Supplemental Information

Supplemental Materials and Methods

Western Blot Analysis

Expression levels of FL, Core, and T490 stably expressed in 6312 RAG2$^{-/-}$ pro-B cells were assessed by Western blot analysis. The clones were cultured in the absence and presence of 5 μM STI-571 for 16 hrs, which arrested the cells in G1 and induced expression of endogenous RAG1. Treatment with STI-571 was limited to 16 hrs, since longer incubation times resulted in STI-571-induced apoptosis. Rabbit polyclonal anti-RAG1 (EPRAGR1, abcam), rabbit monoclonal anti-RAG2 (EPRAGR239, abcam), and biotinylated anti-rabbit secondary antibodies were used for RAG1 and RAG2 blotting assays. ECL Prime (Sigma-Aldrich) was used for chemiluminescence detection.

Recombination activity

To assess V(D)J recombination activity at the endogenous Igk locus, stable clones of FL and T490A RAG2, as well as the control GFP, expressed in the RAG2$^{-/-}$ R2K2 cells (generously provided by Barry Sleckman) were generated. The R2K2 cells contain the Eμ-Bcl2 transgene, which renders the cells resistant to STI-571-induced apoptosis. This property allows extended growth in STI-571 such that V(D)J recombination activity can be detected. The R2K2 FL-RAG2, T490A, or GFP-only stable clones were cultured in media containing RPMI with glutamine, 10% FBS, 1% non-essential amino acids, 1% antimycotic antibiotic, and 0.1% beta-mercaptoethanol with 5

μM STI-571. 5 million cells were harvested 0 and 48 hours after STI-571 treatment and washed twice with PBS, resuspended in 500 μL digestion buffer (10 mM Tris pH 7.5, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, 0.1 mg/mL proteinase K), incubated at 37° overnight, followed by 2 hr incubation at 50°C with periodic mixing. DNA was precipitated with ethanol, and the DNA was subsequently resuspended in 100 μL TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5). Igκ recombination products were PCR amplified using the primer pair VκD (CCGAATTCGSTTCAGTGGCAGTGGRTCWGGRAC) and Jκ2-3' (CACAAAACCCTCCCTAGGTAGACAATTA) (1). This primer pair will amplify the recombined V-Jκ1 and V-Jκ2 to yield ~800 bp and ~400 bp PCR products, respectively (1). A diagnostic restriction digest was used to validate the PCR product identity by adding 20 units of *BamH*I to 15 μL of the PCR product, followed by 1 hr incubation at 37°C. *BamH*I should yield an ~100 bp cleavage product by cleaving near the V-J junction, depending on coding end processing. PCR products were separated on an 8% polyacrylamide gel in TB buffer (40 mM tris-borate, pH 8.3), the gel stained with SYBR safe DNA gel stain, and visualized using a BioRad Chemidoc MP gel imaging system. The identity of the PCR product was validated by digesting the PCR products.

Mass Spectrometry analysis

FL-expressing clones (in 6312 cells) were either untreated, or exposed to 4 Gy irradiation. The samples were subsequently incubated at 37°C for 30 minutes, the cells lysed in buffer containing 10 mM Tris/Cl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, and then incubated overnight at 4°C with GFP-Trap magnetic beads

(Chromotek, Munich, Germany). Samples were eluted with SDS-PAGE sample buffer, run on 10% SDS-PAGE, and stained with Coomassie blue dye. Following destaining overnight, each lane was excised, subjected to in-gel tryptic digestion, and analyzed using a Maldi Synapt mass spectrometer (Waters, Milford, MA) at the OUHSC Mass Spec/Proteomics core facility.

Localization of FL versus a nuclear localization mutant of RAG2:

Localization of FL versus a nuclear localization signal (NLS) mutant of RAG2 was evaluated in HEK293T cells, HEK293T cells were seeded on 35mm plates with a glass bottom. Cells were transiently transfected using Fugene, OptiMEM, and plasmids encoding GFP-tagged RAG2 (FL or NLS mutant). In the NLS mutant, residues K518, K519, R523, and R524 were mutated to alanine residues. Following 48 hrs, cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes, and the cells stained with DAPI and subsequently imaged with a Nikon Eclipse Ti fluorescence microscopy at 100x.

Supplemental Figures:

Supl Fig. 1: A) Schematic of FL and T490A GFP-RAG2. The GFP fusion is at the N-terminus of the FL and T490A RAG2 proteins. RAG2 is shown as a black bar with the core (residues 1-352) and PHD (residues 414-485) domains of the 527 full length murine protein shown as boxes. The relative position of the T490 residue is shown in the bar for the T490A mutant. **B)** Expression of FL and T490A in stable clones. The

GFP only clone is shown as a negative control, bands specific to the expression of FL and T490A GFP-RAG2 are indicated. **C)** Expression of RAG1 and FL in stable clones in the absence or presence of STI-571. Endogenous RAG1 is evident in the absence of STI-571, although it is significantly enriched in cells treated with STI-571. FL is also enriched following STI-571 treatment likely due to arrest of cells in G1. **D)** Expression of endogenous RAG2 in A70 pre-B cells or stably expressed FL, in the absence or presence of STI-571.

Supl Fig. 2: V(D)J recombination of the Igκ locus in FL- and T490A-expressing pre-B cells. **A)** GFP control, FL, and T490A expressing cells were treated with STI-571 for 0 and 48 hrs. Genomic DNA was isolated, and V(D)J recombination at the Igκ locus assessed by PCR as described in Supplementary Materials and Methods. Recombination at the Igκ locus is evident by the production of 400 and 800 bp PCR products, as labeled to the right of the gel. Representative of n=3 experiments. The increased level of the recombination products in the T490A versus FL samples is likely due to increased stability and nuclear localization of the T490A protein relative to FL GFP-RAG2. **B)** The identity of the Igκ recombination proteins were confirmed by restriction digest of the PCR products with *BamH*I. Incubation of the amplified recombination products in the FL and T490, but not GFP control, samples with the *BamH*I enzyme yielded the expected ~100 bp product following, as indicated to the right of the gel. **C)** Igκ V(D)J recombination assay in the absence of STI-571. Overexposure of the gel shows the 400 and 800 bp PCR products for T490A, but not for FL, indicating detectable V(D)J recombination activity for the T490A clone in cycling pre-B cells.

Supl. Fig. 3: Flow chart of RAG2 pull down/mass spectrometry experimental procedure (top panel). Table of centrosomal proteins preferentially pulled down by FL RAG2 in irradiated vs nonirradiated cells (bottom panel). The centrosomal proteins shown localize to distinct regions of the centrosome during interphase and several function in centrosome assembly, duplication, and elongation (2-5).

Supl. Fig. 4: A) Expression of FL and Core fused to RAG2 in stable clones. **B)** Averaged ratios of Annexin V positive to negative cells using flow cytometry. Results show measurements of GFP fluorescence in cells that expressed FL or Core, before and 24 hrs following IR. Plots are representative of 3 separate trials (Annexin, Annexin V) for each sample. Error bars represent SD, n=3. *, $p<0.05$ by student's *t* test. **C)** Detection of histone 3 (H3) phosphorylation at Ser10 and Ser28. At 24 hr post-IR, cells were immunostained with antibody to phospho-H3, and then measured by flow cytometry. Error bars represent SD (n=3). Methods for panels B and C were done as described in the main text.

Supl. Fig. 5: Localization of GFP-RAG2 FL **(A)** and NLS mutant **(B)** in HEK 293T cells that were transiently transfected with the RAG2 expression vectors, and visualized after 48 hrs. Two representative cells are shown for each sample. Images are DAPI stained (left), GFP fluorescence (middle), and merged images (right).

References

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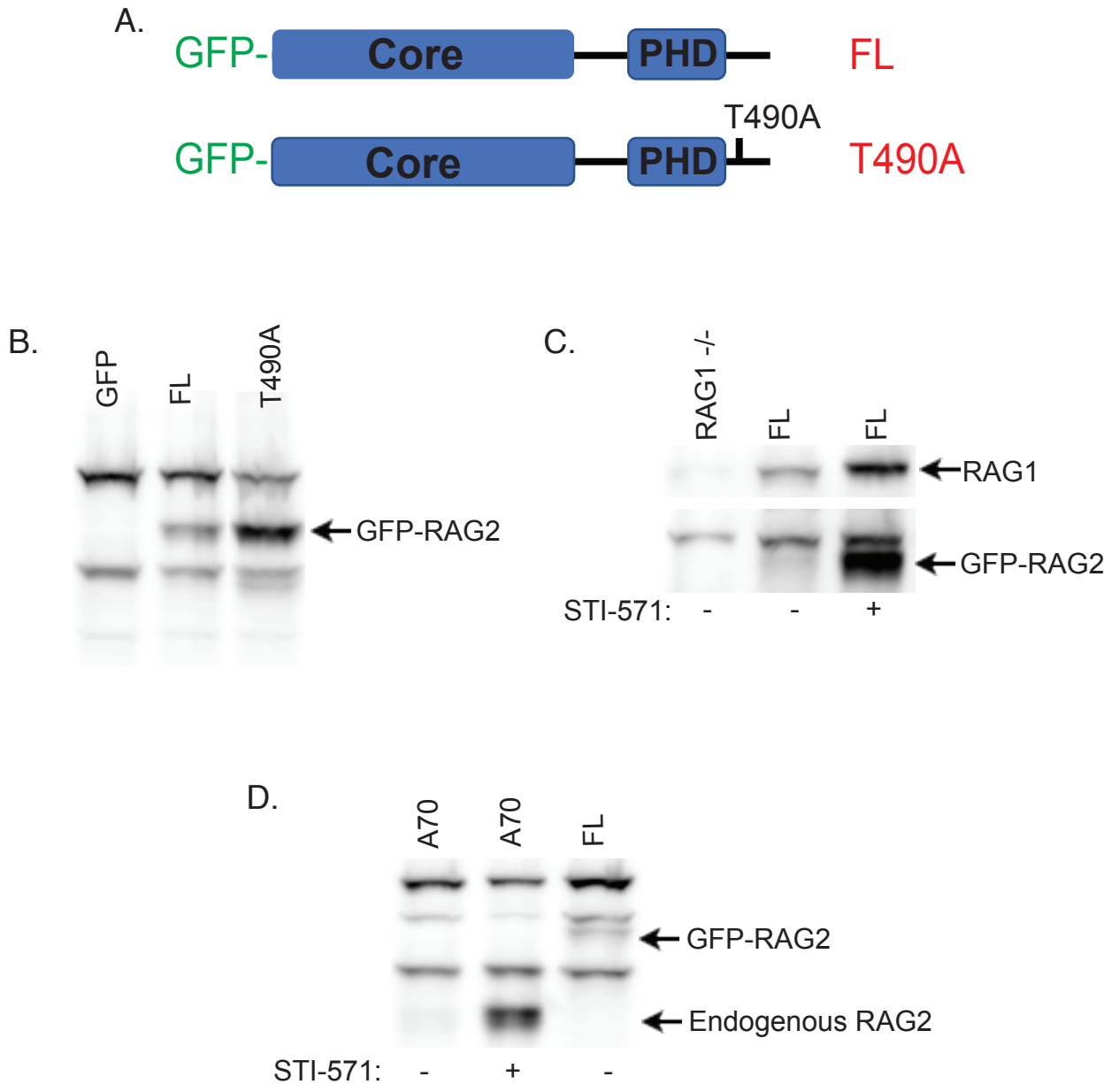
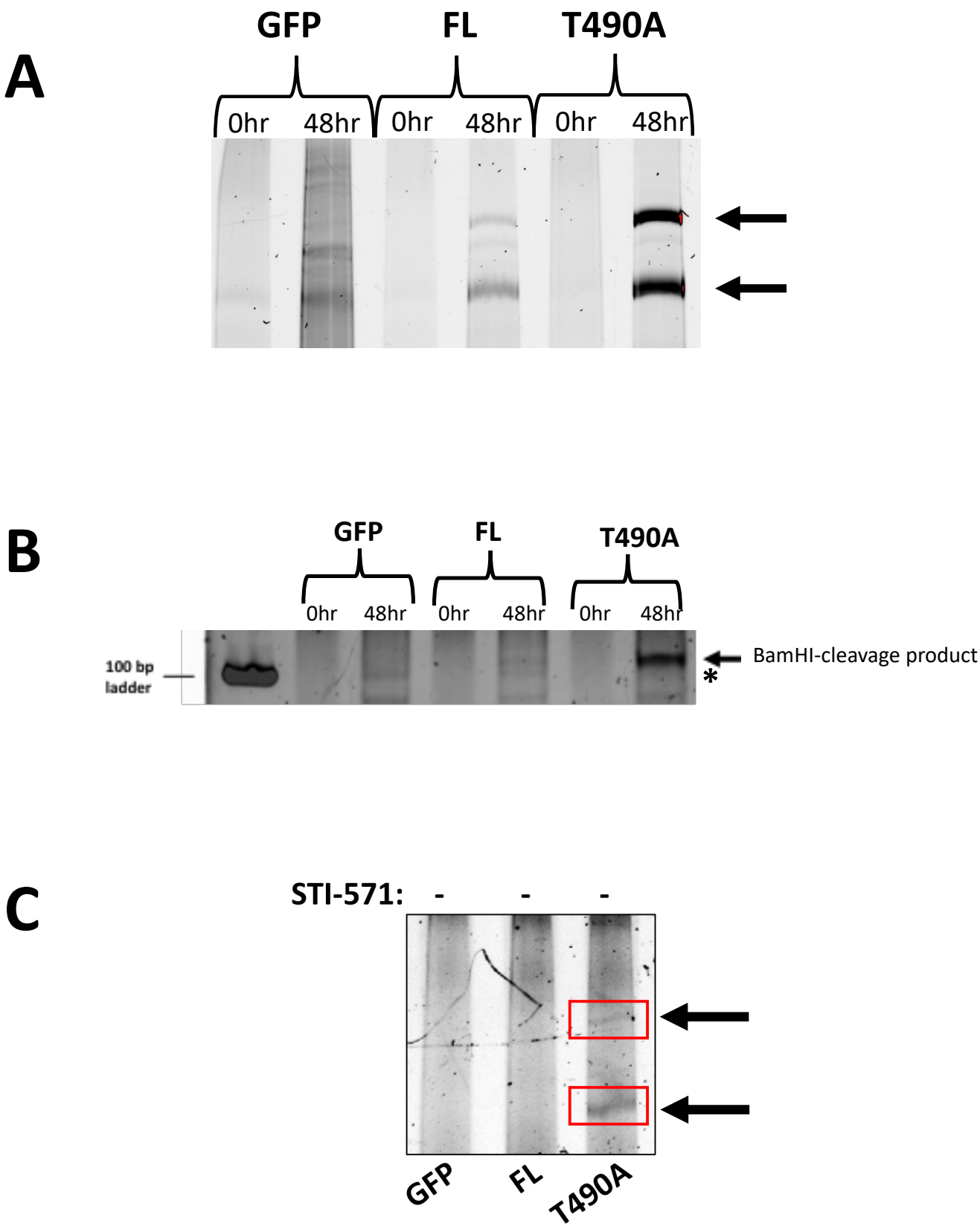
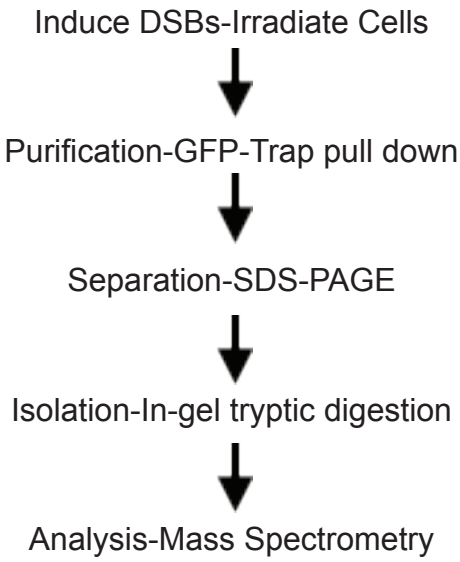


Supplemental Figure 1, Byrum et al.

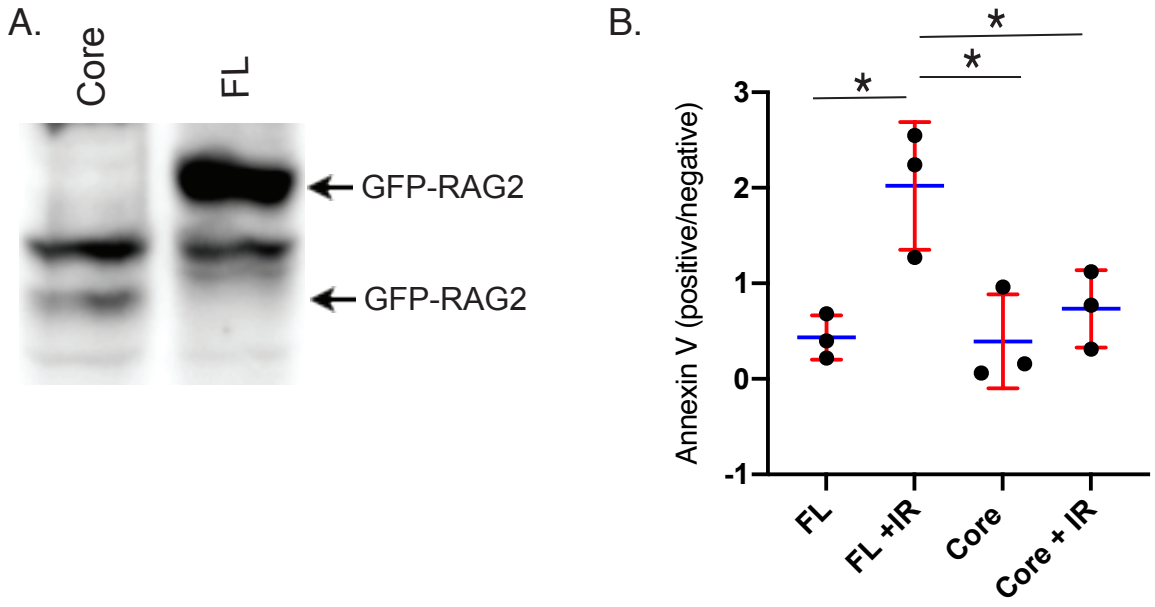


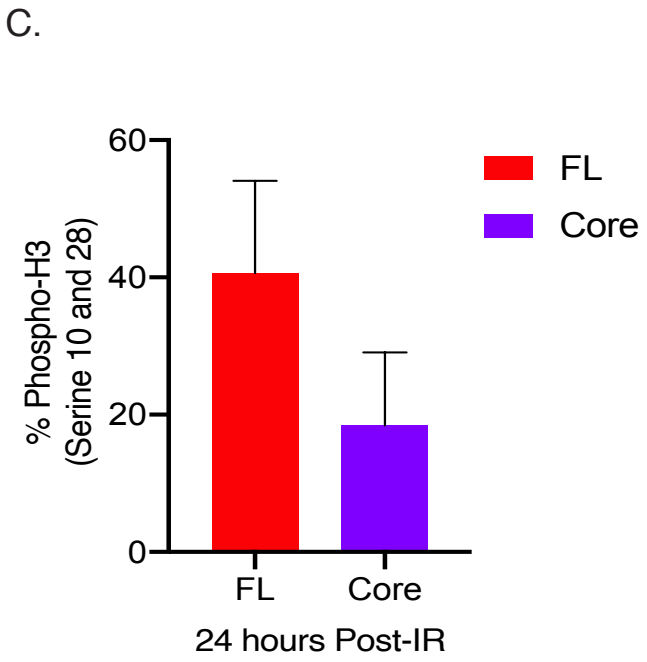
Supplemental Figure 2, Byrum et al.



Protein	# unique peptides	% coverage	Protein Function
CEP120	8	8.5	Centriole duplication/elongation
CEP152	16	11.0	Centriole duplication
CEP162	9	6.3	Localized at centriole ends
CEP55	8	20.0	Mitotic exit/cytokinesis
CEP135	12	10.0	Centriole duplication/elongation

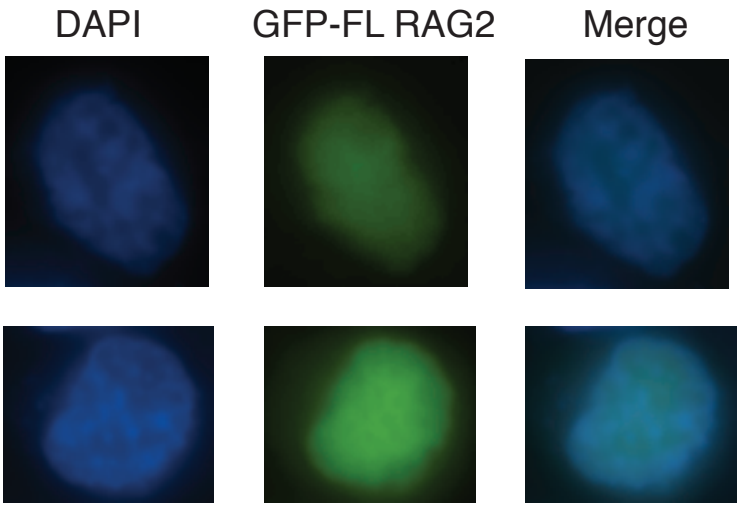
Supplemental Figure 3, Byrum et al.





Supplemental Figure 4, Byrum et al.

A.



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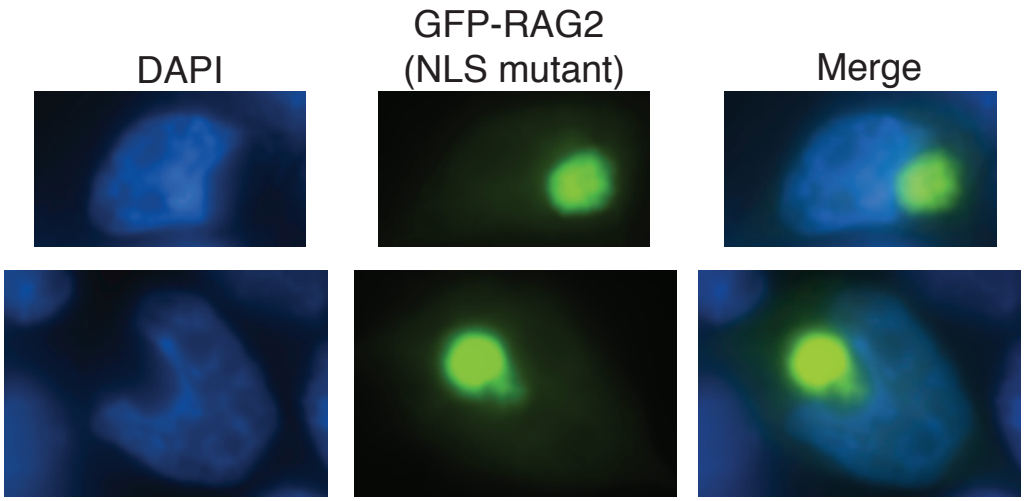

Supplemental Figure 5, Byrum et al.