# CCR6 activation links innate immune responses to mucosa-associated lymphoid tissue lymphoma development

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## Supplementary materials and methods

### CCR6 expression constructs

The full-length coding sequence of CCR6 in pcDNA3.1 (GenScript) was used to generate CCR6 mutants (R159S and W335X) by PCR. The wild type and mutant CCR6 sequences were cloned separately into pcDNA5/FRT (Figure S1) and pIRESpuro vectors using NEBuilder HiFi DNA Assembly Cloning Kit (New England BioLabs), and their sequences were verified by Sanger sequencing. All the above constructs contained a HA-tag at the N-terminus of CCR6, and a separate set of these constructs with an additional C-terminal GFP-tag were also generated for analysis of CCR6 cellular localization.

### Generation of a single copy CCR6 stable expression isogenic cell lines

Flp-InTRex293 host cells (a gift from Dr Yvonne Vallis, MRC, Cambridge) were co-transfected with each of the above CCR6 pcDNA5/FRT expression vectors together with the recombinase producing pOG44 plasmid using TransIT-LT1 transfection reagent (Mirus).[1] Expression of recombinant CCR6 in positive colonies (at least 3 for each construct) was confirmed by flow cytometry analyses using anti-HA antibody (Figure S2).

### Generation of CCR6 enriched expression DG75 line

DG75 cell line originated from an EBV-negative Burkitt lymphoma was chosen due to its ease for *in vitro* transfection among various B-cell lines tested, as there is no established cell lines from MALT lymphoma. DG75 cells ($5x10^6$) were transfected with 2µg of each of the above CCR6 pIRESpuro expression constructs using nucleofector (Lonza kit V, program M13), and then cultured in RPMI 1640 medium. under puromycin selection (2µg/ml) for 2 weeks, and then expanded with 1µg/ml puromycin. CCR6 expression was confirmed by flow cytometry analyses using anti-HA and anti-CCR6 antibodies (Figure S3).

### Effect of CCR6 expression on cytotoxic challenge

The Flp-InTRex293 cells ($1x10^6$) that carried a single copy of the wild type or mutant CCR6 expression construct, and the DG75 B-cells ($1x10^6$) that expressed wild type or mutant CCR6 were treated with 1.5nM staurosporine in the presence of CCL20 (50nM) or vehicle for 16 hours. Cell death was determined by flow cytometry analysis of Annexin V binding according to manufacturer's protocol (eBioscience) and analyzed using FlowJo10 software.

### Soft agar colony formation assay

The Flp-InTRex293 cells (1250) that carried a single copy of the wild type or mutant CCR6 expression construct, together with parental cells, were cultured on standard soft agar in DMEM medium in a 24 well tissue culture dish for 3 weeks, and resulting colonies were visualized by staining with crystal violet, and quantified.

### Wound scratch healing assay

The Flp-InTRex293 cells with a single copy of the wild type or mutant CCR6, together with parental cells, were seeded in 6-well plates and cultured for 24 hours to allow 100% confluence. A scratch wound was introduced using a 10µl pipette tip, the cell debris was removed and the plate was cultured in the presence of 50nM CCL20 or vehicle for 60 hours with the wound image taken every 12 hours. The wound area was measured using the wound healing tool (https://github.com/MontpellierRessourcesImagerie/imagej_macros_and_scripts/wiki/Wound-Healing-Tool), and expressed as the percentage of the total area scratched.

### Transwell migration assays

The Flp-InTRex293 cells ($5x10^4$) that carried a single copy of the wild type or mutant CCR6 expression construct, together with parental cells, were seeded in a Transwell® insert with a 8.0µm pore size (Corning), and cultured in the presence of 50nM CCL20 or 1µM HBD2 or vehicle for 24 hours. The cells that migrated to the bottom of the well were stained with crystal violet and quantified using a haemocytometer.

**Analysis of CCR6 internalization**

For time-lapse microscopy, the Flp-InTRex293 cells that carried a single copy of wild type or various mutant CCR6 construct with C-terminal GFP-tag were seeded in a glass bottom dish (MatTek Life Science) overnight. The cells were then treated with CCL20 (50nM) in FluoroBrite medium (Gibco) while videoed by time-lapse microscopy for 30 minutes (Figure S4). The CCR6 expression was quantified using Image J

(https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html).

For flow cytometry analysis, the above Flp-InTRex293 cells with a single copy of the wild type or mutant CCR6 expression construct with an N-terminal HA-tag were suspended in a fasting medium at a density of $3 \times 10^6$ cells/ml. The cells were treated with CCL20 (50nM), and an aliquot (100µl) was taken out at the indicated times and immediately incubated in a dry ice/ethanol bath to terminate the reaction. The cells were then fixed with 4% formaldehyde for 15 minutes, incubated with an anti-HA antibody (1/125 dilution) at 4°C overnight, and subsequently with a secondary antibody (Alexa Fluor 488 goat anti-mouse) for 30 minutes at room temperature. Finally, the cells were routinely analyzed using an Accuri C6 flow cytometer with FlowJo software.

The data were presented as proportion of CCR6 expression at the 0 time point following normalization against secondary antibody control.[1, 2]

**Dual luciferase reporter assay**

The firefly reporter plasmids for CRE (cAMP/PKA), SRF-RE (RhoA), SRE (MAPK/ERK), NFAT-RE (Calcium/Calcineurin), TCF/LEF-RE (Wnt), ISRE (JAK/STAT1/2), AP1 (MAPK/JNK), NFκB, CSL (NOTCH) and the Renilla pRL-TK control vector were from our previous studies.[1, 3, 4] Reporter assays were optimized before data collection (Figure S5).

DG75 cells ($5x10^6$) were co-transfected with 5µg of the pcDNA5/FRT expression construct containing either wild type or mutant CCR6, together with 2.5µg each of the above firefly reporter plasmids and 1.0µg renilla using Lonza transfection kit V (program M13), and cultured in RPMI medium for 24 hours. The cells were harvested and divided into two equal parts, with one part treated with 50nM CCL20 and the other treated with vehicle for 5 hours. The cells were collected and dual luciferase reporter assays were performed according to the manufacturer's protocol (Promega, Southampton). Each experiment was performed in three replicates.

Where indicated, reporter assays were similarly performed using the Flp-InTRex293 cells that carried a single copy of wild type or mutant CCR6 construct under CCL20, HBD2 and HD5 stimulation.

**Prediction of coupling probabilities of CCR6**

Coupling probability of G protein α to CCR6 wild type and its various mutants was estimated by using an online machine-learning program PRECOG (predicting coupling probabilities of G-protein coupled receptors) (http://precog.russelllab.org).[5] A value above 0.5 suggests a high probability of G-protein binding (Figure S6).

**Immunohistochemistry**

The expression of CCR6, CCL20, HBD2, TFF2 (trefoil factor 2) and HD5 in MALT lymphoma, lymphoepithelial sialadenitis and normal salivary glands, where indicated, was investigated by immunohistochemistry on formalin-fixed paraffin-embedded tissue sections. These were carried out using the Bond-III system (Leica Biosystems) with the Bond Polymer Refine Detection Kit. The staining conditions including antibody dilution and antigen retrieval

were systematically optimized before data collection and the optimized conditions were detailed in Table S1. Local ethical guidelines were followed for the use of archival tissues for research with ethical approval (05-Q1604-10).

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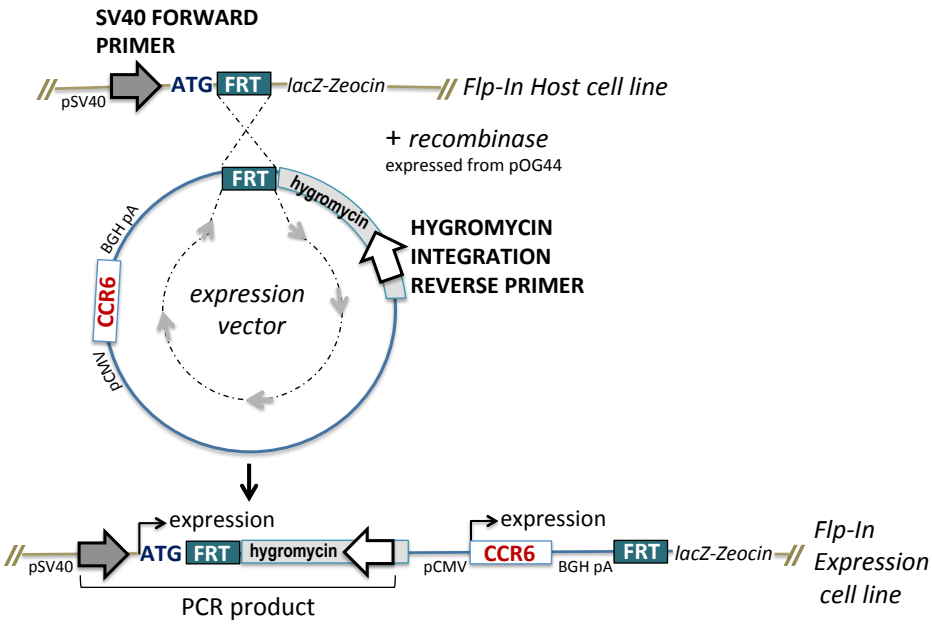
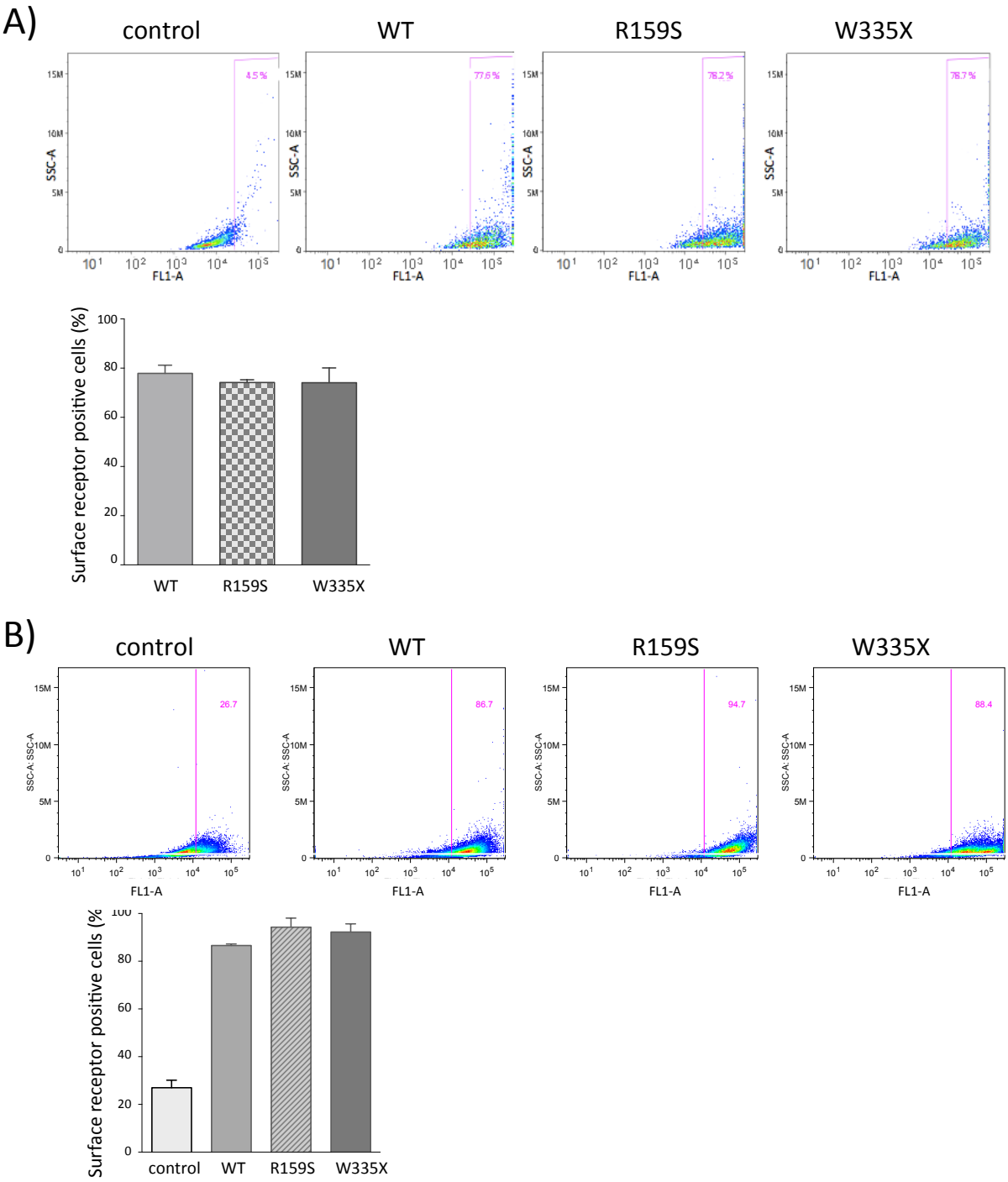


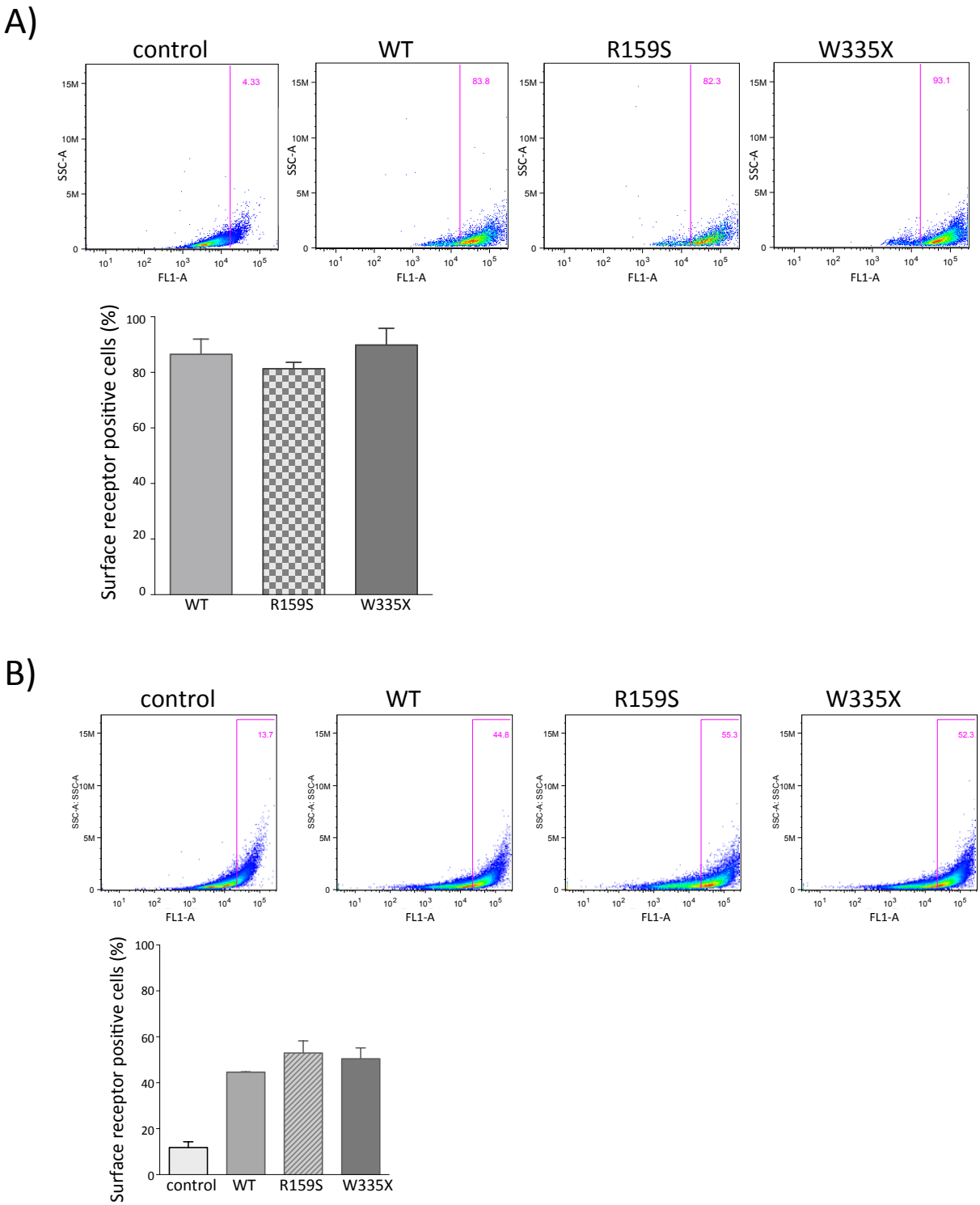

**Supplementary Figure S1**. Schematic illustration of generation of CCR6 pcDNA5/FRT expression vector through recombination in Flp-InTRex293 host cells (for details, please refer to Thermo Fisher online manual). Positive colonies are screened by PCR using primers targeting SV40 and hygromycin sequences as indicated.




**Supplementary Figure S2.** Confirmation of CCR6 expression in Flp-InTRex293 stably expressed cell line.

A) Examples of flow cytometry analysis for each of the investigated cell lines using anti-HA antibody. Results from 3 independent experiments (mean + standard deviation) are summarized in the lower panel.

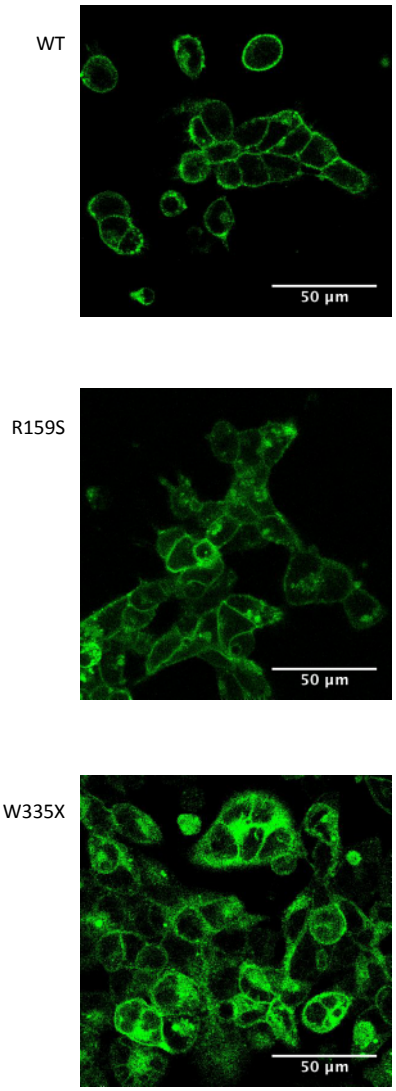
B) Examples of flow cytometry analysis for each of the investigated cell lines using anti-CCR6 antibody. Results from 3 independent experiments (mean + standard deviation) are summarized in the lower panel.




**Supplementary Figure S3.** Confirmation of CCR6 expression in DG75 transiently expressed cells.

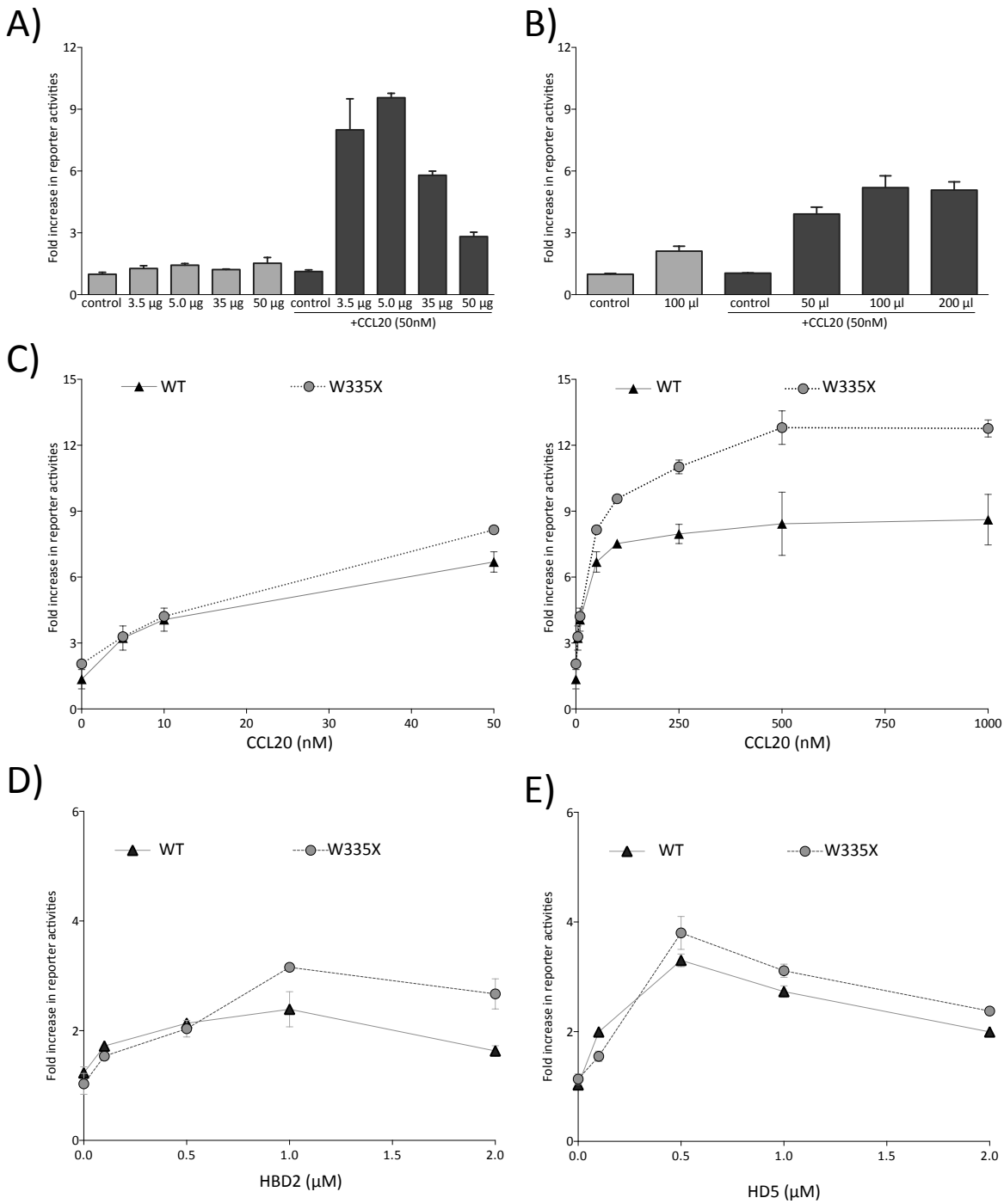
A) Examples of flow cytometry analysis for each of the investigated cell lines using anti-HA antibody. Results are from 3 independent experiments (mean + standard deviation) are summarized in the lower panel.

B) Examples of flow cytometry analysis for each of the investigated cell lines using anti-CCR6 antibody. Results are from 3 independent experiments (mean + standard deviation) are summarized in the lower panel.




**Supplementary Figure S4.** Analysis of sub-cellular localization of CCR6 and its representative mutants by time-lapse microscopy (n=3). Isogenic Flp-InTRex293 cell lines that stably express a single copy of CCR6-GFP or its mutants were treated with CLL20 (50nM), and CCR6 expression was monitored over a 30 minute period by time-lapse microscopy.

Time-lapse microscopy movies of representative CCR6: WT, R159S, W335X are uploaded as separate files.




**Supplementary Figure S5**. Optimization of dual luciferase reporter assays.

A) Testing the optimal amount of CCR6 expression plasmid for MAPK/ERK luciferase reporter assays. DG75 lymphoma cells were transiently transfected with CCR6 wild type pcDNA5/FRT expression or control vector as indicated using Lonza transfection kit V (program M13).

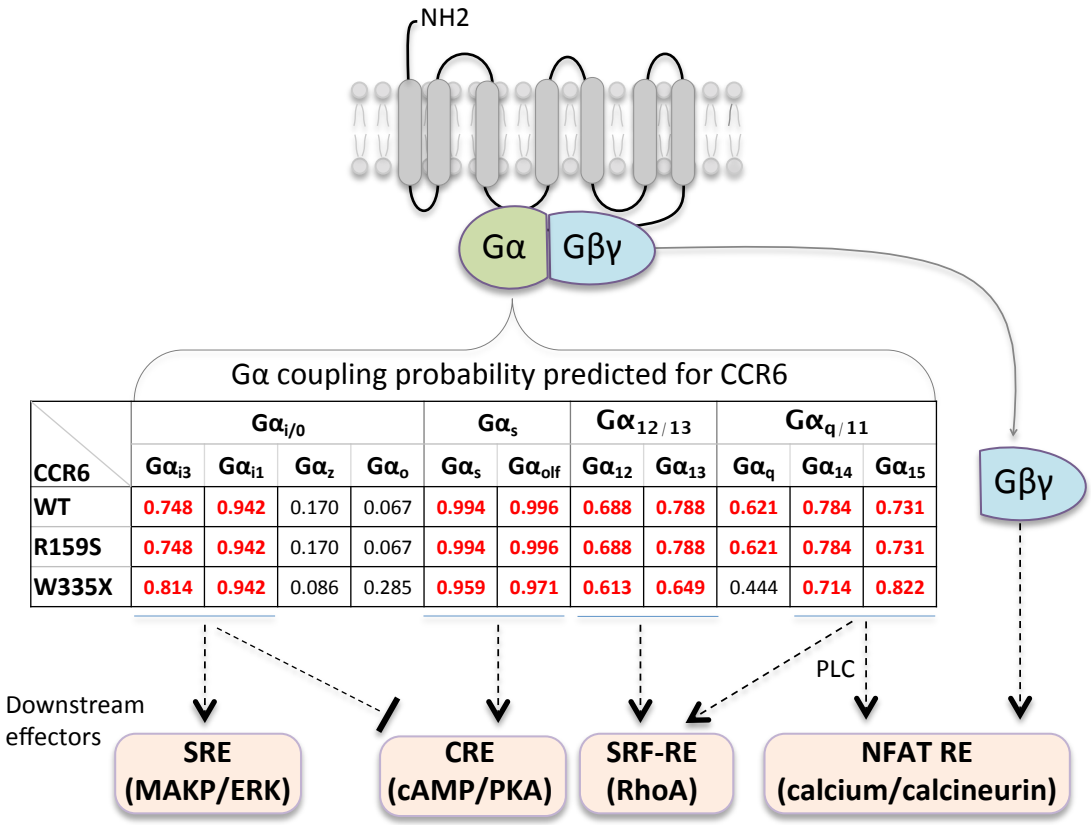
B) Testing the optimal volumes of reactions for CCL20 stimulation using the MAPK/ERK luciferase reporter assay in DG75 cells.

C) Testing the optimal dose of CCL20 for MAPK/ERK reporter assays with both CCR6 wild type and W335X truncation mutant in DG75 cells.

D) Testing the optimal dose of HBD2 for MAPK/ERK reporter assays with both CCR6 wild type and W335X truncation mutant in Flp-InTRex293 cells.

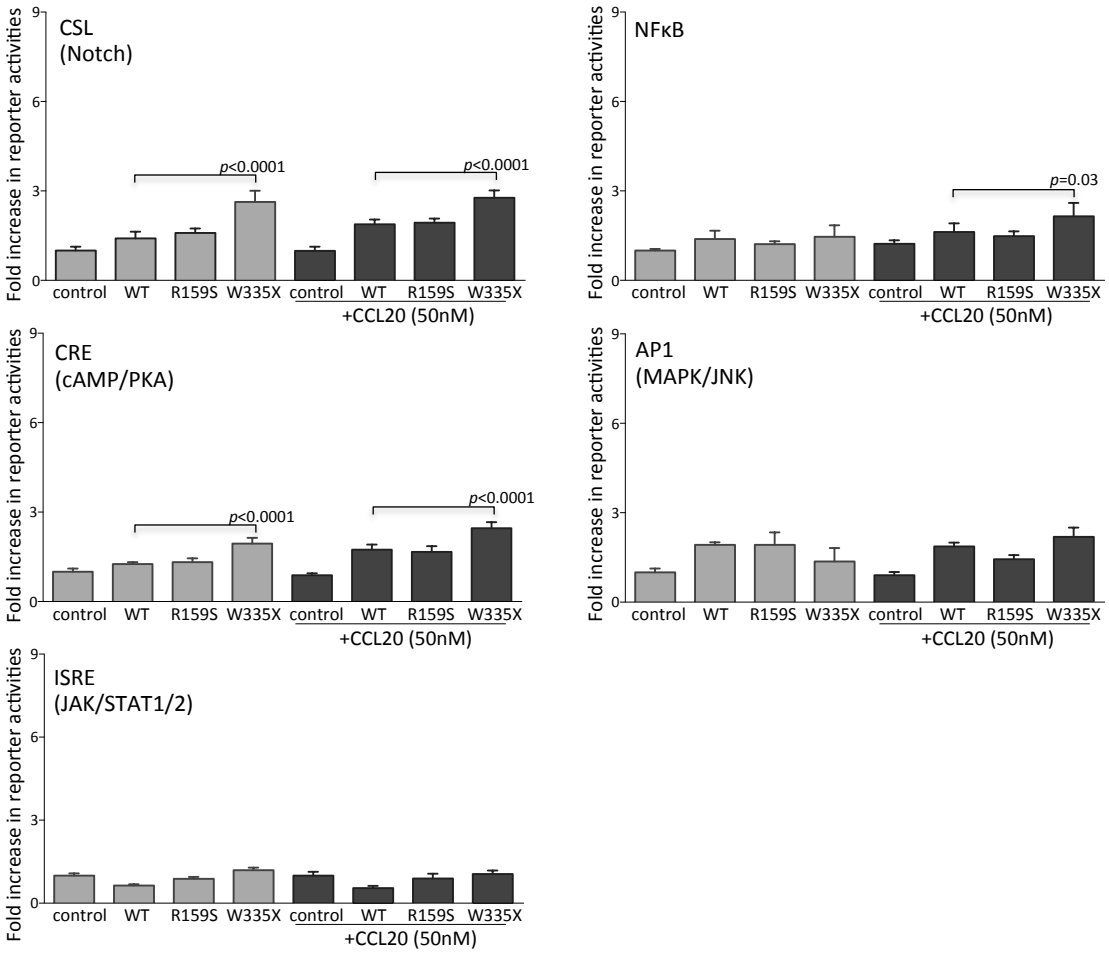
E) Testing the optimal dose of HD5 for MAPK/ERK reporter assays with both CCR6 wild type and W335X truncation mutant in Flp-InTRex293 cells. .

Each experiment was carried out in triplicate, and data are normalized to control without CCL20 stimulation and presented as a mean + standard deviation.

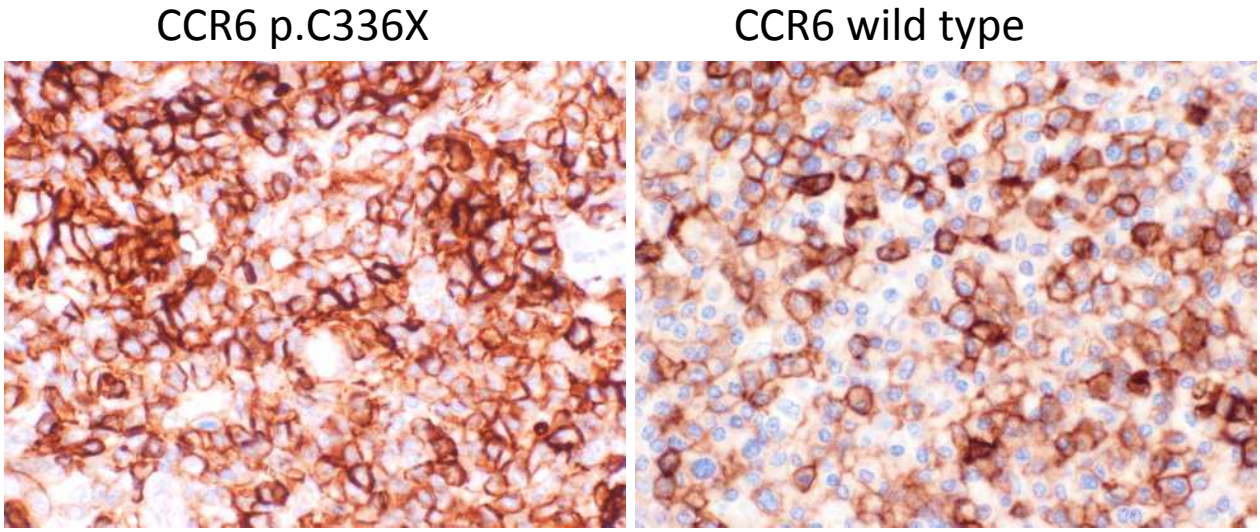



| | $G\alpha_{i/0}$ | | | | $G\alpha_{s}$ | | $G\alpha_{12/13}$ | | $G\alpha_{q/11}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CCR6 | $G\alpha_{i3}$ | $G\alpha_{i1}$ | $G\alpha_{z}$ | $G\alpha_{o}$ | $G\alpha_{s}$ | $G\alpha_{olf}$ | $G\alpha_{12}$ | $G\alpha_{13}$ | $G\alpha_{q}$ | $G\alpha_{14}$ | $G\alpha_{15}$ |
| WT | 0.748 | 0.942 | 0.170 | 0.067 | 0.994 | 0.996 | 0.688 | 0.788 | 0.621 | 0.784 | 0.731 |
| R159S | 0.748 | 0.942 | 0.170 | 0.067 | 0.994 | 0.996 | 0.688 | 0.788 | 0.621 | 0.784 | 0.731 |
| W335X | 0.814 | 0.942 | 0.086 | 0.285 | 0.959 | 0.971 | 0.613 | 0.649 | 0.444 | 0.714 | 0.822 |

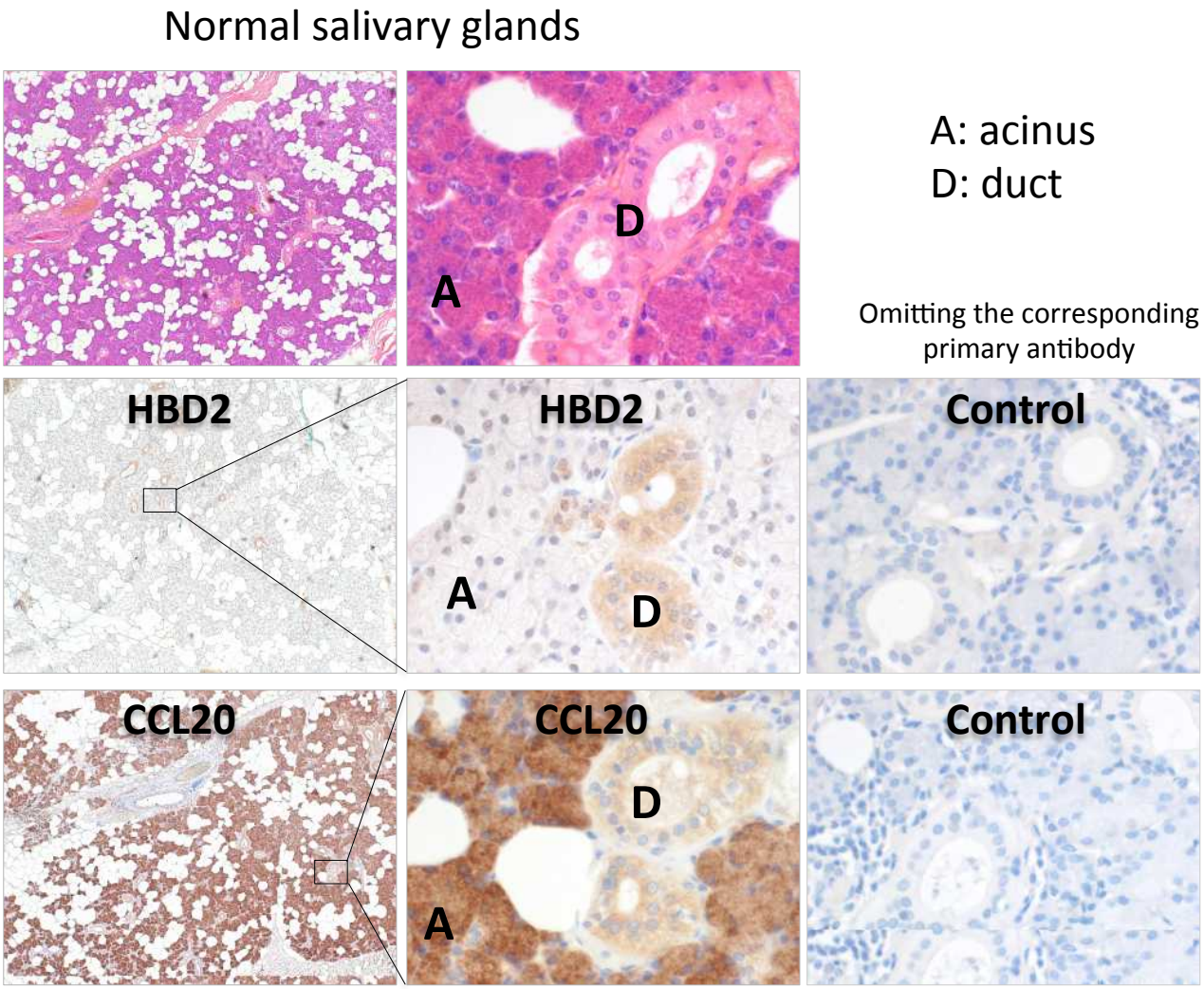
**Supplementary Figure S6**. Prediction of Gα coupling probabilities of CCR6 and its variants by the machine-learning program PRECOG (http://precog.russelllab.org). [5] A high probability of G-protein binding property with a value greater than 0.5 is depicted in red. The suggested effectors/signaling pathway activations downstream of Gα and Gβγ are based on previous literature. [6-10]



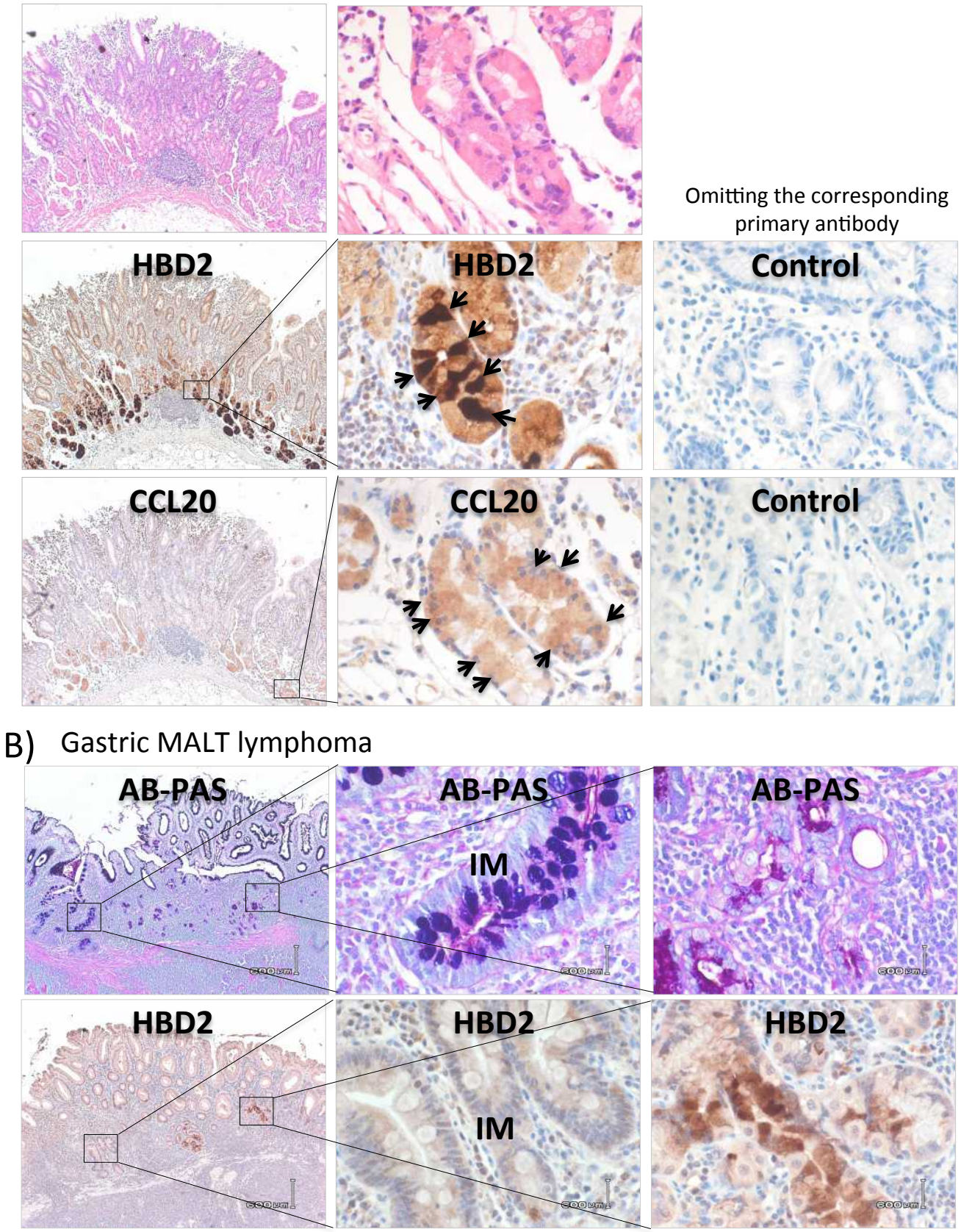

**Supplementary Figure S7.** Comparison of CCR6 and its representative mutants in activation of various cellular signaling using reporter assays. This was carried out in DG75 B-cell lymphoma cell line transiently co-transfected with CCR6 expression constructs and reporter plasmids. Data (mean + standard deviation) are from at least 2 independent experiments performed in triplicate. Comparisons between two groups were assessed using one-way ANOVA with significant differences indicated.



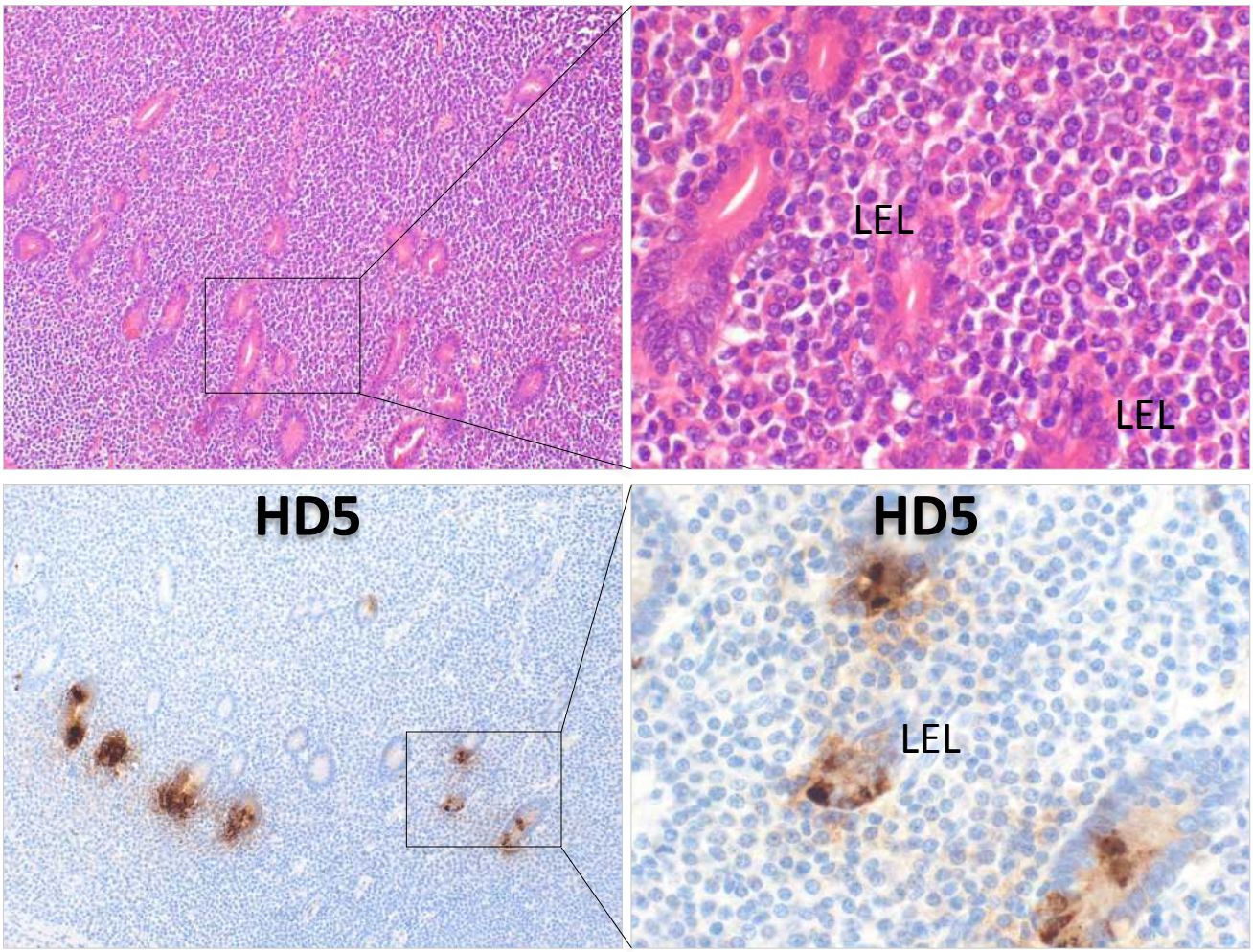

**Supplementary Figure S8**. An example of CCR6 expression cases of thyroid MALT lymphoma with or without CCR mutation.



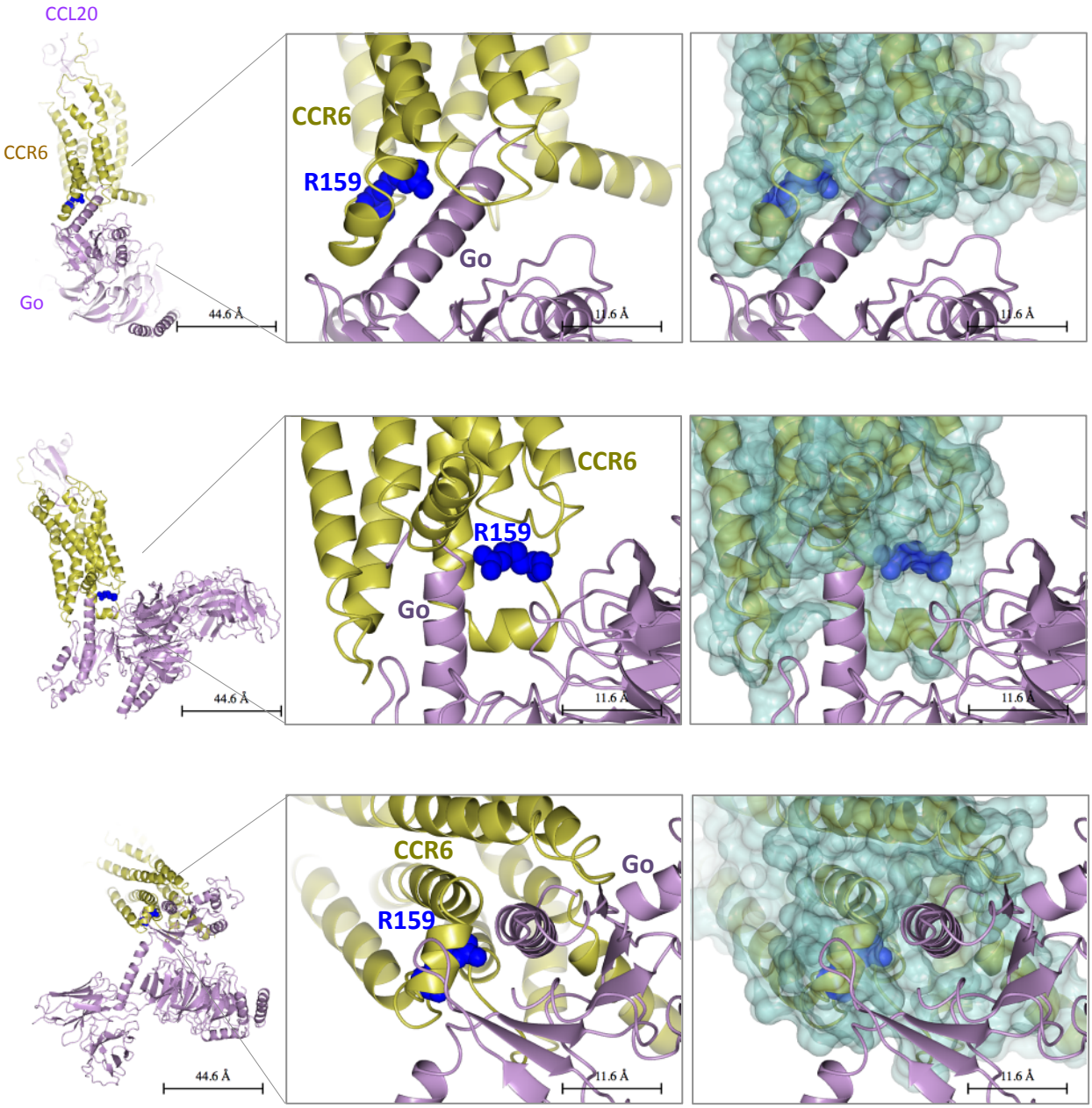

**Supplementary Figure S9**. HBD2 and CCL20 expression in normal salivary glands. HBD2 is expressed moderately in the ductal (D), but not in acinar (A) epithelial cells, while CCL20 is expressed moderately in the ductal, and strongly in acinar epithelial cells.




**Supplementary Figure S10**. **A)** HBD2 and CCL20 expression pattern in chronic gastritis. Hematoxylin and eosin staining shows inflamed gastric mucosa. HBD2 is variably expressed in gastric epithelial cells, with strong expression in a subset of epithelial cells, while CCL20 is weakly to moderately expressed in gastric epithelial cells. Arrows indicate the cells with higher HBD2 or CCL20 expression. **B)** HBD2 expression in gastric MALT lymphoma. AB-PAS (Alcian blue/Periodic Acid–Schiff) staining highlights the intestinal metaplasia (IM). HBD2 staining displays strong positivity in a subset cell population of basal glands consistent with pyloric metaplasia as indicated by high TFF2 (trefoil factor 2) expression (not shown), but negative in the intestinal metaplasia (IM).

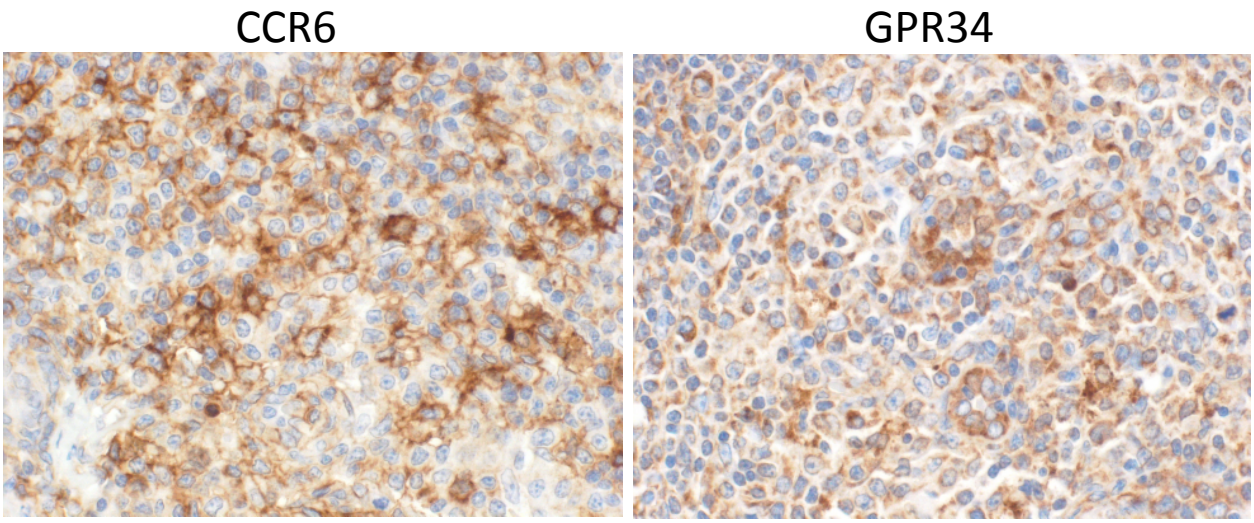



**Supplementary Figure S11**. An example of intestinal MALT lymphoma showing extensive lymphoepithelial lesions (LELs). Immunohistochemistry show strong HD5 staining in the Paneth cells of residual glands including those involved in LELs.




**Supplementary Figure S12**. R159 residue on a cryo-electron microscopy structure of the human CCR6 in complex with CCL20 and a Go protein (PDB: 6WWZ). [11]

R159 residue (blue spheres), CCR6 structure (gold) without/with a contour of an electrostatic surface (semi-transparent light green), Go protein and CCL20 (both in lilac).




**Supplementary Figure S13**. An example of CCR6 and GPR34 expression in a case of salivary gland MALT lymphoma. Both CCR6 and GPR34 are potentially expressed in the same lymphoma cells and may cooperate in their oncogenic activities in the pathogenesis of MALT lymphoma. The GPR34 immunohistochemistry was performed as previously. [1]

**Supplementary Table S1.** Antibodies and conditions used for immunohistochemistry.

| Antibodies | Source | Specification | Immunohistochemistry condition* |
|---|---|---|---|
| CCR6 | Abcam | Rabbit polyclonal (ab227036) | 1/1000 dilution of primary antibody<br>Bond Epitope Retrieval 2 solution for 20 minutes |
| CCL20 | BIORAD | Rabbit polyclonal (AHP788) | 1/400 dilution of primary antibody<br>Bond Epitope Retrieval 1 solution for 20 minutes |
| Beta defensin 2 | Abcam | Rabbit polyclonal (ab63982) | 1/300 dilution of primary antibody<br>Bond Epitope Retrieval 1 solution for 40 minutes |
| Trefoil factor 2 | Abcam | Rabbit monoclonal (ab267474) | 1/10000 dilution of primary antibody<br>Bond Epitope Retrieval 1 solution for 20 minutes |
| Alpha human defensin 5 | Sigma-Aldrich | Mouse monoclonal (8C8) | 1/400 dilution of primary antibody<br>Bond Epitope Retrieval 1 solution for 20 minutes |

*All immunohistochemistry was carried out using the Bond-III system (Leica Biosystems) with the Bond Polymer Refine Detection Kit.

## Legend for the videos

Analysis of sub-cellular localization of CCR6 and its representative mutants by time-lapse microscopy (n=3). Isogenic Flp-InTRex293 cell lines that stably express a single copy of CCR6-GFP or its mutants were treated with CLL20 (50nM), and CCR6 expression was monitored over a 30 minute period by time-lapse microscopy. Representative time-lapse microscopy movies for CCR6 wild type (WT) and mutants R159S and W335X are shown in separate files.