

# AID-Induced Genotoxic Stress Promotes B Cell Differentiation in the Germinal Center via ATM and LKB1 Signaling

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## SUMMARY

During an immune response, B cells undergo rapid proliferation and activation-induced cytidine deaminase (AID)-dependent remodeling of *immunoglobulin (IG)* genes within germinal centers (GCs) to generate memory B and plasma cells. Unfortunately, the genotoxic stress associated with the GC reaction also promotes most B cell malignancies. Here, we report that exogenous and intrinsic AID-induced DNA strand breaks activate ATM, which signals through an LKB1 intermediate to inactivate CRTC2, a transcriptional coactivator of CREB. Using genome-wide location analysis, we determined that CRTC2 inactivation unexpectedly represses a genetic program that controls GC B cell proliferation, self-renewal, and differentiation while opposing lymphomagenesis. Inhibition of this pathway results in increased GC B cell proliferation, reduced antibody secretion, and impaired terminal differentiation. Multiple distinct pathway disruptions were also identified in human GC B cell lymphoma patient samples. Combined, our data show that CRTC2 inactivation, via physiologic DNA damage response signaling, promotes B cell differentiation in response to genotoxic stress.

## INTRODUCTION

DNA double-strand breaks (DSBs) are generated during the assembly and diversification of antigen receptor genes in developing lymphocytes. During early B cell maturation in the bone

marrow, the recombinase activating gene (RAG) endonuclease generates complete antigen receptor genes by the process of *V(D)J* recombination (Fugmann et al., 2000; Tonegawa, 1983). The generation of a diverse repertoire of high-affinity antibodies (Abs) requires further modifications of the *immunoglobulin (IG)* genes (Rajewsky, 1996; Revy et al., 2000) in secondary lymphoid follicles within compartments known as germinal centers (GCs). GCs are sites within lymphoid tissues where mature B cells rapidly proliferate, modify *IG* gene sequences, and differentiate in response to a stimulating antigen. A key feature of *IG* remodeling is class switch recombination (CSR), a process that modifies the effector function of an Ab by replacing one constant region of the *IG* gene with another. CSR requires activation-induced cytidine deaminase (AID)-generated DSB intermediates (Chaudhuri et al., 2003; Muramatsu et al., 2000) and subsequent repair of distal severed ends. This genomic remodeling is critical for a robust Ab response, but genotoxic stress associated with the GC reaction also promotes most human lymphomas (Küppers and Dalla-Favera, 2001).

In order to preserve genomic integrity, mammalian cells undergoing genotoxic stress usually respond by activating a complex DNA damage response (DDR). This response, which is required to prevent tumor formation, includes inhibition of cellular proliferation and/or induction of apoptosis (Khanna and Jackson, 2001). In GC B cells, the DDR is coordinated by the ATM serine/threonine kinase, which senses DSBs in concert with the MRN (MRE11-RAD50-NBS1) complex (Kastan and Bartek, 2004). This response is critical for humoral immunity and evasion of tumorigenesis because defects in CSR and increased chromosomal lesions occur in activated mature B cells from mice lacking ATM (Lumsden et al., 2004; Reina-San-Martin et al., 2004) or its target proteins 53BP1 (Manis et al., 2004; Ward et al., 2004), H2AX (Franco et al., 2006), NBS1 (Kracker et al., 2005; Reina-San-Martin et al., 2005), and MDC1 (Lou et al., 2006).

During the GC reaction, B cells express the BCL6 oncoprotein, which functions as a transcriptional repressor of the *PRDM1* gene encoding BLIMP-1 (Shaffer et al., 2000), the master regulator of plasma cell differentiation (Turner et al., 1994). Importantly, BCL6 also suppresses key components of the DDR in the GC by repressing the expression of *ATR* (Ranuncolo et al., 2007), *TP53* (Phan and Dalla-Favera, 2004), and *CDKN1A* (*P21*) (Phan et al., 2005). This suppression may enable GC B cells to proliferate rapidly without triggering cellular senescence or apoptosis programs, although the resulting modified DDR increases the susceptibility of GC B cells to malignant transformation. Accordingly, BCL6 downregulation is required for post-GC B cell differentiation and evasion of tumorigenesis (Cattoretti et al., 2005).

ATM promotes and BCL6 represses the DDR, representing antagonistic forces in the life of a GC B cell. To terminate the GC reaction, rapidly proliferating B cells must tip this balance toward exiting the cell cycle to allow for terminal differentiation, although a mechanism initiating this shift has not been identified. Previously, we showed that B cell antigen receptor (BCR) engagement led to cytoplasmic sequestration and inactivation of the CREB transcriptional coactivator CRTCC2 (TORC2), causing downregulation of the *TCL1* oncogene in GC B cells (Kurashy et al., 2007). Studies of glucose metabolism regulation have shown that CRTCC2 inactivation results from phosphorylation at S-171 (Screaton et al., 2004) and/or S-275 (Jansson et al., 2008) by members of the AMPK family, promoting a physical association between CRTCC2 and the cytoplasmic chaperone 14-3-3. However, the physiologic event(s) that inactivate CRTCC2 in GC B cells are unknown. Because GC B cells experience both DNA damage and CRTCC2 inactivation-dependent *TCL1* repression, we hypothesized that CRTCC2 is inhibited by the DDR and that CRTCC2 controls an extended gene program beyond *TCL1*. Testing of this hypothesis led to the discovery of a DDR pathway in GC B cells, with exogenous or intrinsic AID-induced DSBs activating ATM signaling to LKB1, a master kinase for AMPK family member proteins (Lizcano et al., 2004), which then resulted in the inactivation of CRTCC2. Suggesting a role as a key homeostatic regulator, changes in gene expression resulting from CRTCC2 inactivation were essential for cessation of the GC reaction, plasma cell differentiation, and suppression of tumorigenesis.

## RESULTS

### DNA Double-Strand Breaks Inactivate CRTCC2

To determine whether DNA damage inactivates CRTCC2, we induced DSBs in the Ramos human GC B cell line by using etoposide (Eto) or  $\gamma$ -irradiation (IR), which are known to generate  $\gamma$ -H2AX foci (Phan et al., 2007). Subcellular fractionation showed that DSBs caused a shift in CRTCC2 localization from the nucleus to the cytoplasm (Figure 1A). This change in CRTCC2 location was accompanied by an increased association between CRTCC2 and the cytoplasmic chaperone 14-3-3 (Figure 1B) (Jansson et al., 2008; Screaton et al., 2004). Chromatin immunoprecipitation (ChIP) showed a >4-fold reduction in the association between CRTCC2 and the CRTCC2-responsive *TCL1* promoter with DSBs (Figure 1C). DSBs also repressed expression of the *TCL1*

promoter (Figure 1D and Figures S1A–S1C, available online). Combined, these data show that DSBs inactivate CRTCC2, leading to repression of CRTCC2-dependent gene expression.

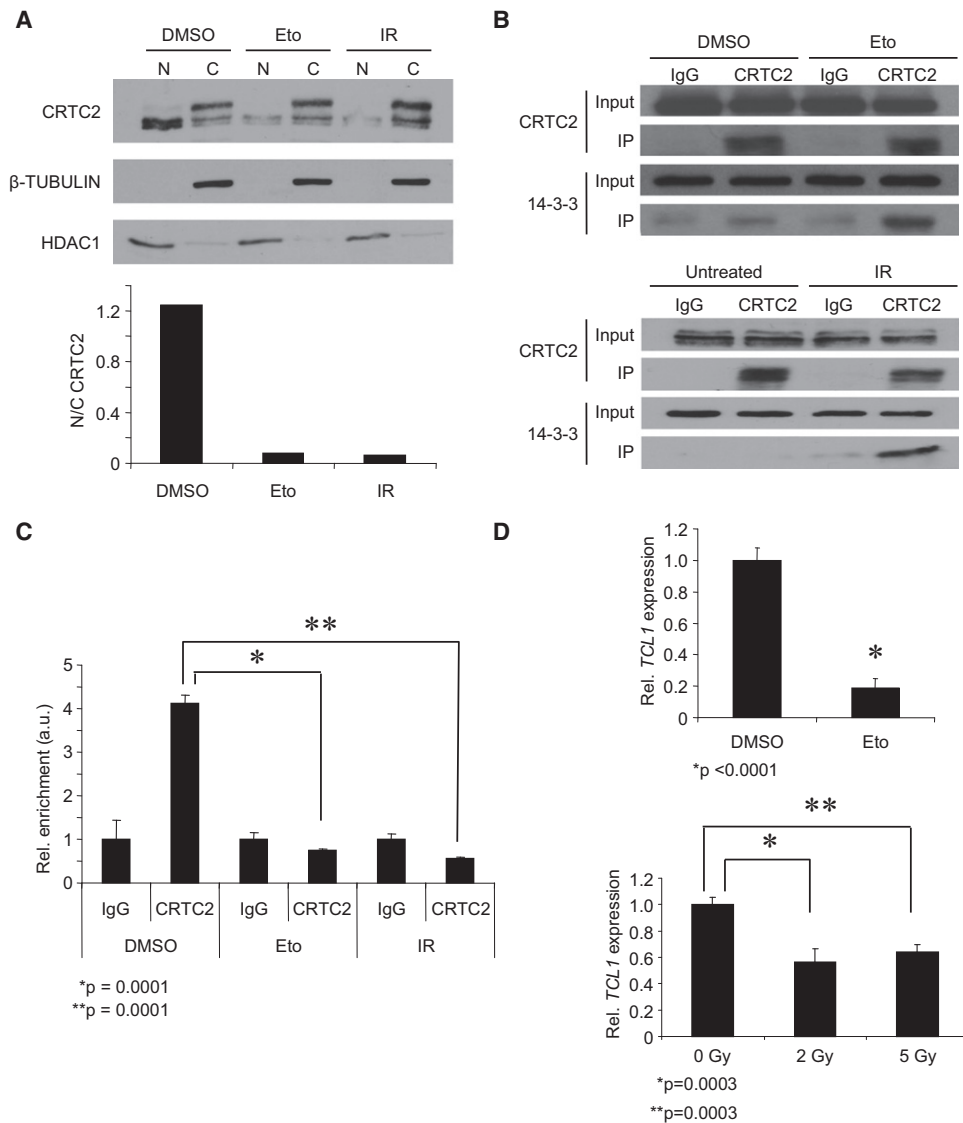
### DSB-Induced CRTCC2 Inactivation Requires Activation of ATM and LKB1

We next tried to identify a link between DSBs and CRTCC2 inactivation. Since the DNA damage-sensing kinase ATM is required for CSR (Lumsden et al., 2004; Reina-San-Martin et al., 2004), we evaluated ATM for a role in CRTCC2 inactivation. Induced DSBs in Ramos cells activated ATM (Figure S2A). ATM loss of function, using two different shRNA sequences targeting *ATM*, pharmacological inhibition with the ATM inhibitor Kudos, and the use of B cell lines from ATM-deficient ataxia-telangiectasia (A-T) patients showed a requirement for ATM in DSB-induced CRTCC2 inactivation (Figures 2A–2D and Figures S2B–S2D). ATM phosphorylates multiple substrates during the DDR (Matsuoka et al., 2007), potentially including T366 of the tumor suppressor LKB1 (Fernandes et al., 2005; Sapkota et al., 2002). In turn, LKB1 phosphorylates and inactivates CRTCC2 through AMPK family members (Fu and Screaton, 2008; Kato et al., 2006; Shaw et al., 2005), suggesting a pathway from DSBs to CRTCC2 inactivation. DSBs caused ATM-dependent phosphorylation of LKB1 T366 (Figures S2E and S2F). Similarly, DSBs induced LKB1 phosphorylation in primary B cells (Figure S2G). Metformin is an antidiabetic drug that promotes LKB1-dependent activation of AMPK (Shackelford and Shaw, 2009; Shaw et al., 2005). Ramos cells exposed to metformin showed reduced nuclear localization of CRTCC2 and *TCL1* repression (Figures S2H–S2J). shRNA knockdown of *LKB1* with two different sequences lessened CRTCC2 inactivation in response to DSBs in Nalm-6 pre-B cells (Figures S2K and S2L) and Ramos cells (Figures 2E–2G, Figures S2M and S2N). These data demonstrate that DSBs inactivate CRTCC2 via ATM and LKB1 signaling, providing a gene regulation mechanism during the DDR.

### CRTCC2 Inactivation Occurs during CSR in GC B Cells

To determine the role of CRTCC2 in GC B cells, we evaluated changes in CRTCC2 activity and direct target gene expression over the course of a GC reaction. For this, we modified an in vitro B cell differentiation system starting with naive human tonsil B cells (Figure 3A) (Arpin et al., 1995; Fluckiger et al., 1998). Rapid B cell expansion and correct modulation of established GC B and plasma cell markers (BCL6, MYC, OCA-B, and BLIMP-1) occurred over 7 days, as expected for a GC-like reaction (Figures 3B–3D) (Allman et al., 1996; Greiner et al., 2000; Shaffer et al., 2008). Though undetectable on day 3, soluble and membrane-bound IgG (32% of cells) was detected by day 7 (Figure 3E and Figure S3A), preceded by  $\gamma$ -H2AX focus formation by day 5 (Figure S3B) (Petersen et al., 2001). These results indicate that CSR followed by plasma cell differentiation was induced during a GC-like reaction between days 3 and 7 of culture.

CRTCC2 activity was evaluated during the interval in which CSR occurred. Nuclear CRTCC2 decreased between days 3 and 7 (Figure 3F) with a coinciding decrease in the association between CRTCC2 and the *TCL1* promoter and decreased *TCL1* expression, as observed in vivo (Figures 3G and 3H) (Said



**Figure 1. DNA Double-Strand Breaks Inactivate CRTC2**

(A) Top: immunoblot showing CRTC2 protein expression in the nucleus (N) and cytoplasm (C) of Ramos B cells exposed to DMSO (control), Eto (20  $\mu$ M, 1 hr), or IR (5 Gy). Upper bands in the cytoplasm lanes indicate phosphorylated CRTC2.  $\beta$ -TUBULIN (cytoplasm) and HDAC1 (nucleus) are loading controls. Bottom: nuclear/cytoplasmic ratios for CRTC2 are plotted from densitometry.

(B) Top: immunoprecipitation of CRTC2 or rabbit IgG from lysates of Ramos cells exposed to DMSO or Eto (20  $\mu$ M, 1 hr). Bottom: untreated cells were compared to cells exposed to IR (5 Gy). Immunoblots for lysates (Input) and CRTC2 or 14-3-3 immunoprecipitates (IP) are shown.

(C) ChIP for CRTC2 or rabbit IgG using chromatin from Ramos cells after DMSO, Eto (20  $\mu$ M, 1 hr), or IR (5 Gy). Immunoprecipitates were analyzed by qPCR for the *TCL1* promoter. Values were normalized to the *ACTB* promoter and shown as arbitrary units (a.u.). Values are expressed as the mean  $\pm$  SEM for three independent experiments.

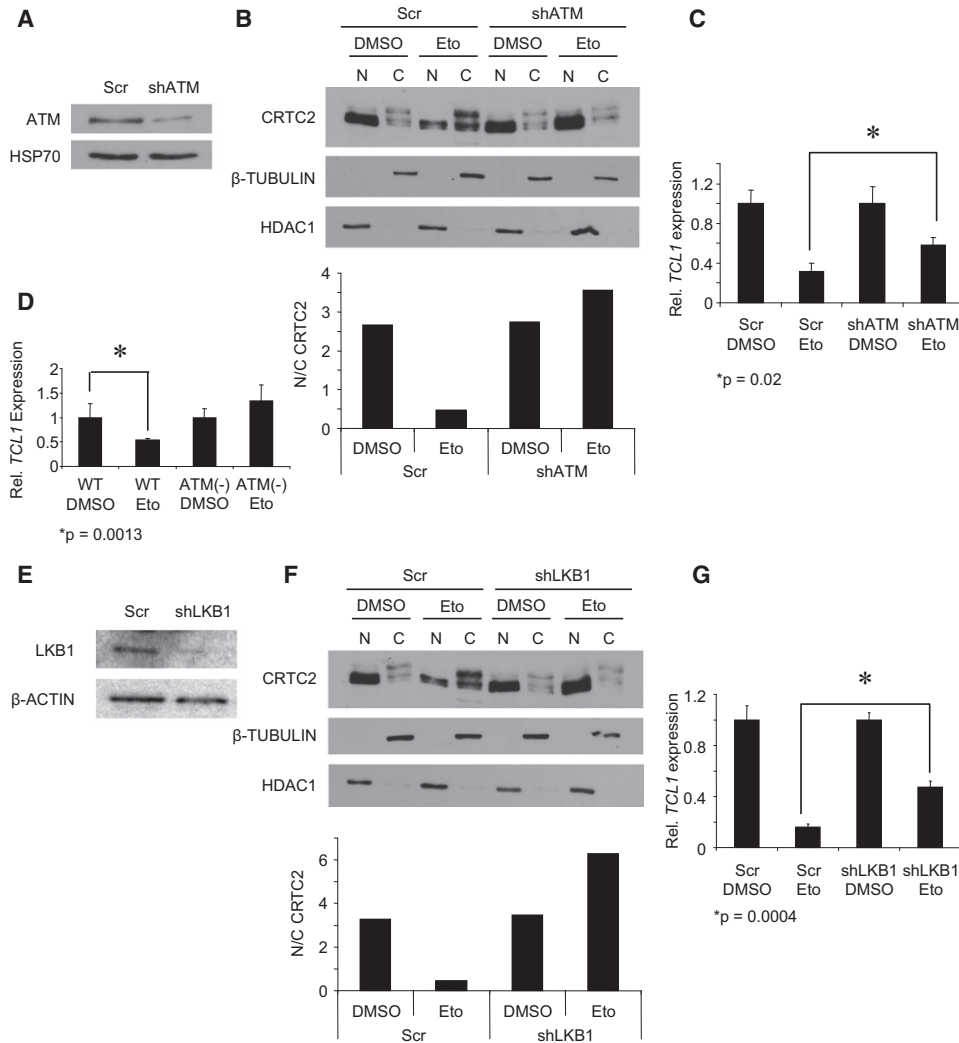
(D) Top: qPCR for *TCL1* in Ramos cells after DMSO or Eto (20  $\mu$ M, 6 hr). Bottom: untreated cells were compared to cells exposed to the indicated doses of IR. Values were normalized to *36B4*. Values are expressed as the mean  $\pm$  SEM for three independent experiments.

et al., 2001; Teitell et al., 1999). Importantly, similar CRTC2 modulation was observed during GC B cell development in vivo (Figure S3C) (Klein et al., 2003; Said et al., 2001), with  $\sim$ 70% of plasma cells containing entirely cytoplasmic CRTC2 and  $\sim$ 30% negative for CRTC2 protein expression. Combined, these results strongly suggest that CRTC2 becomes phosphorylated and sequestered in the cytoplasm and inactivated during

CSR in vivo, resulting in reduced expression of CRTC2-dependent target genes, such as *TCL1* (Figure S3D).

#### AID-Dependent ATM to LKB1 Signaling in GC B Cells Inactivates CRTC2

The requirement for ATM and LKB1 as upstream regulators of CRTC2 inactivation was assessed in the modeled GC-like



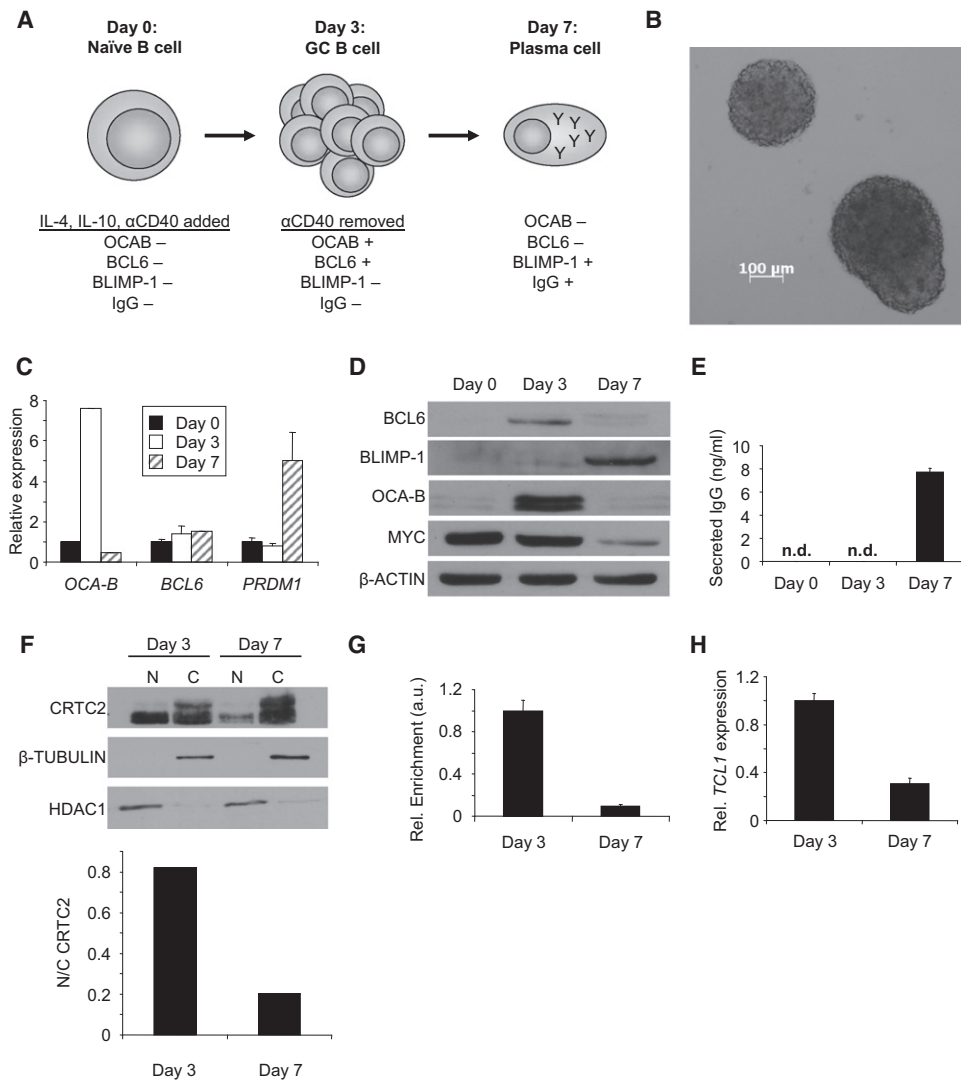
**Figure 2. DSB-Induced CRTC2 Inactivation Requires Activation of ATM and LKB1**

(A) Immunoblot showing ATM knockdown in Ramos cells 4 days after transduction. HSP70 is a loading control.  
 (B) Top: immunoblot showing CRTC2 protein expression in the nucleus (N) and cytoplasm (C) of Ramos cells transduced with control (Scr) shRNA or shATM after exposure to DMSO or Eto (20 μM, 1 hr). β-TUBULIN (cytoplasm) and HDAC1 (nucleus) are loading controls. Bottom: nuclear/cytoplasmic ratios for CRTC2 are plotted from densitometry.  
 (C) qPCR for *TCL1* in shATM or control (Scr) Ramos cells after DMSO or Eto (20 μM, 6 hr) exposure. Values were normalized to *36B4*. Values are expressed as the mean ± SEM for three independent experiments.  
 (D) qPCR for *TCL1* in WT or ATM-deficient lymphoblastoid cells exposed to DMSO or Eto (20 μM, 6 hr). Values were normalized to *36B4*. Values are expressed as the mean ± SEM for three independent experiments.  
 (E) Immunoblot showing LKB1 knockdown in Ramos cells 4 days after transduction. β-ACTIN is a loading control.  
 (F) Top: immunoblot showing CRTC2 protein expression in the nucleus (N) and cytoplasm (C) of Ramos cells transduced with control (Scr) shRNA or shLKB1 after exposure to DMSO or Eto (20 μM, 1 hr). β-TUBULIN (cytoplasm) and HDAC1 (nucleus) are loading controls. Bottom: nuclear/cytoplasmic ratios for CRTC2 are plotted from densitometry.  
 (G) qPCR for *TCL1* in shLKB1 or control (Scr) Ramos cells after DMSO or Eto (20 μM, 6 hr) exposure. Values were normalized to *36B4*. Values are expressed as the mean ± SEM for three independent experiments.

reaction. An increase in phospho-ATM S1981 was detected by day 7 (Figure 4A), coinciding with DSB generation during CSR. ATM knockdown (Figure 4B) resulted in increased CRTC2 nuclear localization during B cell differentiation compared to control cells (Figure 4C). Similar results were obtained for LKB1 (Figures 4D–4F). These data strongly suggest that GC-like B cells responding to physiologic DSBs activate ATM to

LKB1 signaling to inactivate CRTC2. This pathway is engaged during the period of CSR, further suggesting a link to Ab production and B cell maturation.

To directly determine whether CSR drives CRTC2 inactivation, we compared the subcellular localization of CRTC2 in B cells from wild-type (WT) and *AID* knockout mice. *AID*-deficient B cells cannot produce DSBs at *IG* loci and therefore CSR does



**Figure 3. CRTC2 Inactivation Occurs during CSR in GC B Cells**

(A) Schematic of primary human B cell differentiation system.

(B) Phase-contrast image of expanding human B cell clusters 24 hr after stimulation (at a magnification of 5 $\times$ ).

(C) qPCR for *OCA-B*, *BCL6*, and *PRDM1* in human B cells at 0, 3, or 7 days of differentiation. Values are normalized to *36B4* and are expressed as the mean  $\pm$  SEM for three independent experiments.

(D) Immunoblot for BCL6, BLIMP-1, OCA-B, and MYC in primary human B cells after 0, 3, or 7 days of differentiation.  $\beta$ -ACTIN is a loading control.

(E) ELISA for total IgG in human B cell culture media harvested after 0, 3, or 7 days of differentiation. n.d. = none detected. Values are expressed as the mean  $\pm$  SEM for three independent experiments.

(F) Top: immunoblot showing CRTC2 protein expression in the nucleus (N) and cytoplasm (C) of primary human B cells on days 3 or 7 of differentiation.  $\beta$ -TUBULIN (cytoplasm) and HDAC1 (nucleus) are loading controls. Bottom: nuclear/cytoplasmic ratios for CRTC2 are plotted from densitometry.

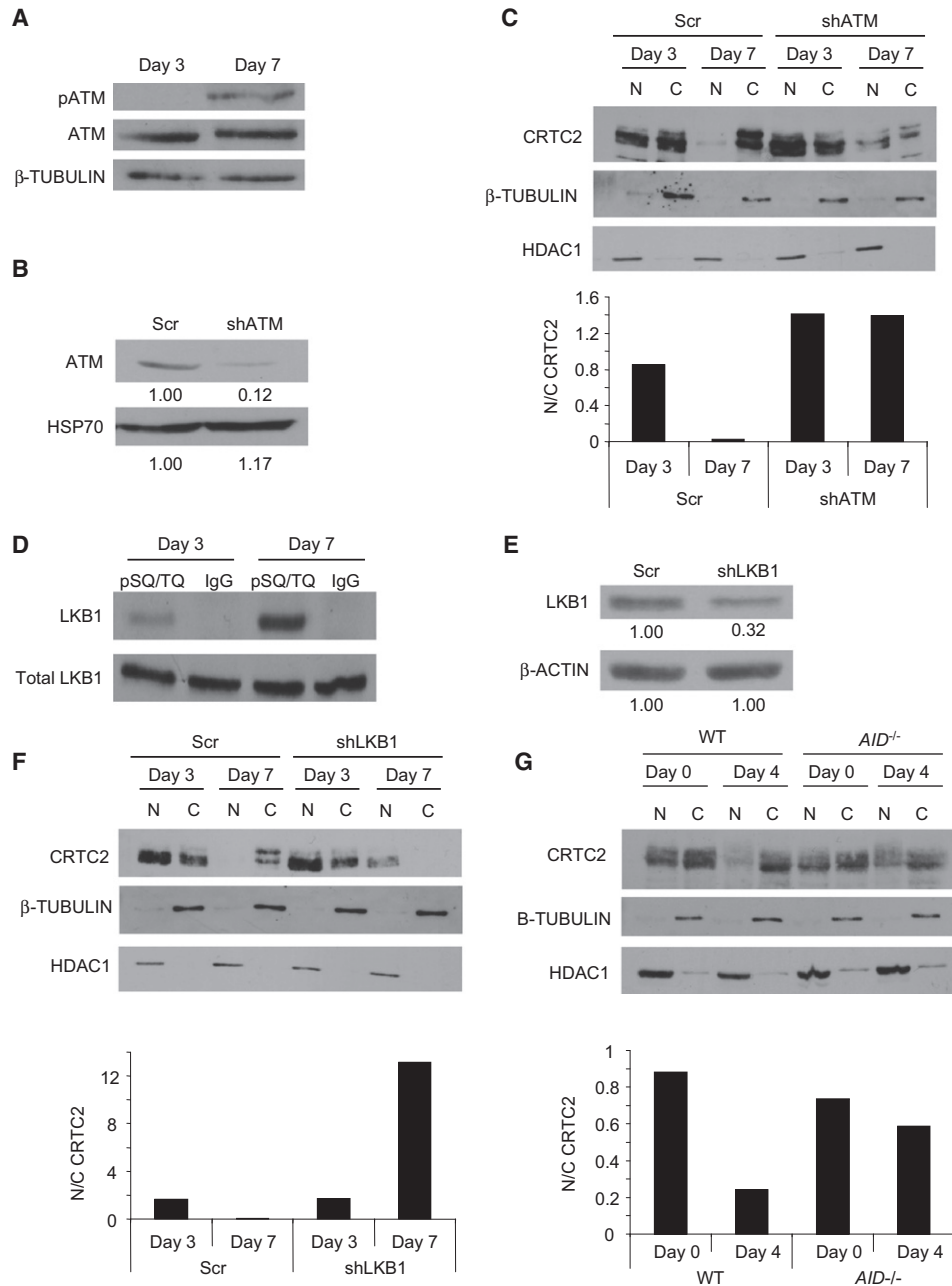
(G) ChIP for CRTC2 using chromatin from human B cells after 3 or 7 days of differentiation. Immunoprecipitates were analyzed by qPCR for the *TCL1* promoter. Values are normalized to an intergenic region and shown as a.u. Values are expressed as the mean  $\pm$  SEM for three independent experiments.

(H) qPCR for *TCL1* in human B cells after 3 or 7 days of differentiation. Values are normalized to *36B4*. Values are expressed as the mean  $\pm$  SEM for three independent experiments.

not occur (Muramatsu et al., 2000).  $\alpha$ CD40 and IL-4 induced CSR in  $\sim$ 43% of WT but not in AID knockout B cells (Figure S4). Most importantly, CRTC2 was retained in the nucleus of AID knockout B cells compared to its nuclear depletion in WT B cells (Figure 4G). This result strongly supports the requirement for physiologic DSBs induced by AID during CSR for ATM- and LKB1-dependent CRTC2 inactivation.

### CRTC2 Regulates Genes that Control B Cell Development

The effect of CRTC2 inactivation on global gene expression in GC B cells was evaluated. ChIP-on-chip for CRTC2 target genes in Ramos cells was performed with two Abs that recognize distinct CRTC2 epitopes (Figure S5A). The genomic DNA bound to CRTC2 and total input DNA were distinctly labeled and



**Figure 4. AID-Dependent ATM to LKB1 Signaling in GC B Cells Inactivates CRTC2**

(A) Immunoblot of pATM (S1981) and ATM in human B cells after 3 or 7 days of differentiation. β-TUBULIN is a loading control.

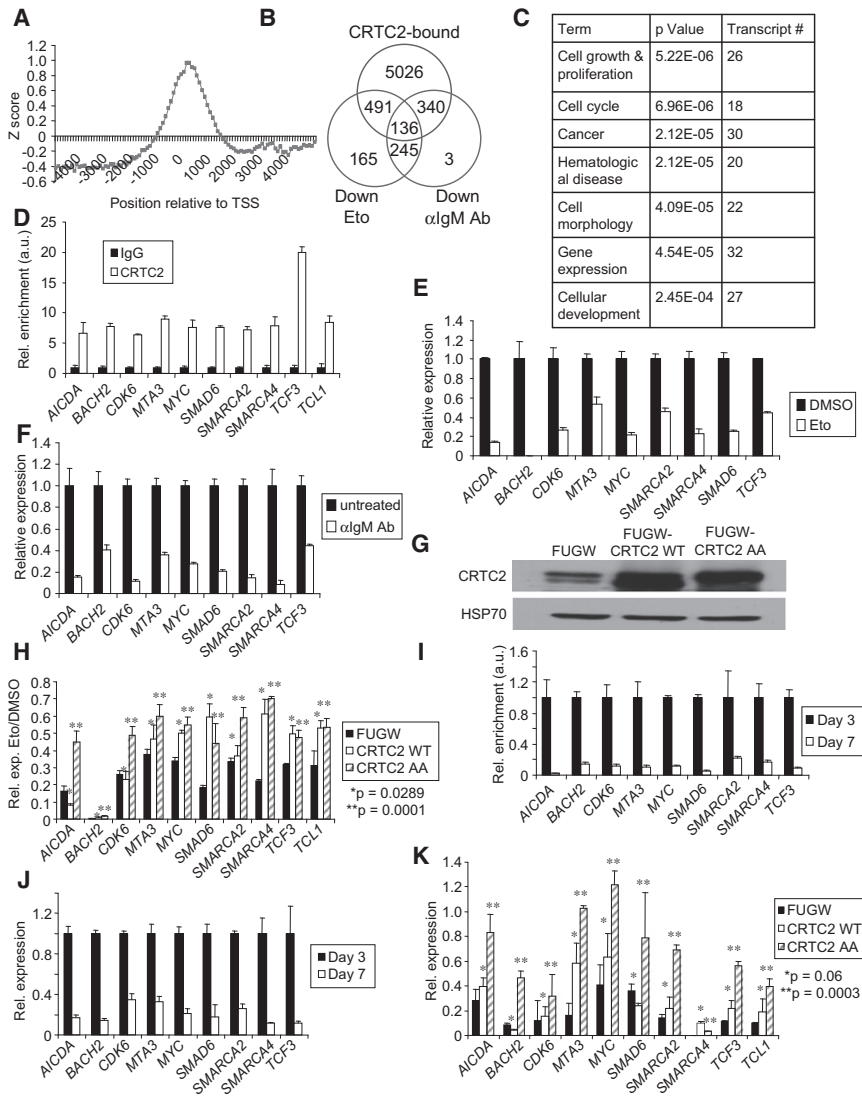
(B) Immunoblot showing ATM knockdown in human B cells 3 days after shATM transduction. HSP70 is a loading control. Densitometric values are indicated below each band.

(C) Top: immunoblot showing CRTC2 protein expression in the nucleus (N) and cytoplasm (C) of primary human B cells, transduced with control (Scr) or shATM-expressing lentiviruses, on days 3 or 7 of differentiation. β-TUBULIN (cytoplasm) and HDAC1 (nucleus) are loading controls. Bottom: nuclear/cytoplasmic ratios for CRTC2 are plotted from densitometry.

(D) Immunoprecipitation of phosphorylated ATM/ATR substrates (pSQ/TQ) or a rabbit IgG control from lysates of human B cells after 3 or 7 days of differentiation. Lysates (Total LKB1) or immunoprecipitates (LKB1) are analyzed by immunoblot for LKB1.

(E) Immunoblot showing LKB1 knockdown in human B cells 3 days after shLKB1 transduction. β-ACTIN is a loading control. Densitometric values are indicated below each band.

(F) Top: immunoblot showing CRTC2 protein expression in the nucleus (N) and cytoplasm (C) of primary human B cells, transduced with control (Scr) or shLKB1-expressing lentiviruses, on days 3 or 7 of differentiation. β-TUBULIN (cytoplasm) and HDAC1 (nucleus) are loading controls. Bottom: nuclear/cytoplasmic ratios for CRTC2 are plotted from densitometry.



**Figure 5. CRTC2 Regulates a Gene Program that Controls B Cell Development**

(A) Plotted z scores for CRTC2 binding to human promoter regions relative to the predicted transcriptional start sites (TSS).

(B) Venn diagram displaying putative CRTC2 direct target genes as defined by downregulation with exposure to  $\alpha$ -IgM Ab and Eto and CRTC2 binding to the promoter region.

(C) Gene ontology (GO) analysis of candidate CRTC2 target genes. p values are based on a hypergeometric distribution.

(D) ChIP for CRTC2 or rabbit IgG in Ramos cells. Immunoprecipitates were analyzed by qPCR for the promoters of CRTC2 target genes. Values are normalized to intergenic regions and shown as a.u. Values are expressed as the mean  $\pm$  SEM for three independent experiments.

(E) qPCR for CRTC2 target genes in Ramos cells after DMSO or Eto (20  $\mu$ M, 6 hr) exposure. Values are normalized to *36B4* and are expressed as the mean  $\pm$  SEM for three independent experiments.

(F) qPCR for CRTC2 target genes in Ramos cells with or without  $\alpha$ -IgM (10  $\mu$ g/ml, 6 hr) exposure. Values are normalized to *36B4*. Values are expressed as the mean  $\pm$  SEM for three independent experiments.

(G) Immunoblot for CRTC2 protein expression in Ramos cells 48 hr after infection with the indicated lentivirus. HSP70 is a loading control.

(H) qPCR for CRTC2 target genes in Ramos cells transduced with the indicated lentivirus. DMSO or Eto (20  $\mu$ M, 6 hr) exposures were initiated 48 hr after infection. Values are normalized to *36B4* and are expressed as the mean  $\pm$  SEM for three independent experiments.

(I) ChIP for CRTC2 using chromatin from human B cells after 3 or 7 days of differentiation. Immunoprecipitates were analyzed by qPCR for the promoters of CRTC2 target genes. Values are normalized to intergenic regions, are shown as a.u., and are expressed as the mean  $\pm$  SEM for three independent experiments.

(J) qPCR for CRTC2 target genes in human B cells after 3 or 7 days of differentiation. Values are normalized to *36B4* and are expressed as the mean  $\pm$  SEM for three independent experiments.

(K) qPCR for CRTC2 target genes in human B cells transduced with the indicated lentivirus after 3 or 7 days of differentiation. Values are normalized to *36B4* and are expressed as the mean  $\pm$  SEM for three independent experiments.

cohybridized to Agilent 244K human promoter microarrays that contained 60-mer oligonucleotide probes covering the region from  $-5.5$  kb to  $+2.5$  kb relative to the transcriptional start sites for  $\sim 17,000$  annotated human genes. The vast majority of bound sequences were located within 1 kb of transcriptional start sites (Figure 5A), consistent with a previous global analysis of CREB promoter occupancy (Zhang et al., 2005). This approach revealed that CRTC2 occupied the promoters of 5993 genes (Figures S5B and S5C). Motif analysis among the bound sequences identified conventional CRE half-sites as the two most highly enriched of all possible 6-mers ( $p = 10^{-22.5}$ ;

Figure S5D), as anticipated for a coactivator of CREB. Gene expression profiling was also performed with Ramos cells treated with Eto or anti-IgM Ab, which was also shown to inactivate CRTC2 (Kuraishy et al., 2007). This screen identified 136 putative CRTC2-regulated genes (Figure 5B, Table S1) implicated in cellular growth and proliferation, the cell cycle, and cancer (Figure 5C).

To validate these findings, we selected ten candidate CRTC2 target genes that have functional relevance for GC B cell development and/or lymphomagenesis. Gene-specific ChIP demonstrated enrichment for promoter regions of these ten candidate

(G) Top: immunoblot showing CRTC2 protein expression in the nucleus (N) and cytoplasm (C) of spleen B cells from WT or *AID* knockout (*AID*<sup>-/-</sup>) mice on days 0 and 4 of differentiation.  $\beta$ -TUBULIN (cytoplasm) and HDAC1 (nucleus) are loading controls. Bottom: nuclear/cytoplasmic ratios for CRTC2 are plotted from densitometry.

CRTC2 target genes with a CRTC2 Ab compared to an isotype control (Figure 5D). Gene expression changes were measured with Eto or anti-IgM treatment, with quantitative polymerase chain reaction (qPCR) validating the expression array results (Figures 5E and 5F). To determine a causal relationship between CRTC2 inactivation and downregulation of candidate CRTC2 target genes, we used a lentiviral expression system to transduce Ramos cells with WT or mutant CRTC2 in which serines 171 and 275 were mutated to alanines (CRTC2-AA; Figure 5G and Figure S5E). CRTC2-AA should remain in the nucleus because both serines require phosphorylation by AMPK to exclude CRTC2 from the nucleus (Jansson et al., 2008; Screaton et al., 2004). CRTC2-WT overexpression or continued activation by CRTC2-AA caused a derepression of the ten target genes with DSBs or with anti-IgM exposure (Figure 5H and Figure S5F), indicating that expression of these genes is dependent upon CRTC2 activity. Similar results were obtained from primary human B cells (Figures 5I–5K). Supporting the connection between genotoxic stress and CRTC2 target gene regulation, pretreatment of Ramos cells with the ATM inhibitor Kudos caused a significant depression of CRTC2 target genes with DSBs (Figure S5I). In addition, a significant derepression of CRTC2 target genes occurred when DSBs were induced in immortalized B cells from A-T patients, which lack functional ATM, compared to WT controls (Figure S5G). A-T cells also exhibited a striking retention of CRTC2 in the nucleus after Eto exposure compared to WT controls (Figure S5H).

### CRTC2 Inactivation Is Required for Plasma Cell Differentiation

To determine the effect of signaling that inactivates CRTC2 on B cell development, we stimulated naive tonsil B cells efficiently transduced with CRTC2-WT or CRTC2-AA to generate a GC-like reaction. Proliferating GC B cells, or centroblasts, are among the fastest proliferating cells in the body (Klein and Dalla-Favera, 2008), and plasma cell differentiation is characterized in part by repression of pro-proliferative gene expression (Shaffer et al., 2002). Hyperactive or overexpressed CRTC2 caused a marked increase in proliferation (Figure 6A), a decrease in soluble IgG production (Figure 6B), impaired induction of the plasma cell master regulator BLIMP-1 (Figure 6C), and sustained expression of the GC B cell master regulator BCL6 (Figure 6D) on day 7 of culture. Similar results were obtained when *ATM* or *LKB1* expression was decreased by shRNAs (Figures 6E–6H). Interestingly, transduced CRTC2 did not impair CSR, as equivalent levels of productive *IGG* transcripts were generated in CRTC2-overexpressing and control cells (Figures S6A and S6B). These data strongly suggest that the DDR pathway leading to CRTC2 inactivation is required for efficient termination of the GC reaction and Ab secretion.

### CRTC2 Inactivation Is Disrupted in GC-Derived B Cell Lymphomas

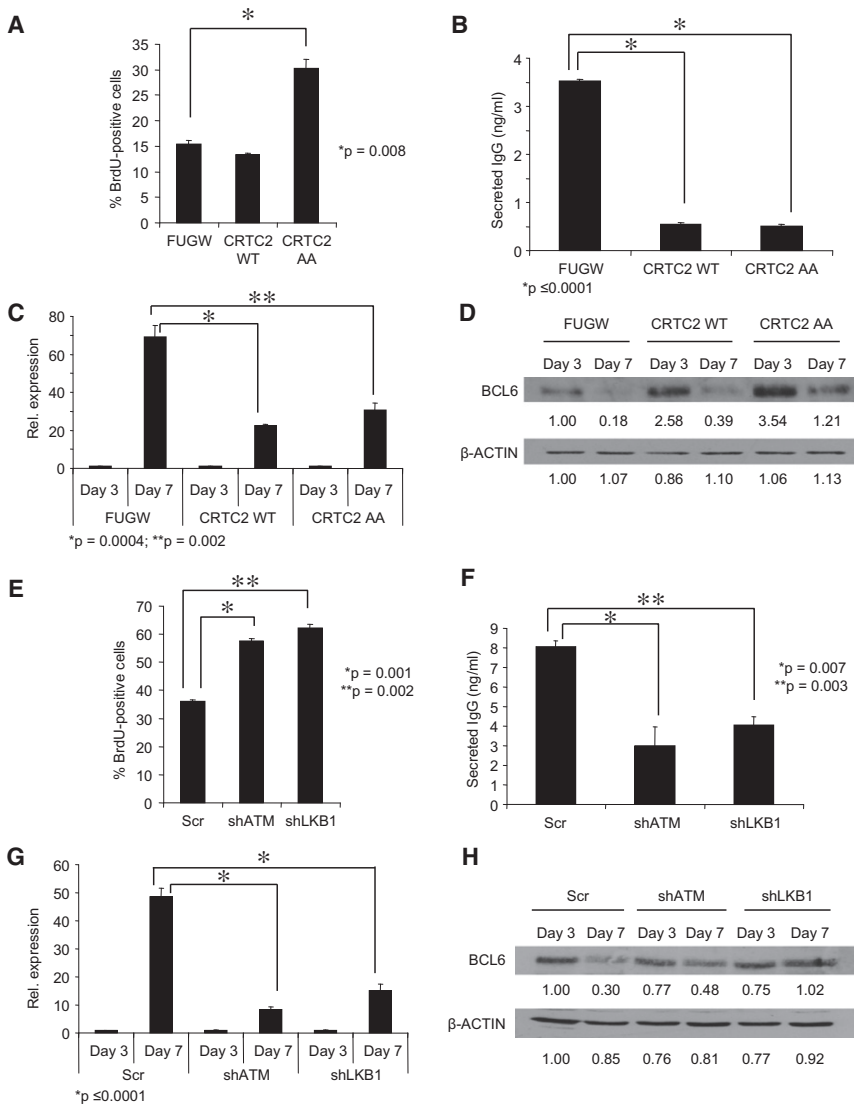
Since *TCL1* is often overexpressed in GC B cell lymphomas (Klein et al., 2001; Narducci et al., 2000; Said et al., 2001), we assessed this signaling pathway in human lymphoma samples. qPCR analysis revealed a 10-fold or greater loss of *ATM* expression in 6/17 (35%), or *LKB1* expression in 7/17 (41%), clinical

samples (Figures 7A and 7B). *CRTC2* expression was not altered in these tumors (data not shown), so the *CRTC2* coding sequence was evaluated for alterations. A C→T missense mutation was identified in 10/17 tumor samples, compared with 0/14 normal tonsil samples ( $p < 0.0005$ , one-sided Fisher's exact test) (Figure 7C and Figure S7). This change results in a L→F amino acid substitution in the AMPK recognition sequence of the CRTC2 protein (Screaton et al., 2004). Although this alteration is conservative, it prevented inactivation of CRTC2 in Ramos B cells subjected to DSBs (Figures 7D and 7E), perhaps by disrupting AMPK-CRTC2 or CRTC2-14-3-3 interactions. Consistent with this result, *TCL1* expression was maintained in 11 of 13 B cell lymphomas that harbored disruptions in the ATM→LKB1→AMPK→CRTC2 signaling pathway (Figure 7F). These results provide evidence for aberrant CRTC2 activity in human lymphomas from multiple, distinct defects in a DDR pathway.

## DISCUSSION

Here, we describe a mechanism in which exogenous and physiologic DNA damage in GC B cells leads to CRTC2 inactivation, which is required for Ig secretion and plasma cell differentiation. Although a prior study showed a BCR signaling requirement for Ab affinity-driven plasma cell development (Phan et al., 2006), the cues that cause GC B cells to differentiate into plasma cells are unknown. Previously, we showed that CRTC2 is also inactivated by BCR engagement (Kuraishy et al., 2007), suggesting that CRTC2 inactivation is a response to both genotoxic stress and BCR signaling, which terminates the GC reaction. More broadly, these results also implicate LKB1 as a central kinase with the potential to integrate metabolic and now AID-initiated genotoxic stress signaling in a pathway that can terminate with CRTC2 inactivation to drive terminal cell differentiation.

Our results, along with aspects of two recent studies, provide an important direction for the DDR by coupling genotoxic stress to non-DNA-repair-related physiologic or pathologic cellular maturation. One recent study in pre-B cells demonstrated that RAG-induced DSBs during *V(D)J* recombination activated transcription by NF- $\kappa$ B, leading to the expression of mature lymphocyte-specific genes (Bredemeyer et al., 2008). However, this study required genetically sustained RAG-induced DSBs to detect mature lymphocyte gene expression, leaving open the question of physiologic relevance. A second recent study showed that genotoxic stress opposed self-renewal in melanocyte stem cells (MSCs) and caused the MSCs to aberrantly differentiate into ectopically pigmented melanocytes, resulting in irreversible hair graying (Inomata et al., 2009). This form of abnormal differentiation resulted from pathologic DNA damage accumulation from the environment, which led to lineage degeneration and aging by an unknown mechanism. In contrast, our study shows a mechanism linking physiologic, AID-induced DSBs to ATM and LKB1 signaling in order to inactivate CRTC2, with CRTC2 inactivation required for the differentiation of plasma cells. This regulatory function exceeds the established response to DSBs that maintains genomic integrity, and it provides evidence that the DDR influences normal cell development and physiology. A potential ontologic reason for coupling genotoxic stress with differentiation is that the forced elimination



**Figure 6. CRTC2 Inactivation is Required for Plasma Cell Differentiation**

(A) BrdU incorporation in human B cells, transduced with the indicated lentivirus, on day 7 of differentiation. BrdU-FITC-positive cells were detected by flow cytometry. Values are expressed as the mean ± SEM for three independent experiments.

(B) ELISA for total secreted IgG in human B cell culture media harvested after day 7 of differentiation. Cells were transduced with the indicated lentivirus prior to the initiation of differentiation. Values are expressed as the mean ± SEM for three independent experiments.

(C) qPCR for *PRDM1* expression in human B cells transduced with the indicated lentivirus after 3 or 7 days of differentiation. Values are normalized to *36B4* and are expressed as the mean ± SEM for three independent experiments.

(D) Immunoblot for BCL6 in human B cells transduced with the indicated lentivirus after 3 or 7 days of differentiation. β-ACTIN is a loading control. Densitometric values are indicated below each band.

(E) BrdU incorporation in human B cells, transduced with the indicated lentivirus, on day 7 of differentiation. BrdU-FITC-positive cells were detected by flow cytometry. Values are expressed as the mean ± SEM for three independent experiments.

(F) ELISA for total secreted IgG in human B cell culture media harvested after day 7 of differentiation. Cells were transduced with the indicated lentivirus prior to the initiation of differentiation. Values are expressed as the mean ± SEM for three independent experiments.

(G) qPCR for *PRDM1* in human B cells transduced with the indicated lentivirus after 3 or 7 days of differentiation. Values are normalized to *36B4* and are expressed as the mean ± SEM for three independent experiments.

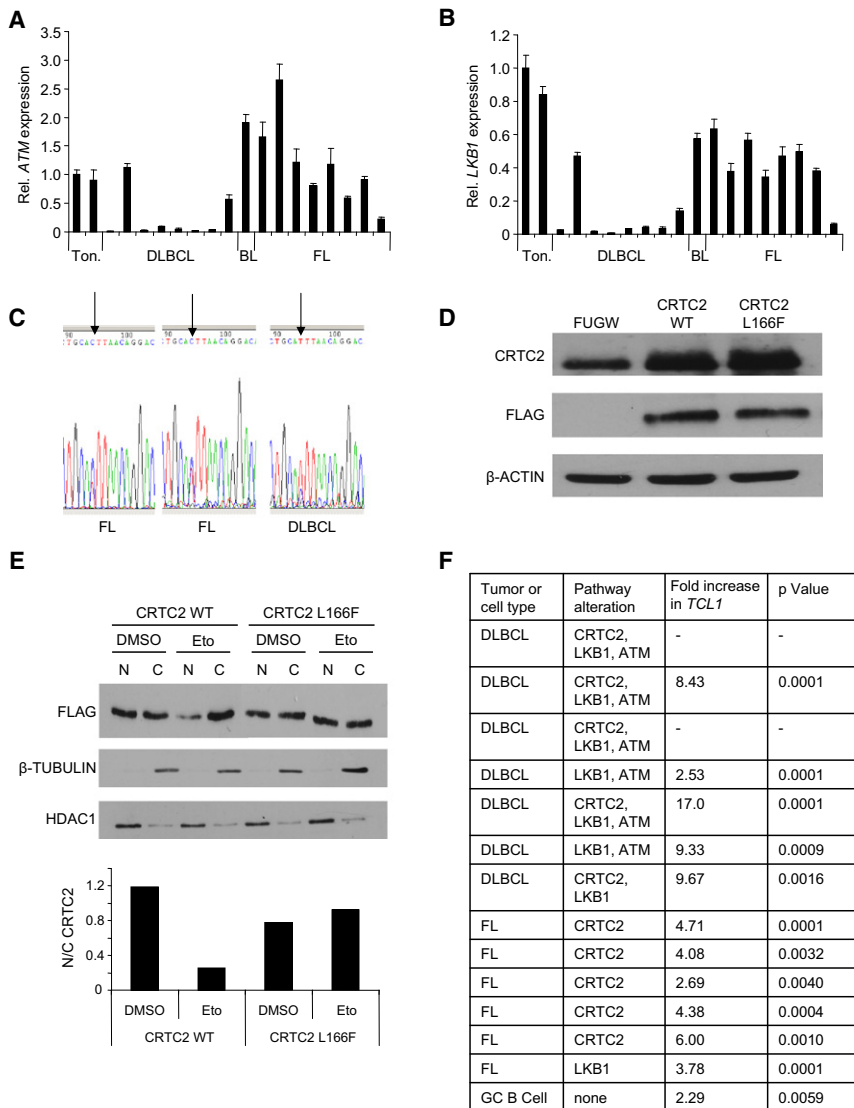
(H) Immunoblot for BCL6 in human B cells, transduced with the indicated lentivirus, after 3 or 7 days of differentiation. β-ACTIN is a loading control. Densitometric values are indicated below each band.

of damaged cells from stem or precursor cell pools, such as the GC, may be an intrinsic mechanism to preserve the integrity of preterminal cell types and prevent tumorigenesis.

A consistent theme that re-emerges from studies of hematopoietic development is that a block in differentiation seems to promote a malignancy that reflects the stage in development at which the block occurs. Here, we show that disruption of the signaling pathway leading to CRTC2 inactivation and plasma cell differentiation occurs in GC-derived lymphomas. Because ATM is required for CRTC2 inactivation, defects in this pathway may contribute in part to the IG deficiencies (Nowak-Wegrzyn et al., 2004; Staples et al., 2008) and increased susceptibility to lymphoma (Taylor et al., 1996) observed in patients with A-T. In addition to mutations, aberrant *ATM* repression in multiple B cell lymphoma subtypes has also been reported (Basso et al., 2005). Like ATM, the LKB1 tumor suppressor is inactivated

in a number of human malignancies (Hezel and Bardeesy, 2008; Shaw, 2008). Furthermore, a small population-based case-control study showed that diabetics taking metformin, which activates LKB1 and results in CRTC2 inactivation, had a reduced risk of cancer (Evans et al., 2005). In mice, a hypomorphic mutation in *Lkb1* present on a *Pten* haploinsufficient background markedly accelerated the development of marginal-zone B cell lymphoma (Huang et al., 2008). Interestingly, PTEN deficiency is similar to aberrantly sustained TCL1 expression for mature B cells because both alterations hyperactivate AKT signaling (Teitell, 2005).

In summary, CRTC2 plays a powerful and previously unknown role in normal GC B cell differentiation, and its inactivation by the DDR is critical for downregulation of a genetic program that maintains the GC reaction. These findings place CRTC2 in a regulatory pathway that controls GC exit and plasma cell



**Figure 7. CRTC2 Inactivation Is Disrupted in GC-Derived B Cell Lymphomas**

(A) qPCR for *ATM* in 17 human lymphoma samples and two tonsils. Values are normalized to *36B4* and are expressed as the mean  $\pm$  SEM for three independent experiments. The following abbreviations are used: Ton., tonsil; DLBCL, diffuse large B cell lymphoma; BL, Burkitt lymphoma; FL, follicular lymphoma.

(B) qPCR for *LKB1* in 17 human lymphoma samples and two tonsils. Values are normalized to *36B4* and are expressed as the mean  $\pm$  SEM for three independent experiments.

(C) Representative chromatograms showing a CRTC2 496 C  $\rightarrow$  T alteration in human GC B cell lymphomas. Arrow indicates nucleotide 496 in the coding region of CRTC2.

(D) Immunoblot showing expression of CRTC2 and FLAG in Ramos cells 48 hr after infection with the indicated lentivirus.  $\beta$ -ACTIN is a loading control.

(E) Top: immunoblot showing exogenous CRTC2 localization in Ramos cells, transduced with the indicated lentivirus, after DMSO or Eto (20  $\mu$ M, 1 hr) exposure.  $\beta$ -TUBULIN (cytoplasm) and HDAC1 (nucleus) are loading controls. Bottom: nuclear/cytoplasmic ratios for CRTC2 are plotted from densitometry.

(F) qPCR for *TCL1* expression in 13 human B cell lymphoma samples and in GC B cells at day 3 of culture. Values are normalized to *36B4* and compared to *TCL1* expression in differentiated plasma cells at day 7 of culture. Dashes indicate tumors with undetectable *TCL1* expression.

**Primary Human B Cell Culture System**

Fresh tonsils were used to isolate naive B cells as described (Said et al., 2001). Tonsils were minced, and mononuclear cells (MCs) were isolated by Ficoll-Paque (GE Healthcare, USA) density centrifugation. MCs were incubated with  $\alpha$ -IgD-PE (BD PharMingen, USA) on ice, washed, incubated with  $\alpha$ -PE beads (Miltenyi Biotec), washed, and collected using the MidiMACS system (Miltenyi Biotec, Germany). Lentiviral transduction was performed at this stage, as indicated. Cells were

seeded  $5 \times 10^5$  cells/ml and cultured in complete RPMI 1640 plus 20 ng/ml IL-4, 20 ng/ml IL-10 (BD PharMingen), and 2  $\mu$ g/ml  $\alpha$ -CD40 Ab.

**ELISA**

ELISA was performed with a human IgG ELISA quantification kit (Bethyl Laboratories, USA).

**RNA Analysis**

Total RNA was extracted with Trizol (Life Technologies, USA). cDNA was made with the Superscript First-Strand Synthesis System (Invitrogen, USA). qPCR was performed as described previously (Kuraishy et al., 2007). Expression was normalized to a *36B4* mRNA control sequence.

**Immunoblot, Immunoprecipitation, and Antibodies**

Immunoblots were performed as described (Kuraishy et al., 2007). Briefly, 20–50  $\mu$ g whole-cell extract for each sample was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Blocked membranes were incubated with primary Abs in TBS-Tween and 5% milk (or 5% BSA for phosphospecific Abs) overnight (Ab sources provided upon request). Immunoprecipitations were performed with whole-cell extracts and primary Ab overnight,

differentiation during terminal B cell development and herald future studies to interrogate the role of CRTC2 as a potential oncogenic factor and therapeutic target in B cell lymphoma.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Tissues, and Reagents**

Wild-type, *Atm*<sup>-/-</sup>, *Lkb1*(T366A), and *Lkb1*<sup>-/-</sup> MEFs (N. Bardeesy, Massachusetts General Hospital) were grown in Dulbecco's modified Eagle's medium (GIBCO, USA) with 20% fetal bovine serum (FBS) plus antibiotics. Nalm-6, Ramos, PBL, and ATM-deficient lymphoblastoid cells were grown in RPMI 1640 (GIBCO) with 10% FBS plus antibiotics. Fresh-frozen human tissues and fresh human tonsils were obtained from the University of California, Los Angeles (UCLA) Tissue Procurement Core Laboratory in accordance with institutional guidelines and Institutional Review Board approval. Reagents included etoposide, mitomycin C, and metformin (Sigma, USA) and the ATM inhibitor KU55933 (Kudos Pharmaceuticals, UK).  $\alpha$ -CD40 (mouse anti-human IgG) was purified from a culture medium of G28-5 mouse hybridoma cells (K. Zhang, UCLA).

followed by precipitation of immune complexes with Protein G beads (Santa Cruz Biotechnologies, USA). Subcellular fractionations were performed with the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, USA) according to the manufacturer's protocol.

#### Chromatin Immunoprecipitation

ChIP assays were performed as described (Kuraishy et al., 2007).

#### Immunofluorescence Microscopy

Ramos or primary B cells were plated on poly-L-lysine coverslips and used for immunofluorescence studies as described (Kuraishy et al., 2007).

#### siRNA Electroporation

*LKB1* siRNA (2  $\mu$ M; Dharmacon) or a scrambled control siRNA (2  $\mu$ M) was electroporated into Nalm-6 cells with the Amaxa Nucleofector I (program C-05) and Nucleofector kit T (Amaxa, Germany).

#### Retrovirus and Luciferase Assays

Luciferase assays were performed as described (Kuraishy et al., 2007). A *pBABE-FLAG-LKB1* retroviral construct, expressing FLAG-tagged *LKB1*, was generated by standard methods. Viral supernatant from HEK293T cells was collected 48 and 72 hr after transfection. PBL cells ( $1 \times 10^5$ /well) were incubated with 1 ml of virus supplemented with 2  $\mu$ l polybrene and centrifuged at 2500 rpm for 1 hr at 30°C. One day after repeat infection, puromycin (0.5  $\mu$ g/ml) was added to the media. FLAG-LKB1 expression was determined by western blot.

#### Lentivirus

For shRNA, the *H1* promoter and RNAi sequences for *LKB1*, *ATM*, or scramble were subcloned into *FUGW* at the PacI site upstream of a ubiquitin promoter-driven *EGFP* sequence (Lois et al., 2002). For expression vectors, full-length *CRTC2* cDNA was cloned into *FUGW* downstream of the ubiquitin promoter. *CRTC2* mutants were generated with the Quikchange site-directed mutagenesis kit (Stratagene, USA). Virus produced by HEK293T cells was concentrated by ultracentrifugation, resuspended in RPMI 1640, and used to spin-infect  $2 \times 10^6$  cells/well in a 24-well plate for 2 hr.

#### In Vitro Proliferation Assays

In vitro proliferative kinetics were assayed with the BrdU flow kit (BD Pharmingen) according to the manufacturer's protocol.

#### ChIP-on-Chip

ChIP was performed with two different Abs against *CRTC2* (EMD Biosciences, USA; Cell Signaling, USA). Biological duplicate experiments were performed with each Ab. Array details are in the [Supplemental Experimental Procedures](#).

#### Gene Expression Arrays

RNA was isolated from Ramos cells without treatment or after 6 hr of Eto (20  $\mu$ M) or  $\alpha$ -IgM (10  $\mu$ g/ml) exposure using Trizol, followed by clean-up with the QIAGEN RNeasy kit. Array details are in the [Supplemental Experimental Procedures](#).

#### Gene Ontology Analysis

Gene ontology analysis was performed with Ingenuity Pathways Analysis.

#### Statistical Analyses

Data are presented as the mean  $\pm$  standard error of the mean (SEM). A two-tailed t test was used for most comparisons, with  $p < 0.05$  considered significant.

#### ACCESSION NUMBERS

All ChIP-on-chip and gene expression microarray data have been deposited in the Gene Expression Omnibus (GEO) under the submission number GSE23171.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at [doi:10.1016/j.molcel.2010.08.019](https://doi.org/10.1016/j.molcel.2010.08.019).

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