# Supplementary Material for

## T cell help controls the speed of the cell cycle in germinal center B cells

Alexander D. Gitlin, Christian T. Mayer, Thiago Y. Oliveira, Ziv Shulman, Mathew J.K. Jones, Amnon Koren, Michel C. Nussenzweig

## Material and Methods

### Mice

C57BL/6 and DsRed mice were purchased from Jackson Laboratories. B1-8$^{hi}$ and DEC205$^{-/-}$ mice were described previously (*16, 40*). Fucci transgenic mice were obtained from T. Kurosaki and A. Miyawaki (*18, 19*). All experiments were performed with authorization from the Institutional Review Board and the IACUC at The Rockefeller University.

### B cell transfers

Resting B cells were purified from mouse spleens by forcing tissue through 40 μm mesh into complete RPMI media (Gibco) containing 6% serum. Cell suspensions were purified using CD43 magnetic beads (MACS), according to manufacturer's protocols (Militenyi Biotec). 1.5-10 x$10^6$ B cells (≈(1.5-10) x $10^5$ Igλ$^+$, NP-specific B cells) were transferred intravenously into recipient mice. In figures 2 and 4, adoptive transfers utilized ratios of 15:85 or 50:50 of B1-8$^{hi}$ DEC205$^{+/+}$ and B1-8$^{hi}$ DEC205$^{-/-}$ B cells followed by injection of either αDEC-OVA or control treatment (αDEC-CS or PBS), respectively, on day 6 after boosting with NP-OVA.

### Immunizations and treatments

C57BL/6 mice were primed by intraperitoneal immunization with 100 μl containing 50 μg of OVA (Grade V, Sigma) precipitated in alum at a 2:1 ratio. Two to six weeks later, mice received the indicated adoptive transfers followed the next day by subcutaneous boosting with 25 μg of $NP_{16}$-OVA (Biosearch Technologies). Draining lymph nodes were collected and processed for flow cytometric analysis. Polyclonal GCs were generated by intraperitoneal immunization of C57BL/6 mice with 50 μg of

$NP_{16}$-OVA in alum. αDEC-OVA and αDEC-CS fusion antibodies were produced by transient transfection in 293T cells, as described previously (*17*). 5 μg of fusion antibody in PBS was injected subcutaneously at the indicated time points. For cell cycle analysis in Fig. 2, 1 mg EdU (Life Technologies) in PBS was injected intravenously followed 0.5 hours later by an intravenous injection of 2 mg BrdU (Sigma-Aldrich) in PBS. Cells were then stained for flow cytometry and processed using an anti-BrdU-FITC kit (BD Biosciences) and Click-iT EdU-Pacific Blue kit (Life Technologies) according to manufacturers' instructions. DNA content was generally analyzed by staining cells with 7-AAD (BD Biosciences), except in fig. S1 in which DAPI was used to assess DNA content. In Fig. 3, mice were treated with 1 mg EdU intravenously followed 0.5 hours later with 5 mg BrdU intraperitoneally. For DNA fiber analyses, mice were injected with 1.25 mg and 0.125 mg of IdU (Sigma-Aldrich) in PBS intraperitoneally and subcutaneously, respectively. After 20 minutes, mice were injected with 1 mg thymidine (EMD Millipore) immediately before lymph nodes were isolated and processed for cell sorting.

**Flow Cytometry and Cell Sorting**

Single cell suspensions were processed in PBS that was maintained at 4°C and contained 2% serum and 1 mM EDTA. Samples were treated at 4°C for 10 minutes with 1 μg/ml of anti-CD16/32 (2.4G2, Bio-X-Cell) and then stained for 25 minutes at 4°C. Anti-B220, CD38, CD86, CD45.1, CD45.2 antibodies were from eBioscience. FAS, CXCR4, CD45.1, CD45.2, Igλ1-3, GL7, streptavidin-phycoerythrin and streptavidin-allophycocyanin were from BD Biosciences. Streptavidin-Alexa Fluor 488 was from Invitrogen. Antibody for intracellular staining of pRb-S807/811 was from Cell

Signaling Technology (clone: D20B12). Cell fixation and permeabilization were performed using the Cytofix/Cytoperm kit (BD Biosciences). Samples were analyzed on a BD Fortessa. GC B cells were gated as live/single and $B220^+$ and either $CD38^-$ $FAS^+$ or $FAS^+GL7^+$. DZ cells were gated as $CXCR4^+CD86^-$, as described previously (*11, 13*). CD45.1 and CD45.2 allotypic markers or DsRed were used to trace transferred B cell populations. B1-$8^{hi}$ $DEC205^{+/+}$ B cells were identified as $CD45.1^+$, and B1-$8^{hi}$ $DEC205^{-/-}$ B cells were identified as $CD45.1^+CD45.2^+$. For RNA sequencing, cells were sorted directly into Trizol LS reagent (Invitrogen) that was maintained at 4°C using a FACS Aria II (Becton Dickinson). Prior to cell sorting in the experiment in Fig. 3, GC B cells were enriched using anti-IgD-biotin (eBioscience) and both anti-biotin and CD43 magnetic beads (MACS).

**Gene expression analysis**

Purified RNA from sorted samples was processed using the SMARTer Ultra Low Input RNA for Illumina Sequencing kit (Clontech Laboratories). High throughput sequencing was performed using a HiSeq 2500 (Illumina). RNA-seq reads were aligned with STAR version 2.3.0 allowing unique alignments and using Mouse Ensembl genes as reference. Differential expression was calculated using Cufflinks with default settings. Gene Set Enrichment Analyses were performed using GSEA v2.2.2.

**Replication timing data**

To generate replication timing data, cell suspensions from draining lymph nodes were stained for surface markers and then fixed and permeabilized as described above. Samples were then incubated in 100 μl of 20 μg/μl RNAse A (Life

Technologies) for 30 minutes at 37°C and subsequently stained for DNA content with 7-AAD. B1-8$^{hi}$ DEC205$^{+/+}$ GC B cells were identified as B220$^{+}$CD38$^{-}$ FAS$^{+}$CD45.1$^{+}$CD45.2$^{-}$ and sorted based on G1 or S phase DNA content. Genomic DNA from sorted cells was isolated and libraries derived from these samples were sequenced on a HiSeq 2500 (Illumina). Sequence reads were then aligned to the mouse reference genome, build mm9, using BWA mem with default settings. Replication timing was computed using normalized sequence tag density values for G1 and S for each 2 kb window of the genome. Only windows containing more than 25% of tags expected by a uniform distribution throughout the genome were used. 2 kb window data were smoothed using the Matlab function Csaps with a parameter of $10^{-17}$. Smoothed data for the two biological replicates of each condition were averaged, and both averaged and single-repeat data were Z-score normalized to a genome-wide mean of zero and standard deviation of 1. Smoothed data was compared to mouse L1210 lymphoblast replication timing data (*41*) by interpolating to the same data coordinates.

**DNA Fiber analyses**

DNA fibers analysis was performed on sorted cell populations as previously described (*38*). In brief, cells were resuspended in PBS at 1x$10^6$ cells/ml and 2 μl of the cell suspension were spotted onto a glass slide and lysed with 10 μl spreading buffer (0.5% SDS in 200 mM TRIS-HCl (pH 7.4) and 50 mM EDTA) for 6 minutes. Slides were tilted at a 15° relative to horizontal, extending DNA fibers. After air-drying, slides were fixed in methanol and acetic acid (3:1) for 2 minutes. DNA was then denatured in 2.5 M HCl for 25-30 minutes at room temperature. Slides were

washed three times in PBS and blocked for 1 hour in PBS containing 0.1% Triton X-100 + 10% goat serum (MP Biomedicals 092939149). IdU labeled tracks were detected using mouse anti-BrdU (Becton Dickinson 347580, 1:100) and Alexa Fluor 488-labelled goat anti-mouse IgG (InvitrogenA11029, 1:350) antibodies. DNA fibers were counterstained with an anti-ssDNA antibody (Millipore MAB3034, 1:600) and Alexa Fluor 647-labeled goat anti-mouse IgG2A (Invitrogen A21241, 1:350). Slides were mounted using Prolong Gold antifade reagent (Invitrogen) and replication tracks were imaged on a DeltaVision Elite system (Applied Precision) and measured using SoftWoRx software (Applied Precision).

**VH186.2 mutation analysis**

Genomic DNA was extracted from GC B cells that were isolated as described above. PCR was performed using Phusion HF (New England Biolabs), and gel-extracted PCR products were cloned into Zero Blunt TOPO vectors (Invitrogen) as described previously (*11, 13*). Antibody gene sequences were analyzed using the IMGT/V-QUEST system to identify W33L and K59R mutations.

**Statistical Analyses**

Statistical significance was assessed using the tests indicated in the figures using Prism software v. 5.0 (Graphpad).

**Supplementary Figures**

**Fig. S1. RNA sequencing of dark zone cells in G1 phase. (A)** Outline of the experimental protocol. OVA-primed WT recipient mice received an intravenous (i.v.) transfer of B1-8$^{hi}$ DsRed$^{+}$ Fucci$^{tg}$ and B1-8$^{hi}$ DEC205$^{-/-}$ B cells at a 15:85 or 85:15 ratio one day before subcutaneous (s.c) boosting with NP-OVA. After 6 days, mice were treated s.c. with either αDEC-OVA or αDEC-CS, respectively. RNA sequencing was performed from sorted B1-8$^{hi}$ DsRed$^{+}$ GC B cells in G1 phase (Fucci$^{-}$) in the DZ (CXCR4$^{+}$CD86$^{-}$) 2 days after antibody treatment. **(B)** Flow cytometry plot showing fluorescent separation of B1-8$^{hi}$ DsRed$^{+}$ Fucci$^{tg}$ GC B cells into G1 and S/G2/M phases.

**Fig. S2. Gene set enrichment analysis. (A-F)** Selected gene sets from the Broad GSEA mSig database that were significantly enriched in experiment described in Fig. 1.

**Fig. S3. EdU/BrdU double-pulse labeling. (A)** Experimental protocol used for analyzing cell cycle phase progression. GCs were induced in mice as described in Fig. 2 and corresponding text. At 48 hours after chimeric antibody treatment, mice were intravenously pulsed with EdU, followed 1 hour later with BrdU and then analyzed by flow cytometry for DNA content at 0.5, 2.5, or 5 hours after the second pulse. **(B)** Representative flow cytometry plots demonstrating EdU and BrdU incorporation in follicular (B220$^{+}$CD38$^{+}$FAS$^{-}$) and GC B cells (B220$^{+}$CD38$^{-}$FAS$^{+}$) at 0.5 hours after double-pulsing. Representative CD45.1/2 gates used to trace B1-8$^{hi}$ DEC205$^{+/+}$ and B1-8$^{hi}$ DEC205$^{-/-}$ GC B cells are shown.

**Fig. S4. T cell help increases the rate of DNA accumulation in GC B cells undergoing DNA replication.** Results are from experiments described in Fig. 2 and corresponding text. Median fluorescence intensity (MFI) of DNA content was measured in $EdU^{-}BrdU^{+}$ GC B cells 2.5 hours after EdU/BrdU double-pulse labeling. Fraction of genome replicated was calculated as (MFI of $EdU^{-}BrdU^{+}$ cells)/(MFI of G1 phase cells) – 1. Paired t-test was used to determine significance. *** p = 0.0007.

**Fig. S5. EdU/BrdU labeling of polyclonal GCs. (A)** C57BL/6 mice were treated with either EdU alone (right) or with BrdU 5 minutes before EdU treatment (left). Flow cytometry plots demonstrate inhibition of EdU uptake in the presence of BrdU among GC B cells in peyer's patches. **(B)** Experimental protocol used in Fig. 3.

**Fig. S6. Replication timing analysis in the GC. (A)** Representative histogram showing gates used to sort G1 and S phase GC B cells for replication timing analyses. **(B)** Scatter plot showing fraction of $B1\text{-}8^{hi}$ $DEC205^{+/+}$ GC B cells in S phase 48 hours after treatment with αDEC-CS or αDEC-OVA. Each dot represents one mouse. **(C)** Correlation matrix showing degree of similarity between replication timing profiles of independent biological replicates of αDEC-CS and αDEC-OVA treatment and L1210 mouse lymphocytic leukemia cells (*41*). **(D)** Outline of experimental protocol for Fig. 4C-E. At 48 hours after αDEC-OVA or αDEC-CS treatment, mice were pulsed with IdU and $B1\text{-}8^{hi}$ $DEC205^{+/+}$ GC B cells were sorted and processed for replication fiber imaging.

**Fig. S1**

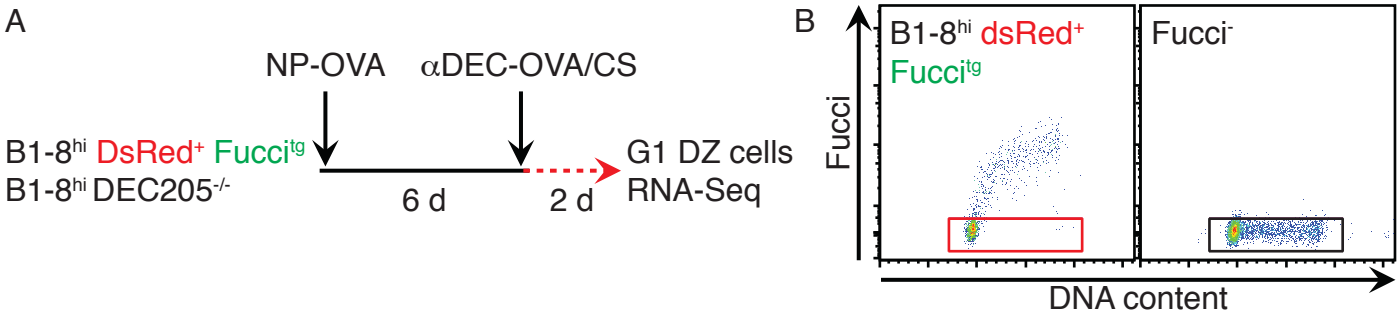
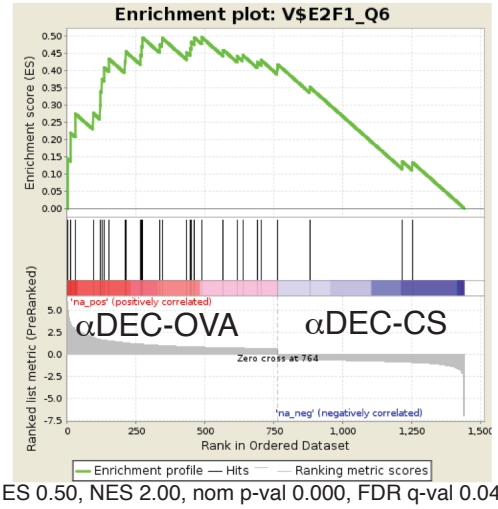

A
NP-OVA
αDEC-OVA/CS
B1-8hi DsRed+ Fucci tg
B1-8hi DEC205-/-
6 d
2 d
G1 DZ cells
RNA-Seq
B
B1-8hi dsRed+
Fucci tg
Fucci-
Fucci
DNA content

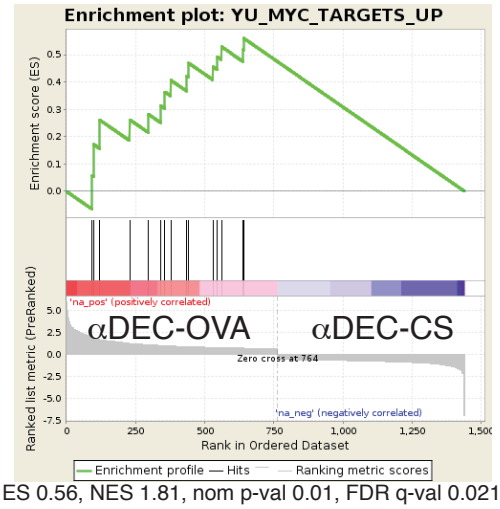
**Fig. S2**

A



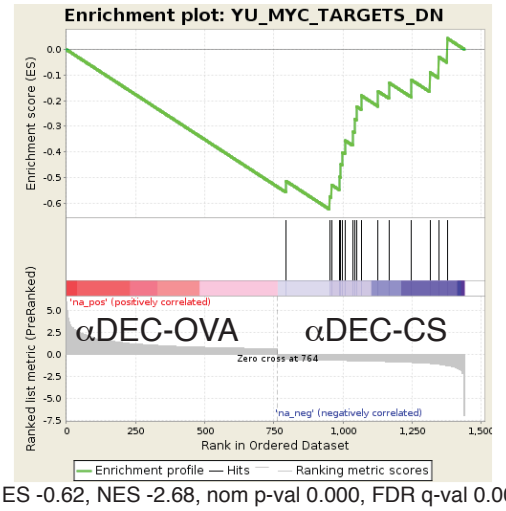

ES 0.50, NES 2.00, nom p-val 0.000, FDR q-val 0.043

B



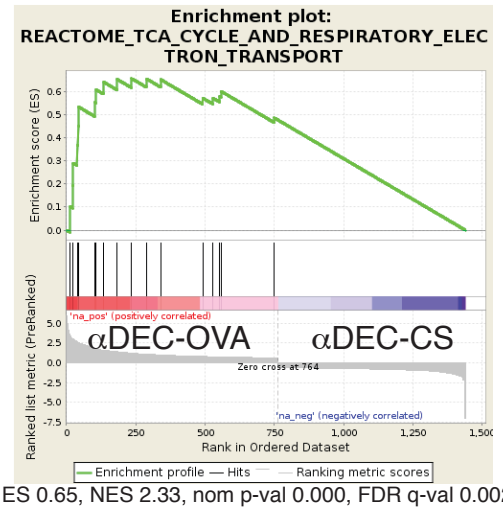

ES 0.56, NES 1.81, nom p-val 0.01, FDR q-val 0.021

C



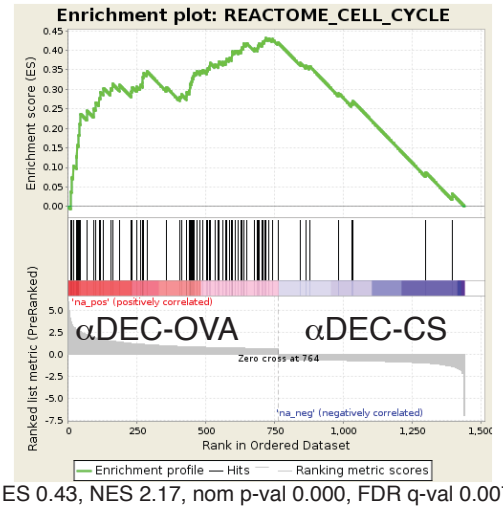

ES -0.62, NES -2.68, nom p-val 0.000, FDR q-val 0.000

D



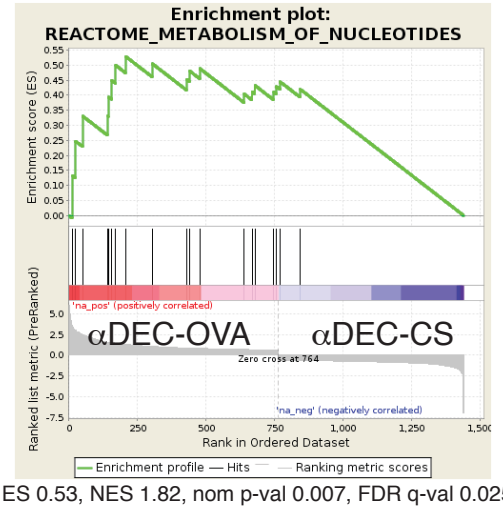

ES 0.65, NES 2.33, nom p-val 0.000, FDR q-val 0.002

E




ES 0.43, NES 2.17, nom p-val 0.000, FDR q-val 0.007

F




ES 0.53, NES 1.82, nom p-val 0.007, FDR q-val 0.025

**Fig. S3**

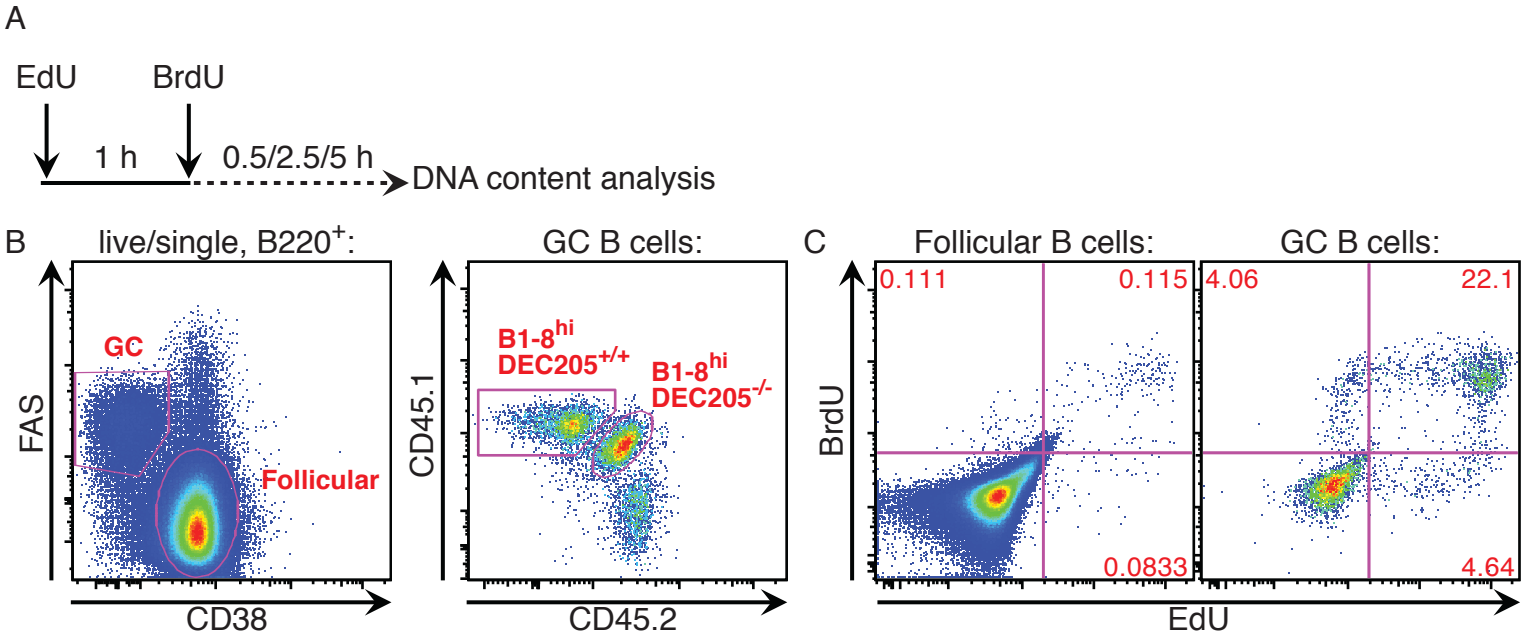

A
EdU
BrdU
1 h
0.5/2.5/5 h
DNA content analysis
B
live/single, B220+:
GC
Follicular
FAS
CD38
GC B cells:
B1-8hi DEC205+/+
B1-8hi DEC205-/-
CD45.1
CD45.2
C
Follicular B cells:
GC B cells:
0.111
0.115
0.0833
4.06
22.1
4.64
BrdU
EdU

**Fig. S4**

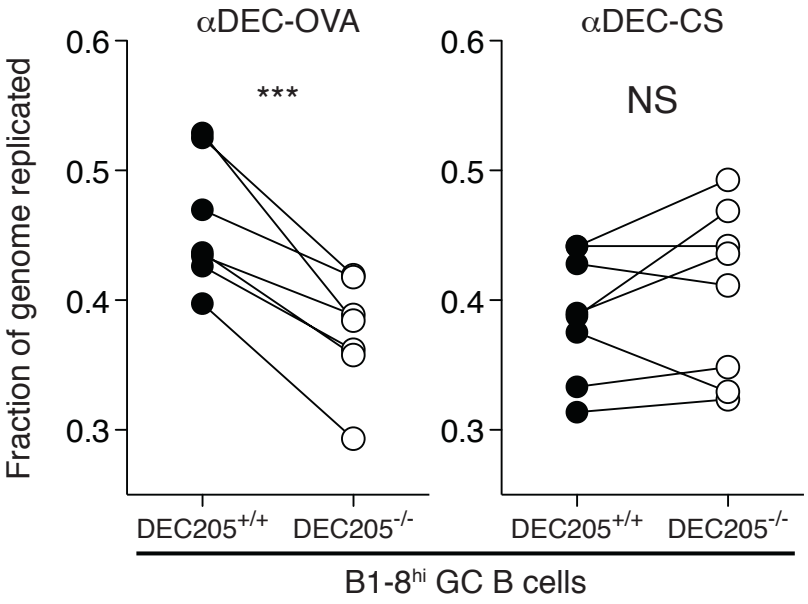

αDEC-OVA
αDEC-CS
***
NS
Fraction of genome replicated
0.6
0.5
0.4
0.3
DEC205+/+
DEC205-/-
B1-8hi GC B cells

**Fig. S5**

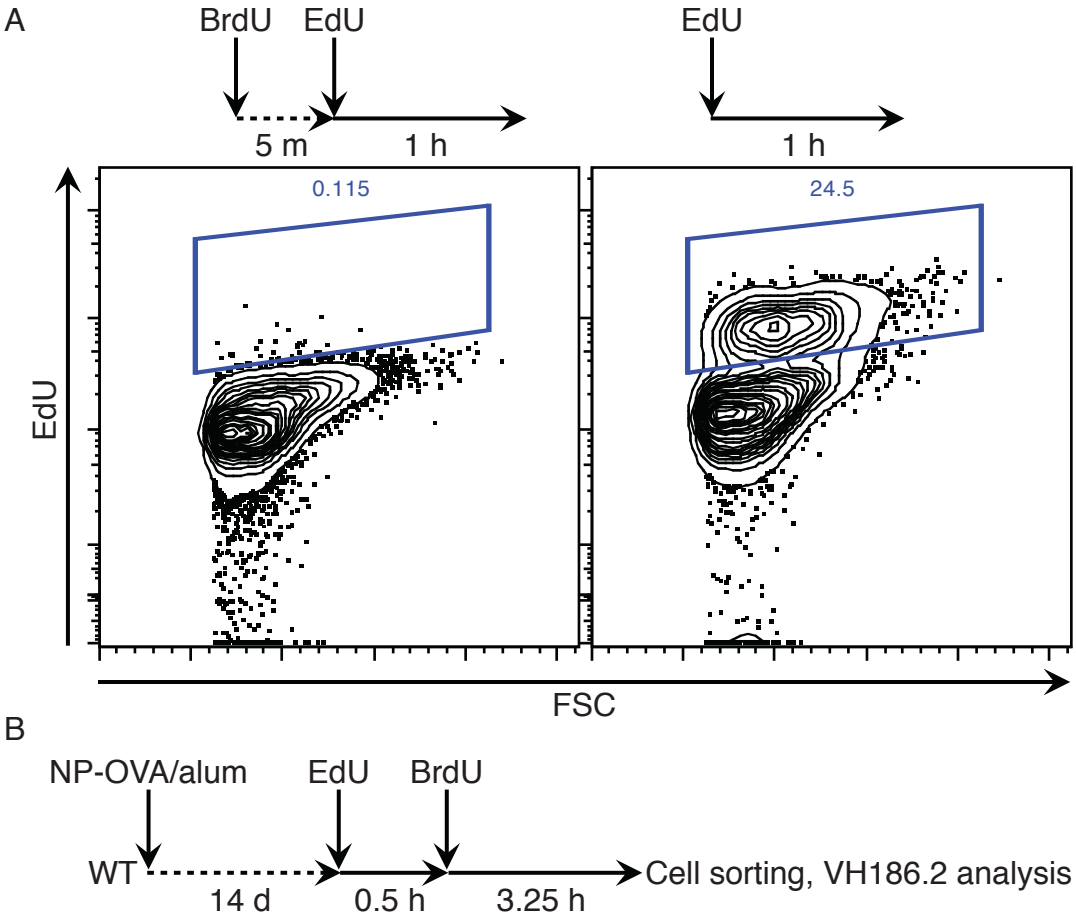
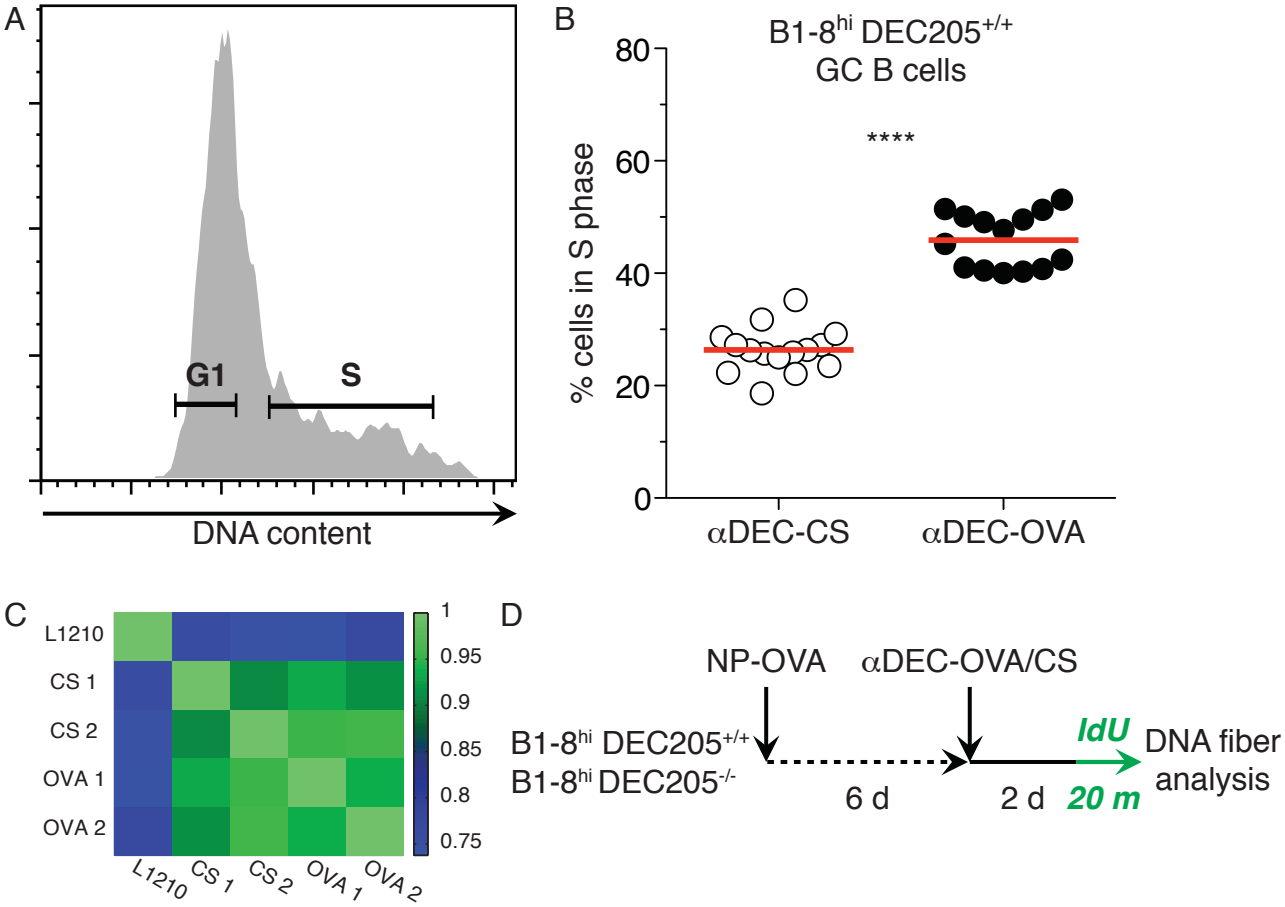

A
BrdU
EdU
5 m
1 h
EdU
1 h
0.115
24.5
EdU
FSC
B
NP-OVA/alum
EdU
BrdU
WT
14 d
0.5 h
3.25 h
Cell sorting, VH186.2 analysis

**Fig. S6**


A
G1
S
DNA content
B
B1-8hi DEC205+/+
GC B cells
****
% cells in S phase
80
60
40
20
0
αDEC-CS
αDEC-OVA
C
L1210
CS 1
CS 2
OVA 1
OVA 2
1
0.95
0.9
0.85
0.8
0.75
D
NP-OVA
αDEC-OVA/CS
B1-8hi DEC205+/+
B1-8hi DEC205-/-
6 d
2 d
IdU
20 m
DNA fiber
analysis