Supporting Information

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SI Materials and Methods

Cell Lines and Transfection. Human embryonic kidney 293 cells containing complete EBV/B95-8 bacmids (2089 cells) (1) or EBV bacmids with insertional inactivation of the EBV *BZLF1* gene (BZKO) (2) were grown in DMEM with 10% (vol/vol) FBS, antibiotics, and hygromycin (100 μg/mL). The HH514-16 subclone Burkitt lymphoma cells (3, 4) and HKB5/B5, a somatic cell hybrid between 293 and HH514-16 cells (5, 6), were grown in RPMI medium 1640 with 8% FBS and antibiotics; 293 cells and HKB5/B5 cells were transfected with the DMRIEC reagent (Invitrogen). HH514-16 (Cl16) cells were transfected by nucleofection. DNA (5 μg) was introduced per 2×10^6 cells resuspended in Nucleofector solution. Transfection was accomplished with program A-023 of Nucleofector II (Lonza). RPMI medium 1640 (5 mL) with 8% FBS was added immediately after transfection. Cells were harvested for analysis 48 h after transfection.

Expression Vectors. Expression vectors for the wild-type (wt) EBV *BZLF1* gene or the Z(S186A) mutant containing EBV genomic DNA driven by the CMV immediate-early (IE) promoter in pHD1013 have been described (7, 8). To construct a c-Jun expression vector full length (993 bp), c-Jun cDNA was amplified by RT-PCR from total HeLa cell RNA. The forward primer had a synthetic EcoRI site at the 5′ end, and the reverse primer had a synthetic XbaI site at the 3′ end. The cDNA was cloned into pCMV/Flag2 (Sigma-Aldrich) using these restriction enzyme sites. The cDNA of c-Fos (1,143 bp) in the pCMV6-XL5 vector was purchased from Origene (SC116873). The cDNA was transferred to the pCMV/Flag2 vector using a PCR fragment with a 5′ forward primer with an EcoRI site and a 3′ reverse primer with a BamHI site. Mutants were made using the QuickChange mutagenesis strategy (Qiagen). In c-Jun(A266S), a codon for alanine (GCC) was changed to serine (AGC); in c-Fos(A151S), a codon for alanine (GCA) was changed to serine (AGT). The wt human c-Jun and c-Fos and the mutants were confirmed by DNA sequencing. The amino acid numbering was based on UniProtKB database (www.uniprot.org): c-Fos accession no. P01100; and c-Jun accession no. P05412.2. Expression of ZEBReplication Activator (ZEBRA) and the Activating Protein-1 (AP-1) proteins was determined by immunoblotting with antibodies to ZEBRA or FLAG (Fig. S1).

Northern Blotting. Preparation of total cellular RNA, radioactive probes, gel conditions, and transfer have been described (9, 10). The probes were double-stranded DNA prepared by PCR or, in one instance, a HincII/SacI restriction fragment of a plasmid containing the EBV BamHI H fragment. The probes were purified by agarose gel electrophoresis. The positions of the probes on the sequence of the complete EBV genome (GenBank accession no. NC_007605) are available upon request. Northern blots were also probed with the H1 component of RNase P to control for RNA loading (11).

Immunoblotting. The methods for preparation of cell extracts, electrophoresis, transfer, and detection of antigens with antibodies and ^{125}I protein A have been described (12). Primary antibodies included polyclonal rabbit anti-sera raised against EB viral proteins that were expressed in *Escherichia coli* and purified on nickel affinity (4, 8, 13) columns and mouse monoclonal antibodies to early antigen-diffuse (EA-D) (14) and FLAG. Secondary antibodies were purchased (Table S3).

Southern Blotting. The method for analysis of the abundance of EB viral DNA using a probe from the BamHI W region has been described (8).

Indirect Immunofluorescence. BZKO or 2089 cells that were transfected 43 h previously with plasmid DNA were fixed in chilled methanol for 30 min at −20C, washed with PBS, and incubated in 10% human serum in PBS for 1 h at room temperature. Cells were stained with primary antibodies to R transactivator (Rta), FLAG, or lamin B for 1 h in humidified chambers. Cells were washed with PBS and incubated with appropriate secondary antibodies conjugated to fluors (Table S3) for 1 h at room temperature in opaque humidified chambers. Cells were washed with PBS, briefly rinsed in distilled water, and mounted on glass slides with Vectashield media (Vector Laboratories). Digital images were obtained with a Zeiss LSM510 confocal laser-scanning microscope.

Sorting of Transfected Cells by Fluorescence-Activated Cell Sorting Using a Membrane-Targeted GFP. HH514-16 cells were transfected by nucleofection with 10 μg of wt ZEBRA or Jun(A266S) together with 2.5 μg of membrane-targeted EGFP-farnesylated (15) per 2×10^6 cells. GFP fluorescence in unfixed cells was measured using fluorescence-activated cell sorting (FACS) on a FACSVantage SE sorter (BD Biosciences). Cell fragments and debris were excluded based on forward and side scatter profiles. Unstained cells were used to set the GFP-negative and GFP-positive sorting gates. Intermediately positive cells were excluded from the sorts. Cell extracts from collected GFP-negative and GFP-positive cells from each sample were subjected to Northern blot analyses in the experiment presented in Fig. S4*C*.

Electrophoretic Mobility-Shift Assays. The methods for preparation of cell extracts and gels have been described (12, 16). The probes (Table S2) were double-stranded oligonucleotides-radiolabeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Cold competitor DNA at the desired concentration was diluted in TE [0.01 M Tris, 0.001 M EDTA (pH 7.5)]. The *BRLF1* promoter response element (ZRE)-2 and Rp/ZRE-3 probes were synthesized in an unmethylated or methylated state. The methylation state of these probes was confirmed by mass spectroscopy at the Yale Keck facility. EMSA binding reactions in 20 μL contained 10 μg of protein from BZKO or HKB5/B5 cells, 0.05 ng of radioactive probe, 500 ng of poly(dI-dC), and the desired concentration of cold-competitor DNA in buffer [10 mM Hepes (pH 7.5), 50 mM NaCl, 2 mM MgCl2, 2.5 μM ZnS04, 0.5 mM EDTA, 1 mM DTT, 15% glycerol]. The samples were held at room temperature for 15 min and electrophoresed in a 4% polyacrylamide gel in 0.5× tris-borate-EDTA (TBE) buffer. The gel was fixed in 10% methanol and 10% acetic acid for 20 min, vacuum dried at 80 °C for 2 h, and exposed to Kodak XAR5 film. The fraction of probe shifted was determined by densitometry of autoradiographs.

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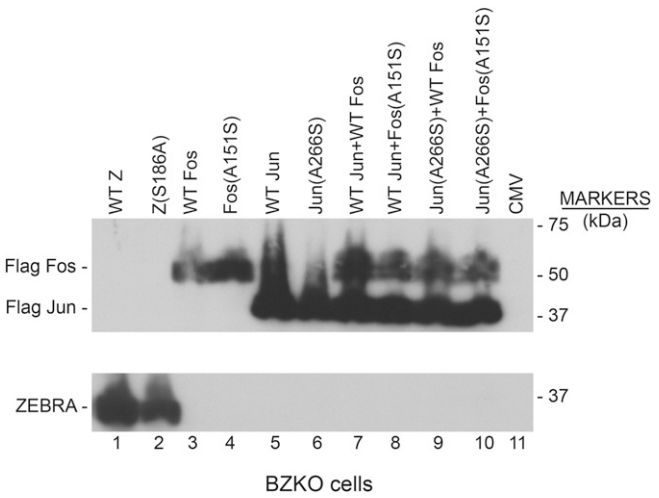

Fig. S1. Expression of wt ZEBRA, c-Fos, c-Jun, and corresponding mutant proteins Z(S186A), Fos(A151S), and Jun(A266S). BZKO cells were transfected with the indicated expression vectors. Cell extracts prepared in parallel from cells analyzed for mRNA shown in Fig. 2*A* and Fig. S3 were examined for expression of wt and mutant ZEBRA, c-Fos, and c-Jun proteins by immunoblotting with polyclonal rabbit antibody to ZEBRA or mouse monoclonal antibody to FLAG.

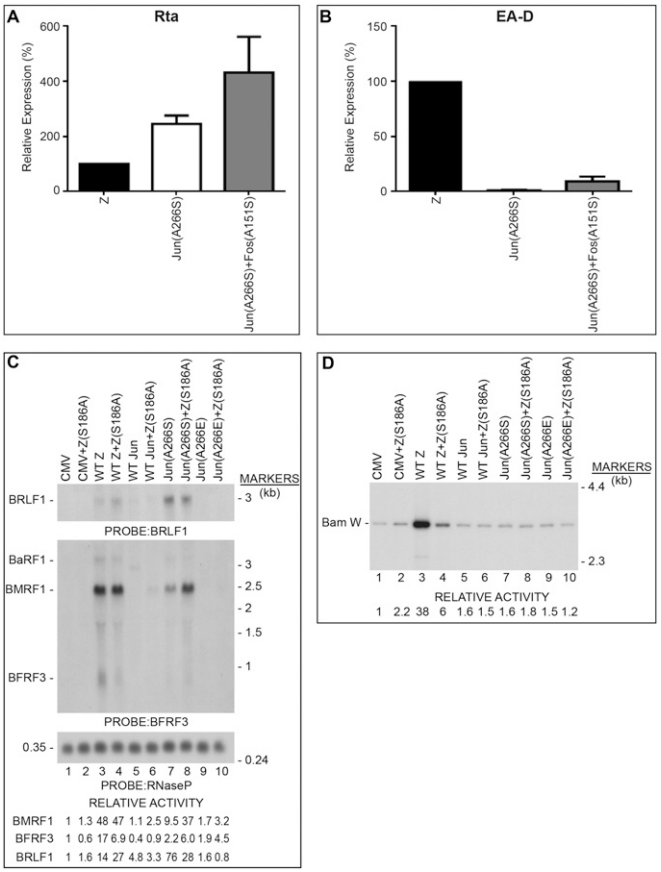

Fig. S2. AP-1A/S mutants activate EBV early lytic proteins in a gene-specific manner (supplement to Fig. 3). The relative expression of Rta (*A*) and EA-D (*B*) were compared following introduction of ZEBRA, Jun(A266S), and Jun(A266S) plus Fos(A151S) into BZKO cells. The data were derived from replicate experiments [$n = 6$ (*A*); $n = 5$ (*B*)]. The defect in the capacity of c-Jun(A266S) to activate expression of EBV early protein EA-D can be complemented by ZEBRA mutant Z(S186A). BZKO cells were transfected with plasmids encoding wt or mutant c-Jun(A266S) in the presence or absence of Z(S186A), a ZEBRA mutant that by itself is unable to activate the lytic cycle. The transfected cells were examined by Northern blotting for *BRLF1*, *BaRF1*, *BMRF1*, and *BFRF3* mRNAs (*C*); the abundance of EB viral DNA was measured by a Southern blot for the large internal repeat encoded in EBV BamHI W (*D*).

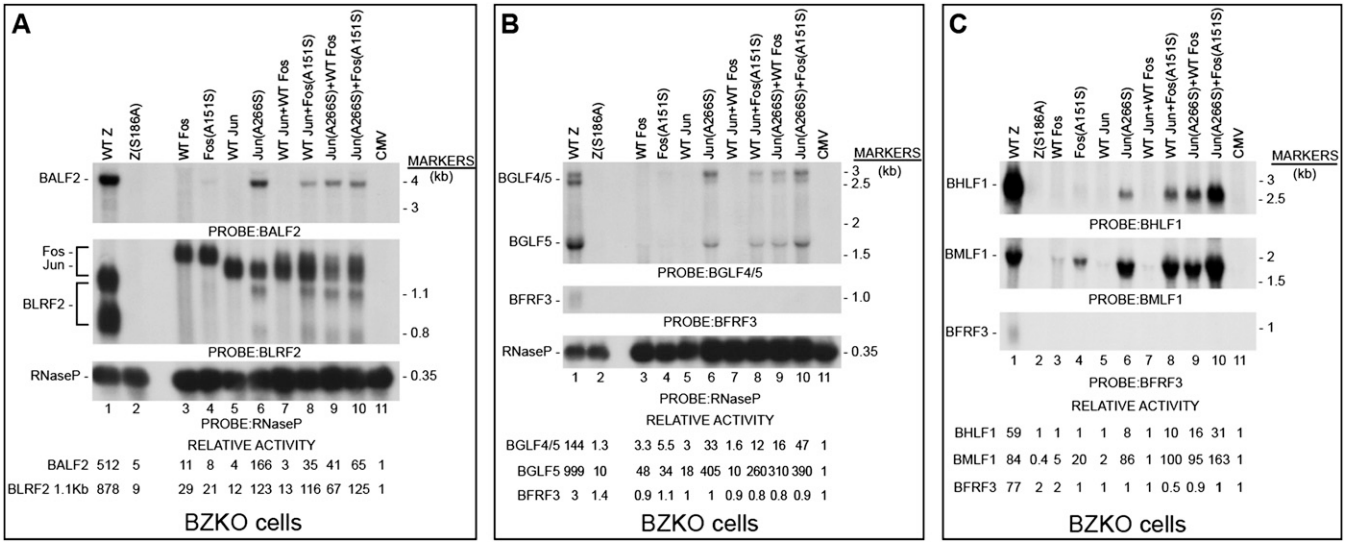

Fig. S3. Point mutants in the basic domain of c-Jun and c-Fos activate transcripts of early but not late EBV lytic genes in BZKO cells (supplement to Fig. 2*C*). Expression vectors for wt ZEBRA, c-Fos, and c-Jun and corresponding point mutants in the basic domain of these proteins Z(S186A), Fos(A151S), and Jun(A266S) were transfected into BZKO cells. The cells were assessed for expression of EBV *BALF2* (early) and *BLRF2* (early/late) and Fos/Jun mRNAs (*A*); *BGLF4* (early), *BGLF5* (early), and *BFRF3* (late) mRNAs (*B*); and *BHLF1* (early/late), *BMLF1* (early), and *BFRF3* (late) mRNAs (*C*).

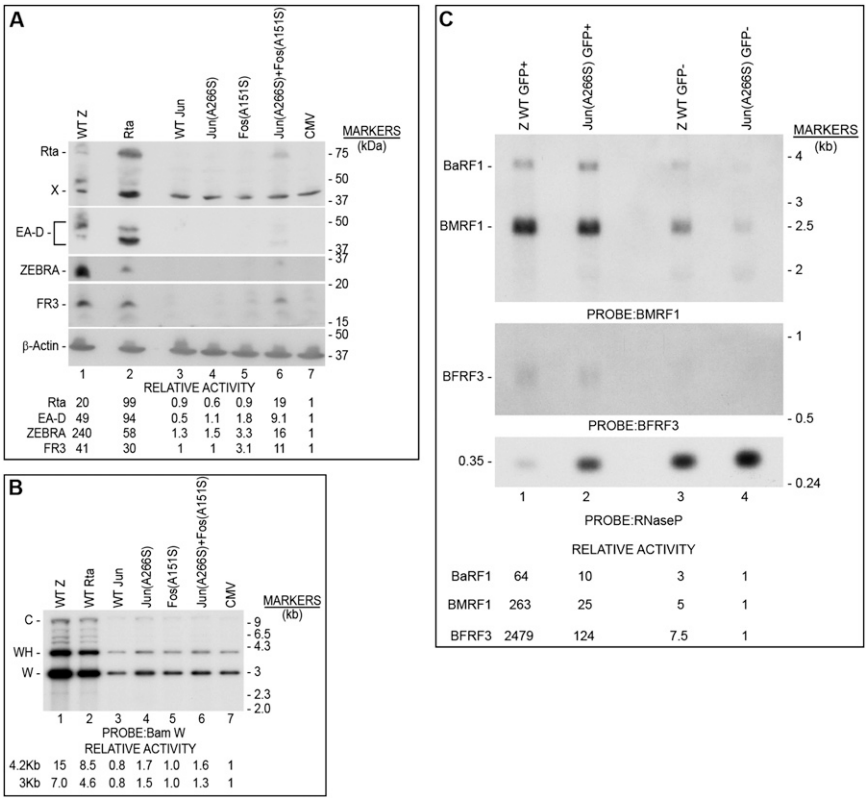

Fig. S4. Burkitt lymphoma cells transfected with c-Jun(A266S) express the mRNA and protein products of the EBV late gene *BFRF3* (supplement to Fig. 4). The abundance of Rta, EA-D, ZEBRA, and FR3 proteins (*A*) and the abundance of EBV DNA (*B*) was measured after nucleofection of HH514-16 cells with the indicated plasmids. (*C*) HH514-16 cells were nucleofected with plasmids expressing wt ZEBRA or c-Jun(A266S) together with mGFP. The cells were sorted into GFP-positive or GFP-negative populations and scored for expression of early (*BaRF1*/*BMRF1*) and late (*BFRF3*) mRNAs by Northern blotting. RNaseP controlled for loading of total RNA.

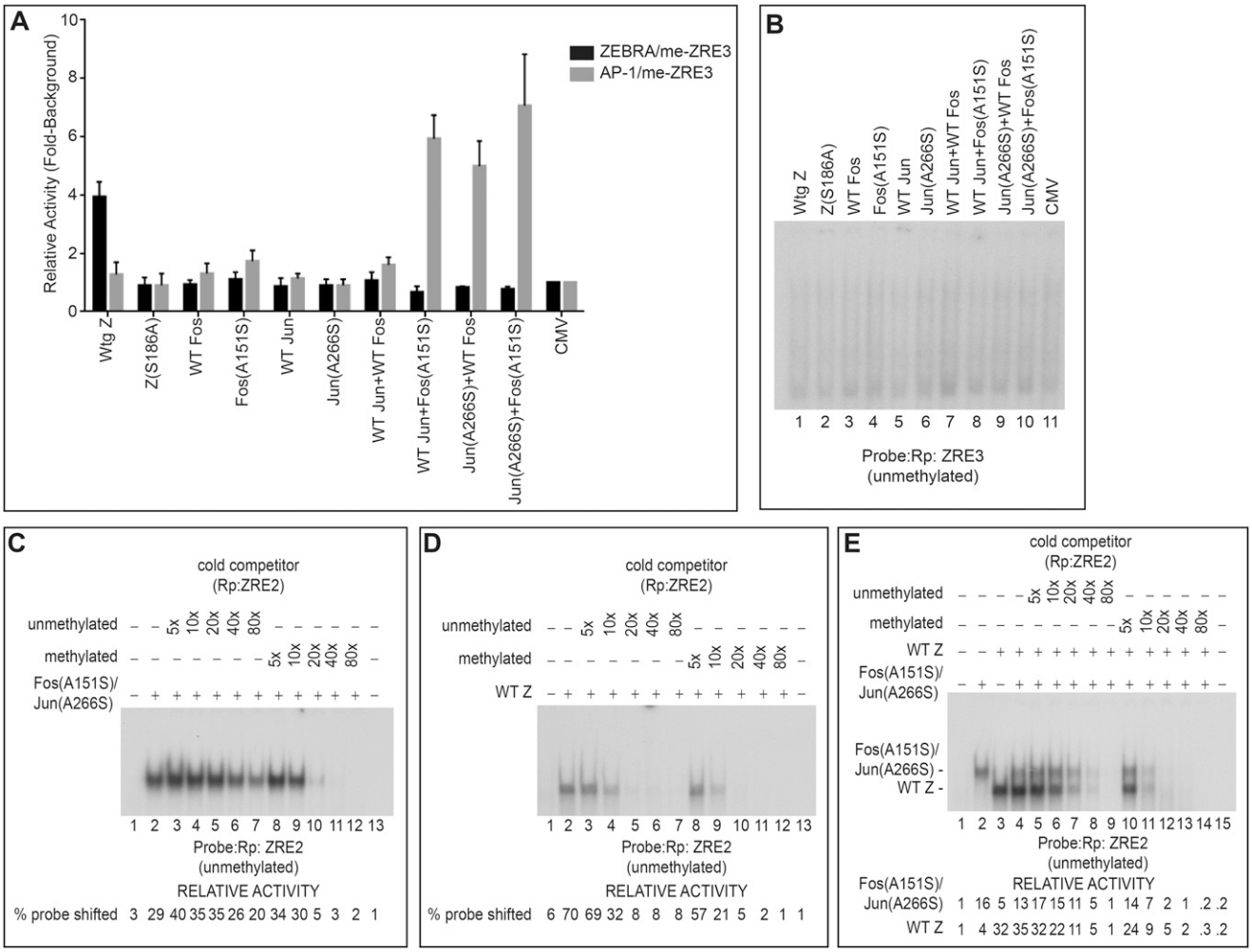

Fig. S5. AP-1(A/S) mutants are enhanced in binding to methylated ZREs from the promoter of the *BRLF1* gene (supplement to Fig. 5). (*A*) Data derived from three replicate EMSA experiments in which extracts expressing the indicated proteins were mixed with a radiolabeled oligonucleotide probe of methylated ZRE-3. The ZEBRA/meZRE-3 (black) and AP-1/meZRE-3 (gray) complexes were quantitated separately by densitometry. The results were expressed as percentage of probe shifted. The value from cell extracts expressing the indicated proteins was compared with the value from cell extracts transfected with empty CMV vector. (*B*) BZKO cells were transfected with the indicated expression vectors; extracts prepared for EMSA were incubated with a radiolabeled probe of unmethylated ZRE-3. The cell extracts were the same ones used in the EMSA experiments illustrated in Fig. 5 and *A* in this figure. Cell extracts for EMSA were prepared in HKB5/B5 cells transfected with expression plasmids for ZEBRA or the mutant AP-1 proteins. Control cell extracts were transfected with empty vector. The extracts were mixed radiolabeled probes containing unmethylated ZRE-2 in the absence or presence of either unmethylated or methylated ZRE-2 cold competitors that were added simultaneously with the radiolabeled probes. The binding proteins were the AP-1 mutants (*C*), ZEBRA (*D*), or a mixture of mutant AP-1 proteins and ZEBRA (*E*).

Table S1. Summary of activation of EBV lytic gene products by AP-1$^{(A/S)}$ mutants in 293 Cells containing EBV bacmids

Gene	Kinetic class	Function	Activated by AP-1 mutants
BRLF1	Very early	Transcription/replication	+/+
BZLF1	Very early	Transcription/replication	–/–
BaRF1	Early	Ribonucleotide reductase	+/ND*
BMRF1	Early	DNA polymerase processivity factor	+/+[†]
BGLF5	Early	Alkaline endonuclease/host shut off	+/+
BALF2	Early	Single-stranded DNA-binding protein	+/ND*
BMLF1	Early	mRNA processing and transport	+/+
BLRF2	Early/late	Tegument protein	+/ND*,[‡]
BHLF1	Early/late	noncoding RNA	+/ND*
BFRF3	Late	Small viral capsid protein	–/–

+, mRNA shown to be activated; +/+, both mRNA and protein shown to be activated; ND, not done.
*Detection of protein ND.
[†]*BMRF1* protein weakly activated (Fig. 3).
[‡]Activation of early but not late expression of *BLRF2*.

Table S2. Probes used for electrophoretic mobility-shift assays

Name	Sequence
Rp/ZRE-1	5′-TCTTTTATGAGCCATTGGCA-3′
Rp/ZRE-2	5′-AAGCTTATGAG**CG**ATTTTAT-3′
Rp/ZRE-3	5′-GTCAAAATT**CGCG**ATGCTAT-3′
*BMLF1*P/AP-1	5′-GAAGCACTGACTCATGAAG-3′

One strand is shown. The ZRE and AP-1 sites are underlined. CpGs are bold.

Table S3. Antibodies used in immunoblotting and immunofluorescence

Target protein	Type of antibody	Source
Rta (*BRLF1*)	Rabbit polyclonal	8
EA-D (*BMRF1*)	Mouse monoclonal	14
BGLF5	Rabbit polyclonal	This report
BFRF3	Rabbit polyclonal	13
ZEBRA (*BZLF1*)	Rabbit polyclonal	4
BMLF1	Rabbit polyclonal	This report
FLAG	Mouse monoclonal	Sigma-Aldrich (F1804)
Lamin B	Goat polyclonal	Santa Cruz (sc-6216)
Mouse IgG	Rabbit polyclonal	Invitrogen (81-6700)
Mouse IgG	Donkey polyclonal-FITC	Jackson (715-045-150)
Rabbit IgG	Donkey polyclonal-DyLight 549	Jackson (711-505-152)
Goat IgG	Donkey polyclonal-Cy5	Jackson (705-175-147)