNA mutagenesis by the nucleotide excision repair (NER) pathway may also provide AID a mechanism to initiate and spread mutations in the genome. NER components are associated with factors that promote "promoter escape." However, we note that patients with NER pathway mutations do not manifest any defects in SHM, the physiological target of AID activity (Kim et al. 1997).

Proper distribution and rapid resolution of AID-induced mutations at switch sequences and variable region genes is important, since they can otherwise be intermediates for deleterious chromosomal translocations. On the other hand, it is also important to remove residual stalled RNA polII resident genome-wide, which may have been generated during the G1 phase of the cell cycle and prior to the onset of DNA replication during S phase. Indeed, a low level of AID-promoted mutations may induce RNA polII stalling at various regions of the B-cell genome during the G1 phase of the cell cycle. In addition, stalled RNA polII may also induce AID-independent mutations in the B-cell genome (Unniraman et al. 2004; Barlow et al. 2013). In either case, failure to remove stalled RNA polII may induce collision of these transcription complexes with components of the replication machinery during S phase and generate DNA double-strand breaks. Thus, the role of Nedd4 in destabilizing stalled RNA polII molecules that are stalled genome-wide in an AID-dependent or AID-independent manner may have significant implications in preventing genomic instability. HECT domain E3 ligases have been implicated in the prevention of various oncogenic events by catalyzing proteasomal degradation of various oncogenes like PTEN, p53, Notch-1, etc.; thus, understanding its mechanism of function in B cells is important (Bernassola et al. 2008). Our work points toward a novel role of Nedd4 as a checkpoint of oncogenesis by prevention of aberrant mutagenesis of the B-cell genome.

Materials and methods

Antibodies and plasmids

AID antibodies were generated as described (Chaudhuri et al. 2003). Anti-exosome subunit antibodies were purchased from GenWay Biotech, Inc., or Abcam, Plc. Details of additional antibodies are as follows: Actin was purchased from Sigma-Aldrich; Nedd4 was purchased from R&D Systems; rabbit IgG and RNA polII (4H8) were purchased from Abcam; Spt5, TFIIS, and TFIIH p52 were purchased from Santa Cruz Biotechnology; and IgG1, IgA, and B220 were purchased from Becton Dickinson. All secondary HRP-conjugated antibodies were purchased from Sigma. shNedd4 (TRCN0000092433), shNedd4L (TRCN0000086869), nonmammalian shRNA control (SHC002), and MISSION Lentiviral Packaging Mix (SHP001) were all purchased from Sigma.

Cell culture, transfection, infection, and selection

Splenic B cells were prepared with CD43-negative selection and cultured in RPMI1640 medium containing 15% FBS plus 20 μg/mL LPS and/or 20 ng/mL IL-4. CH12F3 cells were maintained in RPMI1640 medium containing 10% FBS or were stimulated for IgA CSR with the addition of 20 μg/mL LPS, 20 ng/mL IL-4, and 1 ng/mL TGFβ1. HEK293T cells were cultured in DMEM with 10% FBS. HEK293T cells were transfected with specific plasmids for protein production. All cells were incubated at 37°C in a 5% CO_2 humidified incubator. HEK293T cells were cotransfected with shRNA plasmid and MISSION Lentiviral Packaging mix for lentivirus production. (For details of lentiviral knockdown assays, see the Supplemental Material.)

RNA extraction, protein preparation, immunoprecipitation, and ChIP

For RNA extraction, protein preparation, immunoprecipitation, and ChIP, see the Supplemental Material.

Real-time qPCR and primers

Real-time PCR was performed with SYBR Green ROX (Roche Applied Science) using Eppendorf Realplex2. For quantification, a standard curve was established with a serial dilution of samples with each primer pair. Assays for germline S region transcript levels were performed according to published protocols (Muramatsu et al. 2000). Primer pairs for germline transcripts were as follows: Iμ, 5′-CTCTGGCCCTGCTTATTGTTG-3′ and 5′-GAAGACATTTGGGAAGGACTGACT-3′; Iα, 5′-CCTGGCTGTTCCCCTATGAA-3′ and 5′-GAGCTGGTGGGAGTGTCAGTG-3′; and Iγ1, 5′TATGATGGAAAGAGGGTAGCATT-3′ and 5′-CTGGGCTGGTCTGTCAACTCCTT-3′.

Other q-PCR primers were GAPDH, 5′-TGGCCTTCCGTGTTCCTAC-3′ and 5′-GAGTTGCTGTTGAAGTCGCA-3′; Nedd4, 5′-GTGGGAAGAGAGGCAGGATGTC-3′ and 5′-GCGAATTCACAGGAAGTGTAGGC-3′; and Nedd4L, 5′-GAGGCTCCAGTTCATGTGGG-3′ and 5′-GGATACGGGATTCTCCCTGTC-3′. Primer pairs for switch region ChIP were 5′Sμ, 5′-TAGTAAGCGAGGCTCTAAAAAGCAT-3′ and 5′-AGAACAGTCCAGTGTAGGCAGTAGA-3′; and Sα, 5′-TGAAAAGACTTTGGATGAAATGTGAACCAA-3′ and 5′-GATACTAGGTTGCATGGCTCCATTCACACA-3′.

High-throughput sequencing

Splenic B cells of Nedd4$^{-/-}$ and Nedd4$^{+/+}$ were cultured with LPS and IL-4 for 3 d, and rRNA-depleted total RNA was prepared from these cells as described in the Supplemental Material. Libraries were prepared with Illumina's TruSeq RNA prep kit and then sequenced with 50 million to 60 million 2 × 100-bp paired raw passing filter reads on an Illumina HiSeq 2000 V3 instrument at the Columbia Genome Center. We mapped the pass filter reads to the genome (human, NCBI build37; mouse, UCSC mm9) using TopHat version 2.0.4 (Trapnell et al. 2009). TopHat infers novel exon–exon junctions ab initio and combines them with junctions from known mRNA sequences (refgenes) as the reference annotation. For each read, we allowed up to two mismatches and 10 multiple hits during the mapping. The gene expression level was calculated using FPKM (fragment per kilobase transcriptome per million mapped reads) by Cufflinks version 2.0.2. The RNA sequencing data from Nedd4$^{-/-}$ and Nedd4$^{+/+}$ cells can be obtained from Gene Expression Omnibus (accession no. GSE49027).

Nedd4-deficient mouse B cells

The generation of Nedd4$^{+/+}$ and Nedd4$^{-/-}$ fetal liver chimeras was described previously (Yang et al. 2008). Heterozygous mice encoding a gene trap inserted between the first two WW domains of Nedd4 (Bay Genomics, XA398) were crossed in timed matings, and fetal livers were harvested at day 16 of fetal development. Fetal liver cell suspensions were frozen in medium containing 90% FCS and 10% DMSO, while DNA samples from the fetuses were genotyped. Fetal liver suspensions from Nedd4$^{+/+}$ and Nedd4$^{-/-}$ embryos were transferred by tail vein injection into lethally irradiated 6- to 10-wk-old Rag1$^{-/-}$ recipients that had received a "split dose" of 800 and 400 rads separated by 2–4 h. Each fetal liver was used to reconstitute five lethally irradiated recipients. Recipient mice were analyzed between 7 and 9 wk post reconstitution.

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