p52:ETS1: a transcriptional complex essential for germinal center formation

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Keywords: p52, ETS1, germinal center, antigen-specific antibody, B cell class switching recombination, T cell dependent immunity

ABSTRACT

The NF-κB family comprises five transcription factors - RELA, RELB, C-REL, NF-κB1(p50) and NF-κB2 (p52) - that form homo- or hetero-dimers amongst themselves to regulate gene expression by binding DNA. Here we show that p52 activates transcription without directly binding DNA but as a hetero-tetrameric complex with ETS1, a transcription factor outside the NF-κB family. By generating a knock-in mouse model (*p52*$^{ki/ki}$) with three mutated residues on p52 required for its interactions with ETS1 but not RELB, we demonstrate that the p52:ETS1 complex regulates expression of transcription factors, OCT1 and OBF1, known to be critical for the germinal center (GC) program. Consequently, B cell-intrinsic expression of p52:ETS1 complex was indispensable for splenic GC B cell formation and T cell-dependent antibody responses. Functionally, loss of p52:ETS1 interaction led to diminished antigen-specific IgE, thereby protecting mice from allergic responses. Collectively, our findings expand current knowledge of NF-κB signaling and may provide new therapeutic targets for the treatment of allergic diseases.

INTRODUCTION

Activation of non-canonical NFκB signalling drives dimerization of p52 and RELB, a complex critical for regulating adaptive immunity[1]. Both proteins contain a Rel homology domain (RHD), a common feature amongst NFκB family of transcription factors including RELA (p65), C-REL, and p50[2]. RHD comprises a DNA-binding subdomain (DBSD) and a dimerization subdomain (DSD)[2-4]. While DSD domain allows formation of homo-dimers or hetero-dimers, DBSD allows these dimers to directly bind κB sites on DNA to regulate transcription[5, 6]. Although 15 possible homo/hetero-dimers are formed amongst NFκB family members, complexes like RELA:p50 and p52:RELB featured more prominently in various physiological roles[5, 6]. Interestingly, not all p52 functions can be explained by its partnership

with RELB[7-11]. Notably, deletion of *p52* but not *Relb* in Immunoglobulin G1 (IgG_1)-expressing B cells resulted in impaired development of antigen-specific antibodies[10], suggesting that p52 might partner other transcription factors for certain functions *in vivo*.

Indeed, NFκB family members could complex with transcription factors outside their family including Twist, Stat, IRF, NFAT, AP-1 and ETS leading to activation or inhibition of NFκB activity[12]. Recently, p52 and ETS1 were found to bind and activate the C250T mutant human telomerase reverse transcriptase (*hTERT*) promoter, leading to its reactivation in glioblastoma cell lines[13]. Currently, there is no genetic evidence that partnerships of NFκB DNA-binding subunits outside its family have a physiological function *in vivo*. In this study, we examined whether p52:ETS1 complex exists *in vivo* and plays a physiological role. Given that the *TERT* promoter sequence and architecture in humans and mice differ significantly[14, 15], we evaluated the functions of p52:ETS1 complex in antibody production wherein genetic and biochemical evidence vouches for non-redundant physiological roles for both proteins[10, 16].

ETS1, a member of the ETS family of transcription factors, functions downstream of Ras-ERK signaling through interactions with several transcription factors, including PAX5 and RUNX1[17-19]. While both subunits in p52:RELB dimer are known to contact DNA[20], structural determination of p52:ETS1 interaction with C250T *hTERT* promoter revealed that this hetero-tetrameric complex comprises an ETS1 dimer that directly contacts DNA, while the p52 dimer bridges the two ETS1 subunits[21]. Consistent with observations that a DNA-binding defective p52 mutant can bind to tumour suppressor p53 and promote gene expression of p21, DR5 and PUMA[7], these studies, albeit *in vitro,* pointed to the fact that some NFκB subunits a) not necessarily bind κB sites to activate transcription, b) can work as co-factors for other transcription factor families, and c) function as tetramers. Therefore, we aimed to understand if p52:ETS1 complex has a transcriptional role that regulates immune responses *in vivo*.

By generating a knock-in mouse model ($p52^{ki/ki}$), wherein p52:ETS1 interaction was specifically disrupted, we provide evidence that this complex is necessary for splenic T follicular helper (T_{FH}) cell differentiation, germinal centre (GC) B cells formation and antigen-specific antibody production during T cell-dependent (TD) immunization[22]. Importantly, p52:ETS1 complex was required in a B cell-intrinsic manner for GC formation and TD antibody responses. At both transcript and protein levels, we found that p52:ETS1 complex regulates OCT1 and OBF1, two transcription factors important for GC program and generation of antigen-specific antibodies[23-27]. Finally, we showed that disruption of p52:ETS1 complex in B cells led to reduced IgE and muted anaphylactic responses in mice, thereby providing new targets for treatment of allergic diseases.

RESULTS

Generation of a model to study p52:ETS1 complex *in vivo*

RHD of p52 consists of a DSD and a DBSD that precedes a glycine rich region (GR)[28] (**Fig.1a**). Important ETS1 domains include the pointed domain (PNT), transactivation domain (TAD), ETS DNA-binding (ETS) domain and an autoinhibitory module consisting of inhibitory helix 1 (HI1), inhibitory helix 2 (HI2), helix 4 (H4), and helix 5 (H5)[29] (**Fig.1a**). Homeostatically, ETS1 is in an autoinhibitory state, unable to bind DNA due to the packing of inhibitory helices in its ETS domain[30]. Upon activation of non–canonical NFκB signalling, DSD domain of p52 (residues 226-328) interacts with ETS domain (residues 332-437) to form the p52:ETS1 complex ,which counteracts autoinhibition of ETS1 in binding DNA[21]. The p52:ETS1 complex is a hetero-tetramer due to interactions via p52 DSD homodimerization[21]. Three residues, R241, M297 and K298 (RMK), within p52 DSD domain are important for binding to ETS1[21] (**Fig.1b**). Indeed, triple mutations of these amino acids (RMK>A) to generate $p52^{RMK>A}$ mutant resulted in less interaction with ETS1 (**Fig.1c**) while binding to RELB remained intact

(**Extended Data Fig. 1a**), as measured by biolayer interferometry (BLI) assays. We also studied previously reported p52 interactions including p52:BCL3[31] and p52:p53[7], whereby p52 has been postulated to function as DNA-binding factor and co-factor respectively. BLI assays documented that $p52^{RMK>A}$ mutant could form homodimer to bind DNA (**Extended Data Fig.1b**) and BCL3 (**Extended Data Fig.1c**). Unfortunately, BLI did not detect any interaction between p52 and p53 (**Extended Data Fig.1d**) possibly due to the interaction being too transient or weak to be captured *in vitro*. Based on these data[21], we replaced these three crucial amino acids, R241, M297 and K298, in p52 with alanine, using CRISPR/Cas9 technology (**Fig.1d**) and generated $p52^{ki/ki}$ mice which express $p52^{RMK>A}$ protein from the endogenous NFκB2 loci. Genotyping of offspring including wildtype (WT), $p52^{+/ki}$ and $p52^{ki/ki}$ (**Fig.1e**) confirmed that they (**Fig.1f**) were born at expected Mendelian ratio (**Extended Data Fig.2a**). In line with our BLI analyses (**Fig. 1c**), compared to p52 protein in WT mice, $p52^{RMK>A}$ protein in $p52^{ki/ki}$ mice showed much less interaction with ETS1, *ex vivo* (**Fig.1g and Extended Data Fig.2b,c)**. Importantly, $p52^{RMK>A}$ mutant protein retained its interaction with RELB (**Fig.1g and Extended Data 2b,c**), consistent with structural determination that amino acids, R241, M297 and K298, in p52 DSD do not mediate interaction with RELB[21]. At steady-state, there were no significant changes in p52 and ETS1 expression in the spleens of WT and $p52^{ki/ki}$ mice, similar to other NFκB family members (**Fig.1h**). There were also no differences in the transcription of *Ets1, Relb* and *Nfκb2* (**Fig.1i)** and known targets of p52:RELB (**Fig.1j**). Since p52 could interact with both ETS1 and ETS2[13], we investigated whether mutant $p52^{RMK>A}$ protein in $p52^{ki/ki}$ mice affects binding to ETS2. However, we did not observe any p52:ETS2 interaction in splenic B cells (**Extended Data Fig.2c**), possibly because ETS2 expression is ~10-fold lower than ETS1 in mouse splenic B cells[32]. To overcome possible technical limitations of BLI in detecting p52:p53 interaction, we examined their target genes[7] and found them to be comparable between WT and $p52^{ki/ki}$ mice (**Fig.1k**).

Next, we studied ETS1 activity in *p52*$^{ki/ki}$ mice. While ETS1 complex formations with known interacting partners, such as PAX5 and RUNX1[18, 33, 34], were comparable between WT and *p52*$^{ki/ki}$ mice (**Extended Data Fig.2d,e**), interactions with p52 and its precursor, p100, were reduced in *p52*$^{ki/ki}$ mice (**Extended Data Fig.2e**). Expression of known ETS1 targets in splenic B cells (*Cd79a*) and T cells (*Tcrβ*) (**Extended Data Fig.2f**) were also equivalent between WT and *p52*$^{ki/ki}$ mice. Next, we investigated if disruption of p52:ETS1 interaction affects ETS1 localization. Using cell fractionation, we found that nuclear and cytoplasmic fractions of ETS1 remain comparable in WT and *p52*$^{ki/ki}$ splenic B cells (**Extended Data Fig.2g-i**). Among the NFκB family members, p52 preferentially dimerizes with RELB while p52:C-REL and p52:p50 have not been observed in cells[7]. To eliminate the possibility of ETS1 interacting with p52:RELA dimer *in vivo*, we assessed ETS1 interaction with RELA (Extended Data **Fig. 2e**) but detected no such interaction. At steady state, no differences were noted in hematopoietic stem/progenitor cells (HSPCs) and immune cells from the bone marrow, thymus and spleen between WT and *p52*$^{ki/ki}$ mice (**Extended Data Fig.2j-r**).

Taken together, we posit that *p52*$^{ki/ki}$ mice could be used to analyse specific functions of p52:ETS1 complex, since biochemical associations or biological activities of known partners of p52, such as RELB, p53, BCL3 and those of ETS1, such as PAX5, RUNX1, are unaltered in these mice. Considering these results, we propose that phenotypic changes observed in *p52*$^{ki/ki}$ mice could be attributed to reduced interaction between p52$^{RMK>A}$ and ETS1.

p52:ETS1 drives T cell-dependent splenic GC and antigen-specific antibody responses

Based on previous phenotypes of *p52*$^{-/-}$ and *Ets1*$^{-/-}$ mice, we assessed the potential roles of p52:ETS1 complex in B cell development and antibody production. In mice, p52 is involved in maintaining marginal zone (MZ) architecture, formation of B cell follicles and GCs in the

spleen and lymph nodes, as well as antigen-specific antibody responses following systemic TD immunization[9, 10, 35, 36]. ETS1 also plays a role in splenic MZ B cells, GC B cells and plasma cells formation as well as autoantibody production in mice[16, 37, 38]. To evaluate if these functions are disrupted in *p52*$^{ki/ki}$ mice, we characterised B cell populations and their precursors in bone marrow and spleen but found no differences in B cell development (**Extended Data Fig.2p,q**), follicular B or MZ B cells populations (**Extended Data Fig.3a-c**). To investigate the role of p52:ETS1 in antibody production via GC responses, we immunized WT, heterozygous (*p52*$^{+/ki}$) and *p52*$^{ki/ki}$ littermates intraperitoneally with NP-OVA adsorbed on alum which is known to induce TD antibody responses. Flow cytometry analyses 9 days post immunization, when GC structure is fully established, revealed severe reductions in both frequencies and numbers of GC B cells in *p52*$^{ki/ki}$ mice compared to WT and *p52*$^{+/ki}$ littermates (**Fig.2a-c**). These results suggest that presumably half the wildtype p52 protein amount (from one normal allele) is sufficient to drive normal GC responses (**Fig.2a-c**). All subsequent experiments with *p52*$^{ki/ki}$ mice were performed with a combination of WT and *p52*$^{+/ki}$ as control mice unless otherwise stated. To examine splenic architectures, we imaged B cell follicles in the spleen and observed intact B cell follicles albeit a complete loss of GC B cells (GL7^{+} IgD^{-}) **(Fig.2d)**. This suggests that unlike *p52*$^{-/-}$ mice[36], disruption of p52:ETS1 complex does not impair formation of B cell follicles. We further probed if the loss of GC was selective to dark (DZ) or light zone (LZ) GC B cells and found that although their proportions are like WT, the absolute numbers of both subsets were severely reduced since there was almost complete ablation of GC B cells in *p52*$^{ki/ki}$ mice **(Fig.2e-g)**. To understand whether p52:ETS1 is required for the initiation or maintenance of GC B cells, we examined GC frequencies at day 5 post immunization and found low frequencies of GC B cells in both control and *p52*$^{ki/ki}$ mice **(Extended Data Fig.3d)**. Despite comparable frequencies, we found diminished splenic GC B cell numbers in *p52*$^{ki/ki}$ mice, suggesting a failure of GC B cells to expand in the absence of intact p52:ETS1 complex

(Extended Data Fig.3d,e). Mesenteric lymph nodes (MLN) and Peyer's patches (PP) contain spontaneous GC at steady state due to constant exposure of antigens from gut microbiome and diet. We found WT frequencies of GC B cells in $p52^{ki/ki}$ mice demonstrating that p52:ETS1 activity is dispensable for GC formation in the gut **(Extended data.3f-i)**. We also immunized control and $p52^{ki/ki}$ mice subcutaneously with NP-OVA and alum to assess GC B cell differentiation in skin-draining inguinal lymph nodes (ILN) to examine if the observed GC defects are tissue-specific. Similar to splenic GCs, there was more than 2-fold decrease in the frequencies and numbers of GC B cells in the ILN **(Extended Data Fig.3j,k)**. Collectively, these data suggest that p52:ETS1 complex is necessary for the formation of both LZ and DZ GC B cells in response to TD immunization. However, p52:ETS1 complex is not required for chronic GC formation in the gut. Unlike our single dose TD immunization model, gut GC arose in response to continuous antigen exposure from diet and microbiome, possibly driven by a distinct transcriptional program.

As IgG_1 is the predominant antibody induced by NP-OVA and alum immunization, we checked the frequencies and numbers of IgG_1 GC B cells and found them to be severely reduced **(Fig.2h-j).** To understand if GC-derived NP-specific antibody-secreting cells (ASCs) were also diminished, we isolated bone marrow cells on day 28 and assessed NP-specific IgG_1 (NP-IgG_1) cells by Enzyme-linked immunospot assay (ELISPOT) and found a significant ablation of both low and high affinity NP-IgG_1 ASCs in $p52^{ki/ki}$ mice (**Fig.2k,l**). Consequentially, sera NP-IgG_1 titres assessed on days 14 and 28 were also severely reduced (**Fig.2m,n**). Taken together, these results demonstrate an essential role of p52:ETS1 activity in GC-dependent responses for production of NP-IgG_1 upon TD immunization.

Splenic GC B cell responses require B cell-intrinsic p52:ETS1 activity

As GC formation requires T_{FH} cells, we explored if GC defects in *p52*$^{ki/ki}$ mice was due to impaired T_{FH} cell differentiation. Indeed, splenic T_{FH} cell frequencies and numbers were reduced in *p52*$^{ki/ki}$ mice 9 days post NP-OVA immunization (**Extended Data Fig.3l-m**). These results suggested that the lack of GC formation in *p52*$^{ki/ki}$ mice could be due to either defects in T_{FH} cell priming, GC B cell differentiation or both. To dissect the cell type-intrinsic or -extrinsic roles of p52:ETS1 complex, we generated mixed bone marrow chimeric mouse (BMC) models using bone marrow cells from B cell-deficient mice (muMT) and mixed them with either wildtype control (Ctrl) or *p52*$^{ki/ki}$ (*B-p52*$^{ki/ki}$) marrows in a 1:1 ratio **(Fig.3a)**. After checking reconstitution in control and *B-p52*$^{ki/ki}$ mice 8 weeks post bone marrow transplantation **(Extended Fig.4a,b)**, we immunized these mice intraperitoneally with NP-OVA and alum to examine if GC defects were B cell-intrinsic. Similar to *p52*$^{ki/ki}$ mice, we observed reduced frequencies and numbers, albeit not to the same extent, of GC B cells in *B-p52*$^{ki/ki}$ mice 9 days post immunization (**Fig.3b-d**). In addition, *B-p52*$^{ki/ki}$ mice exhibited a marked decrease in IgG_1 GC B cells compared to controls (**Fig.3e-g**). Phenocopying *p52*$^{ki/ki}$ mice, NP-IgG_1 titres were severely reduced in sera of *B-p52*$^{ki/ki}$ mice 14 and 28 days post-immunization (**Fig.3h,i**). Lastly, analysis of the T_{FH} cell compartment revealed normal frequencies of T_{FH} cells in *B-p52*$^{ki/ki}$ mice (**Extended Data Fig.4c,d**), demonstrating a B cell-extrinsic role of p52:ETS1 in mediating T_{FH} cell differentiation. Thus, the lack of T_{FH} cell defect observed in *p52*$^{ki/ki}$ could be due to an impairment in T cell priming or differentiation that arose from the loss of p52:ETS1 complex in non-B cells. Consequentially, the severe GC defect observed in the *p52*$^{ki/ki}$ as compared to the *B-p52*$^{ki/ki}$ mice likely arises from a combination of a B cell-extrinsic T_{FH} defect and a B cell-intrinsic impairment of GC B cell differentiation. Despite normal T_{FH} cell frequencies, we observed a potential reduction in T_{FH} cell functionality based on reduced ICOS expression (**Extended Data Fig.4e)**, even though BCL6 expression was normal in these cells (**Extended Data Fig.4f)**. Taken together, these data

suggest that the GC B cell defects observed in $p52^{ki/ki}$ mice are due to a B cell-intrinsic requirement of p52:ETS1 complex for GC B cell differentiation and production of antigen-specific IgG_1 following immunization. Our results help explain the observations in literature that loss of p52 but not RELB in murine GC B cells resulted in impaired development of antigen-specific antibodies[10], by suggesting that p52:ETS1 could be the transcriptional regulator of GC-dependent formation of antigen-specific IgG_1-secreting cells.

p52:ETS1 complex regulates GC transcriptional program post B cell activation

To determine p52:ETS1-regulated transcriptional targets that are required for GC B cell differentiation, naïve ($CD19^+IgD^+$), activated non-GC B cell ($CD19^+IgD^-Fas^-GL7^-$) and GC B cell populations ($CD19^+IgD^-Fas^+GL7^+$) were sorted from immunized control and $p52^{ki/ki}$ mice for bulk RNA-sequencing (RNA-seq) **(Extended Data Fig.5a)**. Differentially expressed genes (DEGs) between control and $p52^{ki/ki}$ GC B cells were analyzed using limma package[39]. There were 225 upregulated and 826 downregulated genes in $p52^{ki/ki}$ GC B cells compared to controls (2-fold change, $p < 0.05$). Our analysis revealed that $p52^{ki/ki}$ GC B cells had less GC-like signatures and differed from control GC B cells in gene expression profiles (**Fig.4a and Extended Data Fig.5b**). Consistent with GC defective phenotype, gene expression of POU domain, class 2, transcription factor 1 (*Pou2f1*) was found to be significantly reduced in $p52^{ki/ki}$ GC B cells (**Fig.4a,b**). As an early response GC-specific gene, *Pou2f1* encodes OCT1 which complexes with its co-activator OBF1, to initiate transcription program essential for maintenance and proliferation of GC B cells[23, 24]. Reduced expression of *Aicda* (**Fig.4c**) in $p52^{ki/ki}$ GC B cells was indicative of defects in CSR, somatic hypermutation (SHM) while decreased *Prdm1* (**Fig.4d**) expression suggested impairment in plasma cell differentiation[40, 41]. As previously reported, beyond its role in GC maintenance, the OCT1:OBF1 complex also regulates genes such as *Prdm1*, which is essential for post-GC plasma cell differentiation

during the GC exit phase[23, 42]. Taken together, *Aicda* and *Prdm1* represent downstream genes that participate in GC transcriptional and GC exit programs respectively, regulated by the OCT1:OBF1 complex.

Furthermore, $p52^{ki/ki}$ GC B cells showed poorer expression of *Mki67* (**Fig.4e**), a cell proliferation marker. In parallel, enrichment analysis[43] using hallmark gene sets from the Molecular Signatures Database (mSigDB) revealed that gene signatures highly enriched in control GC B cells compared to $p52^{ki/ki}$ GC B cells were related to cell cycle (**Fig.4f**). Instead, gene signatures highly enriched in $p52^{ki/ki}$ GC B cells were related to NFκB target genes (**Extended Data Fig.5c**), possibly downstream of CD40 and BCR activation[44]. Therefore, we further analyzed CD40 and BCR signaling gene signatures [45] and found them to be significantly upregulated in $p52^{ki/ki}$ GC B cells (**Extended Data Fig.5d,e**). Notably, elevated and dysregulated CD40 and BCR signaling are known to inhibit GC formation[46].

To understand if the observed defects arose earlier in GC precursors, we performed bulk RNA-seq on pre-GC B cells ($CD19^{+}IgD^{-}CD38^{+}GL7^{+}$) from immunized control and $p52^{ki/ki}$ mice **(Extended Data Fig.5f)**. There were 671 up-regulated and 168 down-regulated genes in $p52^{ki/ki}$ pre-GC B cells compared with controls (2-fold change, $p<0.05$) **(Extended Data Fig.5g)** resulting in a distinct heatmap expression profile (**Extended Data Fig.5h)**. DEG analysis further revealed that *Aicda* **(Extended Data Fig.5i)** and *Mki67* **(Extended Data Fig.5j)** were significantly reduced in $p52^{ki/ki}$ pre-GC B cells whereas expression of *Pou2f1* **(Extended Data Fig.5k)** and *Prdm1* **(Extended Date Fig.5l)** were comparable to control pre-GC B cells. Together, these findings suggested the involvement of p52:ETS1 in CSR and proliferation at the pre-GC B stage, in line with previous reports that CSR can occur prior to GC formation[47].

Given the limited numbers of GC B cells in $p52^{ki/ki}$ mice, we tested our hypothesis that transcriptomic changes driven by the p52:ETS1 complex begin shortly after B cell activation by sorting total activated B cells ($CD19^{+}IgD^{-}$) **(Extended Data Fig.5a)** for real-time qPCR. Indeed, *Pou2f1* expression was also reduced in sorted activated B cells from $p52^{ki/ki}$ mice compared to control mice after immunization with NP-OVA and alum (**Fig.4g**). Since the gene product of *Pou2f1* expression, OCT1 works in concert with its transcriptional co-activator OBF1 to effect GC transcription program[23, 24], we also examined transcripts of *Pou2af1* and found it to be reduced in $p52^{ki/ki}$ mice (**Fig.4h**). Additionally, we further confirmed reductions in *Aicda* (**Fig.4i)** and *Prdm1*(**Fig.4j**) transcripts. In summary, these findings indicate that p52:ETS1 complex regulates expression of key early GC-related genes, *Pou2f1*and *Pou2af1 in vivo*. This regulation likely occurs post B cell activation, influencing key processes such as CSR, proliferation, SHM and their differentiation into GC B cells thereby affecting the eventual development of antigen-specific plasmablasts and plasma cells.

To enrich for GC B cells, while not missing chromatin changes present in naïve B cells, we sorted naïve ($CD19^{+}$ IgD^{+}) and total activated ($CD19^{+}$ IgD^{-}) B cells from immunized control and $p52^{ki/ki}$ mice in the ratio of 1:3 **(Extended Data Fig.5a)** for single cell ATAC-seq (scATAC-seq). We then examined the chromatin states driving B cell fates and functions (**Fig. 4k**). Eight (0-7) distinct B cell clusters were identified **(Fig.4k)**, with cluster 1 being reduced and clusters 4 and 5 absent in the $p52^{ki/ki}$ mice compared to controls **(Fig.4k,l and Extended Data Fig. 6a)**, likely representing GC B cells and their precursors. Indeed, weighted kernel density plot analyses revealed that clusters 1, 4, and 5 exhibit the highest combined gene activities for *Bcl6*, a master regulator of GC B cells, along with other GC-characteristic genes such as *Aicda* and *Cd86* in control mice **(Fig.4m)**. As clusters 4 and 5 were lost while 1 is severely reduced in $p52^{ki/ki}$ mice, the weighted density plot of *Bcl6*, *Aidca* and *Cd86* showed diffused gene activity across all remaining clusters **(Fig. 4m)**. Gene expression patterns were

computationally inferred from chromatin accessibility peak data (scATAC-seq) and visualized on UMAP using Signac[48] and Nebulosa[49]. Clusters 1, 4 and 5 also had the highest activity levels for *Pou2f1*, *Pou2fa1* and *Mki67* in control mice, whereas in $p52^{ki/ki}$ mice, these gene activities were localised to cluster 0 **(Fig.4n)**, corresponding to naive B cells with highest gene activities for *IgM* and *IgD* **(Extended Data Fig.6b).** These results further validated that B cell clusters differentiating to become GC B cells have combined high activity of *Pou2f1*, *Pou2fa1*, *Aicda* and *Mki67*. Subsequently, differential accessibility (DA) analyses were performed to identify genomic regions (peaks) with significantly altered chromatin accessibility between control and $p52^{ki/ki}$ mice within each cluster. Compared to control mice, chromatin accessibility at the *Aicda* gene (position:chr6-122530168-122531175) (**Fig.4o)**, and *Prdm1* (position:chr10-44298334-44299193) **(Extended Data Fig.6c)** loci were significantly reduced across all clusters in $p52^{ki/ki}$ mice (adjusted $p<0.05$). In summary, these findings further highlighted the critical role of p52:ETS1 complex in regulating OCT1:OBF1-induced GC transcriptional and GC exit programs[23, 42].

p52:ETS1 regulates early GC transcription factors OCT1:OBF1 in B cells

Since OBF1 serves as a transcriptional coactivator of OCT1 at the regulatory regions of many B cell-restricted genes and these proteins exhibit reciprocal regulation, we wanted to understand if the reduction in their transcripts **(Fig.4g,h)** translated to reduced protein expression post B cell activation, thereby causing GC defects observed in $p52^{ki/ki}$ and $B\text{-}p52^{ki/ki}$ mice. Total activated splenic B cells were sorted from $p52^{ki/ki}$, $B\text{-}p52^{ki/ki}$ and their respective controls 9 days post immunization **(Extended Data Fig.5a)**. Immunoblotting of OCT1 and OBF1 revealed dramatic reductions in their protein levels in activated splenic B cells from $p52^{ki/ki}$ mice compared to controls (**Fig.5a-c**). Similar phenotypes were observed in $B\text{-}p52^{ki/ki}$ mice as compared to their controls **(Fig.5d-f)**. In parallel, to confirm if OBF1 was decreased in

GC B cells, we used flow cytometry to examine OBF1 expression and also observed a notable decrease in GC B cells of *B-p52$^{ki/ki}$* mice (**Fig. 5g,h**). These findings clearly document a B cell-intrinsic role of p52:ETS1 in driving the expression of OCT1 and OBF1 upon B cell activation by TD immunizations.

As OCT1 and OBF1 could regulate GC B cell proliferation[23, 24, 42], we analyzed if a proliferation defect caused the decrease in GC B cells in *p52$^{ki/ki}$* mice. To test this, we investigated Ki-67 expression in GC B cells and Pre-GC B cells and found the frequencies of Ki-67^{+} cells in the latter to be severely reduced **(Fig. 5i-j and Extended Data Fig.6d)**. This finding corroborated with reduced *Mki67* transcripts in Pre-GC B cells **(Extended Data Fig.5g,j)**, suggesting that OCT1 and OBF1 regulates expansion of GC-committed B cells in the early stages post activation. We also checked if GC B cells in *p52$^{ki/ki}$* mice were more prone to apoptosis and found that on the contrary, there were less GC B cells expressing active caspase 3 in *p52$^{ki/ki}$* mice compared to controls **(Extended Data Fig.6e,f)**. Therefore, apoptosis is unlikely to play a major role in contributing to the GC defect in *p52$^{ki/ki}$* mice.

Lastly, we activated naïve splenic murine B cells with anti-CD40 antibody and recombinant IL-4 mimicking TD activation *in vitro* to study B cell-intrinsic roles of p52:ETS1 in regulating CSR and proliferation. Activated B cells from *p52$^{ki/ki}$* mice had decreased IgG$_1$ B cells (**Fig.5k,l**) and reduced cell proliferation (**Fig. 5m,n**) 4 days post activation. This mirrors *in vivo* data that B cell-intrinsic expression of p52:ETS1 complex is necessary for TD antibody responses. Collectively, our data suggest a B cell-intrinsic role of p52:ETS1 in regulating the expression and function of OBF1 and OCT1, since loss of these proteins in *p52$^{ki/ki}$* mice correlated with notable reductions in proliferation of pre-GC B cells as well as decreased IgG$_1$ switching and proliferation of activated B cells *in vitro*. As OCT1 and OBF1 were shown to be necessary for isotype-switched Ig production through direct regulation of *Aicda* and B cell

proliferation[23, 24, 40, 42], our results emphasize the pivotal role of p52:ETS1 complex in orchestrating CSR and proliferation in B cells likely through the regulation of OBF1 and OCT1.

p52:ETS1 binds to promoters of OCT1 and OBF1 to initiate transcription

Since p52:ETS1 complex can drive transcription[21] in human cancer cell lines, and levels of OCT1 and OBF1 were low in *p52*$^{ki/ki}$ mice, we sought to strengthen the notion that p52:ETS1 directly regulates transcription of *Pou2f1* and *Pou2af1* (**Fig.4g,h**) and consequentially their protein expression (**Fig.5a-f**) post B cell activation. To determine whether OCT1 and OBF1 might be *bona fide* physiologically important direct targets of p52:ETS1, we sourced for ChIP-seq datasets of p52 and ETS1 from online databases. However, the absence of p52 ChIP-seq datasets in mouse and lack of a validated ChIP-grade antibody against mouse p52 currently available, limited our analysis of p52-regulated genes in murine B cells. Nevertheless, in the structural model, ETS1 was found to contact DNA while p52 served as a co-factor at the C250T mutant *hTERT* promoter[21].Thus we focused on ETS1 binding motifs (**Fig. 6a**) and ChIP-seq datasets of ETS1-regulated genes in murine B cells. To distinguish between p52:ETS1 and p52:RELB targets, RELB binding motif (**Fig.6b**) and RELB ChIP-seq datasets in murine B cells were also obtained for comparison.

While ETS1 ChIP-seq peaks were observed at *Pou2f1* and *Pou2af1* promoters, which encode OCT1 and OBF1 respectively, none was detected for RELB (**Fig.6c,d**). Using JASPAR, five putative ETS1 binding sites were identified within *Pou2f1* promoter and four within *Pou2af1* promoter, all clustered within ~0–1000 bp upstream of the transcription start site (TSS) (**Extended data Fig.7a,b**). Next, we performed ChIP-qPCR on activated splenic B cells sorted from BMC mice post immunization (**Fig.6e,f**). ETS1, but not RELB recruitment to the predicted regions (-315 and -232 TSS) of *Pou2f1* promoter was more pronounced in activated B cells from control mice than *B-p52*$^{ki/ki}$ mice (**Fig.6e**). Similarly, ETS1 but not RELB

recruitment to two closely situated predicted sites (-677 and -494 TSS) in *Pou2af1* promoter was higher in activated splenic B cells from control mice than *B-p52*$^{ki/ki}$ mice (**Fig.6f**). ChIP-qPCR for *Icosl* (**Fig.6g**) and *Pax5* (**Fig.6h**) genes, served as positive controls to validate RELB and Ets1 binding respectively to their known promoters. Recruitment of ETS1 to *Pou2f1* (**Fig. 6e**) and *Pou2af1* (**Fig.6f**) promoters, particularly to regions featuring two predicted ETS1 binding motifs in close proximity, aligns with the model where two ETS1 monomers within the p52:ETS1 hetero-tetrameric complex bind DNA. Taken together, these findings suggest that p52 binds and relieves autoinhibition of ETS1 allowing it to directly bind *Pou2f1* and *Pou2af1* promoters to regulate transcription. Subsequently, their gene products OCT1 and OBF1 form a complex driving GC transcriptional program in activated B cells.

To overcome the limitation posed by the current lack of validated ChIP-grade antibodies for mouse p52, we assessed p52:ETS1 binding to *POU2AF1* and *POU2F1* promoters in human OCI-Ly3 cells, an activated B-cell (ABC) like diffuse-large B cell line (DLBCL). ChIP-seq analyses showed that p52 and ETS1 bind preferentially to *POU2AF1* **(Fig.6i)** and *Pou2f1* **(Fig.6j)** promoters at similar positions bound by active histone markers (H3K4Me and H3K27Ac) but not repressor marker (H3K27Me). Notably, some RELB binding was also detected at *POU2F1* promoter **(Fig.6j)** in these cells perhaps due to redundant association. Quantified on right panel, ChIP-seq peaks of p52 and RELB to their known promoter, *Icosl,* acted as positive control **(Fig.6k)**. To validate our ChIP-seq results, we examined publicly-available human B cell ChIP-seq datasets and identified p52 in L1236 cells and ETS1 and RELB in GM12878 cells **(Extended data Fig.7c,d)**. ChIP-seq peaks revealed that p52 and ETS1 exhibited more prominent binding than RELB to *POU2AF1* promoter **(Extended data Fig.7c)**. This pattern was not observed at the *POU2F1* promoter **(Extended data Fig .7d)**, likely due to differences in the origin and transformation of the B cells analyzed and the possibility that p52:ETS1 regulates this gene at a very precise stage of B cell

differentiation. We focused on p52:ETS1 binding to *POU2AF1* promoter in OCI-Ly3 cells. Using JASPAR, five potential ETS1 binding sites on human *Pou2af1* gene were predicted **(Extended data Fig.7e)**. Alignment of mouse *Pou2af1* promoter sequence **(Extended data Fig.8a)** containing the validated ETS1 binding sites (-677 and -494 TSS) **(Fig.6f)** with the corresponding human sequence **(Extended data Fig.8b)**, revealed that the two predicted sites (-642 and -458 TSS) are conserved **(Extended data Fig.8c)**. Next, we conducted ChIP-qPCR to confirm ETS1 and p52 recruitment to these regions within *POU2AF1* promoter **(Fig.6l)** using known targets as positive controls **(Fig.6m,n)**. Subsequently, ChIP-reChIP using p52 first followed by either ETS1 or RELB revealed that the p52:ETS1 complex binds to the *Pou2af1* promoter at the two closely situated ETS1 binding sites (–642 and –458 TSS) **(Fig.6o)**, whereas the p52:RELB complex was detected at their target, *Icosl* instead **(Fig.6p)**. Notably, this human *Pou2af1* promoter region containing the two ETS1 motif sites lacks classical κB-sites or p52 consensus sequence **(Extended data Fig.8b)**, suggesting that p52 is recruited via its interaction with ETS1 rather than direct DNA binding, consistent with p52:ETS1 heterotetrametric structure[21]. These results were further confirmed biochemically through electrophoretic mobility shift assays (EMSA). In line with *in vivo* evidence of reduced ETS1 binding to *Pou2af1* promoter in immunised $p52^{ki/ki}$ mice **(Fig.6e,f)**, EMSA showed cooperative binding between recombinant WT p52:ETS1 to Cy5-labeled *Pou2af1* human promoter **(Extended data Fig.8d)** and this distinct gel shift was not observed with $p52^{RMK>A}$:ETS1 proteins **(Extended data Fig.8d)**. Taken together, these results, derived from all possible assays to overcome the current technical limitations posed by unavailability of validated mouse p52 ChIP-grade antibodies, show that p52:RELB and p52:ETS1 bind to distinct regions, exemplified by *Icosl* and *Pou2af1* promoters.

p52:ETS1 regulates IgE-mediated allergic responses

Having found the importance of p52:ETS1 in regulating OCT1 and OBF1-induced GC transcriptional program to produce high affinity antigen-specific class-switched antibodies, we used an allergy model to investigate the generality of these observations and sought to evaluate the functional consequence of this regulation beyond the confines of immunization assays employed. Given the role of OCT1:OBF1 in transcribing *Aicda* and the necessity of B cell-specific OBF1 for IgE production, we conducted a passive cutaneous anaphylaxis assay (PCA) in mice to determine if antigen-specific IgE production is similarly affected in B-$p52^{ki/ki}$ mice. Sera from mixed BMC mice were isolated on days 14 and 28 post NP-OVA and alum immunization for measurement of high affinity and low affinity NP-specific IgE (**Fig.7a,b**). Sera from $B\text{-}p52^{ki/ki}$ mice had a paucity of both low and high affinity IgE antibodies on day 14 **(Fig.7a)** and day 28 **(Fig.7b)** post immunization. Subsequently, sera from control and $B\text{-}p52^{ki/ki}$ mice were intradermally injected into the right and left ears of wildtype recipient mice respectively as illustrated in **Fig.7c**. IgE in sera will bind to FcεRI on tissue mast cells, priming them for activation and degranulation upon reencounter with the antigen[50]. Upon intravenous injection with Evans blue dye and NP-OVA 24 hours later, vascular leakage of the dye was evident in the right ear of mice, indicative of edema due to degranulation of IgE-primed mast cells (**Fig.7d**). In contrast, the left ear showed little discoloration (**Fig.7d**). To quantify vascular leakage through assessing absorbance of Evans blue dye in the ear tissue as a proxy, ears of the mice were digested in formamide before quantification of resulting supernatant with spectrophotometer **(Fig.7e,f)**. We found lower amounts of Evans blue presence in the ears with $B\text{-}p52^{ki/ki}$ sera, indicative of less vascular leakage during passive anaphylactic response **(Fig.7e,f).** Collectively these data suggest that p52:ETS1 is essential for high affinity antigen-specific IgE production responsible for allergic responses. Therefore, temporal inhibition of p52:ETS1 complex can be considered as a potential therapeutic strategy to protect against allergic responses including potentially fatal ones such as anaphylaxis.

DISCUSSION

Though NFκB family members can interact with transcription factors outside their family *in vitro*, their physiological role *in vivo* remains unexplored. Using a new *p52$^{ki/ki}$* mouse model with disrupted p52 binding to ETS1, we illustrated the *in vivo* significance of p52:ETS1 transcriptional complex in regulating splenic GC responses and antigen-specific antibody production in a B cell-intrinsic manner. Although both p52 and ETS1 have been implicated in the regulation of GC and antibody responses[10, 16, 17, 35-37], the findings in this study align with some but not all phenotypes of mice with single knockout of p52 or ETS1. Both *p52$^{-/-}$* and *Ets1$^{-/-}$* mice have impaired B cell development whereby follicular and MZ B cells are reduced in the former while MZ B are lost in the latter[10, 16, 17, 35-37]. In contrast, peripheral B cell development was normal in *p52$^{ki/ki}$* mice with wildtype levels of follicular and MZ B cells, suggesting that p52:ETS1 is dispensable for these stages of B cell development. Similar to *p52$^{-/-}$* mice, GC formation was impaired in *p52$^{ki/ki}$* mice, leading to decreased production of TD antibodies[35, 36]. De Silva et al. (2016) demonstrated that B cell-intrinsic deletion of *p52*, but not *Relb*, led to reduced NP-specific IgG_1 in response to TD immunization[10]. Corroborating this data, we provide evidence that p52 mediates generation of NP-IgG_1 through partnership with ETS1 instead and further demonstrate that p52:ETS1 is required in B cells to produce high affinity NP-IgE in allergic responses. Interestingly, RELB has been shown to be dispensable for IgE production[51], lending credence to the notion that certain functions attributed to p52 extend beyond its binding to RELB[35, 36].

Contrary to *p52$^{ki/ki}$* mice, *Ets1$^{-/-}$* mice had enhanced GCs, ASCs and autoantibody production at steady state [16, 37, 38]. Besides the B cell-extrinsic nature of enhanced GC responses in *Ets1$^{-/-}$* mice[16, 52], increased sera antibodies in *B-Ets1$^{-/-}$* mice were in response to autoantigens. The B cell-intrinsic contribution of ETS1 during TD antibody responses is unknown. Hence our demonstration of a B cell-intrinsic requirement of p52:ETS1 transcriptional complex for

GC formation upon TD immunization are not contradictory with existing studies on the role of ETS1 in B cells[16, 37, 38].

Our data clearly indicate that p52:ETS1 regulates gene transcription and subsequent protein expression of OCT1 and OBF1 during B cell activation, thereby influencing their transition to GC B cells. ETS1 recruitment to *Pou2f1* and *Pou2af1* mouse promoters, was observed at two predicted ETS1 motifs in close proximity, consistent with the current model where two ETS1 monomers within the p52:ETS1 hetero-tetrameric complex bind DNA[21]. Additionally, ETS1 colocalizes with OCT1, OCT2, and OBF1 in mouse B cells based on ChIP-seq analyses[23] and OCT1 and OBF1 were previously described to reciprocally regulate each other[23, 24]. This extensive colocalization supports our findings that ETS1 directly regulates OCT1 and OBF1 expression in mouse B cells. Moreover, ChIP-reChIP and EMSA analyses on human *POU2AF1* promoter further validated that ETS1 requires p52 interaction to bind two conserved ETS1 binding sites, demonstrating specific p52:ETS1 binding with p52 acting as a co-factor. These assays helped address the limitation posed by the current unavailability of validated ChIP-grade antibodies for mouse p52. Future work will require a reliable mouse ChIP-grade p52 antibody to determine whether p52:ETS1 physically interacts with OCT1 and OBF1 in activated mouse B cells to regulate common downstream targets.

Beyond B cell-specific functions, p52:ETS1 likely regulates gene expression and functions in other cell types as evidenced by a B cell-extrinsic defect in T_{FH} differentiation. The reduction in T_{FH} population in $p52^{ki/ki}$ but not B-$p52^{ki/ki}$ suggests that T_{FH} cells differentiation, maintenance or priming require p52:ETS1 complex in non-B cells, a topic of future investigation. Lastly, the combined B cell-extrinsic T_{FH} cell deficiency and B cell-intrinsic GC defects likely explain the more severe GC impairment observed in $p52^{ki/ki}$ mice compared to *B-*$p52^{ki/ki}$ mice.

In summary, using *p52$^{ki/ki}$* mice with reduced p52:ETS1 binding, we demonstrated the requirement of p52:ETS1 complex in splenic GC B cells differentiation and production of TD antigen-specific IgG_1. Importantly, we also revealed binding of p52:ETS1 at *Pou2f1 and Pou2af1* promoters in activated B cells, driving their gene expression and consequentially, protein expression. The resultant OCT1:OBF1 complex initiates early GC B cell transcriptional program and genes essential for CSR and proliferation. As p52:ETS1 does not bind classical κB-sites on DNA to effect transcriptional changes, this implies that there exist many other genes lacking upstream κB-sites binding sites that can be regulated by stimuli that activates NFκB pathways. These findings have therapeutic implications, particularly in the context of disrupting or activating specific p52 transcriptional complexes that regulate biology beyond the "classical" NFκB target genes.

ACKNOWLEDGEMENTS

The authors thank all members of the Tergaonkar and LKP lab for useful discussion and advice. We thank SIgN Flow cytometry Core, in particular Seri Mustafah and Ong Jia Min, for cell sorting services. We are also grateful to Grace Yeo (GIS, A*STAR) and Yingrou Tan (SIgN, A*STAR) for helpful discussion about scATAC-seq analyses and imaging respectively. This work was supported by MOH-OFIRG21jun-0014, NRF-CRP26-2021-0001 and MOH-OFIRG24jan-0003 awarded to V.T. This work is also supported by SIgN Core Funds and HHP-IAF-PP:H22J1a0046 awarded to K.P.L. D.M and B.Z are supported by A*STAR Graduate Scholarship and National Science Scholarship from the Agency for Science Technology and Research Singapore respectively. B.Z. is supported by A*STAR Career Development Fund C210812013. G.G and S.S were supported by NIH grant number (GM 085490).

AUTHOR CONTRIBUTIONS

D.M and B.Z conceptualised the project, designed and performed all experiments, analysed all data and wrote the manuscript unless otherwise stated. A.R, T.Y.S, J.Y, A.H.W and M.M.H.M assisted with performing the experiments. T.S performed PCA. M.I made the *p52*$^{ki/ki}$ mouse model. D.H and A.B helped with confocal microscopy and image processing. W.P and V.Y.W designed and performed BLI. K.F performed all computational analyses unless otherwise stated. S.W.H and S.H.F helped to design and performed scATAC-seq experiment respectively. N.A, W.L.T and M.C.L analysed scATAC-seq data. S.S and G.G helped to design and optimise ChIP-Seq experiment. I.N.S and J.W.C performed ChIP-seq on human cell lines. P.M performed EMSA. Y.F.P, P.J, A.D.J designed and optimised PLA. I.T optimised cell fractionation. X.S and Y.Z, helped with experimental design. V.T and K.P.L obtained funding for this research and cowrote the manuscript. V.T and K.P.L are joint corresponding authors on this paper and co-supervised this work. All authors approved the final draft of the paper.

COMPETING INTEREST

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1: Generation of *p52*$^{ki/ki}$ mice with disruption of p52:ETS1 interaction.

(a) Schematic diagram of domains present in p52 and ETS1. R241, M297 and K298 (RMK) in p52 DSD mediate interaction with ETS1. **(b)** Structural interface showing side chains of R241 of p52 (green) forming hydrogen bonds with backbone carbonyl group of T346 and salt bridges with D347 of ETS1 (pink) (left). L422 of ETS1 interacts with M297 and K298 of p52 via main chain hydrogen bonds (right). Images from PDB. **(c)** Biolayer interferometry was performed using His-tagged-ETS1$_{331\text{-}441}$ immobilized on Ni-NTA biosensors, incubated with p52$_{1–398}$ WT

(top) and $p52_{1–398}$ RMK>A mutant (bottom) in solution at 2-fold serial dilutions (188 nM to 11.75 nM) to measure binding affinities. Representative data of n=2 experiments are shown. **(d)** 3 regions encoding R241, M297, and K298 in WT *Nfκb2* alleles were mutated to alanine (GCC) to generate $p52^{ki/ki}$ mice. Blue arrows show genotyping primers targeting mutated regions. Resultant 551 bp PCR product was digested with HhaI, which cuts WT alleles (GCGC site), yielding 436 bp and 115 bp bands. **(e)** Genotyping results for $p52^{+/+}$ (WT), $p52^{ki/+}$ and $p52^{ki/ki}$ littermates. Data are representative of multiple biological replicates. **(f)** Image of 8 weeks old WT and $p52^{ki/ki}$ mice. **(g)** Murine splenic B cell lysates were immunoprecipitated using anti-p52 antibody. Lanes 1–2: Input. Lanes 3–4: IP fractions. Lanes 5–6: IgG control. p52, RELB, and HSP90 were detected on one blot. ETS1 and GAPDH were detected on a separate blot using same samples. Data are representative of 3 biological replicates. **(h)** Splenic protein expression of NFκB members and ETS1. Data are representative of 3 independent experiments, n=3 per group. **i-k**, Gene transcription of **(i)** *Ets1*, *Relb* and *Nfκb2,* **(j)** *Cxcl13*, *Ccl19* and *Ccl21*, **(k)** *p21*, *Dr5* and *Puma* in spleen measured by qPCR, n=12 per group. Data are pooled from 3 independent experiments, n=4 mice per group. Data are presented as mean ± sd with each data point representing an individual mouse. Mice were sex-matched per experiment, with both male and female mice aged 8-12 weeks used. Statistical analyses were performed using two-tailed Mann-Whitney U tests in **(i-k)**.

Figure 2: p52:ETS1 interaction is necessary for splenic germinal center B cell responses to T cell-dependent immunization.

a-c, Flow cytometry analyses of splenic germinal center (GC) B cells from wildtype $p52^{+/+}$(WT), $p52^{ki/+}$ and $p52^{ki/ki}$ mice on day 9 post NP-OVA+alum intraperitoneal immunization. **(a)** Representative plots and summary bar graphs of **(b)** frequencies and **(c)** numbers of GC B cells ($GL7^+$ Fas^+). Pooled data from 3 independent experiments, minimum

n=3 mice per group. **d-n**, Analyses in wildtype $p52^{+/+}$ (Ctrl) and $p52^{ki/ki}$ mice post intraperitoneal NP-OVA+alum immunization (**d)** Representative immunofluorescence images of splenic GC B cells on day 9. GC B cells are defined as $GL7^{+}$ (green) IgD^{-} (purple) $TCR\beta^{-}$ (blue) areas. CD169 (yellow) marks metallophilic macrophages. Top panel: whole spleen (10×); middle and bottom panels: single B cell follicles (40×). Scale bars, 250 μm. Representative data of n=3 mice. **(e)** Representative flow plots and summary bar graphs of **(f)** frequencies and **(g)** numbers of dark ($CXCR4^{hi}$ $CD86^{lo}$) and light zone ($CXCR4^{lo}$ $CD86^{hi}$) splenic GC B cells in mice on day 9. Pooled data from 3 independent experiments, minimum n=3 mice per group. **(h)** Representative flow plots and summary bar graphs of **(i)** frequencies and **(j)** numbers of $IgG1^{+}$ GC B cells in mice on day 9. Pooled data from 3 independent experiments, minimum n=3 mice per group. **(k)** Representative enzyme-linked immunospot assay (ELISpot) images and summary bar graphs of **(l)** numbers of NP-specific IgG_1 antibody secreting cells (ASCs) isolated from bone marrow on day 28. High and low affinity antibodies were assessed through binding to NP2-BSA and NP28-BSA respectively. Representative data from 3 independent experiments with n=4 mice per group. Enzyme-linked immunosorbent assay (ELISA) quantification of **(m)** low and **(n)** high affinity NP-specific IgG_1 in the sera of Ctrl and $p52^{ki/ki}$ mice 14 and 28 days after immunization. Representative data from 3 independent experiments with n=4 mice per group. Data were presented as mean ± sd. Statistical analyses were performed using two-tailed Mann-Whitney U tests in **(f,g,j,l,m,n)** or Kruskal–Wallis tests with Dunn's post hoc analysis **(b,c,i)**. **a-n**, Mice were sex-matched per experiment, using both 8-12 weeks male and female mice.

Figure 3: B cell-intrinsic disruption of p52:ETS1 complex impairs splenic GC responses. **(a)** Schematic diagram of mixed bone marrow chimeric mice made using irradiated C57BL/6J wildtype (CD45.1) recipient mice reconstituted with bone marrow cells from muMT (CD45.2) and wildtype (CD45.1.2) or $p52^{ki/ki}$ (CD45.1.2) mice in a 1:1 ratio, set up to study B cell-

intrinsic role of p52:ETS1 interaction. **b-g,** GC and IgG_1 B cell responses were examined by flow cytometry 9 days post intraperitoneal immunization with NP-OVA+alum in MuMT+WT (Ctrl) and MuMT+$p52^{ki/ki}$ (*B-p52$^{ki/ki}$*) BMC mice, n=13 per group. Data are pooled from 3 independent experiments, minimum n= 4 mice per group. **(b)** Representative flow cytometry plots and summary bar graphs of **(c)** frequencies and **(d)** numbers of splenic GC B cells ($GL7^+Fas^+$) gated on live $CD45.1.2^+$ $CD19^+$ IgD^- cells in Ctrl and *B-p52$^{ki/ki}$* mice. **(e)** Representative flow cytometry plots and summary bar graphs of **(f)** frequencies and **(g)** numbers of splenic IgG_1 GC B cells in Ctrl and *B-p52$^{ki/ki}$* mice. IgG_1^+ cells gated on live $CD45.1.2^+CD19^+IgD^-GL7^+$ Fas^+ cells. **h-i,** NP-specific IgG_1 titres were quantified by ELISA in sera obtained from Ctrl and *B-p52$^{ki/ki}$* mice 14 and 28 days after immunization with NP-OVA+alum, n=10 per group for Day 14 and n=9 per group for Day 28. Data are pooled from 3 independent experiments, minimum n=3 mice per group. Summary bar graphs of ELISA quantifications of **(h)** low affinity NP28-specific or **(i)** high affinity NP2-specific IgG_1 in the sera of Ctrl and *B-p52$^{ki/ki}$* mice 14 and 28 days after immunization. Data are presented as mean ± sd with each data point representing biological sample from an individual mouse. All mice used in these experiments were 8 weeks old males at the point of bone marrow reconstitution and 16-18 weeks old at the beginning of each experiment. Statistical analyses were performed using two-tailed Mann-Whitney U tests in **(c,d,f-i)**. nd = not determined as values fall below detection limit of assay at 1.56 arbitrary units (AU) relative to the standard.

Figure 4: Disruption of p52:ETS1 complex alters gene expression during the differentiation of B cells into GC B cells.

(a) Volcano plot of differentially expressed genes (DEGs) (2-fold; p-value ≤0.05) between control (Ctrl) and *p52$^{ki/ki}$* splenic GC B cells ($CD19^+IgD^-GL7^+Fas^+$) sorted from mice 9 days post NP-OVA+alum immunization analyzed by RNA-seq. Normalized gene expression of **(b)**

Pou2f1, **(c)** *Aicda*, **(d)** *Prdm1*, **(e)** *Mki67* presented as $\log_2$ count per million (CPM) in **(a). b-e**, Box plots show median (center line), interquartile range (box: 25th to 75th percentile), and whiskers extending to data points within 1.5× the IQR. **(f)** Gene sets enriched for DEGs between control and p$52^{ki/ki}$ GC B cells analyzed by Enrichr using gene signatures from MSigDB Hallmark (adjusted p-value < 0.05). Top 5 enrichments are shown based on p-value ranking. **a-f,** Data from n=3 Ctrl mice and n=2 $p52^{ki/ki}$ mice. Statistical analyses were performed using empirical Bayes moderated t-test. **g-j**, Transcript levels of **(g)** *Pou2f1,* **(h)** *Pou2af1,* **(i)** *Aicda* and **(j)** *Prdm1* in sorted activated splenic B cells ($CD19^+IgD^-$) from mice 9 days post immunisation with NP-OVA and alum, n=11 per group. Pooled data from 3 independent experiments, minimum n=3 per group. Data presented as mean ± sd with each data point representing an individual mouse. Statistical analyses were performed using two-tailed Mann-Whitney U tests. **k-o**, Naïve and activated splenic B cells were sorted from Ctrl and p$52^{ki/ki}$ mice 9 days post NP-OVA+alum immunization for scATAC-seq**. (k)** Uniform manifold approximation and projection (UMAP) visualization of B cell clusters and **(l)** cell frequencies for each cluster identified in **(k)**. Weighted kernel density plots presenting combined gene activity levels for **(m)** *Bcl6*, *Aicda* and *Cd86* and **(n)** *Bcl6*, *Aicda*, *Cd86 Pou2f1*, *Pou2af1* and *Mki67*. **(o)** Top panel: Normalized scATAC-seq sequencing tracks for *Aicda* gene across all clusters identified in **(k)**. Bottom panel: Combined coverage plot across all clusters for the region in red box presented in upper panel. Two-tailed wilcoxon rank-sum statistical test with adjustment for multiple comparisons correction was used to analyze data, adjusted p < 0.05. Mice were sex-matched per experiment, using both 8-12 weeks old male and female mice.

Figure 5: p52:ETS1 complex is necessary for OCT1 and OBF1 expression in GC B cells.

a-f, Quantification of OCT1 and OBF1 expression in splenic activated B cells ($CD19^+IgD^-$) sorted from mice 9 days after intraperitoneal immunization with NP-OVA+alum. **(a)** Protein

expression in activated B cells from Ctrl and *p52*$^{ki/ki}$ mice, each lane represents an individual mouse. Representative of 3 independent experiments, minimum n=3 per group. Densitometry ratio of **(b)** OBF1/HSP90 and **(c)** OCT1Oct1/HSP90 in Ctrl (n=15) and *p52*$^{ki/ki}$ (n=14) mice. Pooled data from 3 independent experiments, minimum n=4 mice per group. **(d)** Protein expression in activated B cells from Ctrl and *B-p52*$^{ki/ki}$ BMC mice, each lane represents an individual mouse. Representative of three independent experiments, n=3 per group. Densitometry ratio of **(e)** OBF1/HSP90, n=6 per group and **(f)** OCT1/HSP90, n=9 per group. Data in **(e)** and **(f)** are pooled from 2 and 3 independent experiments respectively, n=3 BMC mice per group**. (g)** Representative histograms and **(h)** mean fluorescence intensity (MFI) of OBF1 in GC B cells ($CD19^+IgD^-GL7^+Fas^+$) of Ctrl and *B-p52*$^{ki/ki}$ BMC mice 9 days after NP-OVA+alum immunization. Pooled data (n=8) from 2 independent experiments, n=4 mice per group. **(i)** Representative flow cytometry plots and **(j)** summary bar graph of frequencies of Ki-67-expressing Pre-GC B cells ($CD19^+IgD^-GL7^+CD38^+$) 9 days post intraperitoneal immunization with NP-OVA and alum in Ctrl (n=9) and *p52*$^{ki/ki}$ (n=10) mice. Pooled data from 3 independent experiments, minimum n=3 mice per group. **k-n,** *In vitro* IgG_1 switching and proliferation were assessed on day 4 after activating naïve Ctrl or *p52*$^{ki/ki}$ splenic B cells with αCD40 and IL-4. Data are representative of 3 independent experiments with B cells from n=3 mice per group and 4 technical replicates for each sample**. (k)** Representative flow plot and **(l)** summary bar graph of frequencies of IgG_1^+ B cell. **(m)** Representative histogram and **(n)** summary bar graph of B cell proliferation. Data are presented as mean ± sd. Statistical analyses were performed using two-tailed Mann-Whitney U tests in **(b,c,e,f,h,j,l,n)**. **a-c,i-n,** Mice were sex-matched per experiment, with both male and female mice aged 8-12 weeks used. **d-h**, Male BMC mice reconstituted at 8 weeks old, were used at 16–18 weeks old.

Figure 6: p52:ETS1 binding sites are present in the promoter region of *Pou2f1* and *Pou2af1*

Mouse **(a)** ETS1 and **(b)** RELB binding consensus sequences were obtained from Cistrome and Homer. **a-b**, Top putative markers of ETS1 and RELB were downloaded from Cistrome (ETS1:GSM2218592, GSM2218593, GSM2310033, GSM2310034; RELB: GSM881110, GSM881107, GSM881109, GSM881108) and analysed by Homer software to discover de novo motifs. Motif matrix is used in ggseqlogo software to generate TF motif plot. ETS1 and RELB ChIP-seq tracks on **(c)** *Pou2f1* and **(d)** *Pou2af1* mouse promoters retrieved from Cistrome (ETS1: GSM2218592, GSM2218593; RELB: GSM881110, GSM881107). **e-h**, Enrichment levels of ETS1 and RELB on promoters of **(e)** *Pou2f1*, **(f)** *Pou2af1*, **(g)** *Icosl* and **(h)** *Pax5* promoters in activated splenic B ($CD19^{+}IgD^{-}$) cells sorted from Ctrl and *B-p52*$^{ki/ki}$ BMC mice on day 9 post-immunization with NP-OVA and alum measured via ChIP-qPCR. Each data point represents biological sample from n=4 mice per group for each experiment. Data are pooled from 3 independent experiments with 2 technical replicates. Mice used in these experiments were 8 weeks old males at the point of bone marrow reconstitution and 16-18 weeks old at the beginning of each experiment. **i-k**, ETS1, p52, RELB, IgG, input, H3K27Me, H3K4Me3, H3K27Ac and PolII ChIP-seq tracks on human **(i)** *POU2AF1,* **(j)** *POU2F1* and **(k)** *ICOSL* promoters in OCI-Ly3 B cells. Quantification of ETS1, p52 and RELB binding (normalized to IgG, reads per genome coverage= RPGC within + 2kb of TSS) shown on right panel. **1-m**, Enrichment levels of p52, ETS1 and RELB on **(l)** *POU2AF1* **(m)** *ICOSL* and (n) *PAX5* promoters in human OCI-Ly3 B cells measured via ChIP-qPCR. Pooled data from 4 independent experiments. Enrichment levels of p52:ETS1 and p52:RELB complexes on **(o)** *POU2AF1*and **(p)** *ICOSL* promoters in human OCI-Ly3 B cells measured via ChIP-reChIP qPCR. Pooled data from 4 independent experiments. Enrichment was calculated by % input and normalized to IgG in **(e-h,l-p)**. Data are presented as mean ± sd. Statistical analyses were performed using two-tailed Mann-Whitney U tests in **(e-h,l-p)**.

Figure 7: p52:ETS1 complex is necessary for IgE mediated allergy responses.

a-b, ELISA quantification (Optical Density, OD) of low and high affinity NP-specific IgE antibodies in the sera of Ctrl and B-*p52*$^{ki/ki}$ BMC mice **(a)** 14 days and **(b)** 28 days after immunization with NP-OVA and alum. Data are pooled from 3 independent experiments, minimum n=3 mice per group (Day 14: n = 13 and Day 28: n = 9 per group). **c-f**, Passive cutaneous anaphylaxis (PCA) assays were performed using sera from mice in **a,b**. **(c)** Schematic diagram of PCA experimental model in mice. **(d)** Representative images of recipient mice post PCA experiment. Each recipient mouse received sera from 1 Ctrl and 1 B-*p52*$^{ki/ki}$ mice in the right and left ear pinnae respectively. Summary bar graphs of absorbance (OD) and quantification of evans blue dye in digested ear tissues of mice who received **(e)** day 14 and **(f)** day 28 sera from immunized Ctrl and B-*p52*$^{ki/ki}$ BMC mice. Pooled data from 3 independent experiments minimum n=3 mice per group (Day 14: n = 13 and Day 28: n = 9 per group). Data are presented as mean ± sd with each data point representing biological sample from an individual mouse. All donor mice used in these experiments were 8 weeks old males at the point of bone marrow reconstitution and 16-18 weeks old at the beginning of each experiment while all recipient mice are 8 weeks old male wildtype C57BL/6J mice. Statistical analyses were performed using multiple Mann-Whitney U tests with false discovery rate correction for pairwise comparisons between Ctrl and B-*p52*$^{ki/ki}$ group per dilution in (**a,b)** and two-tailed Mann-Whitney U tests in (**e,f)**.

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MATERIALS AND METHODS

Mice

Newly generated $p52^{ki/ki}$ mice were obtained from Prof Masahito Ikawa (Professor, Research Institute for Microbial Diseases, Osaka University). In brief, C57BL/6N mouse zygotes were injected with CRISPR-Cas9 reagents including double-stranded DNA which functioned as repair templates containing the mutated sequences required for homology-directed repair. Subsequently, founder mice with germ cells carrying p52 RMK>A mutations were backcrossed with CD45.2 C57BL/6J wildtype mice (Strain#: 000664, Jackson's laboratory (Jax)) to produce $p52^{+/ki}$ mice. These $p52^{+/ki}$ mice were backcrossed to CD45.2 C57BL/6J wildtype for at least 10 generations. Subsequently, $p52^{+/ki}$ mice of both sexes were used for breeding to result in littermates with genotypes: wildtype $(p52^{+/+})$, heterozygotes ($p52^{+/ki}$) and *p52* knock-in ($p52^{ki/ki}$). Genotyping details can be found in supplementary methods. For mixed bone marrow chimera (BMC) experiments, CD45.1 C57BL/6 wildtype mice (Strain#: 002014, Jax) were bred with both CD45.2 C57BL/6 wildtype and $p52^{ki/ki}$ mice to produce CD45.1.2 donor

mice (CD45.1.2 WT and CD45.1.2 $p52^{ki/ki}$). CD45.2 C57BL/6 muMT mice (Strain#: 002288, Jax**)** were also used as bone marrow donors, with femurs provided by the Agency for Science, Technology, and Research (A*STAR) Infectious Diseases LabsAll experiments were performed using CD45.2 C57BL/6 control (wildtype), $p52^{+/ki}$ and $p52^{ki/ki}$ mice that were 8-12 weeks old. Mice were sex-matched per experiment, using both 8-12 weeks old male and female mice except for experiments involving mixed bone marrow chimeric mice which were exclusively 8-12 week old males for both donors (MuMT and $p52^{+/+}$ or $p52^{-/-}$ mice) and recipients (Wildtype CD45.1). Recipient CD45.1 C57BL/6 male were 8-12 weeks old males at the point of bone marrow reconstitution and 16-18 weeks old at the beginning of each experiment. Randomization was performed through distribution of mice of each group across multiple cages. Equal number of mice from each genotype were selected for experiment but mice were randomly weaned into individual vivarium prior to genotyping. Mice were immunized and data were acquired in no particular order.All mice were housed in specific-pathogen free rooms maintained at 23ºC, 40-60% humidity with 12-hour light/dark cycle. All animal studies have been approved by the Institutional Animal Care and Use Committee (IACUC) of the Biological Resource Centre (BRC) at the A*STAR and performed in compliance with ethical guidelines.

Mixed bone marrow chimera

Bone marrow cells were harvested using aseptic techniques from both femurs of C57BL/6 donor mice (CD45.2 muMT, CD45.1.2 $p52^{ki/ki}$ and CD45.1.2 WT). For co-transfer, 500,000 cells from each donor were resuspended in 100 µl of PBS and mixed at 1:1 ratio before injection into recipient mice. CD45.1 C57BL/6 wildtype male mice were lethally irradiated at 600 gamma rads twice, 4 h apart. Within 1 h of the second irradiation dose, 1 million bone marrow

898 donor cells in 200 µl of PBS were retro-orbitally injected into the mice. Mice were bled to
899 check for engraftment levels 8 weeks post-reconstitution via flow cytometry.

900 **Mice immunizations**

901 For assessment of germinal centres and T-cell dependent antibody responses, mice were
902 immunized intraperitoneally with 100µg of NP-OVA (LGC Biosearch Technologies)
903 precipitated on 4mg aluminium hydroxide (Sigma) in 200µL of PBS. Spleens were harvested
904 on days 5 and 9 for flow cytometry or FACS while bone marrow was harvested on day 28 for
905 enzyme-linked immunospot (ELISpot) assay. Sera were collected weekly on days 14 and 28
906 by retro-orbital bleeding and frozen at -80ºC before enzyme-linked immunosorbent assays
907 (ELISA) were performed. For germinal centre B cell analyses in inguinal lymph nodes, mice
908 were immunized subcutaneously with 10µg of NP-OVA (LGC Biosearch Technologies)
909 precipitated on 2mg aluminium hydroxide (Sigma) in 200µl of PBS on each flank. After which,
910 inguinal lymph nodes were harvested on day 9 for analyses.

911 **Passive Cutaneous Anaphylaxis**

912 CD45.2 C57BL/6 male recipient mice were passively sensitized once with 20 µl of day 14 or
913 28 sera from NP-OVA and alum-immunized mice in each ear pinnae via intradermal injection.
914 After 24 h, mice were injected retro-orbitally with 200µl of 1% Evans blue containing 100 µg
915 of NP-OVA and observed for 30 min. Subsequently, mice were euthanized, and whole ear
916 pinna were collected. The ear pinna were diced into pieces in an Eppendorf tube and incubated
917 in 240µl of formamide overnight at 55°C. The samples were centrifuged at 21130 x g for 10
918 min. 100µl aliquot of the supernatant from each digested sample were quantified with a
919 spectrophotometer at OD 600 nm.

920 **Harvest and isolation of cells from mice lymphoid organs**

Immune cells were isolated from thymus, bone marrow, spleen, Peyers' patches, mesenteric and inguinal lymph nodes of mice for various downstream experiments. For collection of cells from thymus, bone marrow, spleen, the tissues were meshed on a 100 μm cell strainer placed onto a 50 ml tube. Cells were flushed through cell strainer using ice cold PBS and centrifuged at 250 x g for 5 min. Subsequently, 2ml of AKC lysis buffer was added into each cell pellet to lyse red blood cells for 2 min at 25 °C. To stop the lysis reaction, equivolume of ice cold PBS with 2% of fetal bovine serum (FBS) (FACS buffer) was added before centrifugation at 250 x g for 5 min. Subsequently, cells were resuspended in FACs buffer (for flow cytometry) or PBS (for immunoprecipitation, western blot and qPCR). The cells were then passed through another 70 μm cell strainer and ready to use for downstream experiments. Cells from Peyer's patches, mesenteric, and inguinal lymph nodes were dissociated through homogenization in between two frosted glass slides, followed by washing with ice-cold FACS buffer. The cell suspension was then filtered through a 70 μm strainer into 5 mL tubes. After an additional wash with ice-cold FACS buffer and centrifugation at 250 x g for 5 min, the cells were resuspended in FACS buffer for flow cytometry staining.

Fluorescence-activated cell sorting (FACs)

Briefly, single cell suspension were prepared as described above before cell pellets were resuspended in EasySep™ Buffer (1x PBS, 2% FBS, 1mM EDTA) before filtration into FACS tube with 70 μm filter caps. B cell isolation was performed using negative selection through magnetic separation according to manufacturer's protocol (STEMCELL Technologies, Cat No: 19854). Subsequently, cells were stained with LIVE/DEAD fixable Aqua dead cell stain (Invitrogen) and fluorophore-conjugated antibodies at various dilutions (Supp. Table 2) in FACS buffer (PBS containing 2% FBS) in 4ºC for 30 min in the dark. Stained cells were then washed with ice cold PBS, centrifuged at 250 x g for 5 min before resuspension in 0.5% bovine

serum albumin (BSA) in PBS for cell sorting on BD influx or BD Aria III into appropriate collection media for downstream assays including RNA-seq, qPCR, single cell ATAC-seq, western blot and ChIP.

Flow cytometry

Briefly, single cell suspensions were prepared from lymphoid tissues as described above. For staining, cell pellets were prepared in 96 well plates before incubation with LIVE/DEAD fixable Aqua dead cell stain (Invitrogen) and fluorophore-conjugated antibodies at various dilutions (Supp. Table 2) in FACS buffer at 25 °C or on ice for 30 min in the dark. Stained cells were then washed with FACS buffer, centrifuged at 700 x g for 3 min and re-suspended in FACS buffer. To perform intracellular staining, cells were fixed and permeabilized with BD Cytofix/Cytoperm™ kit or eBioscience™ Foxp3 Transcription Factor Staining Buffer Set for staining of cytoplasmic or transcription factors respectively. All samples were analysed on BD LSRII/FACSymphony™ A5. Flow cytometry data were analysed using FlowJo software (Version 10.10).

Enzyme-linked immunospot (ELISpot) assay

To measure antigen-specific antibody secreting cells (ASCs), 96 well ELISpot multiscreen HTS plates (Merck Millipore) were pre-activated with ethanol before being coated with 10µg/mL of either NP2-BSA or NP28-BSA (LGC Biosearch) dissolved in PBS and incubated at 4°C overnight. Next day, the plates were washed in sterile PBS, blocked with complete media (RPMI supplemented with 10% of FBS, 25mM HEPES (pH 7.2-7.5), 1mM sodium pyruvate, 2mM L-glutamine, 1% of penicillin/streptomycin and 55µM β-mercaptoethanol) for 2 h at 37°C. Single cell suspension were prepared from bone marrow cells isolated from a pair of femurs from each mouse. Briefly, bone marrow tissue were homogenized using the back of a plunger of a syringe on a 100 micron cell strainer placed onto a 50 ml tube. Cells were flushed

through cell strainer using ice cold complete media and centrifuged at 250 x g for 5 min. Subsequently, 2ml of AKC lysis buffer was added into each cell pellet to lyse red blood cells for 5 min at 25 °C. Bone marrow cells were plated at 2 x 10^6/well and diluted 1:2 in three serial dilutions as technical replicates. Cells were cultured at 37° C for 20-24 h before removal. Plates were washed thrice with PBS with 0.1% Tween 20 before addition of AP-conjugated goat anti-mouse IgG_1 antibodies (Southern Biotech, see Supp. Table 2) and incubation at 37°C for 1 h. ASC were visualized using 5-Bromo- 4-Chloro-3-Indolyl Phosphate (BCIP) and quantified using an ImmunoSpot analyzer (Mabtech IRIS).

Enzyme-linked Immunosorbent Assay (ELISA)

For the analysis of antigen-specific antibodies, 96-well Maxisorp plates (Thermo Fisher Scientific) were coated overnight with 10µg/mL of NP2-BSA or NP28-BSA (LGC Biosearch) dissolved in PBS. The following day, plates were washed and incubated with blocking buffer (1% BSA in PBS) at 25 °C for 1 h. Serially diluted serum samples and standards were then added to the plate and incubated at 25 °C for 2 h. Standards for NP-IgG_1 were prepared from pooled sera of wildtype mice hyperimmunized with NP-OVA adsorbed to alum. NP-specific IgG1 and NP-specific IgE were detected using anti-mouse IgG_1-HRP and IgE-HRP (Southern Biotech) respectively (Supp. Table 2) and incubated for 1 h. Finally, plates were developed with stabilized chromogen tetramethylbenzidine (Life Technologies) and stopped with 1N hydrochloric acid before reading at 450nm on a microplate reader (Tecan).

Immunofluorescence

Spleens harvested from immunized mice were serially dehydrated in 10%, 20%, 30% sucrose solution before being frozen in a cryomold with O.C.T. Compound and stored at −80°C. Tissues were then cut into 6-µm sections using a cryostat (Leica) and mounted onto microscope slides before being storage at −80°C. Frozen sections were rehydrated with PBS before

blocking with normal rat serum in 1% BSA in PBS for 30 min. Sections were stained with the following fluorophore-conjugated antibodies (Supp. Table 2) before acquisition on an Olympus IX83 Slide Scanner microscope using 10x (NA 0.3) and 40x (NA 0.95) objectives.

Immunoprecipitation and western blotting

20 million cells were lysed in an eppendorf tube in protein lysis buffer (50 mM Tris at pH 8, 150 mM NaCl, 0.5% NP40, 0.05% SDS, 0.5% deoxycholic acid) supplemented with phosphatase inhibitors (Roche: 4906845001) and protease inhibitors (Roche: 11873580001) for 30 min on ice. Subsequently, the samples were sonicated for 5 min at high output intensity at 4°C, with 30-second intervals (on and off), and then centrifuged at 18,000 x g for 30 min. The aspirated supernatants were measured for protein concentration using the Bradford assay (Bio-Rad), and 1000 µg of each protein sample was used for the pull-down using 5 µg of either NFκB p52 (Santa Cruz: sc-7386), ETS1 (Santa Cruz: sc-55581) and IgG antibodies (Supp. Table 2). For the input, 100 µg of protein samples were set aside with 4x loading dye and denatured at 95°C for 10 min before being stored at -20°C. The immunoprecipitation samples were incubated with their respective antibodies overnight at 4°C on a rotator. The following day, 60 µl of protein G Dynabeads beads (Invitrogen: 10004D) were added to the samples, and incubation took place for 4 h at 4°C on a rotator. Afterward, the protein samples were placed on a magnetic stand, and the supernatants were discarded. The beads were washed three times with a specific buffer (10 mM Tris at pH 8, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, and 1% Triton-X). Following the removal of the supernatant, proteins were eluted by adding 60 µl of 1x loading dye (1X SDS-Sample loading dye containing 10% β-mercaptoethanol) to the beads and boiling the samples at 95°C for 10 min. Eluted immunoprecipitated complexes and input samples were loaded onto a 4–12% Bis-Tris gel (Invitrogen) for western blotting. Primary antibodies diluted (1:1000) in PBS containing 5% BSA were used for probing the protein of

interest and veriblot for IP detection (Abcam: Ab131366) was used as the secondary antibody (Supp. Table 2). Please refer to supplementary methods for more details on western blotting.

RNA isolation and cDNA conversion for qPCR

In an eppendorf tube, harvested cells were washed once with PBS and re-suspended in 800µl of Trizol (Invitrogen). After 10 min of incubation, 160µl of chloroform was added and mixed homogeneously several times. Samples were centrifuged at 16,000 x g for 15 min at 4°C, and 400µl of the aqueous layer was carefully transferred into a new tube containing 400µl of isopropanol. Once again, samples were centrifuged at 16,000 x g for 30 min at 4°C. The supernatant was discarded, and the precipitated pellet was washed twice with 500µl of 70% ethanol and transferred into a new tube. After air-drying the tubes for 5 min, 30µl of RNase-free water was added to resuspend the RNA. RNA concentration was measured using Nanodrop. For cDNA conversion, 1000 ng of RNA was reverse transcribed into cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). The conditions used for this reaction were 10 min at 25°C, 1 h at 50°C, then 20 min at 80°C. After which the samples were diluted to 5ng/µl using RNase-free water and used for Real-Time qPCR (See supplementary methods).

Bulk-RNA sequencing

RNA were extracted from FACS-sorted cells as described above and stored at -80°C. RNA integrity was assessed using a Bioanalyzer with an Agilent RNA 6000 Pico chip, while RNA concentration was measured using Qubit assays (Thermo Fisher Scientific: Q32852). Samples with high RIN values and concentrations were sent to the Genome Institute of Singapore, A*STAR, for library preparation and sequencing. For pre-GC B cells, sorted cells were sent to Singapore Immunology Network (SIgN) Immunogenomics platform, A*STAR, for RNA extraction, library preparation and sequencing. The total RNA was extracted using Arcturus

PicoPure RNA Isolation kit (Thermo Fisher Scientific) according to the manufacturer's protocol. RNA was assessed using either RiboGreen (Thermo Fisher Scientific) or Agilent Bioanalyser. cDNA libraries were prepared using 0.5 ng of total RNA, or half of the RNA sample where concentration was too low to be accurately quantified. The SMARTSeq v2 protocol[53] was used with 17 PCR cycles and the following modifications: 1. Addition of 20 μM TSO; 2. Use of 200 pg cDNA with 1/5 reaction of Illumina Nextera XT kit. The length distribution of the cDNA libraries was monitored using a DNA High Sensitivity Reagent Kit on the Perkin Elmer Labchip. All samples were subjected to an indexed paired-end sequencing run of 2x151 cycles on an Illumina Novaseq 6000 targeting at least 13 million read-pairs/sample. Data processing of bulk RNA-seq can be found in supplementary methods.

In vitro B cell proliferation and class switch recombination assays

For *in vitro* proliferation and class-switching assays, naive B cells were isolated as described above with B cell isolation kit (STEMCELL Technologies, Cat No: 19854) supplemented with biotin anti-mouse CD43 before labelling with CellTrace Violet (Invitrogen) in PBS for 5 min at 37°C. Labelling reaction was quenched with ice cold FBS and cells were washed twice with complete media before *in vitro* assays. Briefly, cells were stimulated for 96h with 1μg/ml anti-mouse CD40 (HM40-3) and 20ng/mL of IL-4 in complete media to induce class switching to IgG_1. Subsequently, B cells were harvested, stained and analyzed via flow cytometry as described above.

Cell fractionation

Total B cells were isolated with mouse B cell isolation kit as described above before cell fractionation using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermofisher, Cat No: 78835) kit. Cytoplasmic and nuclear extracts from splenic B cells were isolated according to manufacturer’s protocol before probing for ETS1 using immunoblotting. Lamin B and

GAPDH were used as housekeeping proteins for nuclear and cytoplasmic fractions respectively.

Single cell ATAC-seq

FACS-sorted naïve and activated mouse B cells were mixed at 1:3 ratio and sent to SIgN Immunogenomics facility to be processed for single cell ATAC-seq (scATAC-seq) on the 10x Genomics Chromium platform. Briefly, nuclei were prepared from sorted cells as per the 10x Genomics Demonstrated Protocol CG000169 followed by processing with the Chromium Single Cell ATAC Kit v2 following the manufacturer's protocol, targeting the capture of up to 5000 nuclei. After quantification on the Agilent Bioanalyzer, ATAC-seq libraries were pooled for sequencing on the Illumina NovaSeq X, with the cycle configuration of Read 1N-151 cycles; i7 index-8 cycles, i5 index-16 cycles; Read 2N-151 cycles, targeting about 200 million reads-pairs/sample. For data processing of scATAC-seq samples, please refer to supplementary methods.

Chromatin immunoprecipitation (ChIP)

FACS-sorted mouse activated B cells or cultured OCI-LY3 human DLBCL cells were used in ChIP experiments. OCI-Ly3 cells, originally gifted by Dr Mark Minden from university health network (UHN, Canada) were thawed and cultured in IMDM media (Gibco) supplemented with 10% of FBS and 1% of penicillin/streptomycin in T75 flasks. OCI-Ly3 cells were also used for ChIP-seq and ChIP-reChIP experiments. Detailed methods for ChIP followed ChIP-qPCR or ChIP-seq, and ChIP-reChIP experiments, as well as data processing of ChIP-seq samples and analyses of publicly available ChIP-seq datasets, are provided in the Supplementary Methods.

Statistical analyses

No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications[10, 52] . Data collection and analyses were not performed blind to the conditions of the experiments. Animals that died prior to experimental endpoint were excluded from the analyses. Overt outliers in dataset were excluded using ROUT method. As our sample sizes are small and some datasets do not follow normal distribution, data were analyzed with non-parametric tests namely Mann-Whitney U test or Kruskal-Wallis test followed by Dunn's test for post-hoc analyses unless otherwise stated. All statistical analysis was performed using GraphPad Prism software (Version 10.0.1) unless otherwise stated.

Please refer to supplementary methods for details on EMSA and BLI experiments.

DATA AVAILIBILITY

Raw and processed data files for bulk RNA-seq, ChIP-seq and scATAC-seq analyses have been deposited in the NCBI Gene Expression Omnibus under accession numbers GSE299466 (scATAC-seq) , GSE300334 (ChIP-seq), GSE300335 (GC B cells RNA-seq) and GSE300336 (Pre-GC B cells RNA-seq).

CODE AVAILIBILITY

The custom codes used in analyzing the datasets are available on Zenodo under https://doi.org/10.5281/zenodo.15628175 and https://doi.org/10.5281/zenodo.15680988 for scATAC-seq and all other datasets respectively.

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