Regulatory architecture of the RCA gene cluster captures an intragenic TAD boundary, CTCF-mediated chromatin looping and a long-range intergenic enhancer

Jessica Cheng[a], Joshua S. Clayton[b,c], Rafael D. Acemel[d], Ye Zheng[e,f], Rhonda L. Taylor[b,c], Sündüz Keleş[f,g], Martin Franke[d], Susan A. Boackle[h,i], John B. Harley[j,k,l], Elizabeth Quail[a,m], José L. Gómez-Skarmeta[d] and Daniela Ulgiati[a,1]

[a]School of Biomedical Sciences, The University of Western Australia, Crawley, WA 6009, Australia; [b]Harry Perkins Institute of Medical Research, QEII Medical Centre, Nedlands, WA 6009, Australia; [c]Centre for Medical Research, The University of Western Australia, Crawley, WA 6009, Australia; [d]Centro Andaluz de Biología del Desarrollo, Consejo Superior de Investigaciones Científicas/Universidad Pablo de Olavide, Andalucía 41013, Sevilla, Spain; [e]Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, United States; [f]Department of Statistics, University of Wisconsin-Madison, Madison, WI 53706, United States; [g]Department of Biostatistics and Medical Informatics, University of Wisconsin-Madison, Madison, WI 53792, United States; [h]Department of Medicine, Division of Rheumatology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045, United States; [i]Rocky Mountain Regional Veterans Affairs Medical Center, Aurora, CO 80045, United States; [j]Center for Autoimmune Genomics and Etiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, United States; [k]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH 45267, United States; [l]US Department of Veterans Affairs Medical Centre, US Department of Veterans Affairs, Cincinnati, OH 45220, United States; and [m]School of Molecular Sciences, The University of Western Australia, Crawley, WA 6009, Australia

[1]Correspondence: daniela.ulgiati@uwa.edu.au

ABSTRACT

The Regulators of Complement Activation (RCA) gene cluster comprises several tandemly arranged genes with shared functions within the immune system. RCA members, such as complement receptor 2 (*CR2*), are well-established susceptibility genes in complex autoimmune diseases. Altered expression of RCA genes has been demonstrated at both the functional and genetic level, but the mechanisms underlying their regulation are not fully characterised. We aimed to investigate the structural organisation of the RCA gene cluster to identify key regulatory elements that influence the expression of *CR2* and other genes in this immunomodulatory region. Using 4C, we captured extensive CTCF-mediated chromatin looping across the RCA gene cluster in B cells and showed these were organised into two topologically associated domains (TADs). Interestingly, an inter-TAD boundary was located within the *CR1* gene at a well-characterised segmental duplication. Additionally, we mapped numerous gene-gene and gene-enhancer interactions across the region, revealing extensive co-regulation. Importantly, we identified an intergenic enhancer and functionally demonstrated this element upregulates two RCA members (*CR2* and *CD55*) in B cells. We have uncovered novel, long-range mechanisms whereby autoimmune disease susceptibility may be influenced by genetic variants, thus highlighting the important contribution of chromatin topology to gene regulation and complex genetic disease.

SIGNIFICANCE

The complement system is a complex network of protein effectors and regulators that play a key role in immunity. Several regulators of complement response are clustered within Regulators of Complement Activation (RCA) gene family. Its members are all functionally, structurally, and genetically related. However, the functional relevance of this close gene organisation is unknown. We show that the clustering of the RCA members is due to shared long-range regulatory elements and physical chromatin looping. We also reveal that the RCA genes are divided into two adjacent chromatin domains and a domain boundary falls within the body of an expressed gene (*CR1*). Overall, our findings in the RCA cluster offer insights into their evolution, biology and roles in disease.

INTRODUCTION

The complement system is a major immune network of soluble proteins and membrane receptors which elicit potent, innate responses against pathogens, immune complexes and apoptotic cells (1). The complement system is activated by one of three major pathways (classical, alternative or lectin), triggering a series of proteolytic cleavage events which ultimately converge to form the C3 convertase. The C3 convertase enzyme catalyses, in part, the formation of complement effector peptides (C3a, C5a, C3b and C5b) which mediate local inflammation, cell lysis and cell clearance (1). Additionally, complement components are capable of binding numerous immune cell types and activating other immune pathways, including adaptive B cell and T cell responses (2, 3). Complement therefore represents an important bridge between the innate and adaptive immune systems and allows for effective co-ordination of immune responses (4).

The complement cascade is intricately controlled to ensure a sufficient immune response is generated while preventing damage to self (1). In humans, a number of these regulatory proteins are located in a gene cluster known as the Regulators of Complement Activation (RCA) on chromosome 1q32.2. This includes the plasma protein C4 binding protein (encoded by alpha (*C4BPA*) and beta (*C4BPB*) subunits), and several membrane receptors; decay-accelerating factor (DAF, *CD55*), complement receptors 2 and 1 (*CR2* and *CR1*), and membrane co-factor protein (MCP, *CD46*) (5). Several duplicated pseudogenes within the RCA cluster have also been identified (6, 7) of which CR1-like (*CR1L)* has been best characterised (8). All members of the RCA gene cluster are composed of tandem 60 – 70 amino acid motifs known as short consensus repeats (SCRs) which bind complement components and primarily regulate the complement response through inhibition or activation of C3 convertase (1, 5). As such, this gene cluster is believed to have been derived from complex duplications of a common ancestral gene, followed by the diversification of function (5). In addition to their important roles in innate immune responses, members of RCA gene cluster are involved in the processes of tissue injury, inflammation and apoptosis. Accordingly, they have been implicated in a range of inflammatory and autoimmune disorders (9-12).

A role for complement receptors CR2 and CR1 in the autoimmune disease, Systemic Lupus Erythematosus (SLE) is well established. SLE is characterised by the presence of antibodies directed against nuclear antigens and has a complex aetiology with a strong genetic component (13, 14). CR2 and CR1 regulate B cell responses by modulating B cell activation and antibody production upon binding of complement-tagged antigens (15, 16). Aberrant expression of CR2 on the surface of B cells has been demonstrated both in mouse models of the disease (17, 18) and SLE patients (19, 20), which functionally contributes to B cell autoreactivity and autoimmune disease susceptibility (21-23). The *CR1* gene contains an 18 kb intragenic segmental duplication, known as ‘low copy repeat 1’ (LCR1). This repeat results in different structural alleles of *CR1* with recognised association to SLE susceptibility, but the functional role of this large genomic duplication is not well understood (24). The *CR2* gene has also been implicated in SLE at the genetic level through linkage analyses (25-27) and

68 association studies (27-29). A SNP within the first intron of *CR2* (rs1876453) was shown to alter the
69 expression of the neighbouring gene (*CR1*) without influencing CR2 expression (29), indicating that
70 expression of these genes in the RCA cluster may be co-regulated. Functionally, rs1876453 was
71 shown to influence the binding affinity of CCCTC-binding factor (CTCF) to *CR2,* suggesting that
72 CTCF may have a role in co-regulating expression of *CR2, CR1* and the RCA gene cluster (29).
73
74 CTCF is an important transcription factor which was first identified as an insulator of gene expression,
75 and is now known to have several roles in gene regulation. Additionally, CTCF has been shown to
76 play a critical role in forming chromatin loops and mediating interactions between distal loci (30).
77 Chromatin loops are organised into genomic compartments known as topologically associated
78 domains (TADs) (31). The current model proposed to explain TAD formation involves CTCF and the
79 cohesin complex, whereby loops are dynamically formed through 'loop extrusion' between distal
80 CTCF sites in convergent or 'forward-facing' orientation (32). TADs are recognised to be constitutively
81 maintained in different cell types but may alternate between active ("A") and inactive ("B")
82 compartment types depending on the cellular context (33, 34). While genes within the same TAD tend
83 to be co-expressed, not all genes within a TAD are necessarily expressed simultaneously. Rather, in
84 a given context, TADs restrict chromatin interactions between genes and distal regulatory elements,
85 such as enhancers, to ensure that gene expression is properly controlled (33, 35).
86
87 Enhancers represent an important class of distal-regulatory elements which are largely responsible
88 for governing cell-type specific gene expression patterns. Enhancers bind transcription factors to
89 upregulate expression of genes and are located distal to gene promoters in the linear genome but are
90 positioned in close proximity by chromatin looping (36). Importantly, the majority of disease-
91 associated SNPs from genome-wide association studies (GWAS) fall within enhancer regions (37).
92 Enhancer elements have been predicted in the genome by the presence of epigenetic marks such as
93 enrichment of H3K27ac and expression of short, bi-directional transcripts termed enhancer RNA
94 (eRNA) (38, 39). However, enhancers can simultaneously regulate expression of multiple genes,
95 regulate genes large distances away and skip their neighbouring gene/s, which has hindered the
96 identification of their target gene/s (36). The mapping of chromatin interactions through high-
97 throughput chromatin conformation capture technologies, such as Hi-C and capture Hi-C (CHi-C), has
98 aided in the identification of enhancer targets. However, these data are still limited by resolution and
99 the physical chromatin interactions detected using these methods may not necessarily be functional.
100 As such, experimental validation of enhancers and physically associating enhancer-gene pairs is
101 imperative to determine their influence on gene expression (36, 40). In addition, large repetitive
102 regions in the genome, such as the LCR in *CR1,* cannot be uniquely aligned and readily analysed
103 using next-generation sequencing technologies. As a result, these regions are under-represented in
104 high-throughput epigenetic and chromatin conformation capture datasets.
105
106 The aim of this investigation was to explore the structural organisation of the RCA gene cluster in
107 order to identity transcriptional elements which may co-regulate the expression of genes in this

important immunomodulatory cluster. In this study, we examined genomic interactions across the RCA gene cluster using chromosome conformation capture and showed that long-range chromatin interactions are involved in the co-regulation and co-expression of several RCA members in the B cell lineage. Further, we identified an intragenic TAD boundary which discretely separates chromatin interactions in the RCA gene cluster into two domains and co-localises to the intragenic segmental duplication in *CR1*. Importantly, we functionally interrogated a putative long-range enhancer and demonstrated that it co-regulates two genes within a TAD in B cells. Collectively, we have revealed how three-dimensional chromatin organisation plays an important role in regulating the RCA gene cluster and have uncovered novel regulatory loci which govern the expression of these genes.

118 **RESULTS**

119

120 **Chromatin interactions within the RCA gene cluster are organised into two TADs**

121 To investigate the structural arrangement of the RCA gene cluster in B cells, we examined raw Hi-C
122 data in the GM12878 B lymphoblastoid cell line from Rao *et al.* (41) at 10 kb resolution. The intergenic
123 region between *CD55* and *CR2,* and loci across the complement receptor genes (*CR2* and *CR1*)
124 engaged in highly frequent interactions with loci more than 400 kb upstream near *C4BPB* and *C4BPA*
125 (**Figure 1A**). No notable interactions between these regions with downstream genes *CR1L* and *CD46*
126 were observed (**Figure 1A**), indicating that chromatin interactions in this region may be directionally
127 constrained and organised to more than one TAD. This pattern of interaction was consistent across
128 the 6 other cell lines also examined at 10 kb resolution in Rao *et al.* (41) Hi-C dataset (K562, HMEC,
129 NHEK, IMR90, KBM7, HUVEC) (**Supplementary Figure 1**). The *CR1* intragenic duplication (*CR1*
130 exon 5 – 20) is included on the human reference genome, but Hi-C interaction data was filtered out at
131 this region in all datasets due to sequence repetitiveness and sequence unmappability (**Figure 1A,**
132 **Supplementary Figure 1**).

133

134 To further clarify the TAD organisation of the RCA gene cluster, we performed 4C-seq in an
135 analogous B lymphoblastoid cell line (B-0028). It is well established that in the B cell lineage, only
136 membrane-bound RCA members (*CD55, CR2, CR1, CD46)* are expressed, not soluble protein
137 members (*C4BPB* and *C4BPA*) (11). We confirmed this expression pattern using qPCR in the B-0028
138 cell line (**Supplementary Figure 2**). As CTCF plays an important role in chromatin looping, we
139 selected 4C viewpoints (VP) from CTCF sites utilising B cell ChIP-seq data. (GM12878) Specifically
140 viewpoints were selected which co-localised to regions of highly frequent interactions observed in the
141 Hi-C data (**Figure 1B**). These viewpoints included; the intergenic region between *CD55* and *CR2*
142 (VP1), intron 1 of *CR2* (VP2), which is the CTCF site influenced by SLE-associated SNP rs1876453
143 (*29*), and intron 1 of *CR1* (VP3) (**Figure 1B**). We also selected a 4C viewpoint from a CTCF binding
144 site within intron 29 of *CR1* (VP4) which did not markedly engage in chromatin interactions with the
145 upstream region of this gene cluster (**Figure 1B**). We confirmed enrichment of CTCF at these VPs in
146 the B-0028 cell line using ChIP-qPCR (**Supplementary Figure 3**).

147

148 4C maps from VP1, VP2 and VP3 yielded consistent 4C signal peaks at upstream CTCF sites near
149 RCA member *C4BPB* and within non-RCA member *YOD1* (**Figure 1C**, asterisks), corresponding to
150 Hi-C data (**Figure 1A**). These CTCF viewpoints also consistently interacted with the CTCF site within
151 intron 6 of RCA gene *CD55* (**Figure 1C**, asterisks). Chromatin interactions from VP1 – 3 did not
152 extend to CTCF sites upstream of *YOD1* or downstream of *CR1* exon 7 (**Figure 1C**). In contrast, VP4
153 produced a unique 4C map whereby interactions were constrained to a 60 kb region downstream of
154 this viewpoint within the *CR1* gene body and did not extend upstream (**Figure 1C**). We replicated 4C-
155 seq from these CTCF viewpoints in another B cell line (B-0056), whereby CTCF interactions in the
156 RCA gene cluster were also organised to two discrete regions (**Supplementary Figure 4**), potentially
157 representing two TADs with an inter-TAD boundary located within the *CR1* gene itself. However, like

158 Hi-C, reads mapping to the *CR1* segmental duplication were filtered out during 4C-seq data
159 processing.
160
161 To refine the location of the TAD boundaries in the RCA gene cluster, we used a customized mHi-C
162 pipeline which probabilistically assigns multi-mapping reads in Hi-C experiments to their most likely
163 genomic position (42). Indeed, mHi-C successfully recovered Hi-C interactions across the *CR1*
164 segmental duplication in the GM12878 cell line at 5 kb resolution (**Figure 2A**). These interactions
165 were visualised as virtual 4C signal from RCA gene 5' upstream promoter regions using Juicebox (43)
166 (**Figure 2B**). In line with our previous observations, interactions from the promoters of upstream RCA
167 genes *C4BPB, C4BPA, CD55, CR2* and *CR1* were localised to a distinct domain region compared to
168 downstream RCA genes *CR1L* and *CD46* (**Figure 2B**). After recovering multi-mapping reads, we
169 used spectralTAD (44) to systematically call TADs. Using this TAD caller, TAD boundaries were
170 assigned at the intergenic region upstream of *YOD1,* intron 11 of *CR1* and the intergenic region
171 downstream of *CD46*, placing RCA genes *C4BPB, C4BPA, CD55, CR2 and CR1* in TAD 1, and *CR1L*
172 and *CD46* in TAD 2 (**Figure 2B**).
173
174 To corroborate these findings, we examined CTCF enrichment and motif binding orientation at the
175 TAD boundaries in the RCA gene cluster. Each TAD was flanked by CTCF enrichment in the
176 GM12878 B cell line and convergent CTCF motifs, characteristic of TAD boundaries. Importantly, the
177 inter-TAD boundary was directly located to the *CR1* segmental duplication. Like other highly
178 repetitive, unmappable genomic regions, CTCF enrichment at this region is underrepresented in the
179 high-throughput datasets such as the ENCODE portal following the typical ChIP-seq processing
180 pipeline. Therefore, we examined raw CTCF ChIP-seq signal in the GM12878 cell lines and observed
181 enrichment of CTCF at this repeat element underlined by reverse orientation CTCF motifs at each
182 repeat segment which has not been previously defined (**Figure 2B**). Enrichment of CTCF at the *CR1*
183 repeat segments (*CR1* intron 7, 15 and 23) was observed across all cell lines in this ENCODE CTCF
184 signal data set (**Supplementary Figure 5**). Taken together, our data shows that the RCA gene
185 cluster is divided into two TADs and reveals that a TAD boundary is located within the *CR1* gene at
186 the intragenic segmental duplication.
187
188 **Putative B cell enhancers in the RCA gene cluster were predicted to regulate multiple RCA**
189 **genes**
190 CTCF plays an important role in establishing long-range contacts within TADs and mediating
191 enhancer-gene interactions (30). As CTCF-mediated chromatin looping was identified in the RCA
192 gene cluster in B cells **(Figure 1**), we speculated that enhancer elements were present in this region
193 that may regulate these genes in this cell lineage. To identify putative enhancers in the RCA, we
194 leveraged a well-known enhancer database, GeneHancer. This integrates enhancer datasets from
195 multiple consortium-based projects and other functional datasets to generate enhancer predictions
196 and identify their potential gene-targets (45). Confidence scores for each enhancer prediction
197 (GeneHancer score) and enhancer-gene prediction (gene-association score) were computationally

198 assigned, based on the level of evidence retrieved. A strength of this database is that predicted
199 enhancers can be classified as “double elite” if both their GeneHancer and gene-association scores
200 were derived from more than one source of data, thus representing a prediction which is more likely to
201 be functional (45). Numerous predicted enhancers on GeneHancer were identified across TAD 1 and
202 TAD 2, but only a subset of these were classified as “double elite” (**Figure 3A**, **Figure 3B**,
203 **Supplementary Table 4**).
204
205 Enhancers are important in cell-type specific regulation of gene expression and act by looping to their
206 target gene promoters (46). To identify active enhancers that were most likely functional in B cells, we
207 examined epigenetic marks characteristic of enhancers, such as H3K27ac and DNase I
208 hypersensitivity (DHS) within candidate regions. We identified four candidate B cell enhancers (BENs)
209 in TAD 1 that showed strong consistent H3K27ac enrichment and DHS in both B cell lines and
210 primary B cells (**Figure 3C**). These candidate enhancers were located within *CD55* (BEN-1) or the
211 intergenic region between *CD55* and *CR2* (BEN-2, BEN-3 and BEN-4) (**Table 1**). Furthermore, each
212 candidate BEN contained binding sites for numerous transcription factors (based on ENCODE ChIP-
213 seq data) including those important in B cell development, such as early B cell factor 1 (EBF1) (47)
214 and PAX5 (48), and general regulatory factors (eg. EP300, CTCF and RNA polymerase II) (**Table 1**).
215 The four BENs identified were supported by multiple lines of evidence to be active enhancer elements
216 in B cells and were prioritised for further investigation.
217
218 Predicted enhancers on GeneHancer were assigned putative gene targets using multiple methods
219 and datasets, including expression quantitative trait loci (eQTL) analysis, enhancer-promoter
220 interactions generated by capture Hi-C in the GM12878 cell line (CHi-C) and eRNA-mRNA co-
221 expression from the FANTOM5 Enhancer Atlas (38, 45, 49). Each candidate BEN was predicted to
222 regulate multiple genes, including RCA genes (*C4BPA, CD55, CR2*, *CR1* and *CD46*) and non-RCA
223 genes (*PIGR, FCAMR, C1orf116*) (**Figure 3D**, **Supplementary Table 5**). However, only interactions
224 between BEN-1 and *CD55*, BEN-2 and *CR2,* and BEN-3 and *CR2*, represented high-confidence
225 (“elite”) associations, being identified by more than one contrasting method (**Figure 3E**,
226 **Supplementary Table 5**). Of these, only BEN-1 was predicted to regulate a gene (*CD46*) located
227 downstream of the intragenic TAD boundary in *CR1* (**Figure 3D**). However, this predicted interaction
228 had the lowest score among all gene-enhancer predictions for these BENs (**Figure 3D**,
229 **Supplementary Table 5**). Although these gene-enhancer interactions were based on bioinformatic
230 predictions, this highlighted the potential for the RCA genes to be co-regulated in B cells.
231
232 **Candidate B cell enhancers in the RCA gene cluster were functional *in vitro***
233 To test the functionality of each BEN, we performed luciferase reporter gene assays using a
234 constitutive minimal promoter (SV40) to drive luciferase expression. Each BEN was cloned upstream
235 of the SV40 promoter in both forward and reverse orientation and the transcriptional effects were
236 assayed in a panel of B cell lines (Reh, Raji, B-0028, SKW) and a non-B cell control (HepG2, liver
237 cell-type) (**Figure 4**). Interestingly, transcriptional activity patterns of BEN-1 and BEN-3 were not

238 consistent with that of an active enhancer, such that activity was unchanged or reduced relative to the
239 control (pGL3-P, no enhancer) across the B cell lines, the latter indicative of silencer activity (**Figure**
240 **4**). BEN-4 displayed some enhancer activity in B cell lines but the relative increase in transcriptional
241 activity was only significant in the SKW cell line in the reverse orientation (p = 0.0368, n = 3). (**Figure**
242 **4**). In contrast, BEN-2 significantly increased luciferase activity by approximately 3-fold relative to the
243 control in SKW, in both forward (p = 0.0219, n = 3) and reverse orientation (p = 0.0436, n = 3), and by
244 1.5-fold in Raji in the forward orientation (p = 0.0003, n = 4) (**Figure 4**). Notably, transcriptional activity
245 of BEN-2 was significantly decreased by 50% in the non-B cell line control (HepG2) in both enhancer
246 orientations (forward p = 0.0321, n = 4; reverse p = 0.0255, n = 3) (**Figure 4**). Together, these data
247 indicated that BEN-2 was the most likely candidate BEN to be active in the B cell lineage.
248
249 To support the functional role of BEN-2 in this cell type, we quantified epigenetic marks characteristic
250 of enhancer regions, such as chromatin accessibility and H3K27ac enrichment, in the panel of cell
251 lines used in the luciferase assays. Nucleosome occupancy was measured using MNase, a
252 micrococcal nuclease which cannot bind and digest nucleosome-bound DNA. At BEN-2, nucleosome
253 occupancy was consistently lower in the B cell lines than in the non-B cell control, HepG2 (**Figure**
254 **5A**). Conversely, chromatin accessibility was consistently high across all B cell lines, but inaccessible
255 in HepG2 (**Figure 5A** and **Figure 5B**), indicating that this region is transcriptionally active in the B cell
256 lineage. Accordingly, H3K27ac enrichment at BEN-2 was not observed in HepG2 but enriched in all B
257 cell lines (**Figure 5C**). Together, these data are in support of BEN-2 acting as a functional B cell
258 enhancer *in vitro.*
259
260 **CRISPR deletion of an intergenic B cell enhancer (BEN-2) decreased *CR2* and *CD55***
261 **expression at the transcript and protein levels**
262 As reporter gene assays remove regulatory elements from their genomic context, which is an
263 important aspect of enhancer function, we sought to assess the functional activity of BEN-2 *in vivo*.
264 We also wished to confirm the predicted gene targets of BEN-2 identified on GeneHancer, including
265 *CD55* and *CR2* which directly flank the enhancer (**Figure 3**). CRISPR deletion machinery was
266 delivered using a plasmid-based method into the Raji mature B cell line. This cell line expresses
267 *CD55, CR2* and *CD46,* although *CR1* is not expressed at levels detectable by qPCR (**Figure 6A**).
268 This pattern of gene expression is in accordance with other B cell lines, such as B-0028
269 (**Supplementary Figure 2**). To efficiently delete the BEN-2 region, we modified the PX458 CRISPR
270 plasmid to express two gRNA sequences that cut either side of BEN-2 (**Figure 6B**). The CRISPR
271 plasmids, containing a GFP marker, were delivered into Raji cells and successfully transfected GFP-
272 positive cells were enriched by fluorescence activated cell sorting (FACS). The resultant polyclonal
273 GFP+ bulk population (bulk) was expanded and used for single cell cloning by limiting dilution.
274 Successful enhancer deletion was qualitatively assessed using PCR (**Figure 6B, Figure 6C**). Indeed,
275 PCR indicated that BEN-2 was deleted in a proportion of cells in the bulk population (**Figure 6C**).
276 After screening expanded single cell clones, we successfully isolated a population containing a

homozygous deletion of the BEN-2 region (**Figure 6C***)*. We confirmed the genotype of the del/del population using Sanger sequencing (**Supplementary Figure 6**).

Remarkably, the homozygous deletion of BEN-2 in Raji cells significantly decreased *CR2* transcript abundance by approximately 90% relative to WT levels ($p = 0.0034$, $n = 3$) and *CD55* transcript abundance by approximately 80% of WT levels ($p = 0.0039$, $n = 3$). We also measured transcript abundance of *CR2* and *CD55* in the bulk population where a proportion of cells contained the enhancer deletion. Accordingly, both transcripts were significantly decreased compared to WT but to a lesser extent than del/del population; *CR2* transcript abundance decreased by approximately 70% of WT levels ($p = 0.0184$, $n = 3$) and *CD55* by approximately 50% of WT levels ($p = 0.0167$, $n = 3$) (**Figure 6D**). We also measured transcript abundance of *CD46,* which was not predicted to be targeted by BEN-2 on GeneHancer and localised to the neighbouring TAD (**Figure 3**). Enhancer deletion did not alter *CD46* transcript abundance (**Figure 6C**). These data confirmed that BEN-2 is a functional enhancer in B cells and demonstrated that BEN-2 regulates *CD55* and *CR2* within this cellular context.

As *CR2* and *CD55* transcript levels were significantly decreased with BEN-2 deletion in the Raji cell line, we next determined if surface protein expression of these receptors was concomitantly affected. We used flow cytometry to assess CR2 surface expression in the bulk and del/del populations. Indeed, CR2 expression was significantly decreased by approximately 3-fold in del/del population relative to the WT control ($p = 0.0257$, $n = 3$) (**Figure 7A**, **Figure 7B**). Surface staining using a CD55 antibody showed that around 2-3% of the population of Raji cells were CD55-positive (**Figure 7B, Figure 7C**). Regardless, the overall CD55 surface expression was also significantly decreased to approximately 75% of WT levels ($p = 0.074$, $n = 3$) (**Figure 7A**). In line with transcript abundance data (**Figure 6D**), CR2 and CD55 surface expression in the bulk population was decreased to levels between WT and del/del; 72% and 90%, respectively, of WT levels. This confirmed that the reduction in CR2 and CD55 transcript expression with CRISPR deletion of BEN-2 reduced surface protein levels. We thus further validated that BEN-2 regulates these genes both at the level of mRNA and subsequent protein expression in a B cell context.

307 **DISCUSSION**

308

309 In this manuscript, we have explored the chromatin architecture of the RCA gene cluster, an important
310 immunomodulatory region. We provide multiple insights into genes and variants within this cluster
311 from a biological, evolutionary and disease perspective. We show for the first time the co-localisation
312 of the RCA genes is in part due to the requirement of shared long-range regulatory elements. Using
313 high-resolution 4C-seq maps in B cell models, we showed that several distal CTCF sites in the RCA
314 gene cluster engage in chromatin looping, including the CTCF site modulated by the SLE-associated
315 SNP rs1876453 (29). We further shine a light onto the enigma of the unmappable segmental
316 duplication within *CR1* (24). Overall, we reveal extensive and complex mechanisms by which the RCA
317 gene cluster may influence gene expression and thereby autoimmunity.

318

319 While Hi-C interaction data are typically sufficient to identify TADs across the genome, the utility of
320 these data to examine the structural arrangement of the RCA gene cluster was hindered by the
321 sequence repetitiveness of the intragenic segmental duplication in *CR1*. Our strategy combined high-
322 resolution 4C-seq and a unique Hi-C pipeline (mHi-C) in conjunction with publicly available Hi-C
323 datasets to uncover a highly intriguing TAD arrangement within the RCA gene cluster in the B cell
324 lineage. We successfully utilised 4C-seq in two representative B cell lines from multiple viewpoints.
325 Further, we were able to show consistent maps of CTCF interactions in the RCA gene cluster and
326 chromatin looping which was constrained to one of two distinct regions across these loci. Importantly,
327 when we examined two CTCF viewpoints within intron 1 and intron 23 of the *CR1* gene, we found that
328 interactions from these viewpoints were directionally constrained in opposite directions (upstream and
329 downstream of *CR1*, respectively). These data indicated that the inter-TAD boundary was located
330 within the *CR1* gene. We corroborated this finding using mHi-C, a novel Hi-C processing pipeline
331 which assigns multi-mapping reads to the most likely genomic location. We thus resolved interactions
332 at this previously unmappable element and refined the location of the inter-TAD boundary to intron 11
333 of *CR1,* falling within the segmental duplication. Previous studies have shown that TAD boundaries
334 are enriched within housekeeping genes (31, 50) and may also be located near gene promoters (51).
335 To our knowledge, a TAD boundary located well within the body of an expressed protein-coding gene
336 has not been delineated to this resolution prior to our study.

337

338 The tandem segmental duplication in *CR1*, also known as 'low copy repeat 1' (LCR1), results in the
339 duplication of 8 exons and introns in *CR1* and has been shown to alter the number of functional
340 domains in the protein (52, 53). There are multiple co-dominant *CR1* alleles in the population defined
341 by copies of LCR1. CR1-A/F (one copy of LCR1) and CR1-B/S (two copies of LCR1) alleles are most
342 common. Alleles that contain zero copies and three copies of LCR1 have also been documented (54).
343 Importantly, this repeat element is known to be associated with SLE and, more recently, late-onset
344 Alzheimer's disease (24, 55). Despite the large size and nature of this repeat element, its biological
345 implication is undefined. Our findings strongly indicate that the LCR1 repeat element in *CR1* co-
346 localises with a TAD boundary and thus has a role in regulating gene expression in the RCA cluster.

347 This is consistent with the presiding hypothesis that complex diseases develop as a result of
348 dysregulated gene expression rather than functional protein changes. Based on the loop extrusion
349 model of TAD formation, it is likely that the increasing copy number of LCR1 results in increased
350 numbers of CTCF sites (reverse orientation) in TAD 1, thereby increasing insulation of chromatin
351 interactions within this TAD (32). Experiments are currently on-going in our laboratory to establish
352 whether the LCR1 copy number influences chromatin interactions and gene expression in the RCA
353 cluster.
354
355 The RCA gene cluster is an exemplar gene cluster as its members are co-localised in the human
356 genome and share protein structure and function. In addition, the genes, gene orientation and gene
357 order of the RCA cluster are well conserved in many species across evolutionary time, including
358 *Xenopus tropicalis* and chicken (56, 57). The RCA gene cluster in mice is also conserved but its
359 members are separated across two chromosomal positions located more than 6 Mb apart (58). This
360 matches closely to the TAD organisation of the gene cluster we describe here. It has been observed
361 that breaks in synteny between species commonly occur at TAD boundaries (59), thus the TAD
362 boundary we identified in *CR1* may represent the breakpoint region for the genomic rearrangement of
363 the RCA gene cluster in humans and mice.
364
365 Our study revealed that the RCA gene cluster is divided into two TADs in the B cell lineage; TAD 1
366 consists of *C4BPB, C4BPA, CD55, CR2* and *CR1*, and TAD 2 consists of *CR1L* and *CD46*. This
367 grouping does not reflect the organisation of active RCA genes in B cells (*C4BPB* and *C4BPA* are not
368 expressed in B cells) or other recognised cell types. Nonetheless, our findings in the RCA gene
369 cluster correspond to the *Six* (60)*, HoxA* (61, 62) and *HoxD* (63, 64) homeobox gene clusters which
370 have also been shown to be separated into two distinct TADs and regulatory regions. Unlike the
371 aforementioned gene clusters, which have highly-restricted and distinct expression patterns (65), the
372 RCA gene cluster is unique in that its members are expressed in numerous cell types and across
373 various stages of cell development. Each member of the RCA has a distinct expression pattern; *CD55*
374 and *CD46* are expressed across nearly all cell types, including non-immune cells (11), whereas *CR2*
375 and *CR1* are predominantly expressed on B cells (66) and erythrocytes (67), respectively. Thus it will
376 be interesting to examine the RCA gene cluster in other cellular contexts to examine the TAD
377 structure and enhancer-gene landscape and determine if these differ from the organisation we define
378 in this study. This will require multiple strategies of experimentation, as we have utilised here, to
379 overcome the caveats of reliance on high-throughput datasets.
380
381 Gene-gene interactions are a long-recognised and important contributor to complex disease
382 susceptibility, often overlooked due to the difficulty in identifying such interactions (68). In this study,
383 we mapped such interactions between genes in the RCA gene cluster at the molecular level using
384 high-resolution chromatin interaction maps and have successfully identified direct chromatin
385 interactions between CTCF sites in *CD55, CR2* and *CR1.* Binding of CTCF at intron 1 of *CR2* was
386 modulated by an SLE-associated SNP (rs1876453) and shown to influence the expression of its

387 neighbouring gene, *CR1,* in B cells (29). Our data now shows that *CR2* and *CR1* form part of the
388 same CTCF-mediated chromatin network in the RCA gene cluster and support the hypothesis that
389 these genes are co-regulated. It is possible that expression of *CR1* is also co-regulated by BEN-2 in
390 TAD 1 as has been predicted by CHi-C in the GM12878 B cell line (49).
391
392 We also uncovered a direct relationship between *CR2* and *CD55*, showing that these genes are co-
393 regulated by an intergenic enhancer, BEN-2. This marks the very first long-range regulatory element
394 identified in this TAD and gene cluster. We characterised this enhancer through multiple lines of
395 evidence *in vitro* and *in vivo* in several B cell models; by characterising transcriptional activity and
396 chromatin marks, as well as utilising CRISPR genomic deletion. Importantly, the reduction in transcript
397 level mediated by this enhancer deletion produced concomitant reduction in surface protein level of
398 both CR2 and CD55. This highlights the importance of BEN-2 in the expression of these complement
399 regulators and improves our understanding of the complex transcriptional control of these genes.
400
401 The surface expression of CD55 (69, 70) and CR2 (19, 20) on B cells is significantly decreased in
402 SLE patients. Genetic variation, such as SNPs, in the BEN-2 region may influence expression of both
403 *CR2* and *CD55* in tandem, thereby exacerbating the effect of variants in their contributions to
404 autoimmunity. The genes of the RCA cluster may also be co-regulated by the other candidate B cell
405 enhancers we identified here. Strategies to map gene-gene interactions and define relationships
406 between genes such as those utilised in this study may open important avenues to better understand
407 how complex diseases, like SLE and Alzheimer's, are influenced by genetic variation in this region.
408
409 We have established for the first time that the RCA gene cluster is transcriptionally co-regulated and
410 comprises of a complex network of enhancer-gene and gene-gene interactions. We have also defined
411 the regulatory architecture of the RCA in the B cell lineage, revealing novel mechanisms by which the
412 RCA gene cluster is controlled and expanding the scope for future investigations in the context of
413 evolution, immunity and complex genetic disease.

414 **MATERIAL AND METHODS**

415

416 **Cell culture**

417 Cell lines Reh (CRL-8286), Raji (CCL-86), SKW (TIB-215), K562 (CCL-243) and HepG2 (HB-8065),
418 were obtained from the American Type Culture Collection. B lymphoblastoid cell lines (B-0028 and B-
419 0056) were derived from healthy individuals and immortalised by Epstein-Barr virus infection (29). All
420 suspension cells were cultured in RPMI-1640 with L-glutamine (Life Technologies), supplemented
421 with 10% FBS, 100 µg/mL penicillin and 100 ng/µL streptomycin. The adherent cell line (HepG2) was
422 cultured in high glucose DMEM (Life Technologies) with 10% FBS, 100 µg/mL penicillin and 100
423 ng/µL streptomycin.

424

425 **Circular chromosome conformation capture (4C-seq)**

426 B-lymphoblastoid cell lines (5 x 10^6 cells) were harvested by centrifugation and resuspended in 5 mL
427 PBS with 10% FBS. To cross-link cells, 5 mL 4% formaldehyde was added, and samples were
428 incubated for 10 min. Cross-linking was quenched by adding 1 M glycine to 125 mM final
429 concentration and cells collected by centrifugation at 300 x *g* for 10 min at 4°C. 4C-seq assays and
430 data processing were performed as previously reported (71, 72). Sequences of primers used as 4C
431 viewpoints are listed in **Supplementary Table 1**.

432

433 **Bioinformatic datasets and pipelines**

434 Hi-C data for GM12878 from Rao et al. (41) were visualised as contact heatmaps and virtual 4C
435 signal using the 3D Genome Browser (73) and Juicebox (43). CTCF orientation calls from GM12878
436 were retrieved from Rao *et al.* (41) and assessed in the *CR1* segmental duplication using CTCFBSDB
437 2.0 (74). Enhancer predictions were retrieved from the GeneHancer database (Version J), which
438 leverages data from multiple sources, including ENCODE, FANTOM5 and Ensembl. Histone
439 modifications and transcription factor enrichment was assessed using ENCODE data and visualised
440 on the UCSC Genome Browser on hg19.

441

442 **Mapping Hi-C reads with mHi-C**

443 Multi-mapping Hi-C sequencing reads from Rao *et al.* (41) were evaluated using the mHi-C pipeline
444 (42) at 5 kb resolution (**Supplementary Table 2**). mHi-C was used as described in Zheng *et al.* (42)
445 with a novel post-mHiC processing strategy. In brief, the genomic distance effect on the contact
446 probabilities is estimated using the univariate spline model based on uniquely mapping reads. Such
447 prior probabilities information is updated iteratively by the local bin-pairs contact counts leveraging
448 both uniquely mapping reads and multi-mapping reads. The posterior probabilities, as the results of
449 mHi-C, quantify the chance for the candidate bin-pair to be the true origin for each multi-mapping read
450 pair. Instead of applying general filtering based on the posterior score by a fixed threshold, the
451 posterior probabilities are interpreted as fractional Hi-C contact counts to incorporate a more
452 significant number of the multi-mapping reads into the analysis. To examine interaction artefacts due
453 to highly repetitive sequences, a stringent multi-mapping allocation strategy was employed which

454 enforced all the multi-mapping reads assigned to corresponding regions have greater or equal to a
455 0.99 posterior score. Subsequent to rescuing multi-mapping reads by mHi-C, TAD boundaries are
456 detected by the state-of-the-art TAD caller spectralTAD (44) which provides nested TAD at different
457 levels. The TADs shown in Figure 2 are first-level TAD boundaries called at 25 kb resolution.
458
459 **Luciferase reporter-gene assays**
460 Candidate enhancers were amplified from human genomic DNA using Q5 Hot-Start High-Fidelity DNA
461 polymerase (New England Biolabs) and directionally cloned into the pGL3-Promoter plasmid (pGL3-
462 P) (Promega) upstream of the SV40 promoter using restriction enzymes. Plasmid DNA was prepared
463 using the EndoFree Plasmid Maxi Kit (QIAGEN) for transfection. Each enhancer construct (1 µg) was
464 transiently transfected with the pRL-TK *Renilla* internal control vector (50 ng) using 4 µL Viafect™
465 transfection reagent (Promega) into suspension cell lines or adherent cell lines. Cell lysates were
466 harvested after 24 h of incubation. Firefly and *Renilla* luciferase activity of cell lysates were
467 sequentially assayed using the Dual-Luciferase Reporter Assay System (Promega) on a GloMax
468 Explorer luminometer (Promega). Firefly luciferase readings were normalised to a co-transfected
469 internal *Renilla* luciferase control, and the activity of each enhancer construct was normalised to a
470 pGL3-P control. Sequences of primers used in this paper are listed in **Supplementary Table 1**.
471
472 **Quantitative PCR**
473 Total RNA was extracted from cells using the RNeasy Mini Kit (QIAGEN) with on-column DNase I
474 treatment. RNA quantity and purity were determined by spectrophotometry. RNA was reverse-
475 transcribed into cDNA using SuperScript III VILO reverse transcriptase (Life Technologies) and
476 diluted with UltraPure dH_2O (Life Technologies). qPCR reactions comprised 1X SYBR Green No-Rox
477 (Bioline), 250 nM forward and reverse primers (**Supplementary Table 1**), and 2 µL diluted cDNA up
478 to a final volume of 10 µL. Cycling and analysis were conducted using a Mic qPCR Cycler
479 (BioMolecular Systems) with the following conditions: 95°C for 10 min, and 35 cycles of 95°C for 15 s,
480 60°C for 15 s, and 72°C for 15 s. Melt curve analysis was used to confirm specific amplification of
481 targets. Relative mRNA expression levels were calculated using the comparative Ct method,
482 normalised to β-actin (*ACTB*).
483
484 **Chromatin immunoprecipitation**
485 Briefly, 4 x 10^7 cells were fixed using 1% formaldehyde (Sigma-Aldrich) for 10 min. Cells were washed
486 in PBS and lysed using NP-40 lysis buffer. Cell nuclei were resuspended in 2 mL 0.4% SDS shearing
487 buffer for sonication with a Covaris S220X sonicator (Covaris) for 7 min. For each
488 immunoprecipitation, 25 µg chromatin was diluted with IP dilution buffer and pre-cleared with Protein
489 A agarose beads (Merck-Millipore) for 1 h at 4°C. Chromatin was incubated with 5 µL anti-CTCF
490 (Merck-Millipore), 5 µg anti-H3K27ac (Abcam), or 5 µg rabbit IgG isotype control antibody (Merck-
491 Millipore) for 16 h at 4°C with rotation. Immune complexes were collected by centrifugation and
492 cleared using Protein A agarose beads (Millipore) and incubated for 1.5 h at 4°C. Complexes were
493 washed and eluted in 500 µL ChIP elution buffer. Crosslinks were reversed by adding 25 µL 4M NaCl

494 and incubation for 16 h at 65°C with shaking (600 rpm). Samples were treated with RNase A and
495 Proteinase K, and DNA was purified using the QIAquick PCR Purification kit (QIAGEN) according to
496 the manufacturer's specifications using 50 µL Buffer EB. For analysis, 2 µL of purified DNA was used
497 for qPCR reactions with a Mic qPCR cycler as described above. Enrichment was determined using
498 the percent input method.
499
500 **Chromatin accessibility by real-time PCR**
501 Chromatin accessibility by real-time PCR (ChART-PCR) was performed as previously described (75)
502 using 20 U DNase I (Promega). To assess nucleosome occupancy, 1000 Gel Units MNase (New
503 England Biolabs) was used. Digested and undigested samples were purified using the QIAquick PCR
504 Purification kit (QIAGEN). For analysis, qPCR reactions consisting of 50 ng DNA, 1X SYBR Green
505 (Bioline), 250 nM primers up to a final volume of 10 µL were cycled using a ViiA7 real-time
506 thermocycler and QuantStudio V1.3 (Applied Biosystems). Cycling conditions were as follows: 95°C
507 for 10 min, 40 cycles of: 95°C for 15 s, 60°C for 15 s, 72°C for 30 s, followed by melt curve analysis.
508 Accessibility levels were determined using the comparative Ct method for undigested and digested
509 samples, normalised to the lung-specific *SFTPA2* promoter (SPA2-P) control locus. For MNase
510 nucleosome occupancy assays, normalised data were transformed such that a value of 1.0
511 represents completely compacted nucleosomes, and lower values indicate reduced nucleosome
512 occupancy.
513
514 **CRISPR deletion**
515 CRISPR plasmid constructs were modified from pSpCas9(BB)-2A-GFP (PX458), a gift from Feng
516 Zhang (76) (Addgene plasmid #48138). To generate a large genomic deletion, PX458 was modified to
517 express two guide RNAs (gRNAs) to cut the 5' and 3' ends of the target region. gRNAs were
518 designed using CRISPRscan (77) to select highest scoring sequences with minimal off-target effects
519 (**Supplementary Table 3**). gRNAs were cloned into the *Bbs*I restriction sites of PX458 using T4 DNA
520 ligase (New England Biolabs). gRNA expression cassette inserts (U6 RNA polymerase III, gRNA
521 sequence and gRNA scaffold) were amplified using PCR with primers containing oligonucleotides with
522 *Acc*65I and *Xba*I restriction ends for sub-cloning (**Supplementary Table 1**). For the negative control
523 construct, the gRNA expression cassette of PX458 was removed using *Pci*I/*Xba*I digestion and
524 purified using the QIAquick Gel Extraction kit (QIAGEN). The linearised plasmid was blunted using T4
525 DNA polymerase (New England Biolabs) and re-ligated.
526
527 CRISPR plasmid constructs (2 µg) were electroporated into 2 x 10^6 Raji cells using the Amaxa Cell
528 Line Nucleofector Kit V (Lonza Bioscience) (Program M-013). Cells were incubated at 37°C with 5%
529 CO_2 for 24 h and then sorted for GFP+ expression using fluorescent activated cell sorting (FACS) on
530 a FACSAria II (BD Bioscience). The GFP+ pool (bulk) was expanded for further analysis. To obtain
531 single cell clones, the bulk sample was plated into 96-well plates at approximately 1 cell per well in
532 conditioned media. Single cell colonies were expanded and cells were cryopreserved. Genomic DNA
533 and total RNA extraction was performed using the QIAamp DNA Blood Mini kit (QIAGEN) and

534 RNeasy Mini kit (QIAGEN), respectively. RNA was reverse-transcribed and transcript abundance was
535 measured by qPCR as previously described.
536
537 **CRISPR deletion screening**
538 DNA was qualitatively screened for the genomic deletion using PCR with oligonucleotides amplifying
539 across the targeted region (deletion; D) and within the target region (non-deletion; ND)
540 (**Supplementary Table 1**). DNA (50 ng) was amplified using 1X GoTaq Green Master Mix (Promega),
541 0.8 μM oligonucleotides and 5% DMSO up to a volume of 20 μL, and cycled as follows: 95°C for 5
542 min, followed by 30 cycles of 95°C for 30 s, 61°C for 18 s, 72°C for 5 s, and a final extension at 72°C
543 for 5 min. Single cell clones that screened positive for genomic deletion were confirmed using Sanger
544 sequencing. The deletion product from the bulk sample was also analysed using Sanger sequencing.
545
546 **Flow cytometry**
547 Cells (1×10^6 cells) were harvested and washed with cold staining buffer (PBS with 5% FBS (v/v)) at
548 300 × *g* for 5 min at 4°C. For surface staining, cells were resuspended in 90 μL staining buffer and
549 incubated with 10 μL of anti-human CD21-PE (Cat #555422, BD Bioscience), anti-human CD55-PE
550 (MHCD5504, Thermo Fisher Scientific) or IgG1κ-PE isotype control (Cat #555749, BD Bioscience) for
551 20 min on ice. After incubation, cells were washed and resuspended in 0.5 mL staining buffer and
552 processed using a BD Accuri C6 flow cytometer (BD Bioscience). Data was analysed using FlowJo
553 software V10.8.0 (Tree Star). Samples were run alongside unstained controls.
554
555 **Statistical analysis**
556 Differences in transcriptional activity, mRNA expression and mean fluorescence intensity were
557 assessed using Student's unpaired t-test with a confidence interval of 95% ($p < 0.05$). Statistics and
558 graphs were generated using GraphPad Prism version 7.0 (GraphPad). Graphed values represent the
559 mean ± SEM of at least three independent experiments.
560

561 **ACCESSION NUMBERS**

562 4C-seq data were deposited in the Gene Expression Omnibus (GEO) database under accession
563 number GSE140127. The mHi-C pipeline can be accessed at https://github.com/yezhengSTAT/mHiC.

564

565 **ACKNOWLEDGEMENTS**

566 First and foremost, we would like to thank and remember our late collaborator, J.L., for his integral
567 and generous scientific guidance in this project, and express our deepest condolences to his family,
568 friends and colleagues. We would like to thank Kevin Li and the FACS Facility at the Harry Perkins
569 Institute of Medical Research for technical assistance with cell sorting. Further, we thank Kathy Fuller
570 and Henry Hui for their technical expertise and reagents for flow cytometry.

571

572 **FUNDING**

573 This work was supported by the National Institutes of Health [R01 AI24717 to J.B.H.], the Australian
574 Government Research Training Program Scholarship at the University of Western Australia [to J.C.
575 and J.S.C.], the Spanish Government [BFU2016-74961-P to J.L.G-.S] and an institutional grant
576 Unidad de Excelencia María de Maeztu [MDM-206-0687 to the Department of Gene Regulation and
577 Morphogenesis, Centro Andaluz de Biología del Desarrollo].

578

579 **CONTRIBUTIONS**

580 J.C., J.S.C., R.D.A. and Y.Z. performed the experiments and conducted the bioinformatic analyses;
581 J.C., J.S.C., J.L.G.-S., R.L.T. and D.U. designed the experiments; J.C., J.S.C., Y.Z., E.Q. and D.U.
582 analysed results; J.S.C. and D.U. conceptualised the project; J.L.G.-S., R.L.T., S.K. M.F. S.A.B. and
583 J.B.H. provided intellectual input and resources; E.Q. and D.U. supervised the project; J.C. wrote the
584 manuscript draft with input from all authors. All authors reviewed the manuscript.

585

586 **CONFLICT OF INTEREST**

587 The authors declare no competing interests.

REFERENCES

1. V. M. Holers, Complement and Its Receptors: New Insights into Human Disease. *Annual Review of Immunology* **32**, 433-459 (2014).
2. M. C. Carroll, D. E. Isenman, Regulation of Humoral Immunity by Complement. *Immunity* **37**, 199-207 (2012).
3. E. E. West, M. Kolev, C. Kemper, Complement and the Regulation of T Cell Responses. *Annual Review of Immunology* **36**, 309-338 (2018).
4. D. Ricklin, E. S. Reis, J. D. Lambris, Complement in disease: a defence system turning offensive. *Nature Reviews Nephrology* **12**, 383-401 (2016).
5. D. Hourcade, V. M. Holers, J. P. Atkinson, The Regulators of Complement Activation (RCA) Gene Cluster. *Advances in Immunology* **45**, 381-416 (1989).
6. D. Hourcade *et al.*, Analysis of the human regulators of complement activation (RCA) gene cluster with yeast artificial chromosomes (YACs). *Genomics* **12**, 289-300 (1992).
7. F. Pardo-Manuel de Villena, S. Rodríguez de Córdoba, C4BPAL2: a second duplication of the C4BPA gene in the human RCA gene cluster. *Immunogenetics* **41**, 139-143 (1995).
8. C. M. Logar, W. Chen, H. Schmitt, C. Yung, D. J. Birmingham, A human CR1-like transcript containing sequence for a binding protein for iC4 is expressed in hematopoietic and fetal lymphoid tissue. *Molecular Immunology* **40**, 831-840 (2004).
9. D. Ermert, A. M. Blom, C4b-binding protein: The good, the bad and the deadly. Novel functions of an old friend. *Immunology Letters* **169**, 82-92 (2016).
10. S. H. Dho, J. C. Lim, L. K. Kim, Beyond the Role of CD55 as a Complement Component. *Immune Network* **18**, 1-13 (2018).
11. P. F. Zipfel, C. Skerka, Complement regulators and inhibitory proteins. *Nature Reviews Immunology* **9**, 729-740 (2009).
12. M. K. Liszewski, J. P. Atkinson, Complement regulator CD46: genetic variants and disease associations. *Human Genomics* **9**, 7-7 (2015).
13. A. Erdei *et al.*, Expression and role of CR1 and CR2 on B and T lymphocytes under physiological and autoimmune conditions. *Molecular Immunology* **46**, 2767-2773 (2009).
14. Y. Deng, B. P. Tsao, Genetic susceptibility to systemic lupus erythematosus in the genomic era. *Nature Reviews Rheumatology* **6**, 683-692 (2010).
15. P. W. Dempsey *et al.*, C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* **271**, 348-350 (1996).
16. M. Jozsi, J. Prechl, Z. Bajtay, A. Erdei, Complement Receptor Type 1 (CD35) Mediates Inhibitory Signals in Human B Lymphocytes. *The Journal of Immunology* **168**, 2782-2788 (2002).
17. B. S. Andrews *et al.*, Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. *Journal of Experimental Medicine* **148**, 1198-1215 (1978).
18. A. N. Theofilopoulos, F. J. Dixon, Murine models of systemic lupus erythematosus. *Advances in Immunology* **37**, 269-390 (1985).
19. H. V. Marquart *et al.*, Complement receptor expression and activation of the complement cascade on B lymphocytes from patients with systemic lupus erythematosus (SLE). *Clinical and Experimental Immunology* **101**, 60-65 (1995).
20. J. G. Wilson, W. D. Ratnoff, P. H. Schur, D. T. Fearon, Decreased expression of the C3b/C4b receptor (CR1) and the C3d receptor (CR2) on B lymphocytes and of CR1 on neutrophils of patients with systemic lupus erythematosus. *Arthritis and Rheumatism* **29**, 739-747 (1986).
21. J. M. Ahearn *et al.*, Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. *Immunity* **4**, 251-262 (1996).
22. K. J. Marchbank, L. Kulik, M. G. Gipson, B. P. Morgan, V. M. Holers, Expression of human complement receptor type 2 (CD21) in mice during early B cell development results in a reduction in mature B cells and hypogammaglobulinemia. *The Journal of Immunology* **169**, 3526-3535 (2002).
23. H. Molina *et al.*, Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2. *Proceedings of the National Academy of Sciences* **93**, 3357-3361 (1996).
24. S. K. Nath, J. B. Harley, Y. H. Lee, Polymorphisms of complement receptor 1 and interleukin-10 genes and systemic lupus erythematosus: a meta-analysis. *Human Genetics* **118**, 225-234 (2005).
25. S. A. Boackle *et al.*, Cr2, a candidate gene in the murine Sle1c lupus susceptibility locus, encodes a dysfunctional protein. *Immunity* **15**, 775-785 (2001).

26. L. Morel *et al.*, Genetic reconstitution of systemic lupus erythematosus immunopathology with polygenic murine strains. *Proceedings of the National Academy of Sciences* **97**, 6670-6675 (2000).
27. H. Wu *et al.*, Association of a common complement receptor 2 haplotype with increased risk of systemic lupus erythematosus. *Proceedings of the National Academy of Sciences* **104**, 3961-3966 (2007).
28. K. B. Douglas *et al.*, Complement receptor 2 polymorphisms associated with systemic lupus erythematosus modulate alternative splicing. *Genes and Immunity* **10**, 457-469 (2009).
29. J. Zhao *et al.*, Preferential association of a functional variant in complement receptor 2 with antibodies to double-stranded DNA. *Annals of Rheumatic Diseases* **75**, 242-252 (2016).
30. C.-T. Ong, V. G. Corces, CTCF: an architectural protein bridging genome topology and function. *Nature Reviews Genetics* **15**, 234-246 (2014).
31. J. R. Dixon, D. U. Gorkin, B. Ren, Chromatin Domains: The Unit of Chromosome Organization. *Molecular Cell* **62**, 668-680 (2016).
32. G. Fudenberg *et al.*, Formation of Chromosomal Domains by Loop Extrusion. *Cell Reports* **15**, 2038-2049 (2016).
33. J. R. Dixon *et al.*, Topological domains in mammalian genomes identified by analysis of chromatin interactions. *Nature* **485**, 376-380 (2012).
34. E. Lieberman-Aiden *et al.*, Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science* **326**, 289-293 (2009).
35. G. Andrey, S. Mundlos, The three-dimensional genome: regulating gene expression during pluripotency and development. *Development* **144**, 3646-3658 (2017).
36. D. Shlyueva, G. Stampfel, A. Stark, Transcriptional enhancers: from properties to genome-wide predictions. *Nature Reviews Genetics* **15**, 272-286 (2014).
37. M. T. Maurano *et al.*, Systematic localization of common disease-associated variation in regulatory DNA. *Science* **337**, 1190-1195 (2012).
38. R. Andersson *et al.*, An atlas of active enhancers across human cell types and tissues. *Nature* **507**, 455-461 (2014).
39. M. P. Creyghton *et al.*, Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 21931-21936 (2010).
40. M. A. Marti-Renom *et al.*, Challenges and guidelines toward 4D nucleome data and model standards. *Nature Genetics* **50**, 1352-1358 (2018).
41. S. S. P. Rao *et al.*, A 3D map of the human genome at kilobase resolution reveals principles of chromatin looping. *Cell* **159**, 1665-1680 (2014).
42. Y. Zheng, F. Ay, S. Keles, Generative modeling of multi-mapping reads with mHi-C advances analysis of Hi-C studies. *eLife* **8** (2019).
43. J. T. Robinson *et al.*, Juicebox.js Provides a Cloud-Based Visualization System for Hi-C Data. *Cell Systems* **6**, 256-258.e251 (2018).
44. K. G. Cresswell, J. C. Stansfield, M. G. Dozmorov, SpectralTAD: an R package for defining a hierarchy of topologically associated domains using spectral clustering. *BMC Bioinformatics* **21**, 1-19 (2020).
45. S. Fishilevich *et al.*, GeneHancer: genome-wide integration of enhancers and target genes in GeneCards. *Database* 10.1093/database/bax028, 1-17 (2017).
46. S. Heinz, C. E. Romanoski, C. Benner, C. K. Glass, The selection and function of cell type-specific enhancers. *Nature Reviews Molecular Cell Biology* **16**, 144-154 (2015).
47. M. Busslinger, Transcriptional Control of Early B Cell Development. *Annual Review of Immunology* **22**, 55-79 (2004).
48. C. Cobaleda, A. Schebesta, A. Delogu, M. Busslinger, Pax5: The guardian of B cell identity and function. *Nature Immunology* **8**, 463-470 (2007).
49. B. Mifsud *et al.*, Mapping long-range promoter contacts in human cells with high-resolution capture Hi-C. *Nature Genetics* **47**, 598-606 (2015).
50. S. V. Ulianov *et al.*, Active chromatin and transcription play a key role in chromosome partitioning into topologically associating domains. *Genome Research* **26**, 70-84 (2016).
51. C. Paliou *et al.*, Preformed Chromatin Topology Assists Transcriptional Robustness of Shh during Limb Development. *Proceedings of the National Academy of Sciences* 10.1007/s11661-997-0172-9, 1-10 (2019).
52. H. Crehan *et al.*, Complement receptor 1 (CR1) and Alzheimer's disease. *Immunobiology* **217**, 244-250 (2012).

53. W. W. Wong, Structural and functional correlation of the human complement receptor type 1. *The Journal of Investigative Dermatology* **94**, 64S-67S (1990).
54. J. M. Moulds, J. D. Reveille, F. C. A. Division, C. Immunogenetics, Structural polymorphisms of complement receptor 1 (CR1) in systemic lupus erythematosus (SLE) patients and normal controls of three ethnic groups. *Clinical and Experimental Immunology* **1**, 302-305 (1996).
55. E. Kucukkilic *et al.*, Complement receptor 1 gene (CR1) intragenic duplication and risk of Alzheimer's disease. *Human Genetics* **137**, 305-314 (2018).
56. H. Oshiumi *et al.*, Regulator of Complement Activation (RCA) Locus in Chicken: Identification of Chicken RCA Gene Cluster and Functional RCA Proteins. *The Journal of Immunology* **175**, 1724-1734 (2005).
57. H. Oshiumi, Y. Suzuki, Regulator of complement activation (RCA) gene cluster in Xenopus tropicalis. *Immunogenetics* **61**, 371-384 (2009).
58. B. Y. S. F. Kingsmore *et al.*, Receptor-Related Genes in the Mouse. *Journal of Experimental Medicine* **169**, 1479-1484 (1989).
59. Q. Szabo, F. Bantignies, G. Cavalli, Principles of genome folding into topologically associating domains. *Science Advances* **5**, eaaw1668-eaaw1668 (2019).
60. C. Gómez-Marín *et al.*, Evolutionary comparison reveals that diverging CTCF sites are signatures of ancestral topological associating domains borders. *Proceedings of the National Academy of Sciences* **112**, 7542-7547 (2015).
61. S. Berlivet *et al.*, Clustering of Tissue-Specific Sub-TADs Accompanies the Regulation of HoxA Genes in Developing Limbs. *PLoS Genetics* **9**, e1004018-e1004018 (2013).
62. M. Rousseau *et al.*, Hox in motion: Tracking HoxA cluster conformation during differentiation. *Nucleic Acids Research* **42**, 1524-1540 (2014).
63. G. Andrey *et al.*, A Switch Between Topological Domains Underlies HoxD Genes Collinearity in Mouse Limbs. *Science* **340**, 123167-123167 (2013).
64. E. Rodríguez-Carballo *et al.*, The HoxD cluster is a dynamic and resilient TAD boundary controlling the segregation of antagonistic regulatory landscapes. *Genes and Development* **31**, 2264-2281 (2017).
65. T. Montavon, D. Duboule, Landscapes and archipelagos: Spatial organization of gene regulation in vertebrates. *Trends in Cell Biology* **22**, 347-354 (2012).
66. J. J. Weis, T. F. Tedder, D. T. Fearon, Identification of a 145,000 Mr membrane protein as the C3d receptor (CR2) of human B lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 881-885 (1984).
67. M. Pascual, J. A. Schifferli, The binding of immune complexes by the erythrocyte complement receptor 1 (CR1). *Immunopharmacology* **24**, 101-106 (1992).
68. T. A. Manolio *et al.*, Finding the missing heritability of complex diseases. *Nature* **461**, 747-753 (2009).
69. A. P. Alegretti *et al.*, Expression of CD55 and CD59 on peripheral blood cells from systemic lupus erythematosus (SLE) patients. *Cellular Immunology* **265**, 127-132 (2010).
70. I. García-Valladares *et al.*, Diminished expression of complement regulatory proteins (CD55 and CD59) in lymphocytes from systemic lupus erythematosus patients with lymphopenia. *Lupus* **15**, 600-605 (2006).
71. A. Fernández-Miñán, J. Bessa, J. J. Tena, J. L. Gómez-Skarmeta, "Assay for transposase-accessible chromatin and circularized chromosome conformation capture, two methods to explore the regulatory landscapes of genes in zebrafish", H. William Detrich, M. Westerfield, L. I. B. T. M. i. C. B. Zon, Eds. (Academic Press, 2016), vol. 135, pp. 413-430.
72. E. Splinter, E. de Wit, H. J. G. van de Werken, P. Klous, W. de Laat, Determining long-range chromatin interactions for selected genomic sites using 4C-seq technology: From fixation to computation. *Methods* **58**, 221-230 (2012).
73. Y. Wang *et al.*, The 3D Genome Browser: a web-based browser for visualizing 3D genome organization and long-range chromatin interactions. *Genome Biology* **19**, 151 (2018).
74. J. D. Ziebarth, A. Bhattacharya, Y. Cui, CTCFBSDB 2.0: a database for CTCF-binding sites and genome organization. *Nucleic Acids Research* **41**, D188-194 (2013).
75. M. Cruickshank, E. Fenwick, L. J. Abraham, D. Ulgiati, Quantitative differences in chromatin accessibility across regulatory regions can be directly compared in distinct cell-types. *Biochemical and Biophysical Research Communications* **367**, 349-355 (2008).
76. F. A. Ran *et al.*, Genome engineering using the CRISPR-Cas9 system. *Nature Protocols* **8**, 2281-2308 (2013).
77. M. A. Moreno-Mateos *et al.*, CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. *Nature Methods* **12**, 982-988 (2015).

FIGURES AND TABLES

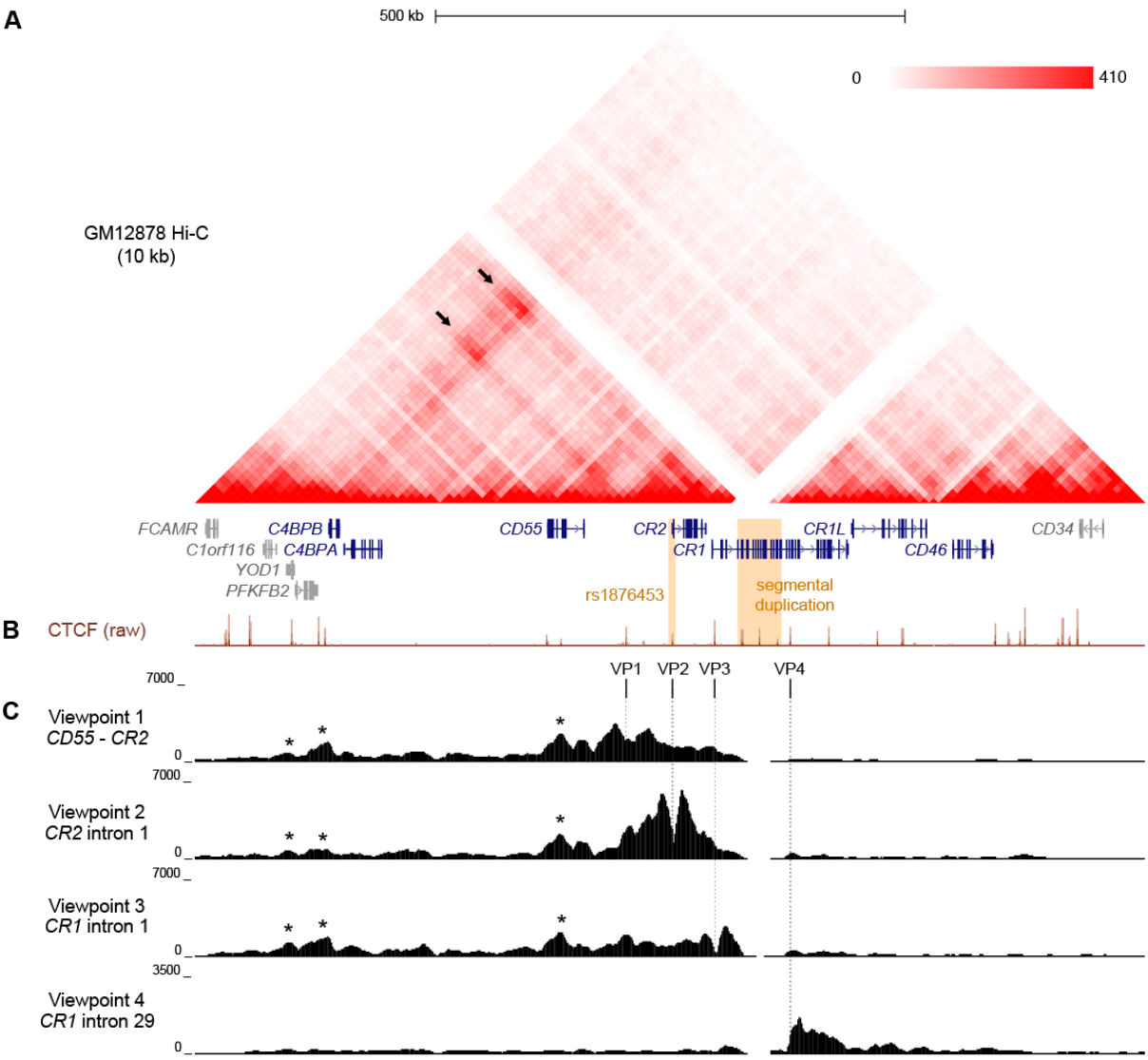

Figure 1: Chromatin conformation of the RCA gene cluster in B cells.

A. Hi-C heatmap matrix (10 kb resolution) for the GM12878 B cell line from Rao *et al.* (41) for the 1 Mb region across the RCA genes (dark blue) on hg19 (chr1:207,120,000-208,130,000). Relative interaction frequencies between two loci are indicated by colour intensity (range 0-410). High frequency long-range interactions (>350 kb) were observed between distal RCA genes *C4BPB* and the complement receptor genes (*CR2* and *CR1*) (arrows), as well as between intervening loci, indicating that these genes reside in the same TAD. SLE-associated variants are indicated in orange.

B. GM12878 ChIP-seq signal for CTCF from ENCODE shows CTCF enrichment at multiple sites across the RCA gene cluster which may engage in long-range chromatin looping.

C. Chromatin conformation of the RCA gene cluster was fine-mapped using 4C-seq in the B-0028 cell line. Maps were generated from four viewpoints on CTCF binding sites in the intergenic region between *CR2* and *CD55* (viewpoint 1, VP1), intron 1 of *CR2* (viewpoint 2, VP2), the intron 1 of *CR1* (viewpoint 3, VP3) and intron 29 of CR1 (viewpoint 4, VP4). Viewpoints are represented by vertical dotted lines. Several 4C-seq peaks were common between VP1 – 3 and aligned with CTCF binding sites within *YOD1,* upstream of *C4BPB* and within *CD55* (asterisks). VP4 showed a distinct interaction profile to all other viewpoints.

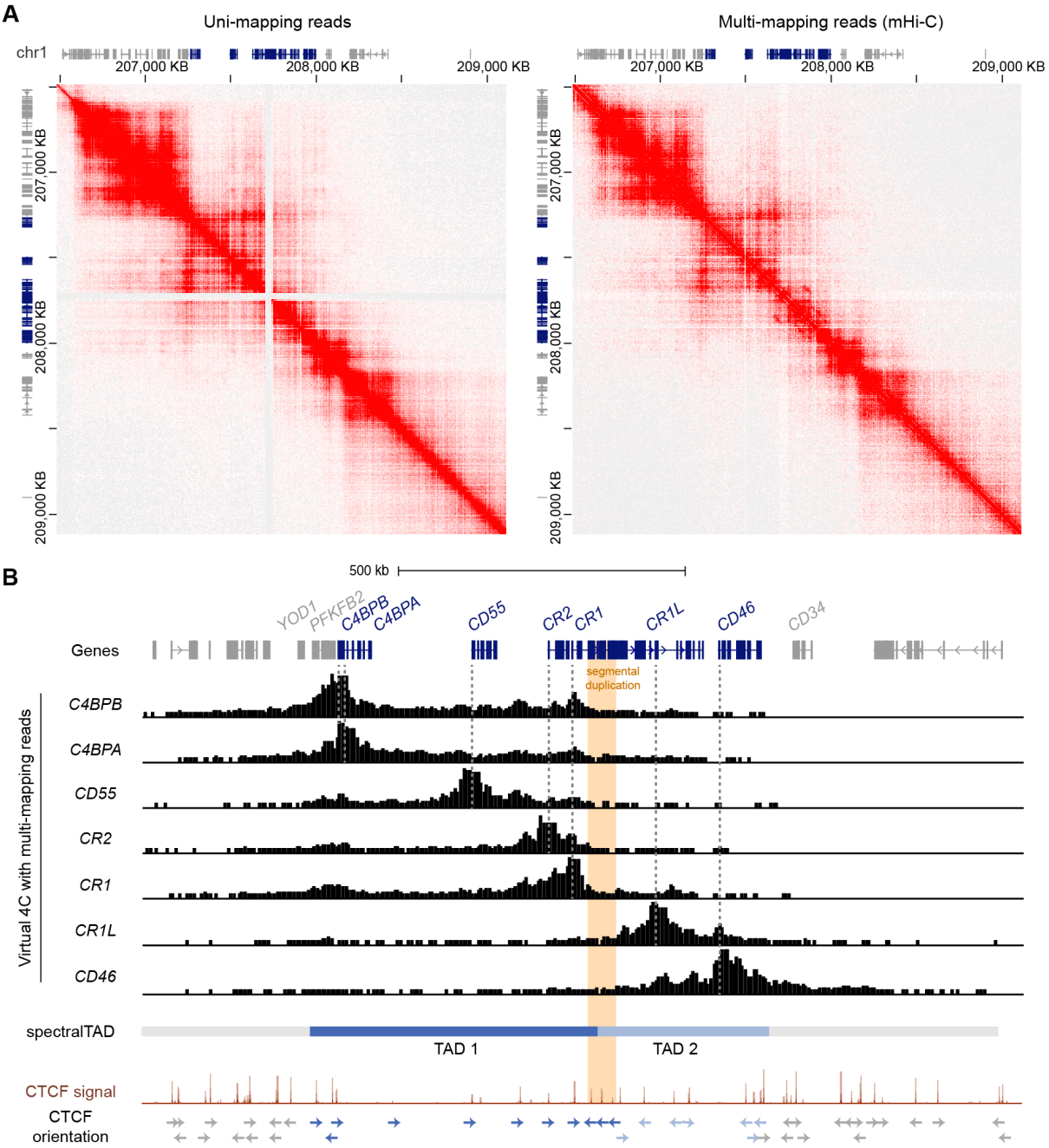

Figure 2: The RCA gene cluster is divided into two TADs and the inter-TAD boundary is located at the CR1 segmental duplication.

A. Hi-C contact maps of GM12878 data at 5 kb resolution from Rao *et al.* (*44*) with only uni-mapping reads and both uni- (left panel) and multi-mapping (right panel) reads assigned with mHi-C. Using mHi-C, interactions across the *CR1* segmental duplication were successfully recovered. Data was visualised using Juicebox.

B. mHi-C interactions were visualised as virtual 4C signal from the promoters of the RCA genes, which showed that interactions of RCA genes were constrained to two distinct regions. SpectralTAD was used to call TADs using Hi-C data with multi-mapping reads recovered, defining two clear TADs in the RCA cluster. Raw GM12878 CTCF ChIP-seq signal from ENCODE showed enrichment of convergent CTCF at the boundaries of both TADs and revealed that the *CR1* segmental duplication (orange) is flanked by repeated reverse orientation CTCF sites (indicated by arrows).

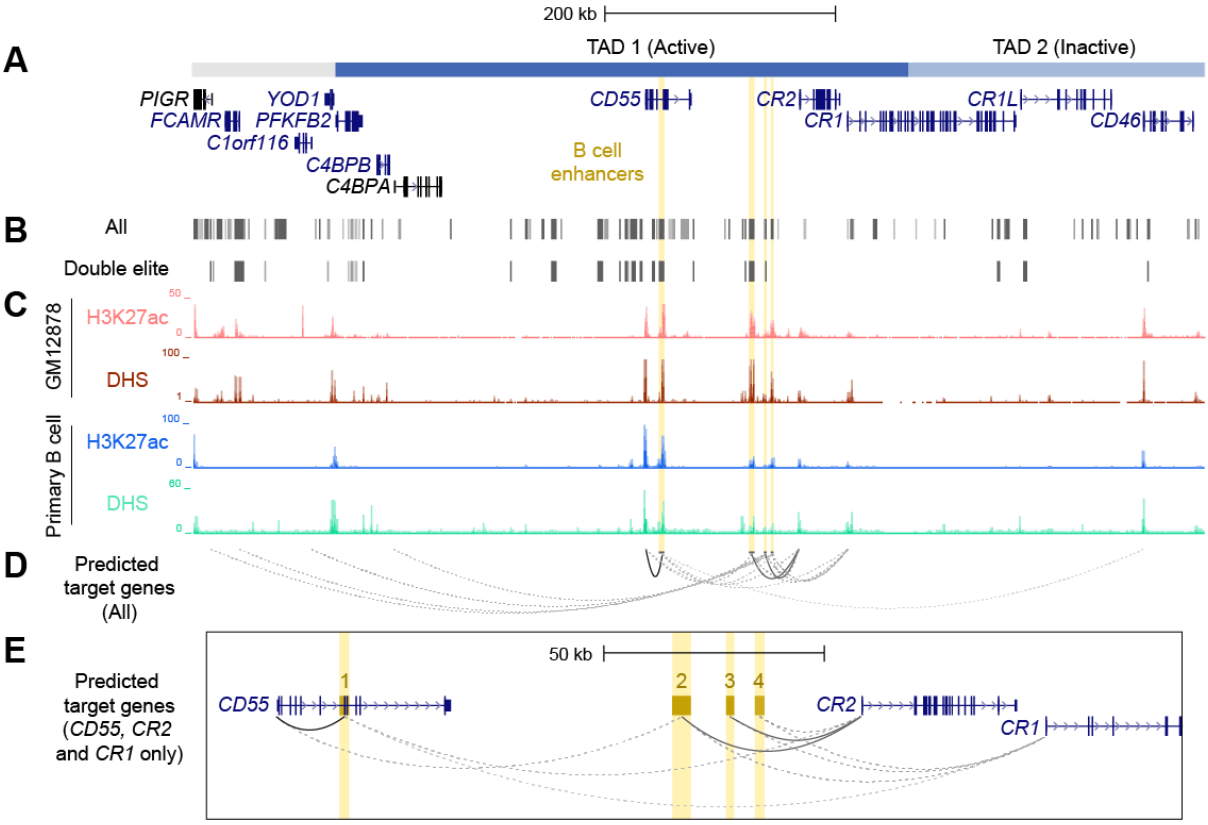

Figure 3: Identification and prioritisation of candidate B cell enhancers in TAD 1.

A. The structural organisation of the RCA genes (dark blue) and upstream genes (grey) *PIGR, FCAMR, C1orf116* and *YOD1* on hg19 (chr1:207,104,491-207,978,031).
B. Putative enhancers were identified by GeneHancer from multiple datasets from different consortia, such as ENCODE, Ensembl and FANTOM5 (GeneHancer). Each putative enhancer was also assigned predicted gene targets based on one or more methods. However, only a subset of putative enhancers were classified as 'double elite' on GeneHancer (Double elite).
C. Four candidate B cell enhancers (yellow) were identified using ENCODE data for H3K27ac enrichment and DNase I hypersensitivity in different B cell samples (GM12878 B cell line and primary B cells from peripheral blood).
D. Candidate B cell enhancers were predicted to regulate multiple genes. Target gene predictions that were identified by more than one method in GeneHancer are represented by a solid line. Predictions that were identified by just one method are represented by a dotted line. The opacity of each line represents the relative score/confidence for each gene-enhancer prediction as determined by GeneHancer whereby higher confidence predictions are darker. Scores determined by GeneHancer are listed in **Supplementary Table 5**.
E. Region across *CD55, CR2* and *CR1* (exon 1 – 6) on hg19 (chr1:207,484,047-207,700,935). Candidate B cell enhancers (BEN) were named based on order of chromosomal position (BEN-1, BEN-2, BEN-3 and BEN-4). Evidence for BENs to regulate *CD55, CR2* and *CR1* was strongest among all gene-enhancer predictions.

Table 1: Candidate B cell enhancers (BENs) on GeneHancer were identified from multiple enhancer databases, contained numerous transcription factor binding sites (TFBS) and were predicted to regulate several genes.

	BEN-1	BEN-2	BEN-3	BEN-4
GeneHancer ID	GH01J207333	GH01J207411	GH01J207424	GH01J207429
Location	*CD55* (exon 5 – 8)	Intergenic (*CD55* – *CR2*)	Intergenic (*CD55* – *CR2*)	Intergenic (*CD55* – *CR2*)
Enhancer sources	FANTOM5 ENCODE Ensembl dbSUPER	FANTOM5 ENCODE Ensembl	FANTOM5 ENCODE Ensembl	ENCODE
GeneHancer score	1.6*	1.2*	1.1*	0.7
TFBS ^	64 EBF1 IRF4 PAX5 RELA SPI1 CTCF EP300 POLR2A	35 EBF1 IRF4 PAX5 RELA EP300 POLR2A	13 EBF1 RELA SPI1 CTCF POLR2A	22 EBF1 RELA SPI1
Predicted gene targets	*CD55* CR2 CR1 CD46*	*CR2* CR1 CD55*	*CR2* CR1 C1orf116 C4BPA*	*CR2 CR1 CD55 FCAMR PIGR*

^ Only transcription factors important in B cell development (EBF1, IRF4, PAX5, RELA, SPI1) and gene regulation or chromatin organisation (CTCF, POL2RA and EP300) assayed using ChIP-seq in ENCODE are listed.

* These predicted enhancers and gene-enhancer interactions were identified using more than one method by GeneHancer.

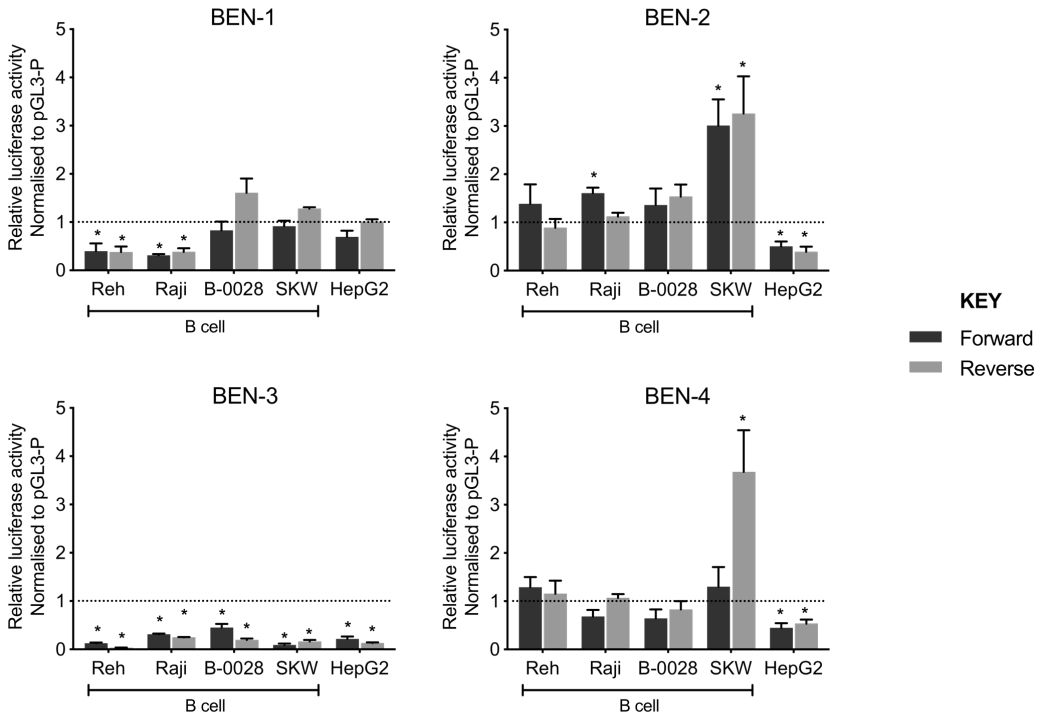

Figure 4: Candidate B cell enhancers demonstrated regulatory potential in luciferase assays, and BEN-2 increased relative transcriptional activity across a panel of B cell lines.

Enhancer constructs for strong candidate B cell enhancers were cloned into the pGL3-P (Promega) luciferase plasmid, upstream of an SV40 minimal promoter in forward (black) and reverse (grey) orientation. Bars represent mean relative luciferase activity ± SEM after normalisation to an empty pGL3-P (no enhancer) control plasmid (n = 3 to 8). Asterisks represent statistically significant differences between normalised values and the pGL3-P control ($p < 0.05$). Dotted line at $y = 1$ represents normalised pGL3-P control value.

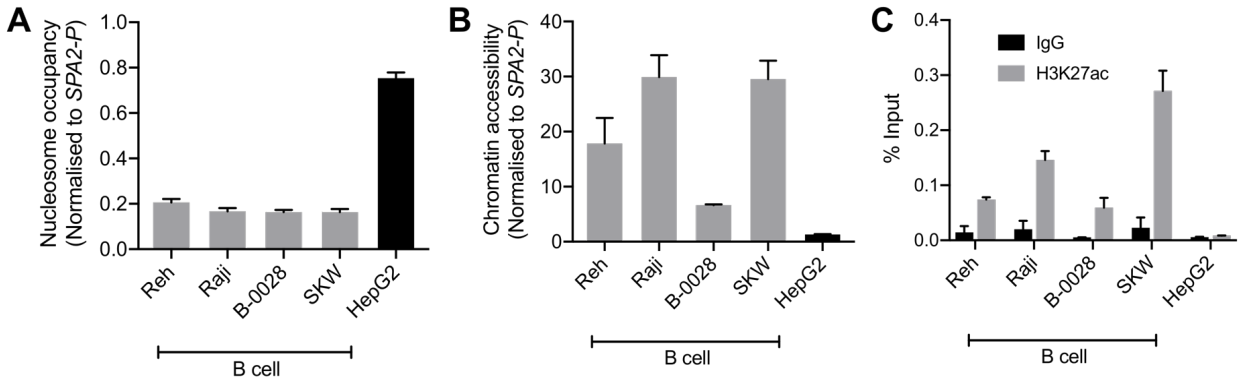

Figure 5: BEN-2 shows B cell-specific nucleosome occupancy, chromatin accessibility and enrichment for the H3K27ac active enhancer histone mark across a panel of B cell lines and non-B control (HepG2, liver).

A. Nucleosome occupancy at BEN-2 as measured by ChART-PCR with MNase digestion. Data was normalised to the inaccessible *SFTPA2* gene promoter such that a value of 1.0 represents fully compacted nucleosomes, and lower values indicate less compacted nucleosomes.
B. Chromatin accessibility at BEN-2 as measured by ChART-PCR with DNase I digestion. Data have been normalised to the inaccessible *SFTPA2* gene promoter.
C. H3K27ac enrichment at BEN-2 as determined by ChIP-qPCR using the percent input method. Grey bars indicate H3K27ac enrichment at the target locus, and black bars show enrichment using a non-specific IgG control antibody. All data are presented as mean ± SEM from at least 3 biological replicates.

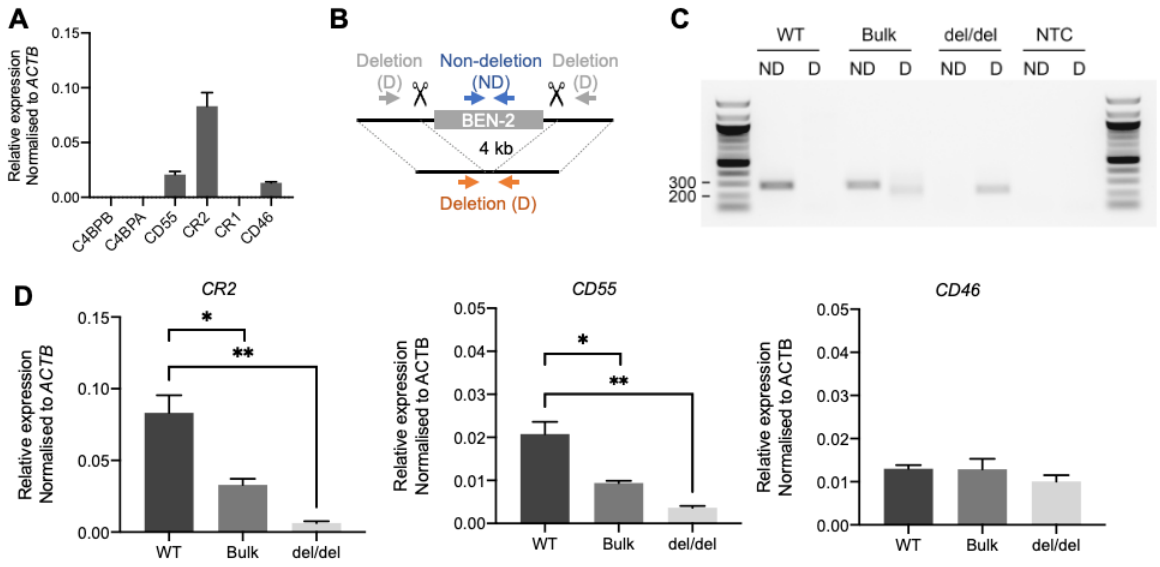

Figure 6: CRISPR deletion of BEN-2 decreased *CR2* and *CD55* mRNA expression in the Raji mature B cell line.

A. Transcript abundance of RCA genes in Raji mature B cell line as measured by qPCR. Values were normalised to the β-actin gene (*ACTB*) using the ΔCt method. Bars represent mean relative expression ± SEM from at least 3 biological replicates.
B. Schematic of enhancer deletion and screening strategy using CRISPR-Cas9. Deletion (4 kb) of BEN-2 was mediated by two gRNAs that cut either side of the enhancer region. Plasmids were modified from PX458 to express the two guides, Cas9 and a GFP marker. Screening was performed using PCR primers that flank the enhancer region (deletion; D) which amplify only in cases where a deletion has occurred (orange arrows). PCR primers that amplify within the enhancer region (non-deletion; ND) were used as a control (blue arrows).
C. PCR deletion screen of wild-type Raji DNA (WT), polyclonal GFP+ bulk population (bulk) and monoclonal single-cell clone containing a homozygous deletion of BEN-2 (del/del). PCRs were run alongside a no-template control (NTC).
D. Transcript abundance of RCA genes in TAD 1 (*CR2* and *CD55*) and TAD 2 (*CD46*) were measured by qPCR. Values were normalised to the β-actin gene (*ACTB*) using the ΔCt method. Bars represent mean relative expression ± SEM from 3 biological replicates. Asterisks represent statistically significant differences between WT, bulk and del/del samples (*$p < 0.05$, **$p < 0.005$).

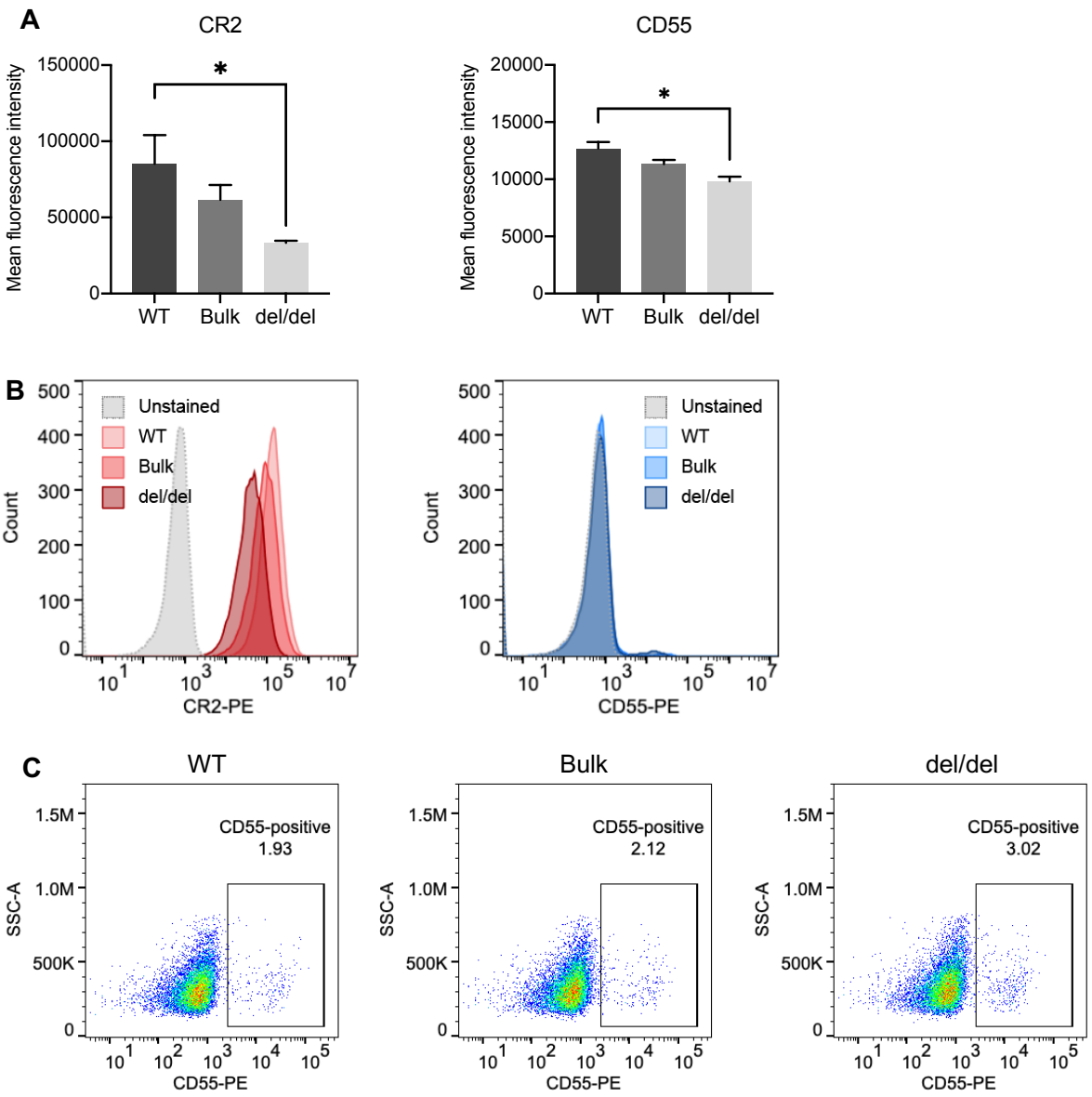

Figure 7: CRISPR deletion of BEN-2 decreased surface expression of CR2 and CD55 in the Raji B cell line.

A. Cell surface expression of CR2 and CD55 protein was determined using flow cytometry. Cells were labelled with PE-conjugated CR2 antibody, PE-conjugated CD55 antibody or PE-conjugated IgG (isotype control) to confirm positive expression. Samples were run alongside unstained controls (not shown). For each sample, 10000 events were collected. Bars represent mean fluorescence intensity ± SEM from 3 biological replicates. Asterisks represent statistically significant differences between WT and del/del samples ($p < 0.05$).
B. Representative histograms from the WT, bulk and del/del samples stained with CR2-PE or CD55-PE, as well as unstained WT control.
C. Representative dot plot from the WT, bulk and del/del samples stained with CD55-PE stained samples. The CD55-positive gate was plotted using the unstained control.