

1 **miR-29b directly targets activation-induced cytidine deaminase in**  
2 **human B cells and can limit its inappropriate expression in naïve B**  
3 **cells**

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5 Running Title: miR29b regulation of AID

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## Abstract

Class-switch recombination (CSR) is an essential B cell process that alters the isotype of antibody produced by the B cell, tailoring the immune response to the nature of the invading pathogen. CSR requires the activity of the mutagenic enzyme AID (encoded by *AICDA*) to generate chromosomal lesions within the immunoglobulin genes that initiate the class switching recombination event. These AID-mediated mutations also participate in somatic-hypermutation of the immunoglobulin variable region, driving affinity maturation. As such, AID poses a significant oncogenic threat if it functions outside of the immunoglobulin locus. We found that expression of the microRNA, miR-29b, was repressed in B cells isolated from tonsil tissue, relative to circulating naïve B cells. Further investigation revealed that miR-29b was able to directly initiate the degradation of AID mRNA. Enforced overexpression of miR-29b in human B cells precipitated a reduction in overall AID protein and a corresponding diminution in CSR to IgE. Given miR-29b's ability to potently target AID, a mutagenic molecule that can initiate chromosomal translocations and "off-target" mutations, we propose that miR-29b acts to silence premature AID expression in naïve B cells, thus reducing the likelihood of inappropriate and potentially dangerous deamination activity.



## 1. Introduction

During the course of the immune response, mature B cells undergo two diversification events at the immunoglobulin loci, somatic hypermutation (SHM) and class-switch recombination (CSR) (Stavnezer, 2011). SHM introduces mutations in the hypervariable loops of the antigen binding site, found within the CDRs of the Immunoglobulin Heavy (*IGH*) and light (*IGL*) chain variable regions. This allows for selection of B cell clones which harbour Igs with increased affinity for antigen, promoting the development of highly specific Ig molecules. In contrast, CSR replaces the C $\mu$  and C $\delta$  heavy chain gene exons (encoding IgM and IgD respectively) with the downstream C-region exons of the  $\alpha$ ,  $\gamma$ , or  $\epsilon$  isotypes (encoding IgA, IgG and IgE) (Stavnezer and Schrader, 2014). This process, which is driven by cytokines released from T helper cell populations, ensures that the effector functions of the antibodies produced by B cells are tailored to the nature of the invading pathogen. Both CSR and SHM require the activity of the mutagenic enzyme, activation-induced cytidine deaminase (AID, encoded by *AICDA*) (Muramatsu et al., 2000; Revy et al., 2000). AID acts to deaminate deoxycytidine residues within the *IG* variable and the *IGH* constant regions, resulting in the production of deoxyuracils (Neuberger et al., 2003). In the case of SHM, the ensuing mismatches initiate low-fidelity DNA repair pathways leading to the incorporation of mutations within the *IGH* and *IGL* variable regions, whereas in CSR the mismatches trigger a deletional-recombination event that replaces the upstream “acceptor” C-region (initially C $\mu$  and C $\delta$ ) with a downstream “donor” C region (C $\gamma$ 1-4, C $\epsilon$  or C $\alpha$ 1-2) (Keim et al., 2013).



67 The mechanisms targeting AID to the Ig genes are incompletely understood. It has  
68 been proposed that transcriptional stalling (Pavri et al., 2010), the formation of  
69 RNA:DNA hybrids called R loops (Shinkura et al., 2003), the exosome (Basu et al.,  
70 2011), super-enhancers (Qian et al., 2014), germline transcript RNA (Wang et al.,  
71 2015), 14-3-3 adaptor proteins (Xu et al., 2010), as well as the sequence location of  
72 the Ig genes (Yeap et al., 2015), recruit AID to the DNA and promote deamination.  
73 However, AID activity is promiscuous, and deamination-induced mutations are  
74 detected at multiple non-Ig sites (M. Liu et al., 2008). This collateral damage has  
75 significant oncogenic potential, as demonstrated by AID-induced chromosomal  
76 translocations that occur in the context of genomic instability (Robbiani et al., 2008;  
77 2009) and AID-induced mutations of non-Ig genes (Duquette et al., 2005; Pasqualucci  
78 et al., 1998; 2001). Therefore, AID expression and activity must be carefully  
79 controlled.

80

81 One mechanism by which cells are able to fine-tune their protein expression is  
82 through microRNAs (miRNAs), short non-coding RNAs that regulate gene  
83 expression by promoting mRNA decay and translational repression (Bartel, 2009). In  
84 mice miR-155 has been shown to directly repress AID (Teng et al., 2008) and  
85 removing the miR-155 binding site within the *Aicda* 3' UTR increases the likelihood  
86 of AID-induced *Igh-Myc* chromosomal translocations (Dorsett et al., 2008), a  
87 transforming event frequently observed in Burkitt's Lymphoma. Similarly, miR-181  
88 can also directly target *Aicda* and is proposed to prevent inappropriate AID  
89 expression in the absence of B cell activation (de Yebenes et al., 2008). Although the  
90 miRNA-mediated regulation of AID expression has been well studied in mice, this  
91 mode of regulation has not been extensively studied in human B cells.



92

93           To identify miRNAs involved in coordinating the B cell response in humans,  
94 we analysed the published literature that had profiled the miRNA pool of distinct  
95 human B cell subpopulations (Basso et al., 2009; Malumbres et al., 2009; Tan et al.,  
96 2009; J. Zhang et al., 2009). These studies consistently reported that the conserved  
97 miRNA miR-29b was less abundant in germinal centre B cells relative to naïve and  
98 memory cell compartments. Similarly, we show that miR-29b has diminished  
99 expression in B cells isolated from tonsil tissue relative to naïve B cells isolated from  
100 peripheral blood. It is of interest to note that miR29b expression has also been  
101 reported to be reduced in mantle cell lymphoma, a lymphoma thought to originate  
102 from Pre-GC mature B cells (Zhao et al., 2010). In this report we investigate  
103 potential miR29b targets and demonstrate that it is able to directly target *AICDA*  
104 mRNA. Enforced overexpression of miR-29b in human B cells reduces overall AID  
105 protein and causes a corresponding diminution of CSR to IgE *in vitro*. Together, these  
106 results suggest that miR-29b is able to silence “leaky” expression of AID, limiting its  
107 expression to appropriately activated B cells and thus helping to maintain  
108 chromosomal integrity.  
109



## 2. Materials and Methods

### 2.1 Ethics

Ethical approval was granted by London Bridge Research Ethics Committee for both peripheral blood donations (09/H0804/77 and 14/LO/1699) and for tonsil donations (08/H0804/94). Full written informed consent was obtained from all donors or the donors' parents or legal guardian.

### 2.2 B cell isolation, transfection and culture

Naïve B cells were isolated from peripheral blood using the Naive B Cell Isolation Kit II (Miltenyi Biotec). B cells were isolated from tonsil tissue as previously described (Cooper et al., 2012). Briefly, mononuclear cells were isolated from dissected tonsil tissue on a density gradient (Lymphoprep, Axis-Shield PoC AS) followed by incubation with aminoethyl isothiuronium bromide-treated sheep red blood cells to rosette T cells. To overexpress miR-29b, 800 nM miR-29b Pre-miR miRNA Precursor (Thermo Fisher Scientific) or the equivalent non-targeting negative control molecules was delivered into  $5 \times 10^6$  freshly isolated primary B cells using the Amaxa Human B cell Nucleofector Kit (LONZA). The Pre-miR miRNA Precursors used were proprietary short (16-28bp) double stranded RNA molecules chemically modified to ensure the desired strand is loaded into the RISC complex (Barnes et al., 2012). To inhibit miR-29b, 800 nM miR-29b of mirVana miRNA Inhibitor (Thermo Fisher Scientific) or the equivalent non-targeting negative control molecule was delivered into  $5 \times 10^6$  freshly isolated primary B cells using the Amaxa Human B cell Nucleofector Kit (LONZA). The mirVana miRNA Inhibitors used were single stranded, chemically modified RNA molecules designed to irreversibly bind and



inhibit endogenous miRNAs (Barnes et al., 2012). Samples were nucleofected using program U-15 on the Nucleofector Device and cultured at a concentration  $5 \times 10^5 \text{ mL}^{-1}$  in RPMI 1640, 10% HyClone Fetal Bovine Serum, 100  $\mu\text{g/mL}$  Streptomycin, 100 U/ml Penicillin, 2 mM L-Glutamine. To stimulate CSR, the medium was supplemented with 1 mg/mL of anti-CD40 antibody, 200 IU/mL of recombinant human IL-4, 5 mg/mL of insulin and 35 mg/mL of transferrin. The human monoclonal IgM<sup>+</sup> IgD<sup>+</sup> CL01 B cell line (Cerutti et al., 1998) was cultured in RPMI 1640, 10% HyClone Fetal Bovine Serum, 100  $\mu\text{g/mL}$  Streptomycin, 100 U/mL Penicillin, 2 mM L-Glutamine and kept at a concentration of between  $1-5 \times 10^5$  cells/mL. To induce expression of AID, the culture medium was supplemented with 1 mg/mL of anti-CD40 antibody and 200 IU/mL of recombinant human IL-4. The CL01 cell line was nucleofected using the Amaxa Cell Line Nucleofector Kit V along with program C-09 on the Nucleofector Device. All cells were cultured in a humidified incubator at 37°C, 5% CO<sub>2</sub>.

### *2.3 RNA Isolation and quantitative (q) RT-PCR analysis*

Total RNA was extracted from cultured cells using the miRNeasy Mini Kit with QIAzol Lysis Reagent (Qiagen). Residual gDNA was removed by incubating the extracted sample with 20 units of TURBO DNase enzyme (Thermo Fisher Scientific) at 37°C for one hour followed by a second phenol-chloroform cleanup. The integrity and yield of isolated RNA was checked on a 2100 Bioanalyzer (Agilent) using the RNA6000 Pico Assay. For analysis of mRNA expression, cDNA was generated from total RNA using random hexamers with RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific) and all genes (with the exception of *AICDA*,  *$\epsilon$ GLT*, *IgG* and *IgE*) were detected expression using TaqMan MGB Gene Expression Assays



(Thermo Fisher Scientific). The primer and probe set for *AICDA* was designed using the Universal Probe Library Assay Design Centre (Roche) while the  $\epsilon$ *GLT*, *IgG* and *IgE* primer and probe sets were designed in house. Individual samples were subjected to qPCR and run in triplicate with TaqMan Universal Master Mix II on the ViiA 7 Real-Time PCR System using 18S rRNA as a normalization control and gene expression was determined using the  $2^{-\Delta\Delta C_t}$  method. For qRT-PCR analysis of miRNA expression, the TaqMan Small RNA Assay for miRNA quantification (Thermo Fisher Scientific) was used. This involved separate cDNA generation for each miRNA, utilising a miRNA-specific, stem-loop primer to facilitate reverse transcription. The small nucleolar RNA RNU6B was used as a normalization control and gene expression was determined using the  $2^{-\Delta\Delta C_t}$  method.

#### 2.4 Gene expression arrays

Fresh tonsil B cells were transfected with a miR-29b mimic or its associated negative control and cultured in class switching stimuli for 24 hours. RNA was isolated using the miRNeasy Mini Kit with QIAzol Lysis Reagent (Qiagen) and then DNase-treated as described. 10 ng RNA was amplified using the Ovation Pico WTA System V2 (Nugen). 4  $\mu$ g of amplified single-stranded cDNA was biotin-labeled using the Encore BiotinIL Module (Nugen). Finally, transcriptomic analysis was performed by hybridizing 750 ng of biotin-labeled single-stranded cDNA onto a HumanHT-12 v4 Expression BeadChip (Illumina) and scanned using the Illumina iScan System. QC analysis and RMA normalization was performed in Illumina's Genome Studio Suite v1.0. Assessment of differential gene expression and statistical analysis was performed in Partek Genomics Suite version 6.6. Data from this study have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible



185 through GEO Series accession number GSE100735  
186 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100735>).

### 187 *Immunoblotting*

188 B cells were stimulated for 60 hours in class-switching stimuli following transfection.  
189 Cells were harvested in protein lysis buffer (pH 7.4, 25 mM Tris, 150 mM NaCl, 1  
190 mM EDTA, 1% NP40, 5% Glycerol, 10% Roche Protease Inhibitor). Total protein  
191 levels were quantified using the bicinchoninic acid assay and approximately 50 ug  
192 protein lysate were run on SDS-polyacridimide gels. Following wet transfer onto  
193 nitrocellulose membranes, the membranes were probed with the following antibodies  
194 for protein expression (AID - EK2 5G9, STAT6 - D3H4, p-STAT6<sup>Tyr641</sup> - C11A12,  
195 p38 MAPK - D13E1, p-p38<sup>Thr180/Tyr182</sup> - D3F9, AKT - 40D4, p-AKT<sup>ser 473</sup> - D9E,  
196 GAPDH - 6C5), and images were developed using the Molecular Imager®  
197 ChemiDoc<sup>TM</sup> XRS System (Bio-Rad). GAPDH (clone 6C5, Abcam) was used as a  
198 loading control.

199

### 200 *2.5 Luciferase Assays*

201 The region of the *AICDA* 3' UTR encompassing the miR-29 binding site was  
202 subcloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector  
203 (Promega), which harbours both a firefly luciferase reporter gene, for analysis of  
204 miRNA-mRNA binding site interactions, and a renilla luciferase reporter gene, which  
205 acts as a transfection efficiency control. For the mismatched construct, the miR-29  
206 binding site in the AID 3' UTR was replaced with a BamHI restriction site using  
207 sequential rounds of mutation cloning. HEK293T cells were seeded onto white 96-  
208 well tissue culture plates and having reached 70% confluence were transfected with  
209 50 ng of one of the luciferase constructs and 10 nM of miR-29 mimic using



Lipofectamine 2000 (Thermo Fisher Scientific). Reactions were performed in triplicate. Luciferase activity was quantified after 24 hours on a GloMax-Multi Microplate Luminometer (Promega, 2 seconds integration time) using the Dual-Glo Luciferase Assay System (Promega). The luciferase signal ratio was calculated by dividing firefly luciferase activity by renilla luciferase activity and normalized to cells transfected with an insert-free pmirGLO vector.

## *2.6 Flow Cytometry*

Apoptosis staining was performed using the Annexin V Apoptosis Detection Kit (eBioscience). For cell proliferation analysis,  $10^7$  transfected CL01 cells were washed with PBS, resuspended in 1 mL PBS containing 1  $\mu$ M CellTrace Violet dye (Thermo Fisher Scientific), and left in the dark at 37°C for 10 minutes. The reaction was quenched by the addition of cell culture media. The cells were washed, resuspended and cultured in a 37°C incubator until analysis via flow cytometry. Intracellular antibody staining of IgG and IgE was performed as previously described<sup>16</sup> using the a fixable viability stain (Zombie Aqua Thermo Fisher Scientific).



### 3. Results

#### *3.1 miR-29b is repressed in tonsil B cells relative to peripheral naïve B cells*

In order to identify dynamically regulated miRNAs within the context of mature human B cells, we analysed the available literature profiling the miRNA pool in different B cell subpopulations (Basso et al., 2009; Malumbres et al., 2009; Tan et al., 2009; J. Zhang et al., 2009). We observed that the evolutionarily conserved miRNA miR-29b was less abundant in germinal centre B cells relative to naïve and memory cell compartments (supplementary Fig. 1A & 1B). miR-29b is a member of the miR-29 family, also including miR-29a and miR-29c, which share the same seed region and thus overlap in their predicted targets (Liston et al., 2012). Interestingly miR29c was also less abundant in GC B cells compared to naïve or memory compartments, potentially reinforcing the effects of this miRNA family (supplementary Fig. 1A & 1B). We analysed the abundance of miR-29b in B cells isolated from tonsils, a large proportion of which have a germinal centre phenotype (CD19<sup>+</sup> CD38<sup>+</sup> CD27<sup>-</sup> or CD19<sup>+</sup> CD38<sup>+</sup> CD27<sup>+</sup>) (supplementary Fig. 2A), and compared its prevalence to naïve B cells (CD19<sup>+</sup> IgD<sup>+</sup> CD27<sup>-</sup>) (supplementary Fig. 2B) isolated from peripheral blood. In line with previous reports (Malumbres et al., 2009), miR-29b was nearly 3 times more abundant in the circulating naïve B cells than in tonsil-derived B cells (Fig. 1).

#### *3.2 Identification of AICDA as a miR-29b target gene in human tonsil B cells*

To gain insight into the role of miR-29b in the context of B cell activation we overexpressed miR-29b in tonsil B cells and investigated global gene expression changes following 24 hours culture with IL-4 and anti-CD40 stimulation using Illumina BeadChip Microarrays (HumanHT-12 v4). Transfection of a miR-29b mimic



into tonsil B cells increased miR-29b abundance by approximately 15-fold 24 hours after transfection, compared to cells transfected with a negative control mimic molecule (supplementary Fig. 3A). No detectable difference was seen in cell viability between the miR29b mimic or negative control as judged by flow cytometry FSC/SSC although cell viability was reduced approximately 2 fold compared to untransfected cells. Analysis of the array data revealed only a moderate effect on overall gene expression, with just 29 genes being modulated by more than 1.2 fold (supplementary table I and Fig. 2A). However, of these genes, 20 (69%) contained miR-29 binding sites within their 3' UTRs, suggesting potential for direct targeting by miR-29 (Fig. 2B). Further analysis of the genes with miR29 binding sites revealed that 16 (80%) of the seed regions contained either 8mers (an exact match to positions 1-8 of the mature miRNA including an A opposite position 1 of the miRNA) or 7mers (an exact match to positions 2-8 of the mature miRNA) (Fig. 2B).

Of particular interest to B cell regulation, we observed that *AICDA* was one of the top target genes repressed by miR-29 overexpression, while the previously validated miR-29b target, *CDK6* (Zhao et al., 2010), was also robustly repressed (Fig. 2A, C). Other validated miR-29 targets repressed following miR-29b overexpression in tonsil B cells included the guanine exchange factor *RCC2* (Matsuo et al., 2013), the thymine glycosylase *TDG* (Morita et al., 2013; P. Zhang et al., 2013) and the trafficking protein *TRAK2* (Kato et al., 2016) (Fig. 2C).

Given its indispensable role in promoting B cell antibody diversification, the repression of *AICDA* following miR-29b overexpression was of particular interest. To further explore the potential regulation of *AICDA* by miR-29b, tonsil B cells were



transfected with a miR-29b mimic and cultured in the presence of IL-4 and anti-CD40 antibody to induce *AICDA* expression CSR and plasmablast differentiation; as previously demonstrated this resulted in an 15 fold increase in miR29b levels, supplementary figure 3A). RNA was isolated 24 hours after transfection and the expression of a series of miR-29 targets associated with either CSR-, GC- and plasma cell differentiation and function, including *AICDA*, were assessed via qRT-PCR.

In line with the array data, *AICDA* was consistently repressed by over 2-fold following miR-29b overexpression (Fig. 3A). Similarly, the bona fide miR-29 target, *CDK6* (Zhao et al., 2010), was also robustly reduced following miR-29b overexpression, demonstrating the biological validity of these experiments (Fig. 3A). However, two previously reported miR-29 targets, *AKT3* (Wei et al., 2013) and *SPI* (Jia et al., 2014), remained unperturbed (Fig. 3A), suggesting that in the context of human B cells, they are not subject to miR-29b repression at the transcript level. The expression levels of *BATF* (Ise et al., 2011), *HOXC4* (Park et al., 2009), *MYC* (Fernandez et al., 2013) and *IRF4* ((Luo and Tian, 2010), previously reported to directly regulate AID transcription, and the expression of factors critical to the GC/PB phenotype (*BCL6*, *PAX5*, *XBP1* and *BLIMP1*) were all perturbed by >20%, although *BATF*, *XBP1* and *MYC* did show small but reproducible decreases.

The repression of *AICDA* mRNA following miR-29b overexpression was also mirrored at the protein level. Immunoblotting 60 hours post transfection using the EK2 5G9 anti-AID monoclonal antibody showed a clear reduction in AID protein (Fig. 3B). Densitometry revealed this to be an approximate 60% decrease, indicating a



strong correlation between mRNA and protein expression changes (Fig. 3B). Overall, the data show that the overexpression of miR-29 in tonsil B cells activated to express AID was indeed capable of repressing AID expression at both the protein and mRNA level.

### *3.3 Knockdown of miR-29b in activated naïve B cells augments AID expression*

To gain further insight into the role of miR-29b in the context of B cell activation, naïve B cells, which express endogenous miR-29b, were transfected with a commercial miR-29 inhibitor (see methods for details) or non-silencing control and cultured with IL-4 and anti-CD40 stimulation. Delivery of the inhibitor repressed endogenous miR-29b by approximately 60% (as assessed by qPCR) when compared to B cells transfected with a negative control, and lasted for approximately 48 hours before beginning to recover (supplementary Fig. 3B). As in total B cell experiments, no detectable difference was seen in cell viability between the miR29b inhibitor or non-silencing control as judged by flow cytometry FSC/SSC although cell viability was reduced approximately 2 fold compared to un-transfected cells. The biological consequences of this inhibition were assessed by monitoring the expression of *AICDA* as well as CSR, GC and plasma cell associated genes and three previously validated miR-29 targets, by qRT-PCR.

In a reciprocal manner to the miR-29b overexpression results, we observed that *AICDA* was significantly increased following 60% miR-29b inhibition (supplementary Figure 3B & Fig. 4A). Similarly, expression of the previously validated miR-29 target, *CDK6* (Zhao et al., 2010) and *MYC*, previously indirectly



linked to miR-29 (S. Liu et al., 2010), were elevated. Although a modest increase in  $\epsilon$ GLT expression was detected following miR29 knockdown this was not found to be significant compared to the control. Also in line with our overexpression analysis, the reported miR-29 targets, *AKT3* (Wei et al., 2013) and *SPI* (Garzon et al., 2009), were unaltered following miR-29b inhibition (Fig. 4A).

To determine whether the elevated *AICDA* mRNA expression was reflected at the protein level, we performed immunoblots 60 hours post transfection. Further confirming the effect at the mRNA level, we observed that AID protein expression was increased by 1.6-fold following miR-29b inhibition, as determined via densitometry (Fig. 4B). Together, these data demonstrate that reducing the physiological levels of miR-29 expression found in naïve B cells is sufficient to de-repress AID in circulating naïve B cells.

#### *3.4 miR-29b directly targets the miR-29 binding site within the AICDA 3' UTR*

To investigate the possibility that AID may be indirectly regulated by miR-29b, the expression of a number of signalling components of the B cell activation pathway were investigated to determine the impact of potential indirect modes of regulation. Following miR-29b manipulation, the expression and phosphorylation of members of the PI3K, MAPK and STAT6 pathway were found to be unperturbed (supplementary Fig. 3C). This further confirmed that miR-29b is likely directly targeting *AICDA*. Bioinformatic analysis of the human *AICDA* 3' UTR revealed the presence of a pronounced miR-29 binding site (Fig. 5A). This site is found in the *AICDA* 3' UTR of other primate species, such as chimpanzees and rhesus macaques, but not in more distantly related mammals, such as mice, rats and rabbits. The miR-29 binding site in the *AICDA* 3' UTR shows complementarity from nucleotides 1-10 at the 5' end of the



miRNA, and includes an adenosine opposite position 1 (Fig. 5A). Seed region complementarity and an adenosine opposite position 1 are both strong indicators of miRNA targeting (Agarwal et al., 2015). In addition, the site is located in a favourable genomic context, it is under 200 nucleotides away from the 3' end of the 3' UTR, and embedded in an AU-rich environment. The miRNA targeting prediction tool TargetScan reported *AICDA* to be in the 4th percentile in terms of favourability for all miR-29 binding sites (not shown).

Since *AICDA* possessed a strong miR-29b binding site in its 3'UTR, the direct targeting of AID by miR-29b was assessed. The region encapsulating the miR-29 binding site of the *AICDA* 3'UTR was sub-cloned into a luciferase reporter vector. Constructs in which the miR-29 binding site was mutated and replaced with a BamHI restriction site (Fig. 5A) and constructs containing no insert were used as negative controls. Each vector was separately cotransfected into HEK293 cells along with a miR-29b mimic molecule. Following 24 hours in culture, miR-29b was shown to be capable of repressing the luciferase activity of the test vector, relative to both negative control vectors (Fig. 5B). This demonstrated that miR-29b was indeed capable of directly interacting with the *AICDA* 3' UTR and suppressing protein expression.

### *3.5 miR-29 dampens CSR to IgE in stimulated tonsil B cells*

In order to mount a diversified humoral immune response, B cells not only undergo CSR and SHM but must also coordinate their proliferative and apoptotic potential (Recaldin and Fear, 2016). As such, the phenotypic consequences of miR-29b overexpression were assessed in both activated tonsil B cells and the CL01 germinal centre cell line. Following anti-CD40 and IL-4 stimulation, overexpression of miR-



375 29b had no detectable effect on apoptosis or cell proliferation (supplementary Fig.  
376 3D-F).

377

378 Given that miR-29b modulates AID levels, we also investigated its effect on *in vitro*  
379 stimulated CSR. Although we show that transient transfection of naïve B cells could  
380 be successfully accomplished and that mir29b over-expression resulted in a robust  
381 diminution of induced AID expression, cells transfected with either the miRNA  
382 mimic or non-silencing control produced very little isotype switched immunoglobulin  
383 following 14 days culture. In order to demonstrate a biological consequence of  
384 miRNA29b diminished AID expression we overexpressed miR29b in total tonsillar B  
385 cells. CSR to IgE is most robustly stimulated in tonsil B cells following activation  
386 with IL-4 and anti-CD40(Ramadani et al., 2017; 2015) and we reasoned that although  
387 miR-29b repression of AID is only transient (approx. 48 hours) in this system, this is  
388 the timepoint at which most AID is functionally active, and thus might affect CSR.  
389 When provided with anti-CD40 and IL-4 stimulation, tonsil B cells maintain their IgG  
390 expression, with switching to and from IgG being balanced, over the course of 7 days,  
391 but significantly increase switching to IgE from negligible to detectable levels, and  
392 thus can be tracked via flow cytometry (Ramadani et al., 2015). Assessment of Ig  
393 gene expression in B cell cultures by qRT-PCR, 5 days after transfection with a miR-  
394 29b mimic, revealed a moderate but reproducible, 30% decrease in IgE mRNA (Fig.  
395 6A). After 7 days in culture, IL-4 and anti-CD40 stimulated B cells transfected with  
396 either the miR29b mimic or non-silencing control were similarly viable (cell viability  
397  $10.3\% \pm 2.2$  versus  $11.2\% \pm 2.0$ ), although viability was reduced 2.5 fold compared  
398 to similarly stimulated un-transfected cells(Ramadani et al., 2015). Intracellular



399 staining of immunoglobulin protein at day 7 reproduced a similar moderate but  
400 statistically significant reduction in the percentage of IgE-expressing cells (Fig. 6B).  
401 The percentage of IgG-expressing cells was not significantly altered (Fig. 6B).  
402 Knockdown of AID through use of a short-interfering RNA to *AICDA* resulted in a  
403 similar pattern of Ig expression; giving a small but reproducible reduction in the  
404 percentage of IgE-expressing cells, but no significant alteration in IgG-expressing  
405 cells (Fig. 6C). Together these results demonstrated that miR-29b, a miRNA  
406 downregulated in activated B cells, is capable of directly targeting *AICDA* mRNA,  
407 and that this decreases CSR to IgE following stimulation with IL-4 and anti-CD40  
408 antibody. We posit that miR 29 represents a previously unrecognised player in human  
409 B cell biology that warrants further investigation.



## 4. Discussion

miRNAs play an important role in fine tuning cells' protein output and have previously been shown to regulate important aspects of B cell biology (Taganov et al., 2007). However, the functional role of miRNAs have not been extensively studied in the activation of human B cells. This study sought to identify miRNAs that were dynamically and temporally regulated between different mature B cell subpopulations, and identify their functional consequences in the development of the humoral response. Previous studies have used PCR, microarray or RNA-seq to monitor the miRNA expression changes that occur during the course of a B cell response, predominantly focusing on the differences between naïve, GC and memory B cells found within the same secondary lymphoid organs (Basso et al., 2009; Malumbres et al., 2009; Tan et al., 2009; J. Zhang et al., 2009). The original data from Malumbres et al., (Malumbres et al., 2009) indicated that miR-29b, a member of the miR-29 family that is important in coordinating the lymphocyte response to intracellular pathogens such as *Listeria monocytogenes* (Ma et al., 2011), was repressed in the GC compartment. Interrogation of data from other papers that analysed the human B cell miRNome (Basso et al., 2009; Tan et al., 2009; J. Zhang et al., 2009) confirmed this observation. The miR-29 family of miRNAs are known to be particularly important in T cells, where they control aspects of development and effector function (Ma et al., 2011; Papadopoulou et al., 2011; Steiner et al., 2011), and in B cells have recently been shown to regulate germinal centre dynamics in a murine model of collagen-induced arthritis (Nieuwenhuijze, 2017). However, the functional consequences of this miRNA in naïve human B cells and B cell activation have not previously been studied.



Here, we found that mature miR-29b was approximately 3-fold less abundant in B cells isolated from tonsil lymphoid tissue than in circulating naïve B cells isolated from blood, confirming that miR-29b was dynamically regulated upon exit from the circulation and entry into the lymphoid tissue. In order to identify miR-29b's function in this context and identify potential target molecules, it was overexpressed through the use of miRNA mimic molecules and global gene expression changes monitored by microarray analysis. This revealed that the second most dynamically regulated gene following miR-29b overexpression, after the eukaryotic initiation factor *EIF4E2*, was found to be *AICDA*, which plays a critical role in B cell biology (Fig. 2A, B). Considering that AID is also a potent genome mutator and has oncogenic potential (Robbiani et al., 2009; 2008) its expression and activity must be carefully regulated. In mice, *Aicda* is post-transcriptionally regulated by miR-155 and miR-181b (de Yebenes et al., 2008; Dorsett et al., 2008; Teng et al., 2008). However, regulation of *AICDA* by different miRNA members has not been fully investigated in human B cells.

The human *AICDA* 3' UTR contains a pronounced miR-29 binding site with extensive complementarity, indicating the possibility of a direct interaction. This is confirmed by our results which show that over expression of a miR-29b mimic represses luciferase activity in HEK293 cells transfected with a luciferase reporter construct containing the region of *AICDA* 3' UTR bearing the miR-29 binding site (Fig. 5B) while miR-29b overexpression in tonsil B cells significantly reduced *AICDA* expression, AID protein levels (Fig. 3) and CSR to IgE (Fig. 6). This raises the question as to the purpose of such an interaction. AID and miR-29b expression are inversely correlated. *AICDA* is detectable in the lymphoid follicles and most abundant



in GC B cells, but absent in circulating naïve B cells. Conversely, miR-29b is reduced in the follicles but prominent in circulating naïve B cells. This indicates that the function of miR-29b may be to suppress premature AID at the early stages of a mature B cell's life cycle, or following inappropriate activation in the absence of suitable T cell help or T-independent "danger" signals.

Upon entry into the lymphoid tissue and subsequently the GC, where AID expression is appropriate, the down regulation of miR-29b would relieve the block on *AICDA* and allow its full regulated expression. Indeed, the potential of endogenous miR-29b to regulate AID in this context was demonstrated by the increase of AID mRNA and protein expression levels when miR-29b was inhibited in IL4 and antiCD40 stimulated naïve B cells (Fig. 4A and 2B). In contrast, in tonsil B cells, where miR-29b is less abundant, miR29b inhibitors did not elevate *AICDA* (data not shown). This strongly suggests that while endogenous miR-29b expression in naïve B cells is sufficient to suppress *AICDA* expression, the low levels of miR-29b in GC B cells is insufficient to do so (although overexpression of miR29b strongly repressing AID and partially blocks CSR). As such, it is likely that miR-29b is able to repress leaky or inappropriate expression of AID prior to B cell activation. In relation to miR29s potential role in preventing inappropriate AID expression, and therefore function, in naïve B cells, it of interest to note that miR29 (a, b and c) was found to be decreased in Mantle cell lymphoma (MCL) (Zhao et al., 2010), a lymphoma with characteristics of CD5<sup>+</sup>, antigen-naïve pregerminal center B-cells. Although Cyclin D1 translocation and the up-regulation of CDK6 (a direct miR29 target) is thought to account for the transformation of MCL cells, they were also found to frequently express AID possibly



accounting for at least some of wide range of chromosomal abnormalities present in this disease(Babbage et al., 2004).

Following B cell exit from the circulation, miR-29b silencing in GC B cells leads to a de-repression of *AICDA* transcription. Such a model has been previously ascribed to miR-181b regulation of *aicda* in mice (de Yebenes et al., 2008). Interestingly, miR29c shares a similar expression pattern to miR-29b, being most highly expressed in naïve B cells and low in GC B cells (Supplementary Figure 1). Since miR29 family members share seed region homology and therefore predicted targets, the expression of these miRs would be expected to reinforce their functional effects. It should be noted that the miR-29 site in the *AICDA* 3' UTR is conserved amongst other primate species, but not in more distantly related mammals, such as mice, rats and rabbits. Thus, it is not an interaction that has been strongly conserved throughout mammalian evolution and may have arisen more recently.

In conclusion, the data in this paper have shown that miR-29b, a member of the conserved miR-29 family, is repressed in tonsil B cells relative to circulating naïve B cells. Enforced overexpression of miR-29b in tonsil B cells led to the direct targeting of *AICDA*, through a pronounced binding site in the *AICDA* 3' UTR and this interaction was sufficient to reduce CSR to IgE. Similarly, inhibition of endogenous miR-29b in naïve B cells resulted in elevated AID expression. We hypothesise that one of the endogenous functions of miR-29 is to silence leaky expression of AID, a mutagenic protein whose expression must be controlled in order to maintain chromosomal integrity.



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## Figure legends

### **Figure 1: miR-29 is less abundant in B cells isolated from tonsillar tissue than in naïve B cells isolated from peripheral blood.**

The expression level of miR-29 in IgD<sup>+</sup> CD27<sup>-</sup> naïve B cells isolated from peripheral blood compared to CD19<sup>+</sup> tonsil B cells, as assessed via qRT-PCR. Values are plotted relative to the small nucleolar RNA RNU6B. \*\* P < 0.01(unpaired t-test).

### **Figure 2: Genome-wide analysis of miR-29b targeting in tonsillar B cells shows an enrichment in miR-29 binding sites within the 3' UTR of downregulated genes.**

(A) Heatmap representing expression changes in the most downregulated genes in tonsillar B cells transfected with a miR-29 mimic and stimulated with anti-CD40 antibody and IL-4 cytokine for 24 hours, as detected by Illumina BeadChip Array. Genes containing predicted miR-29 binding sites in their 3' UTR are listed in red. P values (two-way ANOVA) are also included.

(B) Pie chart displaying the prevalence of miR-29 binding sites within the 3' UTR of genes downregulated following miR-29b overexpression. The percentage of 8mers (an exact match to positions 2-8 of the mature miRNA with an A opposite position 1 of the miRNA), 7mer-m8s (an exact match to positions 2-8 of the mature miRNA) and 6mers (an exact match to positions 2-7 of the mature miRNA) within the miR-29 binding sites is also shown.

(C) Dot plot representations of the change in fluorescence intensity for *AICDA*, as well as previously validated targets including *CDK6*, *RCC1*, *TDG* and *TRAK2*, in tonsil B cells transfected with a miR-29 mimic and stimulated with anti-CD40



antibody and IL-4 cytokine for 24 hours, as detected by Illumina BeadChip Array.

**Figure 3: Overexpression of miR-29 reduces AID abundance in tonsillar B cells.**

tonsil B cells were transfected with a miR-29b mimic or its associated negative control molecule and subsequently activated with IL-4 and anti-CD40.

(A) Gene expression of key B cell molecules after 24 hours of stimulation as assessed via qRT-PCR. Values are normalized to 18S ribosomal RNA and plotted relative to the abundance of each gene in the control transfected cells, which were arbitrarily assigned a value of 1. N = 3 - 12, mean and s.e.m. \* P < 0.05, \*\* P < 0.01, \*\*\*\* P < 0.0001 (paired t-test).

(B) Representative immunoblot and cumulative densitometry analysis (n = 3) of AID protein in follicular B cells following miR-29b overexpression and 60h of culture in IL-4 and anti-CD40 stimulus, compared to AID protein in the control transfected cells. GAPDH served as a loading control.

**Figure 4: Knockdown of miR-29 increases AID abundance in naïve B cells.**

Naive B cells were transfected with a miR-29 inhibitor or its associated negative control molecule and subsequently activated with IL-4 and anti-CD40.

(A) Gene expression of key B cell molecules after 48 hours of stimulation as assessed via qRT-PCR. Values were normalized to 18S ribosomal RNA and plotted relative to the abundance of each gene in the control transfected cells, which were arbitrarily assigned a value of 1. N = 6 - 12. Mean and s.e.m. \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P < 0.0001 (paired t-test).

(B) Representative immunoblot and cumulative densitometry analysis (n = 3) of AID protein in naïve B cells following miR-29b knockdown and 60h of culture in IL-4 and anti-CD40 stimulus, compared to AID protein in the control transfected cells.



GAPDH served as a loading control.

**Figure 5: miR-29 is capable of directly targeting the miR-29 binding site within the AICDA 3' UTR.**

(A) The miR-29 binding site within the human, chimpanzee and rhesus macaque *AICDA* 3' UTR, as well as the mismatched sequence used for luciferase analysis.

(B) HEK293T cells were contranfectected with one of the dual luciferase plasmids (endogenous *AICDA* 3' UTR, mismatched 3' UTR or no insert) and a miR-29 mimic.

Firefly luciferase activity was quantified after 24 hours, normalized to renilla luciferase and plotted relative to the no insert plasmid. \*  $P < 0.05$  (one-way ANOVA).

N = 3, mean and s.e.m.

**Figure 6: miR-29 overexpression dampens induction of class switching to IgE.**

Tonsil B cells were transfected with a miR-29 mimic or negative control molecules and subsequently cultured in class-switching conditions (IL-4 and anti-CD40 antibody).

(A) IgE and IgG mRNA were detected via qRT-PCR following 5 days of culture.

Values are plotted relative to 18S ribosomal RNA. N = 7 for IgE and 5 for IgG, mean and s.e.m.

(B) Intracellular IgE and IgG was analysed following 7 days of culture via flow cytometry. \*  $P < 0.05$  (paired t test).

(C) Intracellular IgE and IgG was analysed following 7 days of culture via flow cytometry. Values were plotted relative to the control transfected cells, which were arbitrarily assigned a value of 1. N = 3 for siAID, and 10 for miR-29 versus the negative control mimic. \*  $P < 0.05$  (paired t test). Mean and s.e.m.



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1 **miR-29b directly targets activation-induced cytidine deaminase in**  
2 **human B cells and can limit its inappropriate expression in naïve B**  
3 **cells**

4  
5 Running Title: miR29b regulation of AID

6  
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## Abstract

Class-switch recombination (CSR) is an essential B cell process that alters the isotype of antibody produced by the B cell, tailoring the immune response to the nature of the invading pathogen. CSR requires the activity of the mutagenic enzyme AID (encoded by *AICDA*) to generate chromosomal lesions within the immunoglobulin genes that initiate the class switching recombination event. These AID-mediated mutations also participate in somatic-hypermutation of the immunoglobulin variable region, driving affinity maturation. As such, AID poses a significant oncogenic threat if it functions outside of the immunoglobulin locus. We found that expression of the microRNA, miR-29b, was repressed in B cells isolated from tonsil tissue, relative to circulating naïve B cells. Further investigation revealed that while enforced overexpression of miR-29b in human B cells precipitated a reduction in overall AID protein and a corresponding diminution in CSR to IgE, miR-29b knockdown in naïve B cells resulted in elevated AID expression. Similarly, miR-29b was able to directly interact with the AID 5'-UTR and modulate expression in reporter assays. Given miR-29b's ability to potently target AID, a mutagenic molecule that can initiate chromosomal translocations and "off-target" mutations, we propose that miR-29b acts to silence premature AID expression in naïve B cells, thus reducing the likelihood of inappropriate and potentially dangerous deamination activity.



## 1. Introduction

During the course of the immune response, mature B cells undergo two diversification events at the immunoglobulin loci, somatic hypermutation (SHM) and class-switch recombination (CSR) (Stavnezer, 2011). SHM introduces mutations in the hypervariable loops of the antigen binding site, found within the CDRs of the Immunoglobulin Heavy (*IGH*) and light (*IGL*) chain variable regions. This allows for selection of B cell clones which harbour Igs with increased affinity for antigen, promoting the development of highly specific Ig molecules. In contrast, CSR replaces the C $\mu$  and C $\delta$  heavy chain gene exons (encoding IgM and IgD respectively) with the downstream C-region exons of the  $\alpha$ ,  $\gamma$ , or  $\epsilon$  isotypes (encoding IgA, IgG and IgE) (Stavnezer and Schrader, 2014). This process, which is driven by cytokines released from T helper cell populations, ensures that the effector functions of the antibodies produced by B cells are tailored to the nature of the invading pathogen. Both CSR and SHM require the activity of the mutagenic enzyme, activation-induced cytidine deaminase (AID, encoded by *AICDA*) (Muramatsu et al., 2000; Revy et al., 2000). AID acts to deaminate deoxycytidine residues within the *IG* variable and the *IGH* constant regions, resulting in the production of deoxyuracils (Neuberger et al., 2003). In the case of SHM, the ensuing mismatches initiate low-fidelity DNA repair pathways leading to the incorporation of mutations within the *IGH* and *IGL* variable regions, whereas in CSR the mismatches trigger a deletional-recombination event that replaces the upstream “acceptor” C-region (initially C $\mu$  and C $\delta$ ) with a downstream “donor” C region (C $\gamma$ 1-4, C $\epsilon$  or C $\alpha$ 1-2) (Keim et al., 2013).



The mechanisms targeting AID to the Ig genes are incompletely understood. It has been proposed that transcriptional stalling (Pavri et al., 2010), the formation of RNA:DNA hybrids called R loops (Shinkura et al., 2003), the exosome (Basu et al., 2011), super-enhancers (Qian et al., 2014), germline transcript RNA (Wang et al., 2015), 14-3-3 adaptor proteins (Xu et al., 2010), as well as the sequence location of the Ig genes (Yeap et al., 2015), recruit AID to the DNA and promote deamination. However, AID activity is promiscuous, and deamination-induced mutations are detected at multiple non-Ig sites (M. Liu et al., 2008). This collateral damage has significant oncogenic potential, as demonstrated by AID-induced chromosomal translocations that occur in the context of genomic instability (Robbiani et al., 2008; 2009) and AID-induced mutations of non-Ig genes (Duquette et al., 2005; Pasqualucci et al., 1998; 2001). Therefore, AID expression and activity must be carefully controlled.

One mechanism by which cells are able to fine-tune their protein expression is through microRNAs (miRNAs), short non-coding RNAs that regulate gene expression by promoting mRNA decay and translational repression (Bartel, 2009). In mice miR-155 has been shown to directly repress AID (Teng et al., 2008) and removing the miR-155 binding site within the *Aicda* 3' UTR increases the likelihood of AID-induced *Igh-Myc* chromosomal translocations (Dorsett et al., 2008), a transforming event frequently observed in Burkitt's Lymphoma. Similarly, miR-181 can also directly target *Aicda* and is proposed to prevent inappropriate AID expression in the absence of B cell activation (de Yebenes et al., 2008). Although the miRNA-mediated regulation of AID expression has been well studied in mice, this mode of regulation has not been extensively studied in human B cells.



94           To identify miRNAs involved in coordinating the B cell response in humans,  
95 we analysed the published literature that had profiled the miRNA pool of distinct  
96 human B cell subpopulations (Basso et al., 2009; Malumbres et al., 2009; Tan et al.,  
97 2009; J. Zhang et al., 2009). These studies consistently reported that the conserved  
98 miRNA miR-29b was less abundant in germinal centre B cells relative to naïve and  
99 memory cell compartments. Similarly, we show that miR-29b has diminished  
100 expression in B cells isolated from tonsil tissue relative to naïve B cells isolated from  
101 peripheral blood. It is of interest to note that miR29b expression has also been reported  
102 to be reduced in mantle cell lymphoma, a lymphoma thought to originate from Pre-GC  
103 mature B cells (Zhao et al., 2010). In this report we investigate potential miR29b targets  
104 and demonstrate that it is able to directly target *AICDA* mRNA. Enforced  
105 overexpression of miR-29b in human B cells reduces overall AID protein and causes a  
106 corresponding diminution of CSR to IgE *in vitro*. Together, these results suggest that  
107 miR-29b is able to silence “leaky” expression of AID, limiting its expression to  
108 appropriately activated B cells and thus helping to maintain chromosomal integrity.  
109



## 2. Materials and Methods

### 2.1 Ethics

Ethical approval was granted by London Bridge Research Ethics Committee for both peripheral blood donations (09/H0804/77 and 14/LO/1699) and for tonsil donations (08/H0804/94). Full written informed consent was obtained from all donors or the donors' parents or legal guardian.

### 2.2 B cell isolation, transfection and culture

Naïve B cells were isolated from peripheral blood using the Naive B Cell Isolation Kit II (Miltenyi Biotec). B cells were isolated from tonsil tissue as previously described (Cooper et al., 2012). Briefly, mononuclear cells were isolated from dissected tonsil tissue on a density gradient (Lymphoprep, Axis-Shield PoC AS) followed by incubation with aminoethyl isothiuronium bromide-treated sheep red blood cells to rosette T cells. To overexpress miR-29b, 800 nM miR-29b Pre-miR miRNA Precursor (Thermo Fisher Scientific) or the equivalent non-targeting negative control molecules was delivered into  $5 \times 10^6$  freshly isolated primary B cells using the Amaxa Human B cell Nucleofector Kit (LONZA). The Pre-miR miRNA Precursors used were proprietary short (16-28bp) double stranded RNA molecules chemically modified to ensure the desired strand is loaded into the RISC complex (Barnes et al., 2012). To inhibit miR-29b, 800 nM miR-29b of mirVana miRNA Inhibitor (Thermo Fisher Scientific) or the equivalent non-targeting negative control molecule was delivered into  $5 \times 10^6$  freshly isolated primary B cells using the Amaxa Human B cell Nucleofector Kit (LONZA). The mirVana miRNA Inhibitors used were single stranded, chemically modified RNA molecules designed to irreversibly bind and inhibit endogenous



miRNAs (Barnes et al., 2012). Samples were nucleofected using program U-15 on the Nucleofector Device and cultured at a concentration  $5 \times 10^5$  mL<sup>-1</sup> in RPMI 1640, 10% HyClone Fetal Bovine Serum, 100 µg/mL Streptomycin, 100 U/ml Penicillin, 2 mM L-Glutamine. To stimulate CSR, the medium was supplemented with 1 mg/mL of anti-CD40 antibody, 200 IU/mL of recombinant human IL-4, 5 mg/mL of insulin and 35 mg/mL of transferrin. The human monoclonal IgM<sup>+</sup> IgD<sup>+</sup> CL01 B cell line (Cerutti et al., 1998) was cultured in RPMI 1640, 10% HyClone Fetal Bovine Serum, 100 µg/mL Streptomycin, 100 U/mL Penicillin, 2 mM L-Glutamine and kept at a concentration of between  $1-5 \times 10^5$  cells/mL. To induce expression of AID, the culture medium was supplemented with 1 mg/mL of anti-CD40 antibody and 200 IU/mL of recombinant human IL-4. The CL01 cell line was nucleofected using the Amaxa Cell Line Nucleofector Kit V along with program C-09 on the Nucleofector Device. All cells were cultured in a humidified incubator at 37°C, 5% CO<sub>2</sub>.

### *2.3 RNA Isolation and quantitative (q) RT-PCR analysis*

Total RNA was extracted from cultured cells using the miRNeasy Mini Kit with QIAzol Lysis Reagent (Qiagen). Residual gDNA was removed by incubating the extracted sample with 20 units of TURBO DNase enzyme (Thermo Fisher Scientific) at 37°C for one hour followed by a second phenol-chloroform cleanup. The integrity and yield of isolated RNA was checked on a 2100 Bioanalyzer (Agilent) using the RNA6000 Pico Assay. For analysis of mRNA expression, cDNA was generated from total RNA using random hexamers with RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific) and all genes (with the exception of *AICDA*, *εGLT*, *IgG* and *IgE*) were detected expression using TaqMan MGB Gene Expression Assays (Thermo Fisher Scientific). The primer and probe set for *AICDA* was designed using the Universal



Probe Library Assay Design Centre (Roche) while the *εGLT*, *IgG* and *IgE* primer and probe sets were designed in house. Individual samples were subjected to qPCR and run in triplicate with TaqMan Universal Master Mix II on the ViiA 7 Real-Time PCR System using 18S rRNA as a normalization control and gene expression was determined using the  $2^{-\Delta\Delta Ct}$  method. For qRT-PCR analysis of miRNA expression, the TaqMan Small RNA Assay for miRNA quantification (Thermo Fisher Scientific) was used. This involved separate cDNA generation for each miRNA, utilising a miRNA-specific, stem-loop primer to facilitate reverse transcription. The small nucleolar RNA RNU6B was used as a normalization control and gene expression was determined using the  $2^{-\Delta\Delta Ct}$  method.

#### *2.4 Gene expression arrays*

Fresh tonsil B cells were transfected with a miR-29b mimic or its associated negative control and cultured in class switching stimuli for 24 hours. RNA was isolated using the miRNeasy Mini Kit with QIAzol Lysis Reagent (Qiagen) and then DNase-treated as described. 10 ng RNA was amplified using the Ovation Pico WTA System V2 (Nugen). 4 µg of amplified single-stranded cDNA was biotin-labeled using the Encore BiotinIL Module (Nugen). Finally, transcriptomic analysis was performed by hybridizing 750 ng of biotin-labeled single-stranded cDNA onto a HumanHT-12 v4 Expression BeadChip (Illumina) and scanned using the Illumina iScan System. QC analysis and RMA normalization was performed in Illumina's Genome Studio Suite v1.0. Assessment of differential gene expression and statistical analysis was performed in Partek Genomics Suite version 6.6. Data from this study have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO



184        Series                                  accession                                  number                                  GSE100735

185        (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100735>).

186        *Immunoblotting*

187        B cells were stimulated for 60 hours in class-switching stimuli following transfection.

188        Cells were harvested in protein lysis buffer (pH 7.4, 25 mM Tris, 150 mM NaCl, 1 mM

189        EDTA, 1% NP40, 5% Glycerol, 10% Roche Protease Inhibitor). Total protein levels

190        were quantified using the bicinchoninic acid assay and approximately 50 ug protein

191        lysate were run on SDS-polyacridimide gels. Following wet transfer onto nitrocellulose

192        membranes, the membranes were probed with the following antibodies for protein

193        expression (AID - EK2 5G9, STAT6 - D3H4, p-STAT6<sup>Tyr641</sup> - C11A12, p38 MAPK -

194        D13E1, p-p38<sup>Thr180/Tyr182</sup> - D3F9, AKT - 40D4, p-AKT<sup>Ser473</sup> - D9E, GAPDH - 6C5), and images

195        were developed using the Molecular Imager® ChemiDoc™ XRS System (Bio-Rad).

196        GAPDH (clone 6C5, Abcam) was used as a loading control.

197

198        *2.5 Luciferase Assays*

199        The region of the *AICDA* 3' UTR encompassing the miR-29 binding site was subcloned

200        into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega),

201        which harbours both a firefly luciferase reporter gene, for analysis of miRNA-mRNA

202        binding site interactions, and a renilla luciferase reporter gene, which acts as a

203        transfection efficiency control. For the mismatched construct, the miR-29 binding site

204        in the AID 3' UTR was replaced with a BamHI restriction site using sequential rounds

205        of mutation cloning. HEK293T cells were seeded onto white 96-well tissue culture

206        plates and having reached 70% confluence were transfected with 50 ng of one of the

207        luciferase constructs and 10 nM of miR-29 mimic using Lipofectamine 2000 (Thermo

208        Fisher Scientific). Reactions were performed in triplicate. Luciferase activity was



quantified after 24 hours on a GloMax-Multi Microplate Luminometer (Promega, 2 seconds integration time) using the Dual-Glo Luciferase Assay System (Promega). The luciferase signal ratio was calculated by dividing firefly luciferase activity by renilla luciferase activity and normalized to cells transfected with an insert-free pmirGLO vector.

## *2.6 Flow Cytometry*

Apoptosis staining was performed using the Annexin V Apoptosis Detection Kit (eBioscience). For cell proliferation analysis, 10<sup>5</sup> transfected CL01 cells were washed with PBS, resuspended in 1 mL PBS containing 1 µM CellTrace Violet dye (Thermo Fisher Scientific), and left in the dark at 37°C for 10 minutes. The reaction was quenched by the addition of cell culture media. The cells were washed, resuspended and cultured in a 37°C incubator until analysis via flow cytometry. Intracellular antibody staining of IgG and IgE was performed as previously described<sup>16</sup> using the a fixable viability stain (Zombie Aqua Thermo Fisher Scientific).



### 3. Results

#### *3.1 miR-29b is repressed in tonsil B cells relative to peripheral naïve B cells*

In order to identify dynamically regulated miRNAs within the context of mature human B cells, we analysed the available literature profiling the miRNA pool in different B cell subpopulations (Basso et al., 2009; Malumbres et al., 2009; Tan et al., 2009; J. Zhang et al., 2009). We observed that the evolutionarily conserved miRNA miR-29b was less abundant in germinal centre B cells relative to naïve and memory cell compartments (supplementary Fig. 1A & 1B). miR-29b is a member of the miR-29 family, also including miR-29a and miR-29c, which share the same seed region and thus overlap in their predicted targets (Liston et al., 2012). Interestingly miR29c was also less abundant in GC B cells compared to naïve or memory compartments, potentially reinforcing the effects of this miRNA family (supplementary Fig. 1A & 1B). We analysed the abundance of miR-29b in B cells isolated from tonsils, a large proportion of which have a germinal centre phenotype (CD19<sup>+</sup>CD38<sup>+</sup>CD27<sup>-</sup> or CD19<sup>+</sup>CD38<sup>+</sup>CD27<sup>+</sup>) (supplementary Fig. 2A), and compared its prevalence to naïve B cells (CD19<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup>) (supplementary Fig. 2B) isolated from peripheral blood. In line with previous reports (Malumbres et al., 2009), miR-29b was nearly 3 times more abundant in the circulating naïve B cells than in tonsil-derived B cells (Fig. 1).

#### *3.2 Identification of AICDA as a miR-29b target gene in human tonsil B cells*

To gain insight into the role of miR-29b in the context of B cell activation we overexpressed miR-29b in tonsil B cells and investigated global gene expression changes following 24 hours culture with IL-4 and anti-CD40 stimulation using Illumina BeadChip Microarrays (HumanHT-12 v4). Transfection of a miR-29b mimic into tonsil



B cells increased miR-29b abundance by approximately 15-fold 24 hours after transfection, compared to cells transfected with a negative control mimic molecule (supplementary Fig. 3A). No detectable difference was seen in cell viability between the miR29b mimic or negative control as judged by flow cytometry FSC/SSC although cell viability was reduced approximately 2 fold compared to un-transfected cells. Analysis of the array data revealed only a moderate effect on overall gene expression, with just 29 genes being modulated by more than 1.2 fold (supplementary table I and Fig. 2A). However, of these genes, 20 (69%) contained miR-29 binding sites within their 3' UTRs, suggesting potential for direct targeting by miR-29 (Fig. 2B). Further analysis of the genes with miR29 binding sites revealed that 16 (80%) of the seed regions contained either 8mers (an exact match to positions 1-8 of the mature miRNA including an A opposite position 1 of the miRNA) or 7mers (an exact match to positions 2-8 of the mature miRNA) (Fig. 2B).

Although, a 15 fold overexpression of miR-29 is non-physiological, the incorporation of miR into “active” RISC complexes is known to be far lower than cytoplasmic levels (Flores et al., 2014). Despite this, these data demonstrate the potential for miR-29 to regulate a number of genes of interest to B cell regulation. We observed that *AICDA* was one of the top target genes repressed by miR-29 overexpression, while the previously validated miR-29b target, *CDK6* (Zhao et al., 2010), was also robustly repressed (Fig. 2A, C). Other validated miR-29 targets repressed following miR-29b overexpression in tonsil B cells included the guanine exchange factor *RCC2* (Matsuo et al., 2013), the thymine glycosylase *TDG* (Morita et al., 2013; P. Zhang et al., 2013) and the trafficking protein *TRAK2* (Kato et al., 2016) (Fig. 2C).



Given its indispensable role in promoting B cell antibody diversification, the repression of *AICDA* following miR-29b overexpression was of particular interest. To further explore the potential regulation of *AICDA* by miR-29b, tonsil B cells were transfected with a miR-29b mimic and cultured in the presence of IL-4 and anti-CD40 antibody to induce *AICDA* expression CSR and plasmablast differentiation; as previously demonstrated this resulted in an 15 fold increase in miR29b levels, supplementary figure 3A). RNA was isolated 24 hours after transfection and the expression of a series of miR-29 targets associated with either CSR-, GC- and plasma cell differentiation and function, including *AICDA*, were assessed via qRT-PCR.

In line with the array data, *AICDA* was consistently repressed by over 2-fold following miR-29b overexpression (Fig. 3A). Similarly, the bona fide miR-29 target, *CDK6* (Zhao et al., 2010), was also robustly reduced following miR-29b overexpression, demonstrating the biological validity of these experiments (Fig. 3A). However, two previously reported miR-29 targets, *AKT3* (Wei et al., 2013) and *SPI* (Jia et al., 2014), remained unperturbed (Fig. 3A), suggesting that in the context of human B cells, they are not subject to miR-29b repression at the transcript level. The expression levels of *BATF* (Ise et al., 2011), *HOXC4* (Park et al., 2009), *MYC* (Fernandez et al., 2013) and *IRF4* ((Luo and Tian, 2010), previously reported to directly regulate AID transcription, and the expression of factors critical to the GC/PB phenotype (*BCL6*, *PAX5*, *XBP1* and *BLIMP1*) were all perturbed by >20%, although *BATF*, *XBP1* and *MYC* did show small but reproducible decreases.



The repression of *AICDA* mRNA following miR-29b overexpression was also mirrored at the protein level. Immunoblotting 60 hours post transfection using the EK2 5G9 anti-AID monoclonal antibody showed a clear reduction in AID protein (Fig. 3B). Densitometry revealed this to be an approximate 60% decrease, indicating a strong correlation between mRNA and protein expression changes (Fig. 3B). Overall, the data show that the overexpression of miR-29 in tonsil B cells activated to express AID was indeed capable of repressing AID expression at both the protein and mRNA level.

### *3.3 Knockdown of miR-29b in activated naïve B cells augments AID expression*

To gain further insight into the role of miR-29b in the context of B cell activation, naïve B cells, which express endogenous miR-29b, were transfected with a commercial miR-29 inhibitor (see methods for details) or non-silencing control and cultured with IL-4 and anti-CD40 stimulation. Delivery of the inhibitor repressed endogenous miR-29b by approximately 2 fold (as assessed by qPCR) when compared to B cells transfected with a negative control, and lasted for approximately 48 hours before beginning to recover (supplementary Fig. 3B). This repression of miR-29 represents a far more physiological, and therefore biologically relevant, manipulation; bringing miR-29 expression down to a similar level to that seen in tonsil B cells (3-fold lower than naïve B cells). As in total B cell experiments, no detectable difference was seen in cell viability between the miR29b inhibitor or non-silencing control as judged by flow cytometry FSC/SSC although cell viability was reduced approximately 2 fold compared to un-transfected cells. The biological consequences of this inhibition were assessed by monitoring the expression of *AICDA* as well as CSR, GC and plasma cell associated genes and three previously validated miR-29 targets, by qRT-PCR.



322

323 In a reciprocal manner to the miR-29b overexpression results, we observed that *AICDA*  
324 was significantly increased following 60% miR-29b inhibition (supplementary Figure  
325 3B & Fig. 4A). Similarly, expression of the previously validated miR-29 target, *CDK6*  
326 (Zhao et al., 2010) and *MYC*, previously indirectly linked to miR-29 (S. Liu et al.,  
327 2010), were elevated. Although a modest increase in  $\epsilon$ GLT expression was detected  
328 following miR29 knockdown this was not found to be significant compared to the  
329 control. Also in line with our overexpression analysis, the reported miR-29 targets,  
330 *AKT3* (Wei et al., 2013) and *SPI* (Garzon et al., 2009), were unaltered following miR-  
331 29b inhibition (Fig. 4A).

332 To determine whether the elevated *AICDA* mRNA expression was reflected at the  
333 protein level, we performed immunoblots 60 hours post transfection. Further  
334 confirming the effect at the mRNA level, we observed that AID protein expression was  
335 increased by 1.6-fold following miR-29b inhibition, as determined via densitometry  
336 (Fig. 4B). Together, these data demonstrate that even reducing the physiological levels  
337 of miR-29 expression found in naïve B cells less than that seen in tonsil B cells (which  
338 express 3 fold less miR-29 than naïve B cells rather than the 2 fold reduction induced)  
339 is sufficient to de-repress AID in circulating naïve B cells.

340

#### 341 3.4 miR-29b directly targets the miR-29 binding site within the *AICDA* 3' UTR

342 To investigate the possibility that AID may be indirectly regulated by miR-29b, the  
343 expression of a number of signalling components of the B cell activation pathway were  
344 investigated to determine the impact of potential indirect modes of regulation.  
345 Following miR-29b manipulation, the expression and phosphorylation of members of



the PI3K, MAPK and STAT6 pathway were found to be unperturbed (supplementary Fig. 3C). This further confirmed that miR-29b is likely directly targeting *AICDA*. Bioinformatic analysis of the human *AICDA* 3' UTR revealed the presence of a pronounced miR-29 binding site (Fig. 5A). This site is found in the *AICDA* 3' UTR of other primate species, such as chimpanzees and rhesus macaques, but not in more distantly related mammals, such as mice, rats and rabbits. The miR-29 binding site in the *AICDA* 3' UTR shows complementarity from nucleotides 1-10 at the 5' end of the miRNA, and includes an adenosine opposite position 1 (Fig. 5A). Seed region complementarity and an adenosine opposite position 1 are both strong indicators of miRNA targeting (Agarwal et al., 2015). In addition, the site is located in a favourable genomic context, it is under 200 nucleotides away from the 3' end of the 3' UTR, and embedded in an AU-rich environment. The miRNA targeting prediction tool TargetScan reported *AICDA* to be in the 4th percentile in terms of favourability for all miR-29 binding sites (not shown).

Since *AICDA* possessed a strong miR-29b binding site in its 3'UTR, the direct targeting of AID by miR-29b was assessed. The region encapsulating the miR-29 binding site of the *AICDA* 3'UTR was sub-cloned into a luciferase reporter vector. Constructs in which the miR-29 binding site was mutated and replaced with a BamHI restriction site (Fig. 5A) and constructs containing no insert were used as negative controls. Each vector was separately cotransfected into HEK293 cells along with a miR-29b mimic molecule. Following 24 hours in culture, miR-29b was shown to be capable of repressing the luciferase activity of the test vector, relative to both negative control vectors (Fig. 5B). This demonstrated that miR-29b was indeed capable of directly interacting with the *AICDA* 3' UTR and suppressing protein expression.



371

372 *3.5 miR-29 dampens CSR to IgE in stimulated tonsil B cells*

373 In order to mount a diversified humoral immune response, B cells not only undergo  
374 CSR and SHM but must also coordinate their proliferative and apoptotic potential  
375 (Recaldin and Fear, 2016). As such, the phenotypic consequences of miR-29b  
376 overexpression were assessed in both activated tonsil B cells and the CL01 germinal  
377 centre cell line. Following anti-CD40 and IL-4 stimulation, overexpression of miR-29b  
378 had no detectable effect on apoptosis or cell proliferation (supplementary Fig. 3D-F).

379

380 Given that miR-29b modulates AID levels, we also investigated its effect on *in vitro*  
381 stimulated CSR. Although we show that transient transfection of naïve B cells could be  
382 successfully accomplished and that mir29b over-expression resulted in a robust  
383 diminution of induced AID expression, cells transfected with either the miRNA mimic  
384 or non-silencing control produced very little isotype switched immunoglobulin  
385 following 14 days culture. In order to demonstrate a biological consequence of  
386 miRNA29b diminished AID expression we overexpressed miR29b in total tonsillar B  
387 cells. CSR to IgE is most robustly stimulated in tonsil B cells following activation with  
388 IL-4 and anti-CD40(Ramadani et al., 2017; 2015) and we reasoned that although miR-  
389 29b repression of AID is only transient (approx. 48 hours) in this system, this is the  
390 timepoint at which most AID is functionally active, and thus might affect CSR. When  
391 provided with anti-CD40 and IL-4 stimulation, tonsil B cells maintain their IgG  
392 expression, with switching to and from IgG being balanced, over the course of 7 days,  
393 but significantly increase switching to IgE from negligible to detectable levels, and thus  
394 can be tracked via flow cytometry (Ramadani et al., 2015). Assessment of Ig gene



395 expression in B cell cultures by qRT-PCR, 5 days after transfection with a miR-29b  
396 mimic, revealed a moderate but reproducible, 30% decrease in IgE mRNA (Fig. 6A).  
397 After 7 days in culture, IL-4 and anti-CD40 stimulated B cells transfected with either  
398 the miR29b mimic or non-silencing control were similarly viable (cell viability 10.3%  
399  $\pm$  2.2 versus 11.2%  $\pm$  2.0), although viability was reduced 2.5 fold compared to  
400 similarly stimulated un-transfected cells(Ramadani et al., 2015). Intracellular staining  
401 of immunoglobulin protein at day 7 reproduced a similar moderate but statistically  
402 significant reduction in the percentage of IgE-expressing cells (Fig. 6B). The  
403 percentage of IgG-expressing cells was not significantly altered (Fig. 6B). Knockdown  
404 of AID through use of a short-interfering RNA to *AICDA* resulted in a similar pattern  
405 of Ig expression; giving a small but reproducible reduction in the percentage of IgE-  
406 expressing cells, but no significant alteration in IgG-expressing cells (Fig. 6C).  
407 Demonstrating that miR-29 OE not only modulates AID expression but is able to illicit  
408 a biologically relevant functional effect. Together these results demonstrated that miR-  
409 29b, a miRNA downregulated in activated B cells, is capable of directly targeting  
410 *AICDA* mRNA, and decreases CSR to IgE following stimulation with IL-4 and anti-  
411 CD40 antibody. We posit that miR 29 represents a previously unrecognised player in  
412 human B cell biology that warrants further investigation.



## 4. Discussion

miRNAs play an important role in fine tuning cells' protein output and have previously been shown to regulate important aspects of B cell biology (Taganov et al., 2007). However, the functional role of miRNAs have not been extensively studied in the activation of human B cells. This study sought to identify miRNAs that were dynamically and temporally regulated between different mature B cell subpopulations, and identify their functional consequences in the development of the humoral response. Previous studies have used PCR, microarray or RNA-seq to monitor the miRNA expression changes that occur during the course of a B cell response, predominantly focusing on the differences between naïve, GC and memory B cells found within the same secondary lymphoid organs (Basso et al., 2009; Malumbres et al., 2009; Tan et al., 2009; J. Zhang et al., 2009). The original data from Malumbres et al., (Malumbres et al., 2009) indicated that miR-29b, a member of the miR-29 family that is important in coordinating the lymphocyte response to intracellular pathogens such as *Listeria monocytogenes* (Ma et al., 2011), was repressed in the GC compartment. Interrogation of data from other papers that analysed the human B cell miRNome (Basso et al., 2009; Tan et al., 2009; J. Zhang et al., 2009) confirmed this observation. The miR-29 family of miRNAs are known to be particularly important in T cells, where they control aspects of development and effector function (Ma et al., 2011; Papadopoulou et al., 2011; Steiner et al., 2011), and in B cells have recently been shown to regulate germinal centre dynamics in a murine model of collagen-induced arthritis (Nieuwenhuijze, 2017). However, the functional consequences of this miRNA in naïve human B cells and B cell activation have not previously been studied.



Here, we found that mature miR-29b was approximately 3-fold less abundant in B cells isolated from tonsil lymphoid tissue than in circulating naïve B cells isolated from blood, confirming that miR-29b was dynamically regulated upon exit from the circulation and entry into the lymphoid tissue. In order to identify miR-29b's function in this context and identify potential target molecules, it was overexpressed through the use of miRNA mimic molecules and global gene expression changes monitored by microarray analysis. This revealed that the second most dynamically regulated gene following miR-29b overexpression, after the eukaryotic initiation factor *EIF4E2*, was found to be *AICDA*, which plays a critical role in B cell biology (Fig. 2A, B). Considering that AID is also a potent genome mutator and has oncogenic potential (Robbiani et al., 2009; 2008) its expression and activity must be carefully regulated. In mice, *Aicda* is post-transcriptionally regulated by miR-155 and miR-181b (de Yébenes et al., 2008; Dorsett et al., 2008; Teng et al., 2008). However, regulation of *AICDA* by different miRNA members has not been fully investigated in human B cells.

The human *AICDA* 3' UTR contains a pronounced miR-29 binding site with extensive complementarity, indicating the possibility of a direct interaction. This is confirmed by our results which show that over expression of a miR-29b mimic represses luciferase activity in HEK293 cells transfected with a luciferase reporter construct containing the region of *AICDA* 3' UTR bearing the miR-29 binding site (Fig. 5B) while miR-29b overexpression in tonsil B cells significantly reduced *AICDA* expression, AID protein levels (Fig. 3) and CSR to IgE (Fig. 6). Although we cannot rule out additional indirect mechanisms of miR-29b eliciting a response on both AID expression and CSR to IgE, miR-29b OE did not affect the expression of apoptotic markers in either tonsil b cells (as evidenced by the microarray study) or a B cell line (Supplementary figure 3E) and



462 did not affect cell proliferation (Supplementary figure 3F). This raises the question as  
463 to the purpose of such an interaction. AID and miR-29b expression are inversely  
464 correlated. *AICDA* is detectable in the lymphoid follicles and most abundant in GC B  
465 cells, but absent in circulating naïve B cells. Conversely, miR-29b is reduced in the  
466 follicles but prominent in circulating naïve B cells. This indicates that the function of  
467 miR-29b may be to suppress premature AID at the early stages of a mature B cell's life  
468 cycle, or following inappropriate activation in the absence of suitable T cell help or T-  
469 independent "danger" signals.

470  
471 Upon entry into the lymphoid tissue and subsequently the GC, where AID expression  
472 is appropriate, the down regulation of miR-29b would relieve the block on *AICDA* and  
473 allow its full regulated expression. Indeed, the potential of endogenous miR-29b to  
474 regulate AID in this context was demonstrated by the increase of AID mRNA and  
475 protein expression levels when miR-29b was inhibited in IL4 and antiCD40 stimulated  
476 naïve B cells (Fig. 4A and 2B). We believe this to be a far more compelling result  
477 (being carried out at a physiologically relevant level of miR-29b) than the  
478 overexpression study and evidence of the potential for miR-29b to regulate AID  
479 expression. In contrast, in tonsil B cells, where miR-29b is less abundant, miR29b  
480 inhibitors did not elevate *AICDA* (data not shown). This strongly suggests that while  
481 endogenous miR-29b expression in naïve B cells is sufficient to suppress *AICDA*  
482 expression, the low levels of miR-29b in GC B cells is insufficient to do so (although  
483 overexpression of miR29b above physiological levels does indeed strongly repressing  
484 AID and partially blocks CSR). As such, it is likely that miR-29b is able to repress  
485 leaky or inappropriate expression of AID prior to B cell activation. In relation to  
486 miR29s potential role in preventing inappropriate AID expression, and therefore



function, in naïve B cells, it of interest to note that miR29 (a, b and c) was found to be decreased in Mantle cell lymphoma (MCL) (Zhao et al., 2010), a lymphoma with characteristics of CD5<sup>+</sup>, antigen-naïve pregerminal center B-cells. Although Cyclin D1 translocation and the up-regulation of CDK6 (a direct miR29 target) is thought to account for the transformation of MCL cells, they were also found to frequently express AID possibly accounting for at least some of wide range of chromosomal abnormalities present in this disease(Babbage et al., 2004).

Following B cell exit from the circulation, miR-29b silencing in GC B cells leads to a de-repression of *AICDA* transcription. Such a model has been previously ascribed to miR-181b regulation of *aicda* in mice (de Yebenes et al., 2008). Interestingly, miR29c shares a similar expression pattern to miR-29b, being most highly expressed in naïve B cells and low in GC B cells (Supplementary Figure 1). Since miR29 family members share seed region homology and therefore predicted targets, the expression of these miRs would be expected to reinforce their functional effects. It should be noted that the miR-29 site in the *AICDA* 3' UTR is conserved amongst other primate species, but not in more distantly related mammals, such as mice, rats and rabbits. Thus, it is not an interaction that has been strongly conserved throughout mammalian evolution and may have arisen more recently.

In conclusion, the data in this paper have shown that miR-29b, a member of the conserved miR-29 family, is repressed in tonsil B cells relative to circulating naïve B cells. Enforced overexpression of miR-29b in tonsil B cells led to the direct targeting of *AICDA*, through a pronounced binding site in the *AICDA* 3' UTR and this interaction was sufficient to reduce CSR to IgE. Similarly, inhibition of endogenous miR-29b in naïve B cells resulted in elevated AID expression. We hypothesise that one of the



512 endogenous functions of miR-29 is to silence leaky expression of AID, a mutagenic  
513 protein whose expression must be controlled in order to maintain chromosomal  
514 integrity.  
515



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524



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## Figure legends

### **Figure 1: miR-29 is less abundant in B cells isolated from tonsillar tissue than in naïve B cells isolated from peripheral blood.**

The expression level of miR-29 in IgD<sup>+</sup> CD27<sup>-</sup> naïve B cells isolated from peripheral blood compared to CD19<sup>+</sup> tonsil B cells, as assessed via qRT-PCR. Values are plotted relative to the small nucleolar RNA RNU6B. \*\* P < 0.01(unpaired t-test).

### **Figure 2: Genome-wide analysis of miR-29b targeting in tonsillar B cells shows an enrichment in miR-29 binding sites within the 3' UTR of downregulated genes.**

(A) Heatmap representing expression changes in the most downregulated genes in tonsillar B cells transfected with a miR-29 mimic and stimulated with anti-CD40 antibody and IL-4 cytokine for 24 hours, as detected by Illumina BeadChip Array. Genes containing predicted miR-29 binding sites in their 3' UTR are listed in red. P values (two-way ANOVA) are also included.

(B) Pie chart displaying the prevalence of miR-29 binding sites within the 3' UTR of genes downregulated following miR-29b overexpression. The percentage of 8mers (an exact match to positions 2-8 of the mature miRNA with an A opposite position 1 of the miRNA), 7mer-m8s (an exact match to positions 2-8 of the mature miRNA) and 6mers (an exact match to positions 2-7 of the mature miRNA) within the miR-29 binding sites is also shown.

(C) Dot plot representations of the change in fluorescence intensity for *AICDA*, as well as previously validated targets including *CDK6*, *RCC1*, *TDG* and *TRAK2*, in tonsil B cells transfected with a miR-29 mimic and stimulated with anti-CD40 antibody and IL-4 cytokine for 24 hours, as detected by Illumina BeadChip Array.



**Figure 3: Overexpression of miR-29 reduces AID abundance in tonsillar B cells.**

tonsil B cells were transfected with a miR-29b mimic or its associated negative control molecule and subsequently activated with IL-4 and anti-CD40.

(A) Gene expression of key B cell molecules after 24 hours of stimulation as assessed via qRT-PCR. Values are normalized to 18S ribosomal RNA and plotted relative to the abundance of each gene in the control transfected cells, which were arbitrarily assigned a value of 1. N = 3 - 12, mean and s.e.m. \* P < 0.05, \*\* P < 0.01, \*\*\*\* P < 0.0001 (paired t-test).

(B) Representative immunoblot and cumulative densitometry analysis (n = 3) of AID protein in follicular B cells following miR-29b overexpression and 60h of culture in IL-4 and anti-CD40 stimulus, compared to AID protein in the control transfected cells. GAPDH served as a loading control.

**Figure 4: Knockdown of miR-29 increases AID abundance in naïve B cells.**

Naive B cells were transfected with a miR-29 inhibitor or its associated negative control molecule and subsequently activated with IL-4 and anti-CD40.

(A) Gene expression of key B cell molecules after 48 hours of stimulation as assessed via qRT-PCR. Values were normalized to 18S ribosomal RNA and plotted relative to the abundance of each gene in the control transfected cells, which were arbitrarily assigned a value of 1. N = 6 - 12. Mean and s.e.m. \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P < 0.0001 (paired t-test).

(B) Representative immunoblot and cumulative densitometry analysis (n = 3) of AID protein in naïve B cells following miR-29b knockdown and 60h of culture in IL-4 and anti-CD40 stimulus, compared to AID protein in the control transfected cells. GAPDH served as a loading control.



**Figure 5: miR-29 is capable of directly targeting the miR-29 binding site within the AICDA 3' UTR.**

(A) The miR-29 binding site within the human, chimpanzee and rhesus macaque *AICDA* 3' UTR, as well as the mismatched sequence used for luciferase analysis.

(B) HEK293T cells were contranfectected with one of the dual luciferase plasmids (endogenous *AICDA* 3' UTR, mismatched 3' UTR or no insert) and a miR-29 mimic. Firefly luciferase activity was quantified after 24 hours, normalized to renilla luciferase and plotted relative to the no insert plasmid. \*  $P < 0.05$  (one-way ANOVA).  $N = 3$ , mean and s.e.m.

**Figure 6: miR-29 overexpression dampens induction of class switching to IgE.**

Tonsil B cells were transfected with a miR-29 mimic or negative control molecules and subsequently cultured in class-switching conditions (IL-4 and anti-CD40 antibody).

(A) IgE and IgG mRNA were detected via qRT-PCR following 5 days of culture. Values are plotted relative to 18S ribosomal RNA.  $N = 7$  for IgE and 5 for IgG, mean and s.e.m.

(B) Intracellular IgE and IgG was analysed following 7 days of culture via flow cytometry. \*  $P < 0.05$  (paired t test).

(C) Intracellular IgE and IgG was analysed following 7 days of culture via flow cytometry. Values were plotted relative to the control transfected cells, which were arbitrarily assigned a value of 1.  $N = 3$  for siAID, and 10 for miR-29 versus the negative control mimic. \*  $P < 0.05$  (paired t test). Mean and s.e.m.



Figure 1

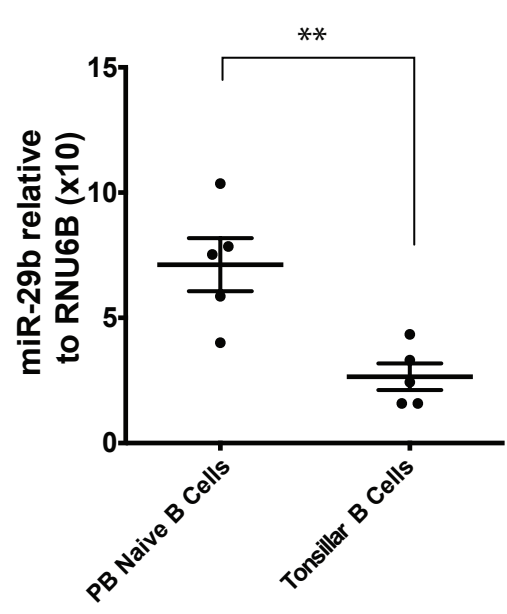




Figure 2

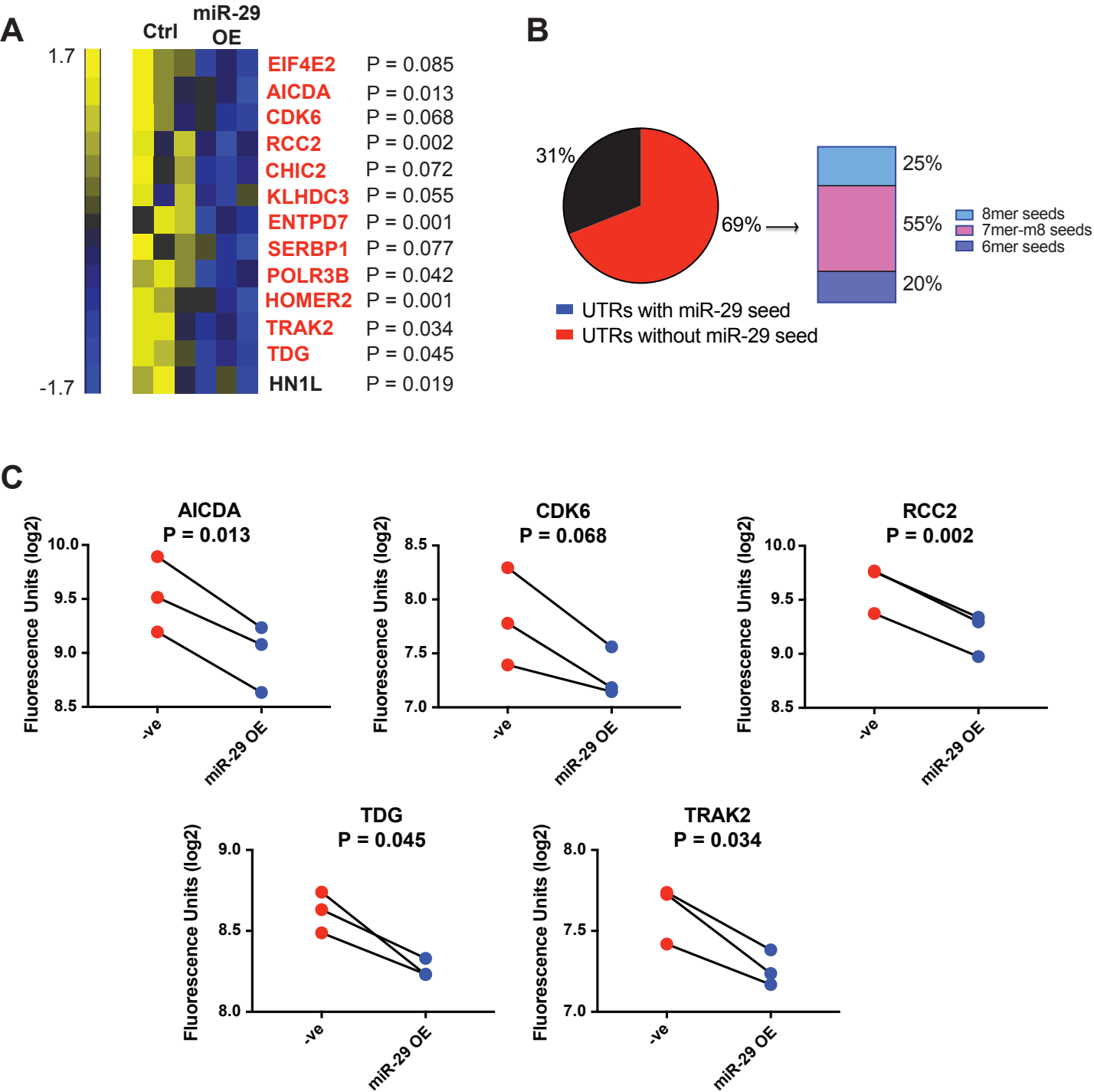
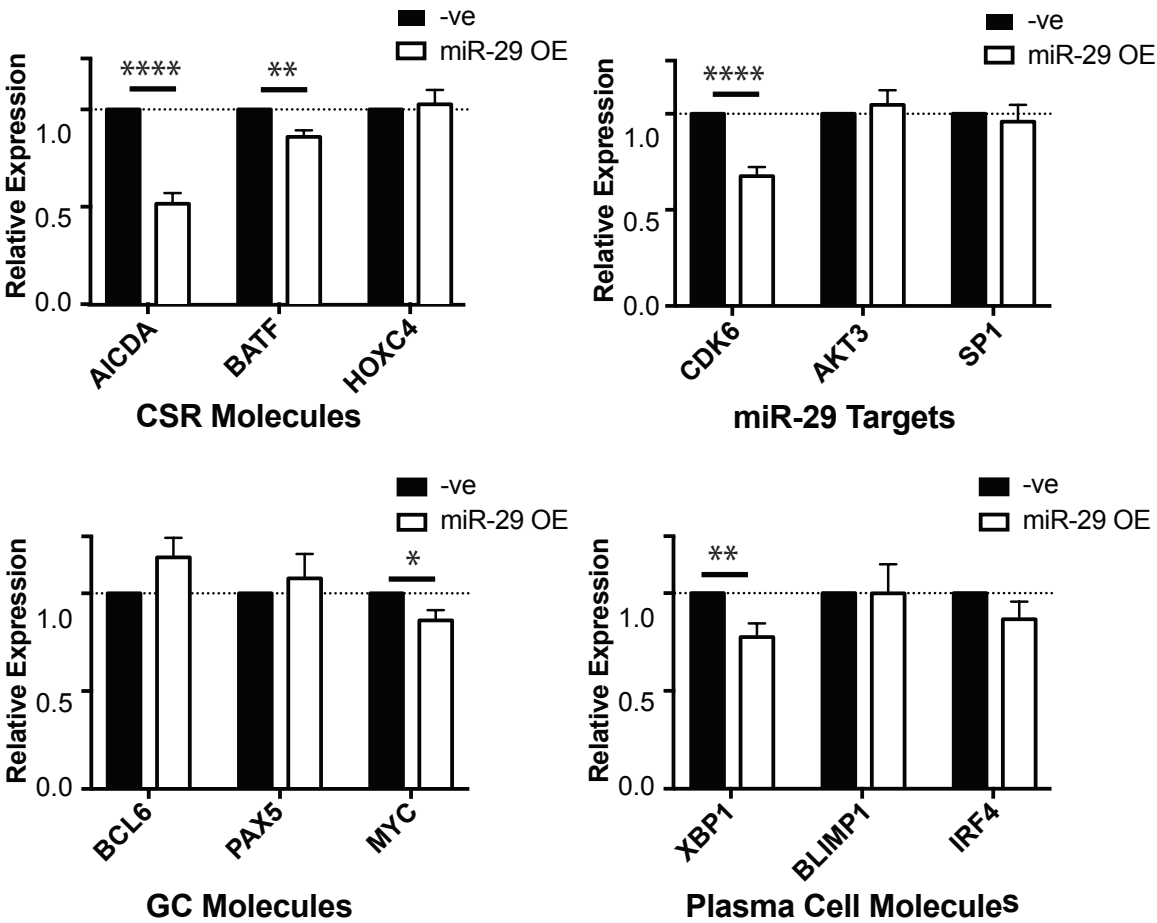




Figure 3

A



B

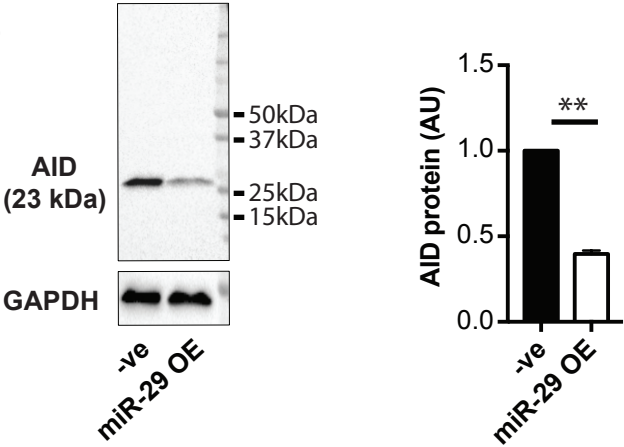




Figure 4

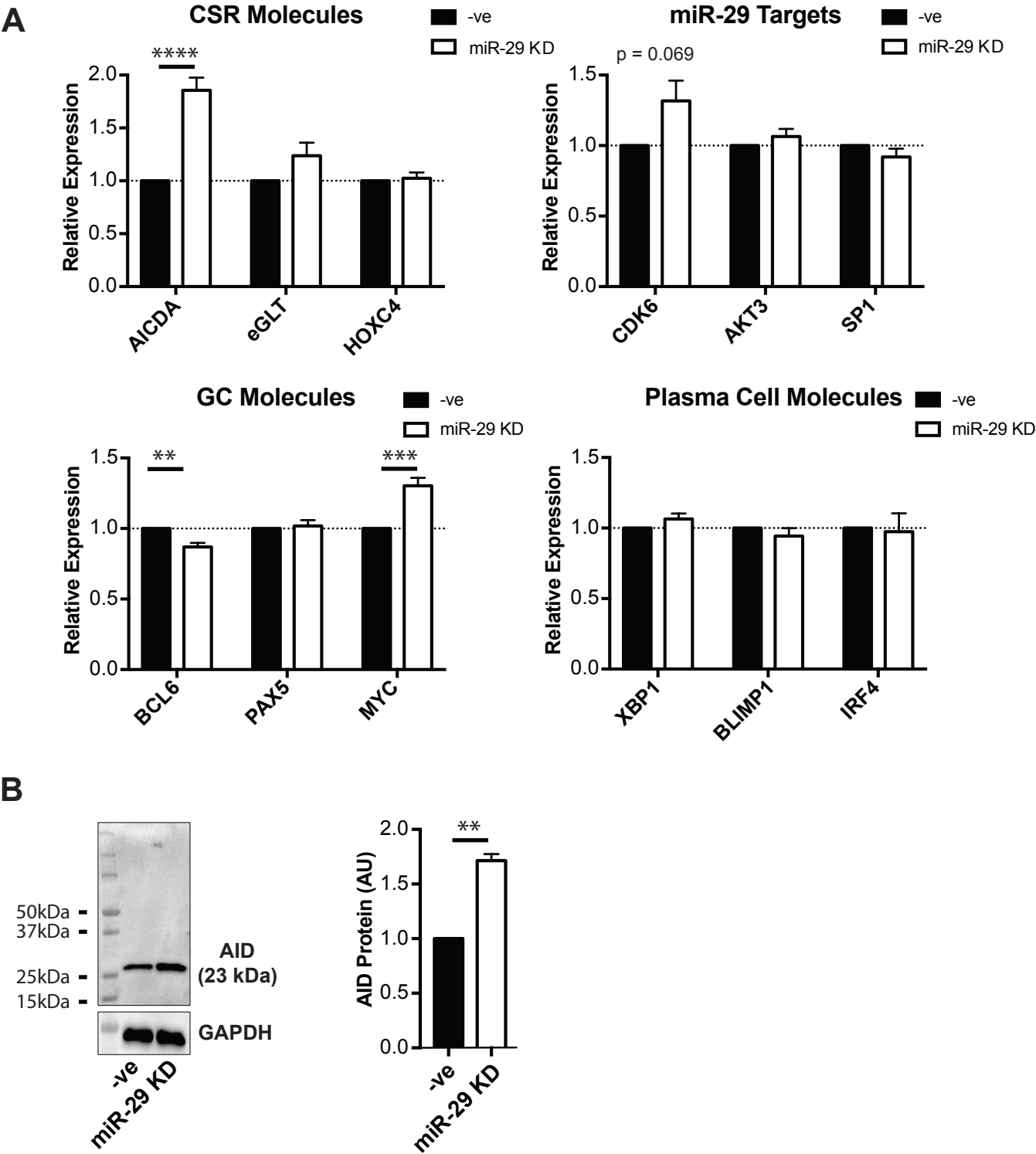




Figure 5

A

5'	...GUAAC <b>AUUGAAGUAAUGGUGCUA</b> ...	<i>AICDA 3' UTR (Rhesus)</i>
5'	...GUAAC <b>AUUGCAGUAAUGGUGCUA</b> ...	<i>AICDA 3' UTR (Chimpanzee)</i>
5'	...GUAAC <b>AUUGCAGUAAUGGUGCUA</b> ...	<i>AICDA 3' UTR (Human)</i>
3'	UUGUGAC <b>UAAAGU</b> ---- <b>UUACCACGAU</b>	<i>miR-29b</i>
	X	
5'	...GUAAC <b>AUUGCAGU</b> ----- <u><b>GGATCC</b></u> -----...	<i>AICDA Mismatched 3' UTR</i>

B

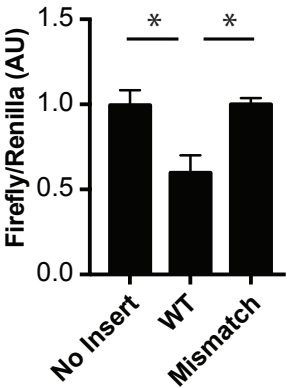
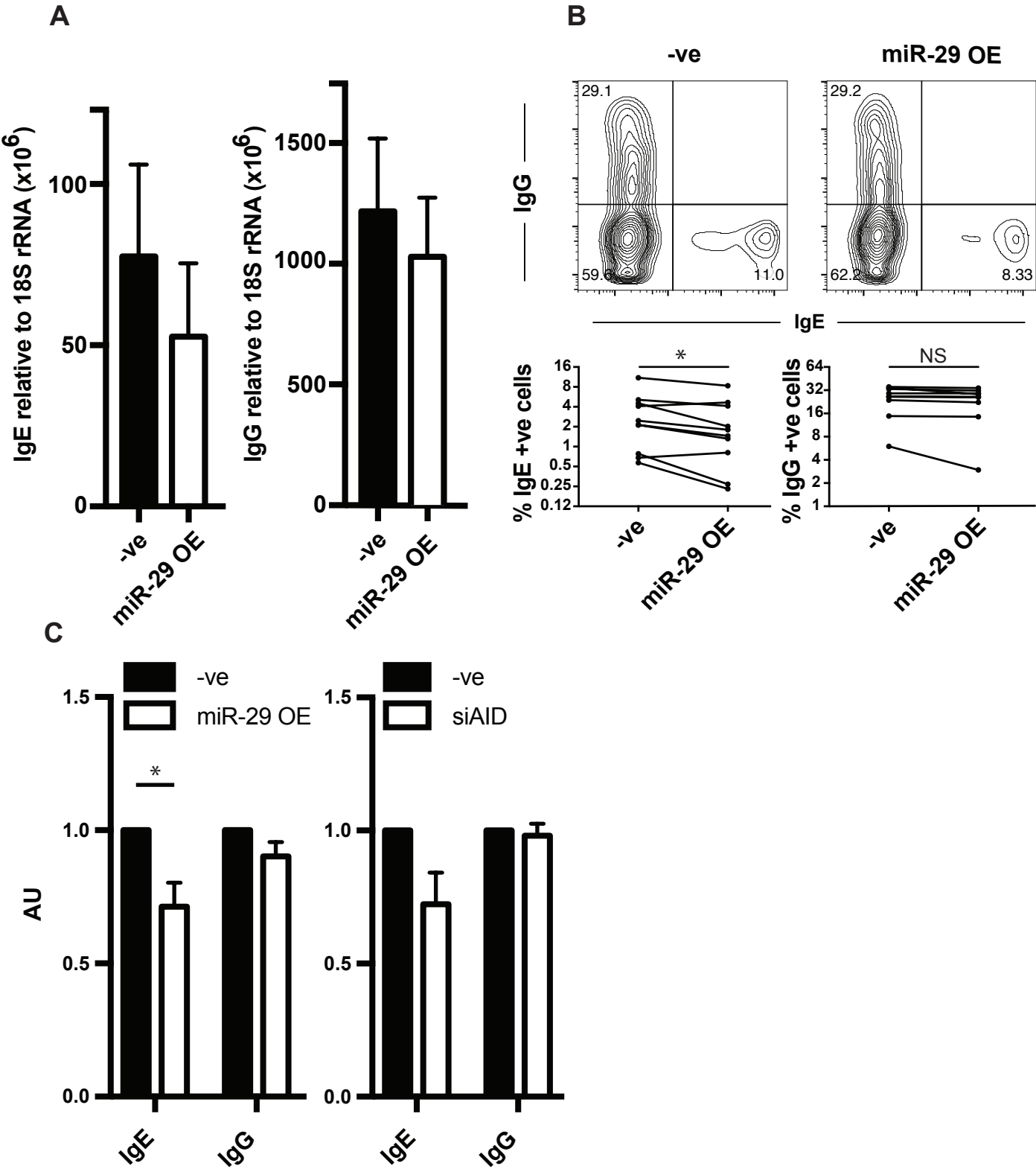




Figure 6





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