Title
A CRTC2 Repressor Role Regulates DNA Damage Response Genes in Germinal Center B-cells

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A CRTC2 Repressor Role Regulates DNA Damage Response Genes in Germinal Center B-cells

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in Cellular and Molecular Pathology

by

Olivia Teresa Schontzler

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ABSTRACT OF THE THESIS

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Master of Science in Cellular and Molecular Pathology

University of California, Los Angeles, 2012

Professor Michael A. Teitell, Chair

Post-DNA damage survival is regulated by ataxia-telengiectaia mutated (ATM) in the germinal center (GC) B-cell and is necessary for continued plasma cell development. Cell survival regulation is critical for the prevention of oncogenic transformation, as the gain of this trait is seen in many cancers. 90% of follicular lymphomas acquire resistance to apoptosis by a chromosomal translocation of the anti-apoptosis *Bcl-2* gene onto the active *IGH* enhancer element. *Bcl-2* is among a set of pro-survival genes that increase in expression at the end of a GC B-cell ATM initiated DNA damage response pathway; these genes will be referred to as DNA damage response (DDR) genes throughout this proposal. Gene ontology (GO) analysis of these genes indicates that they are involved in cellular proliferation, cellular physiological processes,

cell cycle, and apoptosis. ChIP-chip data reveal CREB regulated transcription coactivator 2 (CRTC2) binding to these genes prior to DNA damage, suggesting that CRTC2 has a role to repress gene expression in the absence of damage stimulus. I hypothesize that CRTC2 has a novel role to repress pro-survival genes before DNA damage in GC B-cells, and that CRTC2 repressor function on *Bcl-2* can reverse the transformed state of t(14;18)-positive lymphoma cells. These studies may point to CRTC2 as a novel tumor suppressor, and provide a new target for lymphoma therapy.

The thesis of Olivia Teresa Schontzler is approved.

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2012

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Specific Aims

AIM 1: To investigate a CRTC2 repressor function

CRTC2 is a well-documented co-activator of various transcription factors[1]. Analysis of gene expression and ChIP-chip data led to the detection of a set of genes bound by CRTC2 that are upregulated after DNA damage in GC B-cells, suggesting that CRTC2 has a novel repressor function. In this aim, I will firstly validate the array data by determining CRTC2 binding to and repression of DDR genes. Secondly, repressor function will be examined through loss-of-function experiments by CRTC2 knockdown and gain-of-function experiments with a constitutively active CRTC2 mutant. I hypothesize that CRTC2 has a novel contextual repressor role on DDR genes as part of an ATM pro-survival branch in GC B-cells.

AIM 2: To assess CREB-CRTC2 regulation of germinal center B-cell DNA damage response genes

CREB is phosphorylated by ATM during DNA damage stress[2]. How this modification affects its transcriptional activity on specific target genes was not addressed. Within the pool of GC B-cell DDR genes, a group of genes containing cAMP response element (CRE) in their promoter regions exist[3], implying CREB binding. In this aim, I will determine first whether CREB activates candidate DDR gene expression, and second whether CRTC2 represses this activity, providing a mechanism for its repressor function. I hypothesize that CRTC2 represses CREB transcription of DDR genes in GC B-cells.

AIM 3: To determine whether CRTC2 repressor role sensitizes t(14;18)-positive lymphoma to apoptosis

B cell lymphoma-2 (Bcl-2), an anti-apoptosis gene, is found among the DDR gene set. 90% of follicular lymphomas carry a chromosomal translocation that enhances its expression[4,5].

Targeted *Bcl-2* therapies improve cancer patient responses to chemotheraupetics[6–8]. In this aim, I propose to first determine whether CRTC2 can re-sensitize t(14;18)-positive cells to apoptosis by inhibiting *Bcl-2* expression. Second, I will determine whether CRTC2 overexpression can increase survival in a human lymphoma *scid* mouse model. I hypothesize that CRTC2 repressor role can suppress aberrant *Bcl-2* expression in lymphoma, increasing tumor apoptosis sensitivity and survival of mice.

Background and Significance

1. DNA Damage in Germinal Center B-cells

The normal processes of somatic hypermutation (SHM) and class switch recombination (CSR) experienced by GC B-cells lead to the creation of specific and effective antibodies. SHM introduces point mutations at the variable region of the antibody gene to generate diversity, and CSR produces double-stranded breaks to rearrange the effector portion of the antibody. DNA rearrangements that successfully form a better antibody are eventually followed by clonal expansion to produce large quantities of antibody to fight infection. The coordinated regulation of DNA damage, survival, and proliferation is critical for the development of the B-cell, and in the event of erroneous DNA rearrangements, the prevention of oncogenesis.

Microarray analysis of B-cell chronic lymphocytic leukemias (B-CLLs) revealed that ATM regulates both pro-apoptotic and -survival genes in response to ionizing radiation (IR)[9]. Comparison of differentially expressed IR response genes in *ATM*-mutated, *TP53*-mutated, and *ATM/TP53* wild-type subtypes of B-CLLs identified four clusters of genes regulated by ATM – 1) pro-apoptotic genes dependent on ATM signaling to p53, 2) pro-survival and growth genes that are ATM-dependent and p53-independent, 3) pro-survival genes that are ATM-dependent

and antagonized by p53, and 4) genes that are repressed by ATM (Figure 1A). Whereas ATM signaling through p53 to regulate apoptosis is well studied[10,11], the regulation of pro-survival genes is yet to be understood[12].

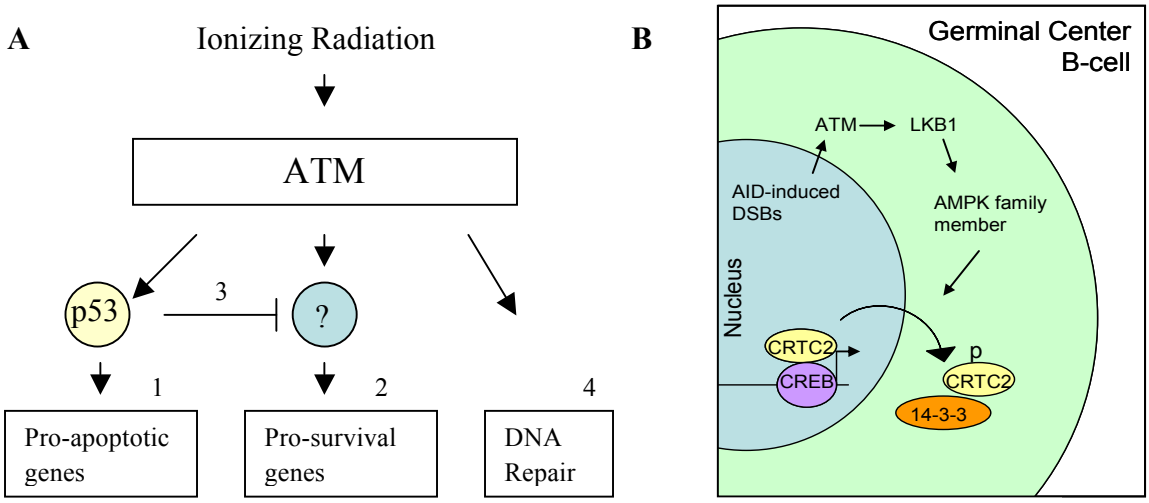

Figure 1. ATM DNA damage response in GC B-cells. A. Clusters of IR response genes regulated by ATM in B-CLLs. **B.** ATM DNA damage response pathway in GC B-cells. Abbreviations: AID – Activation-induced cytidine deaminase; DSBs – double-stranded breaks.

An ATM initiated DNA damage response signaling pathway was shown to affect B-cell differentiation (Figure 1B)[13]. Double-stranded breaks (DSBs) trigger ATM to phosphorylate and activate liver kinase B1 (LKB1). LKB1 signaling through an unidentified AMP-activated protein kinase (AMPK) family member leads to the phosphorylation of CRTC2. Phosphorylated CRTC2 is translocated out of the nucleus, and its activity as a co-activator of gene expression is terminated. The subsequent downregulation of CRTC2 bound genes (Figure 2) allows for further differentiation of a GC B-cell into a plasma cell, as shown by a decrease in GC protein B-cell lymphoma 6 (BCL6) and the increase of the plasma cell transcription repressor B-lymphocyte-induced maturation protein-1 (Blimp-1)[13]. DNA damage triggers plasma cell development through the inactivation of the co-activator CRTC2.

Analysis of the array strategy utilized by Sherman et al. (Figure 2) also revealed a set of genes that are upregulated (Appendix 1) with CRTC2 removal from the nucleus, suggesting a novel repressor role for CRTC2, which I will explore in Aim 1. Gene ontology (GO) analysis of the DDR genes indicates that they are involved in cellular proliferation, cellular physiological processes, and anti-apoptosis, suggesting that they are part of the pro-survival branch of ATM initiated DNA damage response. CRTC2 regulation of these genes would contribute to our understanding of how survival is coordinated in the GC B-cell.

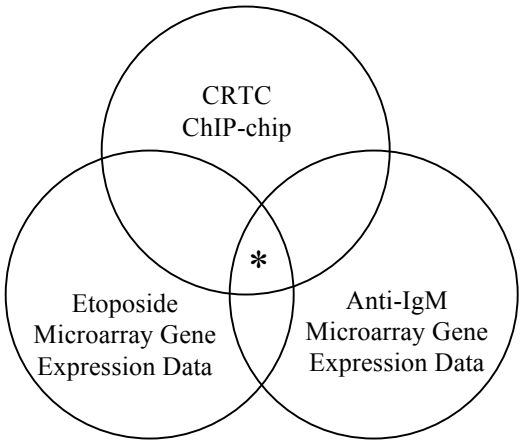

Figure 2. Expression array strategy by Sherman et al. The genetic program regulated by CRTC2 before DNA damage was identified based on an expression array strategy. ChIP-chip data of CRTC2 bound genes in resting Ramos B-cells were combined with the microarray data of etoposide and anti-IgM treated Ramos B-cells.

2. CRTC2 and CREB

CRE binding protein (CREB) is an ATM target during genotoxic stress by IR and H_2O_2[2]. Three residues in its KID domain, Thr-100, Ser-111, and Ser-121 are phosphorylated, leading to a decrease in CREB transactivation potential. How these modifications affect CREB activity and gene expression, however, were not explored. The DDR genes can be used to observe how CREB activity is altered by ATM modifications, since half of them possess a CRE element, suggesting CREB binding[3].

CRTC2 is a well-known co-activator for CREB in metabolism[1]. In fasting conditions, circulating glucagon signals dephosphorylation of hepatic CRTC2[14]. CRTC2 is released from cytosolic sequestration by 14-3-3[14] and binds to phospho-S133 CREB in the nucleus, stabilizing

binding to transcription factor II D (TFIID) and greatly enhancing the transcription of gluconeogenesis genes[3].

CRTC2 function to regulate CREB stress response is not understood. Knockdown of CRTC2 increased CREB regulated gene expression of tetradecanoylphorbol acetate (TPA)-induced stress genes, suggesting that CRTC2 may negatively interfere with CREB expression of stress response genes[15]. In Aim 2, I propose that CREB response to DNA damage is negatively regulated by CRTC2.

3. Bcl-2 in Follicular Lymphomas

A common genetic lesion seen in B-cell malignancies is chromosomal translocation. In fact, 90% of follicular lymphomas derived from the germinal center possess a translocation of the Bcl-2 gene on chromosome 18 onto the IgH gene on chromosome 14 (t(14;18))[4,5]. Bcl-2, along with its binding partners, maintains mitochondrial membrane integrity to prevent the release of cytochrome c during apoptosis[16]. The oncogenic hit thus endows the transformed cell with the ability to evade apoptosis through the increased expression of Bcl-2 under the influence of the IgH enhancer element[4]. Bcl-2 targeted therapy through mechanisms including antisense oligonucleotides[8,17] and BH3 mimetics[6,18] have been heavily investigated. They sensitize tumor cells to apoptosis, and are frequently used in conjunction with other genotoxic drugs or chemotherapies, with some entering clinical trials to treat various cancers such as melanoma, hepatoblastoma, and non-Hodgkin's lymphoma[6,17].

Studies of *Bcl-2* regulation in GC B-cells may provide insight to other mechanisms for targeted therapy. Translocated *Bcl-2* transcripts dominate over wildtype in t(14;18)-positive cells[19], and its expression is reported to be driven by CRE binding protein (CREB)[20]. In Aim 3, I

propose to study the effect of CRTC2 regulation on *Bcl-2* expression in t(14;18)-positive lymphoma cells.

In summary, I hypothesize that CRTC2 has a novel contextual repressor role on CREB DNA damage response genes that enable GC B-cells to survive through the DNA damage in the GC reaction. I hypothesize that this repressor role is potent to suppress the transformative gene alteration t(14;18) by silencing *Bcl-2* expression and re-sensitizing lymphoma cells to apoptosis, making CRTC2 a possible candidate for cancer drug therapy.

Experimental Design

Aim 1: To investigate a repressor function for CRTC2

Rationale: My analysis of the gene expression data employed by Sherman et al. yielded a list of 34 genes that are bound by CRTC2 and increase in expression when CRTC2 exits the nucleus during etoposide and anti-IgM treatment (Appendix 1). These genes regulate cellular proliferation, cellular physiological processes, cell cycle, and apoptosis by GO analysis. The GC B-cell DNA damage pathway is a system in which a physiologically relevant CRTC2 repressor role can be studied. I hypothesize that CRTC2 has a novel contextual repressor role in GC B-cells.

Methodology

Investigate direct binding of CRTC2 on five candidate genes

I will examine CRTC2 gene regulation of five candidate genes: *Bcl-2*, *TNFAIP3, JunB*, *ID2*, and *DUSP5* because of their function in GC B cell development and the DNA damage response. Bcl-2 is an anti-apoptotic protein frequently overexpressed in various cancers and is particularly upregulated in GC B cells[21]. A20, the product of the *TNFAIP3* gene, blocks the

tumor necrosis factor apoptosis pathway[22]. JunB is a member of the Jun family of proteins that form the AP-1 transcription factor complex, and is overexpressed in a range of lymphoma types[23,24]. Id2 with Blimp-1 negatively regulates *aicda* expression[25,26], and DUSP5 inactivates extracellular-signal-regulated kinase signaling downstream of B-cell receptor (BCR) engagement[27]. These genes have a role in permitting plasma cell differentiation.

Ramos B-cells, a Burkitt's lymphoma cell line, will be used because they are germinal center B-cells, and HEK293T cells will be used because they express CRTC2 and are easy to grow and transfect. Resting HEK293T and Ramos B-cells will be fixed with formaldehyde, and their DNA isolated and sonicated. Chromatin Immunoprecipitation (ChIP) will be performed with a CRTC2 antibody. The antibody will be pulled out of solution with agarose beads, washed, and the DNA eluted from the complex. qPCR of eluted DNA will show gene enrichment of the candidate genes over genomic DNA.

Validate candidate gene expression level changes

CRTC2 translocation out of the nucleus and subsequent changes in gene expression levels will be induced by both etoposide, a cancer drug whose inhibition of topoisomerase II activity leads to DNA double stranded breaks, and BCR engagement by anti-IgM[13,28]. Ramos B-cells will be treated with DMSO (control), etoposide (20 uM), and anti-IgM (10uL) for 1hr and 6hr. mRNA will be obtained and qPCR of candidate genes will demonstrate expression level changes compared to housekeeping gene 36B4.

Mouse CH12 B-cells will be induced to class switch by treatment with IL-4, anti-CD40, and TGFb over three days[29]. Class switching will be measured by flow cytometry analysis of surface immunoglobulinA expression, going from low to high[29]. qPCR measurements of candidate gene expression levels before and after class switching.

CRTC2 is responsible for candidate gene repression

To determine if CRTC2 activity is directly responsible for gene repression, loss-of-function and gain-of-function studies will be performed. Knockdown of CRTC2 will be made with shRNA. Scrambled shRNA and shRNA constructs against CRTC2 will be inserted into psiRNAh1.4[30] and FUGW[13] constructs. Scramble- and CRTC2-psiRNAh4.1 will be transiently transfected into HEK293T cells. After 48 hours, cell lysates and mRNA will be obtained to confirm knockdown by western blot and candidate gene expression levels will be measured by qPCR.

Lentivirus expressing the FUGW plasmids will be made by co-transfection of HEK293T cells with HIV-packaging vectors Δ8.9 and VSVG, as previously described[13]. Ramos B-cells will be infected by spin infection of lentivirus expressing FUGW-scramble and -CRTC2 shRNA. After 48 hours, knockdown will be confirmed by western blot and candidate gene expression levels will be measured by qPCR.

For gain-of-function studies, a mutant CRTC2 will be expressed. CRTC2 translocation out of the nucleus is dependent on phosphorylation at Serine-171 by AMPK and S275 by MARK2[1]. An S171A and S275A mutant CRTC2 (CRTC2AA) is constitutively active in that it cannot be phosphorylated and does not move out of the nucleus[31]. CRTC2WT-FUGW, CRTC2AA-FUGW, and empty vector FUGW constructs will be infected into Ramos cells and followed by etoposide or anti-IgM treatment. Candidate gene expression levels will be measured by qPCR.

Expected Results and Possible Difficulties

The gene expression data was validated in Sherman et al., so I expect CRTC2 binding to candidate gene promoters and target gene expression levels to increase upon etoposide and anti-

IgM treatment as measured by ChIP-qPCR and qPCR, respectively. If CRTC2 has a direct repressor function on the target genes, shRNA knockdown of CRTC2 should lead to an increase in target gene expression in resting cells. Expected results from gain-of-function studies with expression of the CRTC2AA mutant will show no change in target gene expression even with etoposide and anti-IgM treatment. Because similar validations were performed by Sherman et al., I do not anticipate any difficulties.

Aim 2: To assess CREB-CRTC2 regulation of germinal center B-cell DNA damage response genes

Rationale: The upstream promoter region of these five target genes contain a cAMP responsive element (CRE), suggesting CREB binding[3]. Knockdown of CRTC2 increased the expression of stress responsive gene FosB by CREB with TPA treatment of HEK293 cells, suggesting that CRTC2 negatively regulates CREB target genes[15]. During DNA damage, CREB is phosphorylated directly by ATM phosphorylation at Ser-121, Thr-100, and Ser-111[2]. How these modifications affect the expression of specific genes, however, was not addressed. I hypothesize that CRTC2 represses CREB on DNA damage response genes in resting GC B-cells.

Methodology

Determine modifications of CREB by ATM during DNA damage in GC B-cells

Ramos B-cells will be treated with etoposide for 1 hr, and western blot analysis with a phospho-CREB S121 antibody will demonstrate DNA damage induced ATM phosphorylation of CREB. ATM knockout by FUGW-ATMshRNA will demonstrate the dependence of CREB activity on ATM sensing of DNA damage. Ramos B-cells will be infected with lentivirus expressing scrambled shRNA and ATM shRNA. After 48 hours, knockdown will be validated by

western blot. The cells will be etoposide treated for 1 hr, and phospho-CREB S121 will be detected by western blot.

Treatment of normal Ramos B-cells with dbcAMP, a cAMP analog that activates CREB by PKA phosphorylation at Ser133, will show stimulus dependence of CREB phosphorylation at Ser-121 on DNA damage.

Determine CREB activity on candidate genes in B-cell DNA damage response

FUGW shRNA constructs will be made against CREB. Ramos B-cells will be infected with lentivirus expressing scrambled shRNA and CREB shRNA. Knockdown of CREB will be measured by western blot analysis. These cells will be treated with etoposide for 6 hr, and qPCR will measure candidate gene expression levels.

CREB3A is a mutant CREB with alanine substitutions at Thr-100, Ser-111, and Ser-121[2]. With ATM phosphorylation at these sites blocked, CREB3A activity will be unaltered by DNA damage. FUGW-CREB3A and FUGW-CREBWT constructs will be made, and introduced by lentivirus into Ramos B-cells. Etoposide treated CREB3A B-cells will show no increase in gene expression by qPCR.

Determine CREB-CRTC2 interactions

CREB-CRTC2 interactions in DNA damage response have yet to be established. Dual tagged CRTC2 will be made by inserting the *CRTC2* gene into p3xFLAG-*Myc*-CMV23 plasmid from Sigma Aldrich. The recombinant protein will have an N-terminus Flag tag and a C-terminus myc tag. The plasmid also contains a preprotrypsin leader sequence that will cause the recombinant CRTC2 to be secreted. The plasmid will be transiently transfected in COS cells, with an expected yield of 1 mg/L. The recombinant CRTC2 will be isolated from the cell media

with an Anti-FLAG M2 antibody, and then treated with enterokinase to cleave off the Flag tag. Co-immunoprecipitation experiments will be performed with this recombinant CRTC2.

Cell lysates will be obtained from resting Ramos B-cells and incubated with the c-myc-CRTC2. c-myc antibody immbolized onto agarose beads will be used to pulldown c-myc-CRTC2 complexes. The proteins will be eluted, and western blot for CREB will show binding. Ramos B-cells will then be treated with etoposide for 1 hr, and the cell lysate will be incubated with c-myc-CRTC2. After pull down and elution, western blot analysis should show no CREB. Lastly, cell lysates from Ramos B-cells treated with etoposide for 1 hr will be treated with alkaline phosphatase. Co-IP and western blot should show a restored presence of CREB.

Expected Results and Possible Difficulties

I expect to see CREB transcription of candidate genes in response to DNA damage that is lost with ATM knockdown, CREB knockdown, or CREB3A mutant. Results from the co-IP experiments with a recombinant CRTC2 are summarized in Table 1 and will show a CRTC2-CREB interaction that is altered with DNA damage signaling.

Etoposide	Alkaline Phosphatase	Is CREB pulled down?
-	-	+
+	-	-
+	+	+

Table 1. Expected results of recombinant CRTC2 binding with CREB. Cell lysates with indicated treatements will be incubated with recombinant CRTC2. Co-IP will be determined by western blot for CREB.

CREB transcription of CRE containing genes varies with context[1], so one possible difficulty is that CREB does not regulate these candidate genes. In this case, CRTC2 repressor mechanism can be determined by altering its other activities besides transcription factor binding. These include looking at its splicing function, O-GlcNac modification, or its acetylation modification[1].

Aim 3: To determine whether CRTC2 repressor role sensitizes t(14;18)-positive lymphoma to apoptosis by rituximab

Rationale: 90% of follicular lymphomas express a *bcl-2-IgH* translocation[32], which outcompetes the native *bcl-2* gene to become the main source of Bcl-2 in these cells[19]. Footprinting experiments reveal CREB binding to the translocated and not the wild-type promoter regions[20]. Treatments of t(14;18)-positive lymphoma cells by antisense targeting of *Bcl-2* mRNA increased apoptosis sensitivity both *in vitro* and *in vivo*[33,34]. I hypothesize that CRTC2 repressor role can limit *Bcl-2* expression in follicular lymphoma cell lines and improve response to rituximab both *in vitro* and *in vivo*.

Methodology

Assess ability of CRTC2 repressor role to suppress Bcl-2 *expression in t(14;18)-positive cells* in vitro

I will overexpress FUGW-CRTC2WT by viral infection in t(14;18)-positive cell lines DoHH2[35], a human B-cell follicular lymphoma, and WSU-FSCCL[36], a human follicular small grade cleaved cell lymphoma, and patient follicular lymphoma samples. Empty FUGW vector will be used as a control. After 24 hours, *Bcl-2* transcript will be measured by qPCR, and the chromosomal translocation measured by PCR. Bcl-2 protein half-life is >10 hours [34,37,38], so time point analysis by western blot will determine when loss of protein is achieved.

I will treat FUGW and FUGW-CRTC2 overexpressing lymphoma cells with rituximab (IDEC-C2B8, 30 ug/mL)[39], a cancer chimeric therapeutic monoclonal antibody directed against CD20 expressed on the surface of B-cells[40]. Induction of cytotoxicity is one of the mechanisms of action by rituximab[41]. Apoptosis will be quantified by flow cytometry analysis of AnnexinV/PI uptake and by western blot for cleavage of caspase-3.

Generate lymphoma cell lines stably expressing a Tet-on system for CRTC2 overexpression

To test the effectiveness of the repressor role of CRTC2 on the t(14;18) translocation *in vivo*, I will firstly generate a lymphoma cell line expressing a Tet-on inducible CRTC2, and secondly use them in a human follicular lymphoma mouse model. The Tet-on inducible system allows temporal control of gene expression through addition of the drug doxycycline or tetracycline. It is comprised of two parts: a transactivator formed by the fusion of the repressor of the tetracycline-resistance operon in *E. choli* and the c-terminal activator domain of viral protein 16 (VP16) of herpes simplex virus, and a response gene that is regulated by the Tet operator sequences. Upon doxycyline or tetracycline treatment, the transactivator binds to the Tet-operator sequences and the downstream gene is transcribed. This system will be used to induce CRTC2 overexpression in lymphoma cells injected into mice.

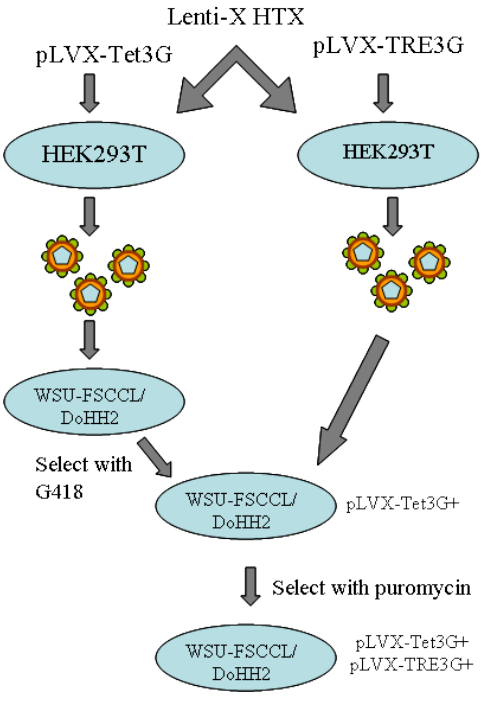

Figure 3. Schematic of the generation of double-stable cell lines.

To generate a Tet-inducible CRTC2 cell line, I will use the Lenti-X Tet-On 3G system from Clonetech. The gene for the VP16-tet fusion protein is carried in the pLVX-Tet3G vector, and CRTC2 will be inserted into the response vector pLVX-TRE3G. The control pLVX-TRE3G construct contains the luciferase gene. The system will be tested in HEK 293T cells by transient transfection and treatment with and without doxycyclin. CRTC2 overexpression will be detected by western blot.

Lentivirus will be made in HEK293T cells by transfection of the Lenti-X HTX system each with the pLVX-Tet3G or pLVX-TRE3G-luciferase or pLVX-TRE3G-CRTC2 vectors (Figure 3). DoHH2 and WSU-FSCCL will be infected with the Tet3G lentivirus. Selection by G418 will form a stable cell line producing the Tet3G transactivator. The cells will then be infected with a lentivirus expressing either the luciferase control or the CRTC2 response vectors. Addition of puromycin to the media will select for cells expressing the response vector, generating cell lines stable for both the transactivator and the response gene.

Whether CRTC2 can suppress *Bcl*-2 expression and sensitize them to rituximab will be tested in the double stable cell lines. Optimal doxycycline concentration (0.1-1 ug/ml) will be determined by detecting protein (luciferase, CRTC2) levels by western blot after 24 hours of treatment. Rituximab (30 ug/mL) will be added to cells, and apoptosis measured by flow cytometry analysis of AnnexinV/PI uptake and by cleavage of caspase-3 by western blot.

Assess ability of CRTC2 repressor role to increase survival in a human lymphoma scid *mouse model*

A CB17 *scid* mouse model of t(14;18) human lymphoma will be made by i.p. injection of 1 x 10^7 WSU-FSCCL cells or 5 x 10^6 DoHH2 cells into 4-6 week old female *scid* mice, as described in Smith et al. Disease will develop between 8-11 weeks with WSU-FSCCL, and at 4-6 weeks with DoHH2 injections, with sick animals displaying adenopathy, splenomegaly, ascites, and lymphocyte infiltration of the liver and bone marrow[34,39].

Mice will be injected with either the double-stable WSU-FSCCL or DoHH2 cell lines expressing luciferase control or CRTC2. To observe increased apoptosis sensitivity, the lymphoma will grow for 5 weeks in the FSCCL model and 3 weeks in the DoHH2 model to allow formation of ascites, or until first signs of illness. Doxycyclin (200 mg/kg)[42] feed will be

given to the mice. Two days later[43,44], rituximab (5 mg/kg)[39] or PBS will be administered by i.p. injection. Ascites fluid will be collected after 16 hrs. Three measurements will be taken: 1) cell count of lymphoma cells in ascites by flow cytometry detection of human anti-CD45 and anti-Bcl-2 staining, 2) apoptosis by flow cytometry detection of Annexin V/PI uptake, and 3) expression of *bcl-2-IgH* in ascites fluid by qPCR.

Survival experiments will be done by administering doxycyclin (200 mg/kg) feed at the time of cell injection. After 1 week, rituximab (5 mg/kg) will be administered by i.p. injection. End-point measurement will be visible illness, at which point the mice will be euthanized.

Expected Results and Possible Difficulties

FUGW-CRTC2 overexpression in t(14;18)-positive cells will decrease Bcl-2 transcript and protein levels, and sensitize them to apoptosis by rituximab compared to FUGW vector only transduced cells. Release of mitochondrial cytochrome c will be restored with *Bcl-2* repression[16], as demonstrated by caspase-3 cleavage detection by western blot. The human lymphoma mouse model will illustrate that CRTC2 repression of *Bcl-2* can reverse the transformed state. Table 2 summarizes expected results. Although drug concentrations were validated in the literature, optimizations may have to be made for doxycycline and/or rituximab.

pLVX-TRE3G-CRTC2	Doxycycline	Rituximab	CRTC2 expression?	Apoptosis sensitive?	Increased survival?
	-	-	No	-	-
	+	-	Yes	+	+?
	+	+	Yes	+++	+++

Table 2. Expected results of CRTC2 overexpression in t(14;18)-positive lymphoma cell lines and mouse model

Appendix 1

DNA damage response genes

Genes upregulated with etoposide and anti-IgM treatment of Ramos B-cells that are bound by CRTC2 prior to DNA damage.

MGC14376
EGR4
DLX2
CDKN2B
ADRA2B
RASL11A
KIAA0895
ITPKA
FLJ31875
FOSL2
SMPD1
JUNB
NR4A3
FZD7
DUSP5
WNT3
NEIL2
NEU1
SGK
CCR7
TNFAIP3
FAM46C
LRRC32
BCL2
TA-NFKBH
FGFR1
FLCN
FN1
IER5L
SQSTM1
ID2
CDKN1A
UPP1
FHL2

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