**Notch signaling induces a transcriptionally permissive state at the *Complement C3d*
Receptor 2 (CR2) promoter in a pre-B cell model**

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Running title: Notch signals regulate transcription of *CR2* in B cells

Abstract

During mammalian lymphoid development, Notch signaling is necessary at multiple stages of T lymphopoiesis, including lineage commitment, and later stages of T cell effector differentiation. In contrast, outside of a defined role in the development of splenic marginal zone B cells, there is conflicting evidence regarding whether Notch signaling plays functional roles in other B cell sub-populations. Complement receptor 2 (CR2) modulates BCR-signaling and is tightly regulated throughout differentiation. During B lymphopoiesis, CR2 is detected on immature and mature B cells with high surface expression on marginal zone B cells. Here, we have explored the possibility that Notch regulates human *CR2* transcriptional activity using *in vitro* models including a co-culture system, co-transfection gene reporters and chromatin accessibility assays. We provide evidence that Notch signaling regulates *CR2* promoter activity in a mature B cell line, as well as the induction of endogenous *CR2* mRNA in a non-expressing pre-B cell line. The dynamics of endogenous gene activation suggests additional unidentified factors are required to mediate surface CR2 expression on immature and mature B lineage cells.

Keywords: B cells; Complement; Gene Regulation; Human; Molecular Biology; Transcription Factors.

Abbreviations: ALL, acute lymphoblastic leukemia; BCR, B cell receptor; ChART-PCR, chromatin accessibility real-time PCR; ChIP, chromatin immunoprecipitation; CR2, Complement Receptor 2; CRS, CR2 intronic silencer; DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; EMSA, electrophoretic mobility shift assay; HPLC, high performance liquid chromatography; NIC, Notch intracellular domain; qPCR, real-time quantitative PCR; SLE, systemic lupus erythematosus; UMOD, Uromodulin; β 2m, β -2-microglobulin.

1. Introduction

The process of Notch activation in mammals can involve four Notch receptors (Notch1-4) and five different ligands (delta-like and jagged proteins). Throughout development, regulation of cell-fate decisions via Notch receptor-ligand engagement has been well documented (Borggreffe and Oswald, 2009; Schroeter et al., 1998). The canonical Notch pathway invokes a model where cleaved Notch intracellular domain (NIC) heterodimerizes with the sequence-specific DNA binding factor RBPJ- κ displacing repressor cofactors and recruiting coactivators (Radtke et al., 2004).

During lymphoid development, Notch signals enforce T cell commitment (Hozumi et al., 2008; Schmitt and Zúñiga-Pflücker, 2002) and differentiation to effectors (Hue et al., 2012; Kuijk et al., 2013). Similar roles for Notch signaling in B cell survival (Yoon et al., 2009), activation (Kang et al., 2014; Thomas et al., 2006) and differentiation (Santos et al., 2007) have been elucidated. A combination of cell intrinsic transcription factor expression (King et al., 2016; Simonetti et al., 2013), and extrinsic microenvironment organization (de Oliveira et al., 2018; Hammad et al., 2017; Magri et al., 2014) within the spleen play critical roles in influencing Notch signal on B lymphopoiesis. It has been established that signaling via Notch2 (Hampel et al., 2011; Saito et al., 2003), DL1 (Hozumi et al., 2004; Tan et al., 2009) and RBPJ- κ (Tanigaki et al., 2002) is necessary for development of marginal zone splenic B cells at the expense of the follicular population. Marginal zone B cells display a skewed antibody repertoire, selected by auto-reactive B cell receptor (BCR)-signaling during transitional stages of maturation, and are characterized by high Complement C3d Receptor 2 (CR2, also known as CD21) surface expression (Allman and Pillai, 2008). Expression of CR2 during B lymphopoiesis is tightly restricted such that induction of CR2 expression occurs after escape from negative selection and is coincident with B cell maturation (Takahashi et

al., 1997; Tedder et al., 1984). We have previously demonstrated that an RBPJ- κ binding site in the first intron of *CR2* is required to maintain developmentally restricted *CR2* expression (Makar et al., 2001; Ulgiati and Holers, 2001). Furthermore, Notch signaling has been shown to influence immature/transitional B cell development in murine cells (Hampel et al., 2011; Roundy et al., 2010). Importantly, elevated *CR2* expression was observed during the maturation of immature/transitional B cells following exposure to Notch signals. However, direct evidence that Notch signaling regulates human *CR2* expression during the pre-B to mature B cell transition in human cells, is required.

At the B cell surface, *CR2* is an important component of the BCR co-receptor complex which amplifies BCR signaling ensuring a robust immune response (Fearon and Carroll, 2000). The correct temporal expression of *CR2* is integral to B cell function as transgenic mice which express human *CR2* prior to the mature stage of B cell development have a 60% reduction in mature B cells and decreased humoral immune response to T-cell-dependent antigens (Marchbank et al., 2002). However, the mechanisms that modulate *CR2* expression in developing B cells are unknown. We have previously reported that polymorphisms within the human *CR2* gene affecting its expression are associated with systemic lupus erythematosus (SLE) (Douglas et al., 2009; Zhao et al., 2016). *CR2* levels are decreased by approximately 50% on B cells from patients with SLE (Marquart et al., 1995; Wilson et al., 1986). Further, SLE-prone MRL/*lpr* mice also show lower *CR2* surface expression prior to clinical evidence of disease (Takahashi et al., 1997). The precise signals that fine-tune *CR2* expression levels during B cell ontogeny are not defined. Additionally, evolutionary divergence of the *CR2* gene from mouse to human means that an appropriate model to elucidate human *CR2* transcriptional regulation is required (Jacobson and Weis, 2008). We hypothesized that Notch signaling could initiate or modify *CR2* transcriptional activation in B cells. To address this

87 hypothesis, we utilized *in vitro* models to examine gene expression and changes in chromatin
88 accessibility surrounding *CR2* regulatory regions in response to Notch signals.

2. Materials and methods

2.1. Cell culture

Cell lines REH (CRL-8286), Ramos (CRL-1596), Raji (CCL-86) and K562 (CCL-243) were obtained from ATCC (Manassas, VA, USA) and were maintained as described (Cruickshank et al., 2009). OP9-DL1 cells were kindly provided by David Izon, derived by Schmitt and Zúñiga-Pflücker as described (Schmitt and Zúñiga-Pflücker, 2002). OP9-DL1 cells were grown at 37°C with 5% CO₂ in MEM- α media, supplemented with L-glutamine, 20% FBS, 50 units/mL penicillin and 50 μ g/mL streptomycin.

2.2. Chromatin immunoprecipitation (ChIP)

Transcription factor binding ChIP assays were performed as described (Cruickshank et al., 2009). Briefly, immunoprecipitation was performed with 40 μ g of α -RBPJ- κ (Santa Cruz Biotechnology, Santa Cruz, CA, USA, Cat#sc-8213X [D20]), α -E12 (Cat#sc-762X [H208]), α -E47 (Cat#sc-763X [N649]), α -Ikaros (Cat#sc-13039X [H100]), α -RNApolII (Cat#sc-899X [N20]), α -CD19 (IgG control; BD Biosciences PharMingen, Franklin Lakes, NJ, Cat#555410) or no antibody. Immune complexes were collected with 60 μ L mixture of Salmon Sperm DNA/Protein A (Millipore, Billerica, MA, USA; Cat#16-157) and Salmon Sperm DNA/Protein G Agarose Slurry (Millipore; Cat#16-201). Enrichment of the *CR2* promoter was determined by qPCR (Forward 5'-gctcacagctgcttgctgct-3' and Reverse 5'-ggtcctcaaagctagcgggagggcg-3', annealing temperature 63°C).

2.3. Plasmid constructs

A *NheI/XhoI* fragment of the *CR2* promoter (-315/+75) was cloned into the pGL3-basic vector (Clontech Laboratories, Palo Alto, CA, USA) as previously described (Ulgiati et al.,

2002). Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA). Successive 5' deletions of the *CR2* promoter were also cloned into the pGL3-basic vector as previously described (Ulgiati et al., 2002). Notch signaling constructs, MigR1-NIC, MigR1-DNMAML1 and the empty vector (MSCV-IRES-GFP), were kindly provided by Warren Pear (Maillard et al., 2004; Pui et al., 1999).

2.4. Transfection assays

Transfections were performed using 3 µg reporter-plasmid DNA, 1 µg expression-plasmid DNA and 120 ng pRL-TK (*renilla*) control vector combined with Superfect reagent (QIAGEN, Valencia, CA, USA) as described (Cruickshank et al., 2012). Co-transfection reactions were prepared with Viafect transfection reagent (Promega, Madison, WI, USA), reporter-plasmid DNA, expression-plasmid DNA, and pRL-TK in a ratio of 8 µL: 1 µg: 1 µg: 100 ng, respectively, per 200 µL of OptiMEM I reduced medium (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at room temperature for 20 min. A total volume of 400 µL of co-transfection reaction mix was prepared to transfect 2×10^6 REH or Ramos cells in RBPJ-κ mutant constructs co-transfection experiments, whilst a total volume of 100 µL of co-transfection reaction mix was prepared to transfect 5×10^5 REH or K562 cells in *CR2* promoter deletion constructs co-transfection experiments. Cell lysates from the transfected cells were assayed for *Firefly* and *renilla* luciferase using the Dual-Luciferase Reporter Assay system according to the manufacturer's instructions (Promega). All transfection data represent the mean of at least three independent transfections expressed as relative *Firefly* luciferase activity normalized against *renilla* luciferase activity.

2.5. Notch Co-Culture

OP9-DL1 cells were grown in T75 flasks to 60 - 70% confluence, media was aspirated to leave 7.5 mL conditioned media to which 3×10^6 REH or Ramos cells suspended in 7.5 mL FBS-free MEM- α media were added. Cells were subcultured every two days and 3×10^6 cells seeded into new T75 flasks containing OP9-DL1 cells grown as described. Co-cultures were performed with the addition of 10 mM N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (Sigma-Aldrich, St. Louis, MO, USA) or 15 μ L DMSO (vehicle) (Sigma-Aldrich) and harvested after 24 h and 11 days of sequential culture. Cells were harvested and re-plated on the third day of co-culture, and subsequently every two days. Remaining cells ($\leq 1 \times 10^7$ cells) were washed in 1 mL of PBS, pelleted and snap-frozen on dry ice for storage at -80°C . Cell pellets were subsequently gently thawed on ice and RNA extracted as described below.

2.6. Real-time quantitative PCR (qPCR)

Total RNA from 5×10^6 cells was isolated with the QIAshredder (Qiagen, Victoria, Australia) and RNeasy mini-kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using 1.5 μ g RNA and the SuperScriptTM VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR (qPCR) was performed using 2 μ L of cDNA or plasmid DNA containing known target sequence, and the Illumina Eco Real-Time PCR system V.4. Primers were directed at *β actin* (Forward 5'-gatgaccagatcatgtttgag-3' and Reverse 5'-gactccatgccaggaaggaa-3', annealing temperature 62°C), *CR2* (Forward 5'-tgctgtaaaaccaactctc-3' and Reverse 5'-agcaagtaaccagattcacag-3', annealing temperature 60°C) or *HES1* (Forward 5'-cggcattccaagctggagaagg-3' and Reverse 5'-acttgggtctgtgtcgcagcgca-3', annealing temperature 65°C). Primers targeting human *immunoglobulin light chain kappa (IgL κ)* and *lambda (IgL λ)* were used as described (Ståhlberg et al., 2003). Transcript abundance was determined by comparison to serial

dilutions of plasmid DNA containing known target sequence and normalized to *β actin* mRNA expression.

2.7. PCR

Conventional PCR was performed using the PTC-100 Thermocycler (MJ Research, Waltham, MA, USA) and Platinum® *Taq* DNA Polymerase (Invitrogen) according to manufacturer's directions. Briefly, 2 µL template was amplified in 20 µL reactions with 0.2 mM dNTPs (Roche Diagnostics, Indianapolis, IN, USA), 1.5 mM MgCl₂, and 0.5 µM each primer. Primers were used to detect *NOTCH1* (Forward 5'-caggcaatccgaggactatg-3' and Reverse 5'-caggcgtgttgttctcacag-3') or *NOTCH2* (Forward 5'-tgagtaggetccatccagtc-3' and Reverse 5'-tggtgtcaggtaggatgct-3') (Nijjar et al., 2001). Thermal-cycling conditions were as follows: 95°C for 5 min; followed by 34 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 1 min; followed by 72°C for 7 min. PCR products were electrophoresed on a 1.5% agarose gel.

2.8. Flow cytometry

Co-cultured REH cells were harvested at days 4 and 11 and 1×10^6 cells washed twice with cold staining buffer (PBS containing 5% FBS). Similarly, 1×10^6 Ramos cells were harvested after 11 days of co-culture. Cells were resuspended in 80 µL staining buffer and incubated with 20 µL of PE-conjugated Mouse anti-human CR2 antibody (BD Biosciences, Cat# 555422) or PE Mouse IgG1 kappa isotype control (BD Biosciences, Cat# 555749), for 20 min. As a control unstained cells were also included. Cells were washed twice with cold staining buffer and resuspended in 0.5 mL staining buffer. REH cells were analyzed using a BD FACSCalibur (BD, San Jose, CA, USA) and FlowJo software V10.0.5 (Tree Star, Ashland, OR, USA), whilst Ramos cells were analyzed using a BD Accuri C6 (BD) and FlowJo software V10.0.5.

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189 *2.9. Chromatin accessibility by real time PCR (ChART-PCR)*

190 Chromatin accessibility assays were performed as previously described with minor
191 modifications (Cruickshank et al., 2008). Approximately 1.25×10^6 nuclei were resuspended
192 in 50 μ L 1x CutSmart buffer (NEB, Ipswich, MS, USA). 50 μ L of pre-warmed 1x RQ1
193 DNase Reaction buffer (Promega) containing 1 U of RNase-Free DNase (Promega) and 50
194 μ g of RNaseA (Thermo Fisher Scientific) was added to the nuclei and reactions incubated at
195 37°C for 10 min. As an “uncut” control, RNase-Free DNase was omitted from the digestion
196 buffer. To terminate the digestion reaction, 10 μ L of RQ1 DNase stop solution (Promega)
197 was added. “Cut” DNA was isolated and purified using the QIAquick PCR purification kit
198 and its recommended protocol (Qiagen), whilst “uncut” DNA was isolated and purified using
199 the QIAamp blood mini kit (Qiagen), as outlined by the manufacturer’s instruction. Both
200 “cut” and “uncut” DNA were diluted to 10 ng/ μ L before analysis by qPCR using the Mic
201 qPCR cycler (Bio Molecular Systems, Queensland, Australia). Primers (Table I) were
202 designed to span across the *CR2* regulatory regions. The *β -2-Microglobulin (β 2M)* and
203 *Uromodulin (UMOD)* genes were used, as internal positive and negative controls for
204 efficiency of digestion and for normalization across cell types, respectively. Student t-test
205 was utilized for statistical analysis between Notch activated or inhibited REH cells and basal
206 REH cells.

207

208 *2.10. Electrophoretic mobility shift assay (EMSA)*

209 Nuclear extract was prepared from 5×10^7 REH or Ramos cells as described previously (Li
210 and Liao, 1991). High performance liquid chromatography (HPLC) purified oligonucleotides
211 spanning -538 to -503 of the *CR2* promoter were obtained from Sigma-Aldrich with the plus
212 strand 5'-biotinylated for labeled probes using the wild-type sequences (forward: 5'-

213 ggtgacaagtatatgggaattctctgcagtactctt-3' and reverse: 5'-aagagtactgcagagaattcccatatacttgtcacc-
214 3') or a 7 bp mutation in the RBPJ- κ /Ikaro core motif (forward: 5'-
215 ggtgacaagtatagcagctctctctgcagtactctt-3' and reverse: 5'-
216 aagagtactgcagagagacgtgctatacttgtcacc-3').

217 Binding reactions were prepared as described (Cruickshank et al., 2012). For competition
218 reactions, unlabeled oligonucleotides in 15x to 60x fold molar excess were incubated with
219 nuclear extract for 10 min on ice, prior to adding 50 fmol biotin-labeled probe for 30 min.
220 For blocking experiments, 2 - 4 μ g of antibodies targeting RBPJ- κ (Abcam, Cambridge, UK.,
221 Cat# ab25949) were added for 45 min on ice prior to addition of 50 fmol biotin-labeled probe
222 for 30 min. Samples were electrophoresed on a Novex® DNA Retardation Gel
223 (6% polyacrylamide) using the XCell SureLock system (Invitrogen) for 1 h at 100 V and
224 transferred onto a nylon membrane for 45 min at 30 V. Biotin-labeled DNA was visualized
225 with the Chemiluminescent Nucleic Acid Detection Module (Pierce) according to the
226 manufacturer's instructions.

227

228 2.11. Statistical analysis

229 All values described represent the mean $\pm$ SEM. Statistics and graphs were generated using
230 GraphPad Prism v6 (GraphPad, San Diego CA). Statistical values for each experiment are
231 shown in representative figure legends.

3. Results

3.1 Notch-associated transcription factors bind to the CR2 promoter in Ramos B cells

We first confirmed expression of *NOTCH1* and *NOTCH2* transcripts in a range of B- and non-B cell lines (K562, REH, Ramos and Raji, data not shown). Our previous studies defined tandem E box elements located near the transcriptional start site of *CR2* that mediate cell-specific transcriptional activity and bind E2A and USF proteins *in vitro* (Ulgiati et al., 2002; Ulgiati and Holers, 2001). These sites are candidate Notch targets as previous studies demonstrate Notch-dependent modulation of E2A activity (Huang et al., 2004; Ikawa et al., 2006; Nie et al., 2003; Zhang et al., 2013). Analysis of the *CR2* promoter using MatInspector (Genomatix GmbH, Munich, Germany) revealed additional sites that could interact with Notch-modulating factors. They include two RBPJ- κ consensus motifs located at -529 to -515 bp (5'-tataTGGGaattctc-3') and at -113 to -98 bp (5'-cgcttgtCCCAccct-3') upstream of the *CR2* TSS. The proximal RBPJ- κ consensus site is directly adjacent to a functional Sp1 binding site (Ulgiati et al., 2002) and the distal site overlaps an Ikaros consensus motif (Fig. 1A). We have previously found that RBPJ- κ binds the *CR2* promoter in Epstein-Barr virus (EBV)-positive/*CR2*-positive Raji B cells using ChIP assays (Cruickshank et al., 2009). We now confirm that finding and show RBPJ- κ also binds the *CR2* promoter in EBV-negative Ramos mature B cells (Fig. 1B, lane 5) indicating that RBPJ- κ binding is not EBV-dependent. Further, we show interaction of additional Notch-associated factors Ikaros, E12 and E47 with the *CR2* promoter in Ramos cells (Fig. 1B, lanes 2 – 4). In agreement with our previous report (Cruickshank et al., 2009), enrichment of *CR2* promoter fragments targeting RBPJ- κ show weaker enrichment (0.05% input) in comparison to antibodies targeting Ikaros (0.1% input), E12 (0.4% input), E47 (0.5% input) and RNA polymerase II (Fig. 1B, Lane 9; 1.6% input) as determined by qPCR.

3.2. Two RBPJ- κ sites have a functional effect on CR2 expression in CR2-negative cells

We next investigated the functional role of the RBPJ- κ binding sites in CR2-negative and CR2-positive B cell lines and a non-B cell line. Luciferase reporter constructs containing the wild-type 1.2 kb CR2 promoter (henceforth termed 1.2-CR2-WT) were cloned with either (RBPJ κ -mut1 or RBPJ κ -mut2) or both (RBPJ κ -mut1/2) of the RBPJ- κ sites ablated (Fig. 2A). Reporter constructs were transfected into a pre-B acute lymphoblastic leukemia (ALL) cell line (REH; CR2-negative), an erythroid precursor cell line (K562; CR2-negative) and a mature B lymphoma cell line (Ramos; CR2-positive). REH cells co-transfected with either the RBPJ κ -mut1 or RBPJ κ -mut1/2 constructs showed a significant 39% ($p=0.005$; $n\geq 4$) or 57% ($p<0.0001$; $n\geq 4$) decrease in basal CR2 transcriptional activity, respectively (Fig. 2B). The RBPJ κ -mut2 construct did not have a significant effect on transcriptional activity in REH cells (Fig. 2B). Similarly, K562 cells co-transfected with the individual RBPJ- κ mutant constructs (RBPJ κ -mut1: $p=0.02$; RBPJ κ -mut2: $p=0.02$, $n=3$) showed a significant decrease in transcriptional activity compared to basal K562 levels, and when both sites were mutated the transcriptional effect was additive (Fig. 2C, white bars; $p=0.004$, $n=3$). In contrast, in Ramos cells ablation of either or both RBPJ- κ binding sites did not significantly reduce transcriptional activity compared to the wild-type promoter (Fig. 2D, white bars; $p>0.05$, $n=3$). These results indicate that the two RBPJ- κ sites appear to have cell-type specific function and had the greatest impact on transcriptional activity in cell lines lacking endogenous CR2 expression.

3.3. *The RBPJ- κ sites differentially regulate the effect of Notch signaling on the CR2 promoter in CR2-negative REH and K562 cells*

We next set out to determine if the two putative CR2-promoter RBPJ- κ sites are responsive to Notch signaling. We performed co-transfections in REH, K562 and Ramos cells using expression constructs that mimic Notch activation (NIC) or suppression (DN-MAML), or with an empty vector control (MigR1), together with either 1.2-CR2-WT or mutant RBPJ- κ constructs (Fig. 3 and Supplementary Fig. 1A-C).

REH cells co-transfected with the 1.2-CR2-WT construct and NIC showed a nine-fold increase in transcriptional activity compared to basal REH transcriptional activity levels ($p=0.0001$; $n\geq 4$), indicating that the CR2 promoter is responsive to Notch signals in REH cells (Fig. 3A). Mutation of either or both RBPJ- κ sites did not ameliorate Notch activation relative to the wild-type promoter (Fig. 3A, black bars). Rather, transcriptional activity was increased 17.6-fold in response to Notch activation when both RBPJ- κ sites were mutated, which was significantly greater than the nine-fold induction observed for the 1.2-CR2-WT construct (Fig. 3A, $p=0.02$, $n\geq 4$).

When K562 cells were co-transfected with the 1.2-CR2-WT construct and Notch activator, there was a 1.7-fold increase in transcriptional activity compared to basal K562 levels (Fig. 3B, black bars, $p=0.04$, $n=3$). This effect was diminished in both single RBPJ- κ mutants and the double mutant ($p>0.05$; $n=3$). These results suggest that the CR2 promoter is responsive to Notch signals in K562 cells, however with a reduced magnitude as compared to REH cells. In contrast, Notch activation had a small but not significant effect on the CR2 promoter in Ramos cells (Fig. 3C, black bars, $p>0.05$, $n=3$). No significant differences in transcriptional activity were observed with any of the RBPJ- κ mutants, however, transcriptional activity was variable and Notch induction in the presence of the RBPJ κ -mut2 construct resulted in an average 2.8-fold increase in transcriptional activity compared to the empty vector control.

Co-transfection of DN-MAML with the wild-type promoter or any of the RBPJ- κ mutant constructs did not have a significant effect on transcriptional activity in any cell type tested (Supplementary Fig. 1A-C, grey bars).

Collectively, these results indicate that the *CR2* promoter is most responsive to Notch signals in the cellular context in which *CR2* is silent. Further, the RBPJ- κ sites appear to mediate this effect in a cell-context-dependent manner.

3.4. Promoter truncation does not abolish Notch-mediated activation of CR2 transcriptional activity

Whilst we observed significant increases in *CR2* promoter transcriptional activity following Notch activation in REH and K562 cells, it was clear that the putative RBPJ- κ sites were not solely responsible for mediating this induction. To this end, we utilized *CR2* promoter deletion co-transfections in REH and K562 cells, to identify regions within the *CR2* promoter that harbored sites governing Notch induction (Fig. 4A).

In REH cells, none of the deletion constructs co-transfected with NIC were able to completely abolish Notch activation (Fig. 4B, black bars). Except for the -135 and -60 constructs, all deletion constructs showed significant 2.6- to 11-fold increase ($p < 0.05$, $n \geq 3$) in transcriptional activity when compared to basal transcriptional activity (Fig. 4B). Interestingly, truncation of the *CR2* promoter did not abolish NIC-activation as compared to the 1.2-*CR2*-WT construct co-transfection, except for the -39 construct (Fig. 4B, $p = 0.03$, $n \geq 3$).

Similarly in K562 NIC co-transfections, truncation of the *CR2* promoter did not abolish Notch activation (Fig. 4C, black bars, $p < 0.05$, $n = 4$). A 3.7- to 9.7-fold increase in transcriptional activity was observed with NIC expression construct co-transfections, when compared to basal transcriptional activity. Only the -53 deletion construct demonstrated a

significant reduction in fold-change below the 1.2-CR2-WT construct in NIC co-transfection (Fig. 4C, $p=0.05$, $n=4$). To determine the effect of these two E-box sites on Notch induction, we performed NIC co-transfections with either wild-type minimal *CR2* promoter (315-CR2-WT) or E box mutants in K562 cells (Fig. 5). Whilst we observed a significant decrease in Notch-induced transcriptional activity with deletion of E box site 2 (Fig. 4C), a site-specific mutation of E box site 2 showed a significant increase in transcriptional activity compared to 315-CR2-WT construct (Fig. 5). Nonetheless, mutation to either E box sites did not significantly decrease Notch induced transcriptional activity, suggesting that the E box sites within the *CR2* promoter do not mediate Notch significantly in this cellular context. Co-transfection of the Notch inhibitor (DN-MAML) construct did not have an effect on any deletion constructs in REH or K562 cells (Supplementary Fig. 1D,E, grey bars). In contrast, co-transfection of either the 315-CR2-WT or E box site 2 mutant construct with DN-MAML resulted in a significant decrease in transcriptional activity compared to basal K562 transcriptional activity (Supplementary Fig. 1F, 315-CR2-WT: $p=0.005$, $n\geq 6$; E-box site 2 mut: $p=0.004$, $n=6$). This suggested that E box site 2 may be functional in repressing Notch signaling in non-CR2 expressing cells. Combined, we were unable to delineate specific transcription factor binding sites that mediate Notch signals, suggesting that regulation of the chromatin landscape may be required for the correct integration of signals mediated through the *CR2* promoter.

3.5. Notch signals activate transcription of endogenous *CR2* in vitro

We next sought to determine if Notch signals could activate endogenous *CR2* expression in REH cells in which *CR2* expression is normally silent. REH cells were originally derived from a pre-B ALL patient and have retained expression of B-lineage stage-specific immunophenotypic markers of pre-B cells such as HLA-DR, CD19, CD22 and

CD79a/CD79b (Matsuo and Drexler, 1998) and have previously been used to characterize Notch-induced activation of the *CD23* promoter (Hubmann et al., 2002). Accordingly, we investigated *CR2* expression in REH cells co-cultured with the adherent Notch-ligand expressing cell line OP9-DL1 (Schmitt and Zúñiga-Pflücker, 2002) in the presence or absence of the γ -secretase inhibitor, DAPT, which blocks cleavage of the Notch receptor. We first analyzed transcript abundance of the canonical Notch target gene, *HES1*, following 24 h of co-culture (Supplementary Fig. 2). As a control, REH cells were cultured independently without the OP9-DL1 cell line and harvested after 24 h (hereafter termed uninduced REH cells). No significant difference was observed in the expression of *HES1* mRNA following 24 h of cell culture. In contrast, REH cells that were co-cultured with Notch activation for 24 h showed a significant 20-fold increase in expression of *HES1* mRNA ($p=0.003$, $n=3$). Notch inhibition blocked *HES1* mRNA up-regulation ($p>0.05$, $n=3$). To assess the effects of prolonged Notch signaling on *CR2* expression in REH cells, a time course co-culture was performed. In the presence of Notch signals, *CR2* transcript was detectable by day 3 of continuous co-culture with significantly increasing expression observed over the 11 days, compared to uninduced REH cells (Fig. 6A, $p<0.05$, $n=5$). This effect was almost completely abolished in the first nine days and reduced on day 11 of co-cultures following Notch inhibition (Fig. 6A, $p>0.05$, $n=5$). In the presence of Notch signals, expression of *HES1* transcript significantly increased approximately 6.5-fold at day 3 of co-culture ($p<0.0001$, $n\geq 6$). However, *HES1* transcript levels gradually decreased over the next 8 days, yet expression levels remained significantly higher than uninduced REH cells (Fig. 6B). However, when Notch signals were inhibited, *HES1* transcript levels did not differ significantly from uninduced REH cells and transcript levels did not vary significantly over the 11 day period ($p>0.05$, $n\geq 6$). Therefore, in a pre-B ALL cell line, we show that Notch signals can activate transcription of endogenous *CR2* which otherwise does not express *CR2*

mRNA. To further extend our investigation, we examined transcript abundance of *immunoglobulin light chains kappa* and *lambda* (*IgLκ* and *IgLλ*, respectively) (expressed in mature B cells) in co-cultured REH cells (Allman and Pillai, 2008). Uninduced REH cells did not express *IgLκ* transcript and co-culturing REH cells in either the presence, or absence of Notch signal, did not induce *IgLκ* expression (data not shown). Conversely, REH cells (Fig. 6C, white bar, n=14) following Notch activation, demonstrated a 1.5- to 3-fold elevated expression of *IgLλ* transcript compared to uninduced REH cells (Fig. 6C, black bars, REH compared with Notch activated REH days 5 – 11: $p<0.05$, $n\geq 3$). This effect was abolished in co-cultured REH cells, where Notch signals were inhibited (Fig. 6C, grey bars, REH compared with Notch inhibited REH days 3 – 11: $p>0.05$, $n\geq 3$).

We next sought to determine if Notch signals modulate *CR2* expression in Ramos cells, an EBV-negative B-lymphoma cell line expressing detectable cell surface CR2 in uninduced conditions. We observe a modest, but consistent, 1.2- to 1.5-fold elevation of *CR2* mRNA following Notch activation in Ramos cells compared to the expression of uninduced Ramos cells, or Notch inhibited Ramos cells (Fig. 6D, Ramos compared with Notch activated Ramos days 3 – 11: $p<0.05$, $n\geq 3$).

However, in contrast to co-cultured REH cells, Notch activated Ramos cells consistently showed a 55 – 67% decrease in *HES1* expression compared to uninduced Ramos cells, across all time points tested and this effect was not reversed by Notch inhibition (Fig. 6E, Ramos compared with Notch activated & inhibited Ramos days 3 – 11, except Notch inhibited Ramos day 9: $p<0.05$, $n\geq 3$).

3.6. Notch signals are permissive for expression of CR2 in a small population of co-cultured REH cells

405 Using our co-culture model, we aimed to determine if endogenous *CR2* mRNA induction
406 resulted in a concomitant detectable cell surface protein expression on REH cells stimulated
407 by Notch signals. Notch inhibited or activated REH cells were harvested at days 4 and 11 for
408 flow cytometric analysis (Fig. 7A-D and 7E-H, respectively). Cells were stained with CR2
409 antibody and compared to background levels of fluorescence using an isotype control (Fig.
410 7A-H) or unstained cells (data not shown). Forward/side-scatter properties were used to gate
411 for viable cells. At both time points a small, but reproducible, increase in the proportion of
412 cells stained positive for CR2 (Fig. 7D, 1.29% at day 4; Fig. 7H, 1.67% at day 11) compared
413 to isotype-matched controls (Fig. 7C, 0.87% at day 4; Fig. 7G, 0.90% at day 11) and cells
414 cultured with DAPT (Notch-inhibitor) (Fig. 7A, 1.00% at day 4 with IgG isotype control; 7B,
415 0.86% at day 4 with CR2-PE and 7E, 0.99% at day 11 with IgG isotype control; 7F, 0.92% at
416 day 11 with CR2-PE) was observed. The number of positively stained cells remained low,
417 however those cells consistently (n=4) expressed detectable levels of surface CR2 which
418 could be abolished by blocking Notch signals. Therefore, Notch signaling induces a change
419 in the *CR2* promoter state from a silenced to a transcriptionally permissive state which is not
420 sufficient for cell surface expression in the vast majority of cells in this system.

421 Ramos cells were also harvested following 11 days of co-culture for cell surface expression
422 by flow cytometric analysis as described above (Fig. 7I-O). An isotype matched control
423 antibody was utilized to set a CR2-positive gate for uninduced (Fig. 7I), Notch activated (Fig.
424 7J) and Notch inhibited Ramos cells (Fig. 7K). We observed a similar percentage of CR2-
425 positive Ramos cells with Notch activation, compared to uninduced Ramos cells (Fig. 7L,M).
426 However, Notch activated Ramos cells displayed a higher mean fluorescence intensity (MFI)
427 than the uninduced Ramos cells (Fig. 7O, Notch activation compared with uninduced Ramos:
428 $p=0.002$, $n=3$) and this was reversed in the presence of the Notch inhibitor (Fig. 7N and 7O,
429 Notch inhibition compared with uninduced Ramos: $p=0.0009$, $n=3$), potentially indicating

that Notch activation leads to a higher number of CR2 molecules on the surface of each cell. The magnitude of this effect is small and concordant with the effect size observed in this cell line at the mRNA level.

3.7. Dynamic chromatin accessibility changes across the distal RBPJ- κ site within the CR2 promoter were observed following continuous Notch activation

Our REH co-culture results indicated that Notch signals create an environment permissible for CR2 transcription, therefore we next investigated the role of chromatin structure in regulating expression of CR2 mRNA following Notch activation. We measured DNase I chromatin accessibility using real-time PCR (ChART-PCR), as previously described (Cruickshank et al., 2008), focusing on the CR2 promoter and intronic silencer (CRS) (Makar et al., 1998) in REH and Ramos cells.

In accordance with the relative expression of CR2 between these cell types, a significant difference in chromatin accessibility was observed at both regions in REH pre-B cells compared to Ramos B cells (Fig. 8A and 8F). Specifically, whilst the CR2 promoter in REH cells was relatively inaccessible, the CR2 promoter in Ramos cells was highly accessible, with increasing accessibility with closer proximity to the TSS (Fig. 8A, REH compared to Ramos at all CR2 promoter regions: $p < 0.05$, $n = 3$). Similarly, we observed regions within the CRS that were significantly more accessible in Ramos cells compared to REH cells (Fig. 8F, CRS2: $p = 0.005$; CRS5: $p = 0.049$; CRS7: $p = 0.007$; CRS8: $p = 0.044$; CRS9: $p = 0.045$; $n = 3$).

Having established a baseline of chromatin accessibility across the CR2 regulatory regions in CR2-negative pre-B and CR2-positive mature B cell lines, we utilized ChART-PCR to explore changes in chromatin accessibility in REH cells harvested following Notch activation in the co-culture system. Basal chromatin accessibility in uninduced REH cells was used for all comparison and statistical analyses.

Our ChART-PCR data demonstrated dynamic changes in chromatin accessibility surrounding the *CR2* regulatory regions. We were particularly interested in regions which became significantly more accessible at time points coincident with increased *CR2* transcription.

Following Notch activation, significantly increased chromatin accessibility was observed at the regions immediately 3' to the distal RBPJ- κ binding site at day 5 (Fig. 8C, CR2pC: $p=0.04$, $n=3$) and day 7 (Fig. 8D, CR2pC: $p=0.02$, $n=3$). Accessibility of the *CR2* promoter returned to a closed state on day 9, where no regions within the *CR2* promoter were significantly increased compared to basal chromatin accessibility (Fig. 8E). No significant change in chromatin accessibility was observed across any regions within the CRS (Fig. 8G-J, $p>0.05$, $n=3$).

In contrast, significant increase in chromatin accessibility was only observed after nine days of Notch inhibition, at the same region 3' of the distal RBPJ- κ binding site (Supplementary Fig.3D, $p=0.02$, $n=3$). No regions within the CRS showed significant change in chromatin accessibility following Notch inhibition (Supplementary Fig. 3E-H, $p>0.05$, $n=3$).

Collectively, these data indicate that alterations to chromatin accessibility surrounding the distal *CR2* promoter RBPJ- κ site are observed in Notch-activated REH cells at time points concordant with transcriptional activation.

3.8. Sequence-specific protein-DNA complexes bound to -538 to -503 *in vitro* contain RBPJ- κ

ChART-PCR analyses indicated that the effect of Notch signaling on *CR2* transcriptional activity in REH cell is, in part, mediated through the distal RBPJ- κ binding site. To investigate if RBPJ- κ can bind to this regulatory element *in vitro*, we performed electrophoretic mobility shift assay (EMSA) using double-stranded oligonucleotides corresponding to the -538 to -503 region of the promoter encompassing this motif. Using REH nuclear extract, we identified three distinct complexes (A, B and C) (Fig. 9A). When

480 REH nuclear extract was pre-incubated with increasing amounts of unlabeled (cold
481 competition) oligonucleotides, only complex A was abolished with 15X molar excess of cold
482 competitor (Fig. 9A). In contrast, pre-incubation with a mutant cold competitor did not
483 abolish complex A (Fig. 9B), indicating that the RBPJ- κ /Ikaros consensus site within the *CR2*
484 promoter binds sequence-specific nuclear factor(s).

485 Next, supershift assays were performed utilizing an α -RBPJ- κ antibody (Fig. 9C) revealing a
486 reduction in the abundance of complex A and the appearance of a novel supershift band (Fig.
487 9C, complex D). Since complex A was not completely abolished even in the presence of 4 μ g
488 of antibody, additional unidentified factors likely participate in the formation of complex A.

489 Similarly, using Ramos nuclear extract, we observed the formation of four complexes, one of
490 which was sequence-specific, as well as the presence of RBPJ- κ binding to the sequence-
491 specific complex (Supplementary Fig. 4).

492 Combined, our EMSA data indicate that RBPJ- κ binds to the distal RBPJ- κ /Ikaros motif with
493 additional factors.

4. Discussion

Collectively, our data reveal the involvement of Notch signals in controlling *CR2* transcriptional activity and initiation of mRNA expression in a cell line model of pre-B lineage cells. We have previously reported *in vitro* induction of *CR2* expression in REH pre-B ALL cells by histone deacetylase inhibitors (Cruickshank et al., 2009). We now show that Notch signals can stimulate *CR2* transcription in non-expressing REH pre-B cells coincident with chromatin conformation changes at specific regulatory regions. Previous studies have revealed increased *CR2* transcript levels in Notch activated cell lines from diverse cancer cell lineages including breast cancer, T-ALL and B cell lymphoma (Petrovic et al., 2019; Ryan et al., 2017). For the first time, we demonstrate that endogenous *CR2* expression can be induced by Notch signaling in a non-expressing pre-B lineage ALL cell line. Further studies are now warranted to determine if Notch signaling also impacts developing pre-B cells as this is a critical window for maintenance of self-tolerance.

Previous reports show increased *CR2* surface and mRNA expression on mature B cells after stimulation via NIC (Chang et al., 2005; Strobl et al., 2000). However, these studies did not delineate regulatory regions mediating this effect, nor identify specific transcription factors involved. We show that RBPJ- κ , Ikaros and E2A bind to the *CR2* promoter in transcriptionally active B cells. These transcription factors have previously been implicated as modulators of Notch signaling (Malinge et al., 2013; Nie et al., 2003; Tani et al., 2001; Toubai et al., 2011). *In vitro*, we observed the greatest magnitude of Notch-mediated increase in *CR2* transcriptional activity in *CR2*-negative pre-B cells compared to myeloid lineage cells and mature B cells. Previous studies have revealed that Notch stimulates endogenous *CR2* expression in mature B lymphoma cells in systems that use a GSI-washout assay to temporarily inactivate endogenous Notch signals, followed by removal of the inhibitor to

reactivate Notch signaling (Petrovic et al., 2019; Ryan et al., 2017). In contrast, we used mutagenized reporter/Notch activator co-transfection assays and Notch-ligand expressing co-culture assays blocked by continuous GSI-exposure to modulate Notch signaling. Our data collectively suggests that Notch activation is associated with increased *CR2* promoter activity with differing cell context-dependent effect sizes.

Interestingly, we observe *CR2* promoter RBPJ- κ sites functioning as activators in unstimulated cells but were unable to abolish Notch activation with RBPJ- κ mutants or deletion constructs. These results suggest that the effects of Notch signaling on *CR2* transcription was not solely mediated by transcription factors binding to RBPJ- κ and may involve the endogenous chromatin environment. Our co-culture and ChART-PCR data show increased *CR2* transcript expression, with significant increases in chromatin accessibility predominantly around the distal RBPJ- κ binding site at early time points of Notch signal. We also note that *CR2* mRNA activation in REH cells follows strikingly different kinetics as compared to *HES1* activation in co-culture assays: *CR2* transcripts increase throughout the 11-day time course while *HES1* transcripts peak at the earliest time point tested, declining thereafter but remaining above baseline. These results may reflect an early transcriptional response involving *HES1* up-regulation (Bonev et al., 2012; Monk, 2003); while sustained signaling may activate a distinct transcriptional network as reported previously in non-B cell context (Kawamata et al., 2002). Accordingly, our data may reflect cell-type specific endogenous chromatin context at the *CR2* promoter that modulates Notch signaling, or alternatively indirect *CR2* promoter activation coinciding with *HES1* feed-back inhibition.

Using a co-culture system, we consistently observed induction of *CR2* surface expression in a minor population of pre-B ALL cells following prolonged γ -secretase-dependent Notch signaling. Additionally, we showed that Notch signals increased *IgL λ* expression in these pre-B ALL cells. Similar to *CR2*, expression of either light chain is crucial for a functional

BCR-mediated response and is characteristic of mature B cells (Nemazee, 2017). Expression of both *CR2* and *IgLλ* mRNA in Notch-activated pre-B ALL cells may reflect an altered gene expression profile skewed towards a more mature state in a sub-population of cells.

We observed dynamic changes to chromatin accessibility at specific sub-regions of the *CR2* promoter that were not always directionally correlated with transcriptional activation. Further work is required to delineate if these changes to chromatin play a role in priming the locus for transcription. Additionally, we did not observe remodeling of chromatin surrounding the CRS. The changes in chromatin accessibility that we observed were small in magnitude, however it should be noted that these data were generated using a bulk population of cells with variable levels of *CR2* mRNA and protein induction, of which only a very minor population had detectable surface levels of CR2.

Isolation and culture of primary human bone marrow-derived pre-B cells is technically challenging. Future studies utilizing single-cell sequencing technology may overcome the limitations associated with harvesting these rare samples. As a step towards resolving some of these challenges, we have successfully utilized an *in vitro* cell line model representative of human pre-B cells. The results presented here represent the first evidence that human *CR2* can be transcriptionally activated in pre-B ALL cells by Notch signals. However, whilst our study utilizing human cell lines in a co-culture system may simulate Notch signals *in vitro*, it does not fully recapitulate the complex bone marrow microenvironment. Therefore, our data support the need for future studies utilizing single-cell sequencing to directly examine human pre-B cells from their native microenvironment to further elucidate biological cues required to elicit robust CR2 expression during B cell development.

4.1. Concluding remarks

568 Combined, our study suggests that Notch signaling functions in a cell-context dependent
569 manner in regulating *CR2* transcription and cell surface expression. Using a unique co-culture
570 system in which pre-B ALL cells are activated by Notch signals, we observe transcription of
571 B cell maturation markers *CR2* and *IgLλ*. Further studies are warranted to determine if Notch
572 signals also play a role in the developmental transition from pre-B to mature B cells, a key
573 step in the maintenance of self-tolerance.

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581

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585

586 **Declaration of Interest**

587 The authors declare no conflict of interest.

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813 **Table**

814 **Table I: List of primers used for ChART-PCR.** Primer positions for *CR2* ChART-PCR are
815 listed in relation to the TSS, whilst *β2M* and *UMOD* primer positions are listed as genomic
816 coordinates.

Primer	Sequence (5' → 3')	Position	Product size (bp)
CR2pF_F	GATTCGCCTATCCCAGTTAACC	-1209/-1188	111
CR2pF_R	AGTTTCTGTGGGTTCGGTCAG	-1099/-1118	
CR2pE_F	TGGACCAGGAAAAACAAAGGTT	-867/-846	180
CR2pE_R	TGGTTCACCTAAGGACTTTCTGTTCC	-688/-712	
CR2pD_F	TCTGGGTGACAAGTATATGGGA	-542/-521	105
CR2pD_R	ATTTAGGAACAGCAGCCACTTC	-438/-459	
CR2pC_F	CAAACCTGGGAGATGAATCCAC	-410/-390	148
CR2pC_R	CACAAGTTAGTACTCACCT	-263/-282	
CR2pB_F	GATTACTAAGGGTGAGTACTAAGTTG	-290/-265	189
CR2pB_R	GTGGGACAAGCGGGAG	-102/-117	
CR2pA_F	TCCCGCTTGTCCCACCCTCA	-116/-97	104
CR2pA_R	AGGCGGGCCCTTAAATAGTGTCC	-13/-35	
CR2pZ_F	CACTATTTAAGGGCCCGCC	-32/-14	165
CR2pZ_R	GACGAGAGCCAAGAAAACCC	+133/+114	
CR2pY_F	ATAAACCGCCTGGTCCTGAT	+300/+319	175
CR2pY_R	GATGAGCACCTGCAGAAATCC	+474/+454	
CRS1_F	TCCTTTGCCAAGCTTCATTT	+3935/+3954	152
CRS1_R	TGGAGAGTTCAAGCAGGAGA	+4085/+4066	
CRS2_F	GGAGCATACCAAATCCATTCCT	+4229/+4250	191
CRS2_R	GTAATGCACTGGAAGGAGAAGG	+4419/+4398	
CRS3_F	CAGTCTGTCTCTTTCTGTTGGATT	+4490/+4513	187
CRS3_R	TGCAATGTTCTATTCCCAAACA	+4676/+4656	
CRS4_F	GAAGGATGTTCTTGTAGGGTTGC	+4850/+4872	102
CRS4_R	AGGGTTCTCATGCTTGTATACCT	+4951/+4929	
CRS5_F	ATAATGATTTTCCCTCCTACCTGC	+5125/+5148	117
CRS5_R	GACTTTACCTGGAATGGCTTGG	+5241/+5220	
CRS6_F	GTGTCTGAATGTCAAGTCACCA	+5329/+5350	98
CRS6_R	ACTGGACCCTCTTCACATCTTT	+5426/+5405	
CRS7_F	TATGATCTGCCTTGTGACTTGG	+5486/+5507	138
CRS7_R	TACGGATCACACTTTCTGGATG	+5623/+5602	
CRS8_F	GCACAGAGTAAGCACTTGGAGA	+5907/+5928	171
CRS8_R	CCTTAGCTGTGACCCCAAATA	+6077/+6056	
CRS9_F	GACTTTCACCCGTACCTGTCTC	+6139/+6160	96
CRS9_R	TCAGTTGTAAGGCCCTCCTAGA	+6234/+6213	
β2M ChART_F	TTCACTAGGACCTTCTCTGAGC	Chr15: 45,003,161 – 45,003,182	173
β2M ChART_R	ATTCGAGAAAACGGAAACCCAG	Chr15: 45,003,312 –	

		45,003,333	
UMOD ChART_F	AGTAACCAAAGGAGATGATGCAA	Chr16: 20,364,821 – 20,364,843	181
UMOD ChART_R	CACTCTCACACTGACTTTTGGAA	Chr16: 20,364,979 – 20,365,001	

817

Figure Legends

Figure 1: E12, E47, RBPJ- κ and Ikaros bind to the *CR2* promoter in Ramos B cells.

(A) Schematic depiction of known and putative transcription factor binding sites within the *CR2* promoter. Starting from the 5' end, motifs include the distal RBPJ- κ site overlapping an Ikaros site, Sp1 site, proximal RBPJ- κ site, AP2, two E box sites and the TATA box. (B) Specific enrichment of *CR2* promoter fragment was observed in chromatin samples from Ramos cells immunoprecipitated using antibodies against E12 (lane 2), E47 (lane 3), Ikaros (lane 4), RBPJ- κ (lane 5) and RNA polymerase II (lane 9; positive control) compared to samples treated with IgG (lane 7) or no antibody (lane 8). No amplification was observed using the no template control (lane 11) and increasing amounts of product was observed using serially diluted input samples (lanes 12-15). Lane 1 contains DNA marker (M), and lanes 6 and 10 contain no samples. These data are representative of at least three independent experiments showing similar levels of enrichment of *CR2* promoter fragments for each antibody tested.

Figure 2: RBPJ- κ sites regulate basal *CR2* transcriptional activity in non-*CR2* expressing cells.

(A) A schematic depiction of RBPJ- κ sites within the *CR2* promoter (upper panel) and sequence of wild-type and mutant sequences (lower panel). The *CR2* promoter contains either the wild-type promoter (1.2-*CR2*-WT), or mutation to the proximal RBPJ- κ site (RBPJ κ -mut1), distal RBPJ- κ (RBPJ κ -mut2), or both RBPJ- κ sites (RBPJ κ -mut1/2), TATA box and a TSS (bent arrow) that transcribes the luciferase reporter gene. The distal RBPJ- κ site (black box) overlaps with an Ikaros consensus site (bold bases); the proximal RBPJ- κ site (black box) is adjacent to a functional Sp1 site (grey box); mutations at both RBPJ- κ sites are

underlined (lower panel). REH ($n \geq 4$) (B), K562 ($n=3$) (C), or Ramos ($n=3$) (D) cells were transfected with either the 1.2-CR2-WT or RBPJ- κ mutants alone (RBPJ- κ -mut1 or RBPJ- κ -mut2) or in combination (RBPJ- κ -mut1/2). Luciferase activity of all constructs were normalized to the 1.2-CR2-WT construct's activity. Significance was calculated using Student's t-test, * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$.

Figure 3: Notch activation of 1.2-CR2-WT constructs is modulated through RBPJ- κ consensus sites in a cell context dependent manner.

Co-transfection of empty vector (white bars) or Notch activation expression construct (NIC) (black bars) expression constructs with either the 1.2-CR2-WT, proximal RBPJ- κ mutant (RBPJ- κ -mut1), distal RBPJ- κ mutant (RBPJ- κ -mut2) or both RBPJ- κ sites mutated (RBPJ- κ -mut1/2) in REH ($n \geq 4$) (A), K562 ($n=3$) (B), or Ramos ($n=3$) (C) cells. Luciferase activity of constructs that were co-transfected with NIC were normalized to the results of corresponding constructs co-transfected with the empty vector for each respective cell line to determine fold change. Results were depicted as means $\pm$ SEM of at least three biological replicates. Significance was calculated using Student's t-test, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 4: The region between both RBPJ- κ sites within the CR2 promoter contains lymphocyte-specific repressor elements that regulate Notch signaling.

(A) Schematic depiction of deletion constructs of the CR2 promoter. Starting at the 5' end, transcription factor motifs within the CR2 promoter include the distal RBPJ- κ site, Sp1 site, proximal RBPJ- κ site, AP2 site, two E box sites and the TATA box. Promoter deletion constructs were named in reference to the remaining regions upstream of the CR2 TSS (bent arrow), from the full length CR2 promoter (1.2-CR2-WT) to the TATA box (-39). REH ($n \geq 3$) (B), or K562 ($n=4$) (C) cells were co-transfected with either the empty vector (white bars), or

NIC (black bars) expression construct and either the 1.2-CR2-WT or promoter deletion constructs. Luciferase activity of constructs that were co-transfected with Notch activation expression construct (NIC) were normalized to the results of corresponding constructs co-transfected with the empty vector for each respective cell line to determine fold change. Results were depicted as means $\pm$ SEM of at least three biological replicates. Significance was calculated using Student's t-test, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 5: Notch activation of -315-CR2-luciferase constructs does not require intact E box sites.

Schematic depiction of E box sites in reporter constructs containing the *CR2* promoter. A wild-type E-box site is represented by a black bar, whilst a mutated E-box site is represented by a grey bar; both E-box sites are 5' to a TATA box and a TSS (bent arrow) that transcribe the luciferase reporter gene. Co-transfection of Notch activation expression construct (NIC) (black bars), or empty vector (white bars) expression constructs, with either wild-type minimal promoter (315-CR2-WT) or E box mutants in K562 cells. Neither E box sites are necessary for transcriptional activation by Notch signaling molecules. Results are expressed as the mean luciferase levels normalized to *renilla* $\pm$ SEM ($n \geq 3$). Significance was calculated using Student's t-test, * $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$.

Figure 6: Notch activation co-cultures induce endogenous REH *CR2* transcription and a modest increase in *CR2* expression in Ramos cells.

(A) Co-cultured REH cells were harvested sequentially every two days, and transcript abundance of target genes analyzed by qPCR. As a control, REH cells were harvested without co-culture (uninduced) (white bar, $n=5$). Notch activated REH cells (black bars, $n=5$) expressed *CR2* mRNA across 11 days of co-culture. Except for day 11, *CR2* mRNA

expression remains silenced in Notch-inhibited REH cells (grey bars, n=5). (B) Expression of *HES1* in basal REH suspension cells (white bar, n=24) and Notch inhibited co-cultures (grey bars, n=6) did not change over 11 days. Notch activated REH cells (black bars, n=6) were induced to express *HES1* transcript by day 3 of co-culture and expression levels diminished over time. (C) Expression of *IgLλ* in basal REH cells (white bar, n=14) and Notch inhibited co-cultures (grey bars, n=3) did not change over 11 days. Notch activated REH cells (black bars, n=3) showed elevated expression of *IgLλ* transcript compared to basal REH cells. (D) Co-cultured Ramos cells were harvested sequentially every two days, and transcript abundance of target genes analyzed by qPCR. As a control, Ramos cells were harvested without co-culture. *CR2* mRNA expression within co-cultured Ramos cells was significantly elevated across 11 days of Notch activation (black bars, n=3), compared to uninduced Ramos cells (white bar, n=15). *CR2* mRNA expression was similar between Notch inhibited Ramos cells (grey bars, n=3) and uninduced Ramos cells (white bar). (E) *HES1* mRNA expression was significantly reduced when Notch signaling was activated (black bars, n=3) or inhibited (grey bars, n=3) compared to uninduced Ramos (white bar, n=15). Significance was calculated using Student's t-test. Significance depicted in (E) were compared between uninduced Ramos cells with each individual time point of Notch activated or inhibited co-cultured Ramos cells. Expression of *CR2*, *IgLλ* and *HES1* were normalized to expression of β -actin and data represented as mean $\pm$ SEM, *p $\leq$ 0.05; **p $\leq$ 0.01; ****p $\leq$ 0.0001.

Figure 7: REH cells and Ramos cells demonstrate differing effects of Notch co-culture on CR2 surface expression.

CR2 cell surface expression was measured by flow cytometry after 4 (A-D) or 11 (E-H) days of co-culture. A small percentage of co-cultured REH cells exposed to Notch signals (Notch activation) stained positive with PE-conjugated *CR2* antibody at day 4 (D) and day 11 (H)

compared to the PE-conjugated isotype control (C, G, respectively). This effect was completely abolished when co-cultured cells were treated with a chemical Notch inhibitor (Notch inhibition) at both day 4 (B) and day 11 (F). Isotype matched controls (A and E, respectively) were unchanged in Notch inhibited co-cultures. The number of cells staining positive after Notch co-culture remained low, but consistent and detectable in four independent experiments. Surface expression of CR2 was measured on Ramos cells and an IgG isotype control was utilized to set the CR2-positive gate for uninduced (I), Notch activated (J), or Notch inhibited (K) cultures. (M) Notch activated Ramos cells showed higher CR2 expression compared to (L) the uninduced control. In contrast, (N) Notch inhibited Ramos cells exhibited lower CR2 expression than the uninduced Ramos. (O) Notch activated Ramos cells displayed increased mean fluorescence intensity (MFI) compared to uninduced Ramos control. Notch inhibited Ramos cells displayed decreased MFI compared to uninduced control. Similar results were observed across 4 independent experiments, and a representative experiment is shown. Results were depicted as mean $\pm$ SEM of three biological replicates. Significance was calculated using Student's t-test, ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 8: Notch activated REH cells show dynamic changes in chromatin accessibility surrounding the distal RBPJ- κ site compared to basal CR2-negative REH cells.

REH cells were harvested at two-day intervals for a total of nine days of co-culture following Notch activation. As a control REH and Ramos cells were cultured separately and harvested for ChART-PCR assay. ChART-PCR was performed across the *CR2* promoter (A-E) and CRS (F-J) in uninduced REH (grey dashes), uninduced Ramos (black dotted lines) and Notch activated co-cultured REH cells (black lines). Each point on the graph represents the mid-point of the amplicon examined. A not-to-scale diagram is depicted below the basal chromatin accessibility graphs at the *CR2* regulatory regions of REH and Ramos cells (A, F),

representing the *CR2* promoter and CRS, including the RBPJ- κ sites (grey triangles), the TSS (bent arrow) and first exon of the gene. The 3' region immediate to the distal RBPJ- κ site showed increased chromatin accessibility on days 5 and 7 (black lines, C and D, respectively) of Notch activation, compared to uninduced REH levels (grey dashes). Chromatin accessibility at all regions examined across the *CR2* regulatory regions were normalized to the negative control (*UMOD*) gene promoter. The horizontal solid lines ($y=1$) represent the baseline of inaccessibility in reference to the negative control, whilst the vertical dotted lines ($x=0$) represent the TSS of the *CR2* gene. The values of the x-axis represent the nucleotide position in relation to the TSS. Results were depicted as mean $\pm$ SEM of three biological replicates. Significance was calculated in comparison to uninduced REH cells using Student's t-test, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 9: Sequence-specific protein complexes bind to the overlapping RBPJ- κ /Ikaros motif within REH cell nuclear extracts.

EMSAs were performed using REH nuclear extract. An oligonucleotide spanning -538 to -503 of proximal promoter sequence incubated with increasing (15x-60x molar excess) amount of (A) unlabeled wild-type cold competitor (from lanes 3 to 5), or (B) a 7 bp mutation within the core RBPJ- κ /Ikaros motif with REH nuclear extract (from lanes 3 to 5). (C) Supershift assays with 2 μ g and 4 μ g of RBPJ- κ antibody (lanes 3 and 4) confirms RBPJ- κ binding within the protein-DNA complexes in REH nuclear extract with the formation of a novel complex D. Data are representative of three independent biological replicates. -NEX: No nuclear extract; +NEX: addition of nuclear extract; Ab: antibody.

Figure 5

Figure 6

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Figure 7

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Figure 9

CRediT authorship contribution statement

Han Leng Ng: Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Rhonda L. Taylor:** Conceptualisation, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Jessica Cheng:** Validation, Formal Analysis, Investigation, Data Curation, Writing – Review & Editing, Visualization. **Lawrence J. Abraham:** Resource, Supervision, Project administration. **Elizabeth Quail:** Resource, Writing – Review & Editing, Supervision, Project administration. **Mark N. Cruickshank:** Conceptualisation, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Daniela Ulgiati:** Conceptualisation, Resource, Writing – Review & Editing, Supervision, Project administration, Funding acquisition

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Supplementary Material

Supplementary Data (Mol. Immunol.) - FINAL.docx