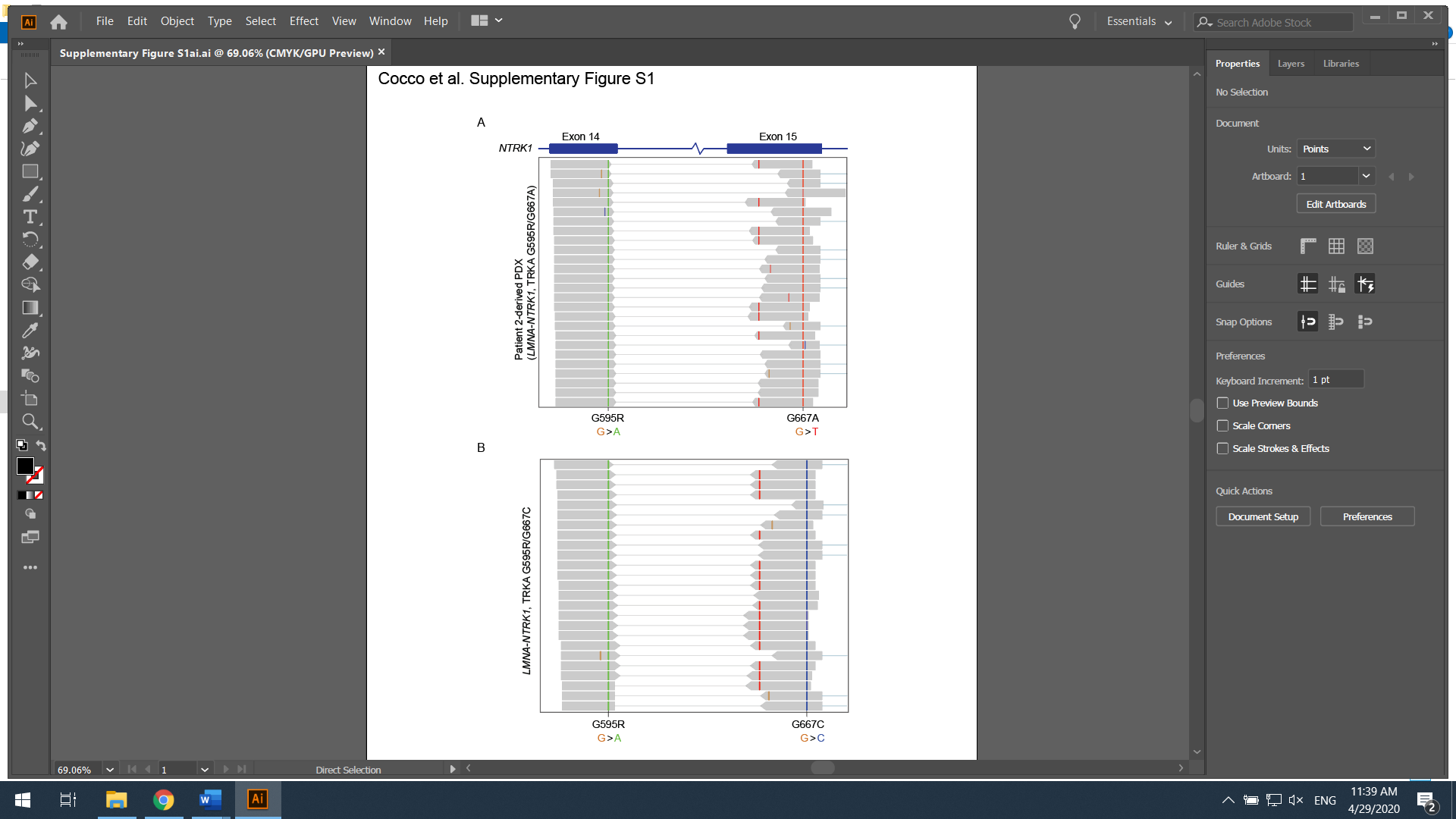
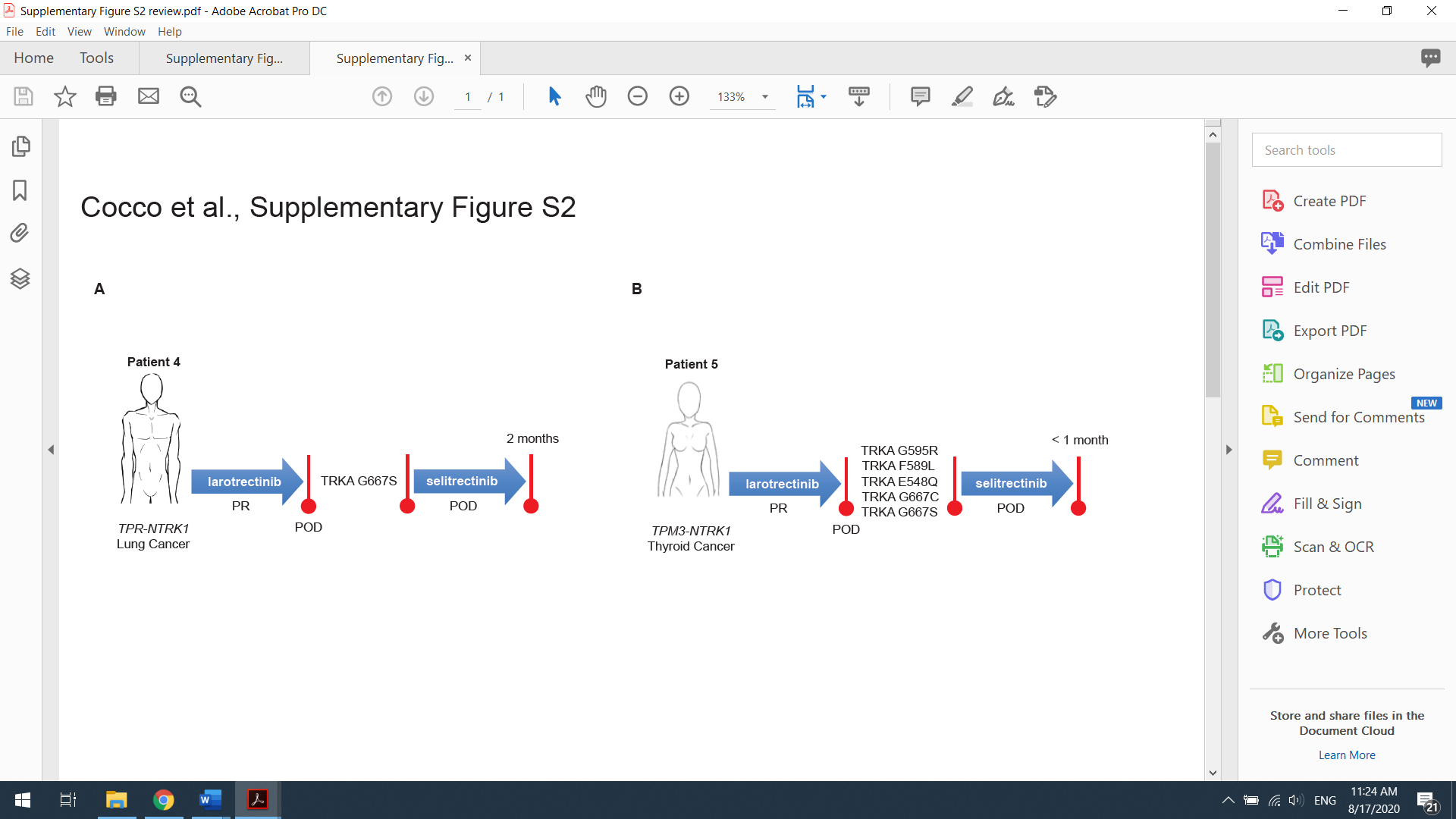
SUPPLEMENTARY FIGURES



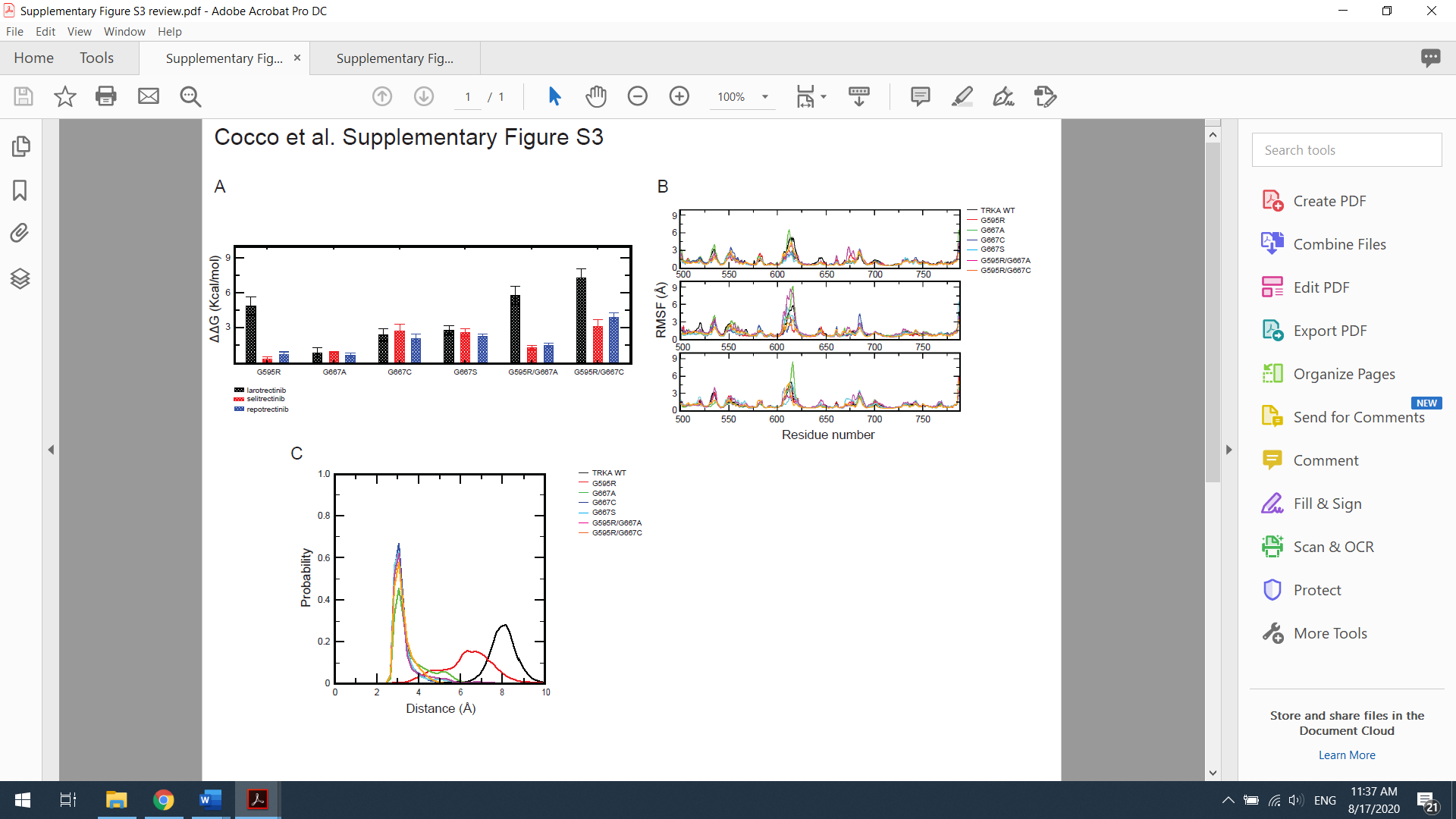
**Supplementary Figure S1. RNA Sequencing Analysis on TRKA Double Mutant Tumors**

Standard RNA sequencing was conducted by GENEWIZ, LLC (South Plainfield, NJ) on RNA samples extracted from the PDX collected from the Patient 2-derived selitrectinib-resistant tumor (harboring a *LMNA-NTRK1*, TRKA G595R/G667A CRC); A) and from the *LMNA-NTRK1*, TRKA G595R/G667C CRC repotrectinib-resistant CRC cell line established in our laboratory (B). IGV schematic showing aligned paired-end RNA sequencing reads of Patient 2-derived PDX and CRC cell line spanning the loci of *NTRK1* exons 14 and 15 containing both variants, conferring evidence for the in-cis configuration of both G595R/G667A (top) and G595R/G667C (bottom) mutations.



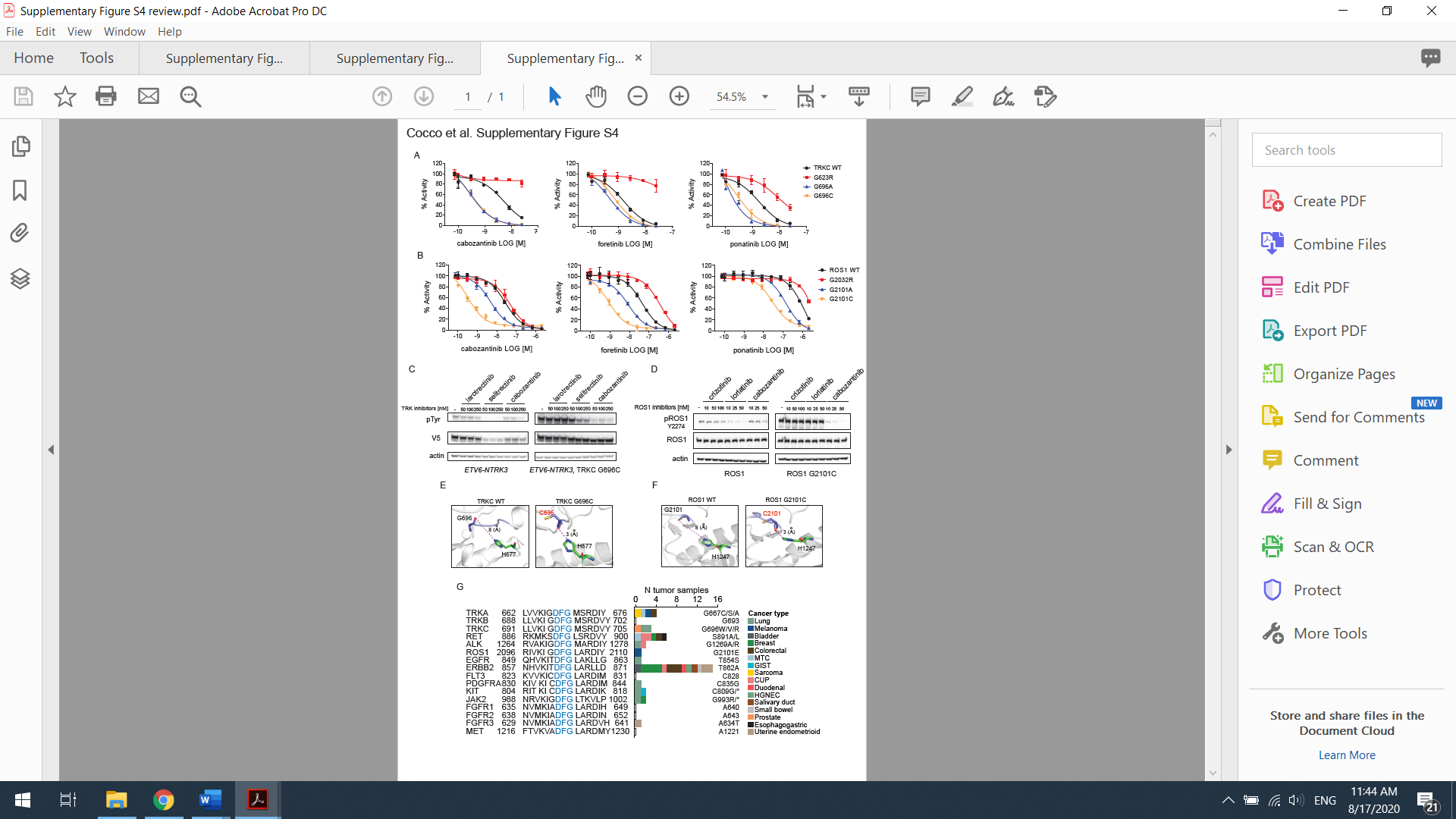
**Supplementary Figure S2. TRKA G667 Mutations Confer Primary Resistance to Selitrectinib in Patients**

Schematic showing the emergence of TRKA G667 mutations at progression on larotrectinib in a *TPR-NTRK1* lung cancer patient (Patient 4, panel A) and a *TPM3-NTRK1* thyroid cancer patient (Patient 5, panel B). The allele frequency of the multiple TRKA mutations identified in Patient 5 were the following: G595R= 0.001; F589L found in two subclones= 0.0182 and 0.0131; E548Q= 0.1694; G667C found in two subclones= 0.0744 and 0.0043; G667S= 0.0195. Both patients were then treated with selitrectinib and experienced POD. PR= Partial Response.



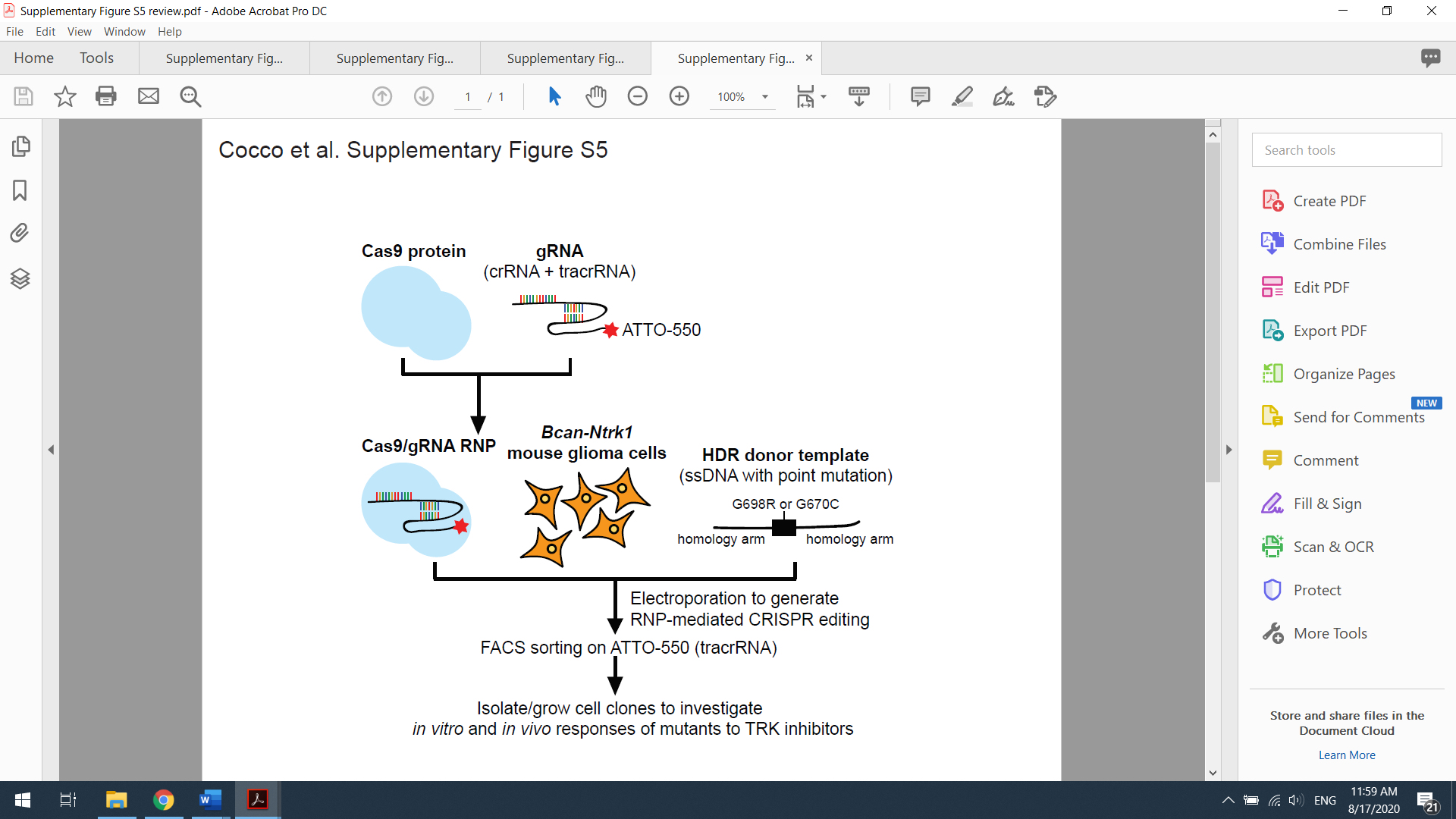
**Supplementary Figure S3. TRKA xDFG Mutants Are Predicted to be More Resistant to Type I but More Sensitive to Type II TRK Inhibitors than TRKA Wild Type and Solvent Front Mutant Kinases**

(A)Differences in the free energies (computed using the FEP/MBAR method, see main manuscript) of binding of larotrectinib, selitrectinib, and repotrectinib to mutant TRKA compared to the WT kinase. (B) Root mean squared fluctuations (RMSF) of WT and mutant TRKA bound to (top) cabozantinib, (middle) foretinib, (bottom) ponatinb sampled during the MD simulations. The flexibility of the mutants is similar to that seen for the WT suggesting that the binding of the drug is tolerated by the mutant TRKA. (C) Distributions of the distances between the residues at position 667 (Gly, Cys, Ser, Ala) and His648 sampled during the MD simulations of the apo states of the inactive conformations of WT and mutant TRKA proteins.

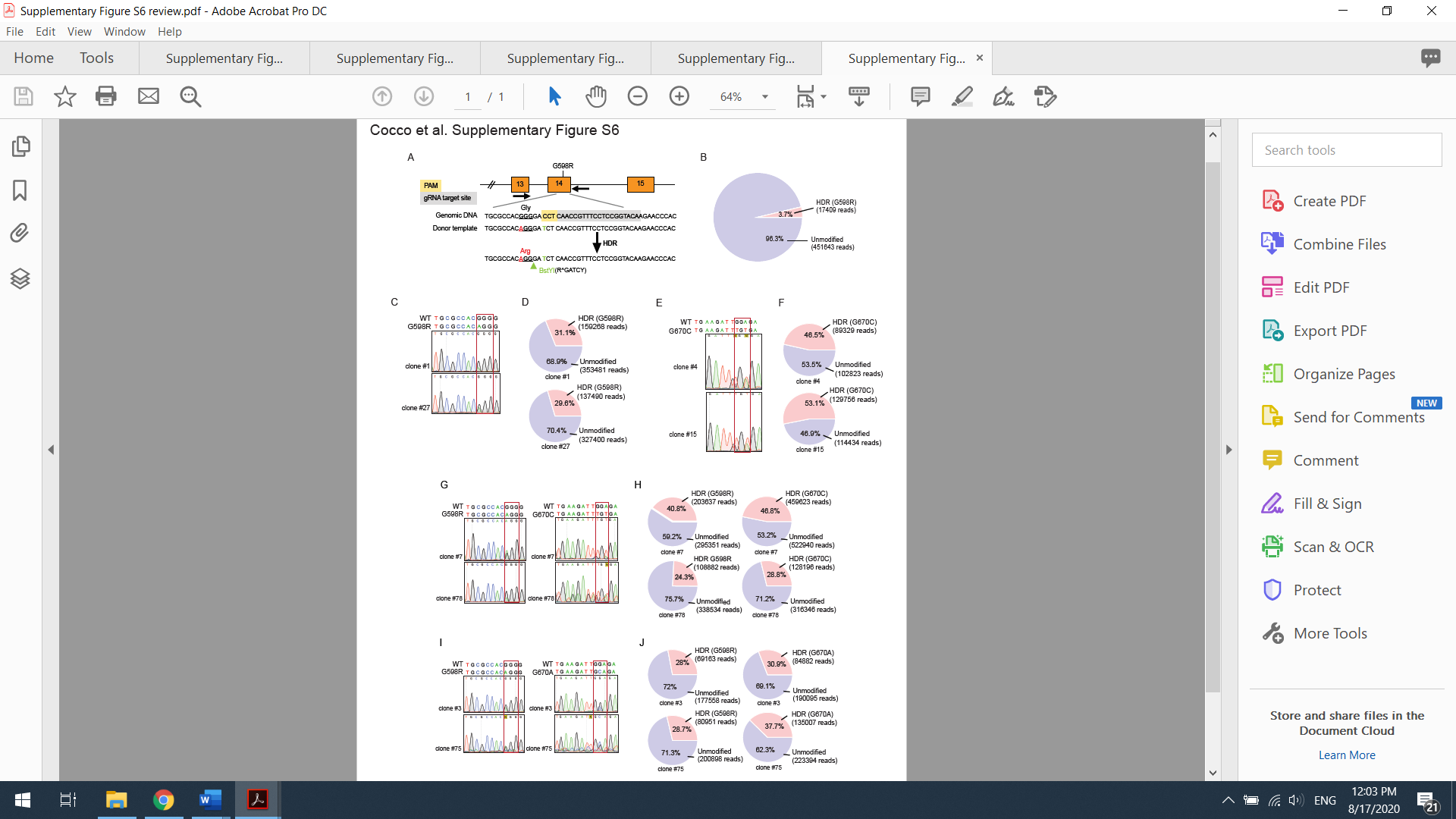


**Supplementary Figure S4. TRKC and ROS1 xDFG Substitutions Sensitize to Type II Inhibitors**

Activity of type II inhibitors against TRKC G696A/C and ROS1 G2101A/C xDFG mutant kinases. *In vitro* radiometric assays of TRKC (A) and ROS1 (B) wild type and mutant kinases treated with a titration of type II drugs. TRKC (C) and ROS1 (D) mediated signaling inhibition following treatment of the wild type or the xDFG Gly to Cys xDFG mutant kinases with type I or type II drugs. Modelling of TRKC (E) and ROS1 (F) in their Inactive states showing that the distance of the interaction between the xDFG residue and the H of the HRD motif reduces about 3-fold when the Glycine at the xDFG position is substituted with a Cysteine. (G) Prevalence of xDFG mutations in the MSK-IMPACT cohort in 16 kinases with high sequence identity.

 **Supplementary Figure S5. Workflow for the Generation of Isogenic Clones through CRISPR-Cas9 HDR Genome Editing**

Schematic of the generation of isogenic *Bcan-Ntrk1* mouse glioma cells harboring the Trka G598R solvent front mutant and the Trka G670C mutant using CRISPR-Cas9 Homology-Directed-Repair (HDR) genome editing technique.

 **Supplementary Figure S6. Validation of *Bcan-Ntrk1* Isogenic Mouse Glioma Cells Harboring Different TRKA Single and Double Mutations**

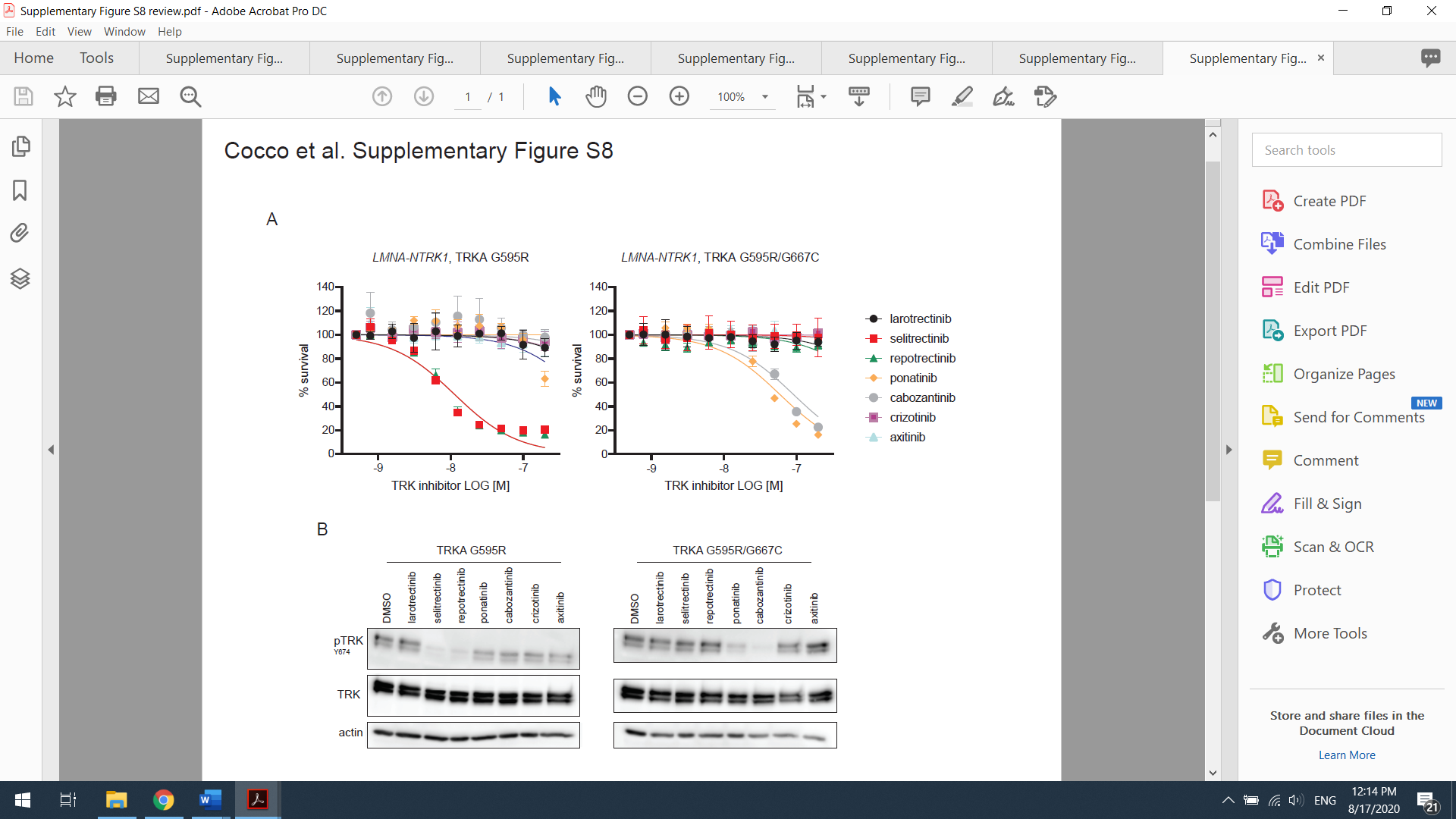
(A-B) Strategy for the generation of knock-in isogenic *Bcan-Ntrk1* glioma cells harboring the TRKA G598R mutation is described. Characterization of *Bcan-Ntrk1* single (C-F) and double (G-J) Trka xDFG mutant glioma cells.

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**Supplementary Figure S7. Trka xDFG mutated *Bcan-Ntrk1* Mouse Glioma Cells Respond to Type II TRK Inhibitors**

Cell-Titer-Glo-based assays (A) and Western blot analyses (B) performed on the *Bcan-Ntrk1* wild type and mutant second set of clones showing the effect of type I and type II TRK inhibitors on cell proliferation and TrkA-mediated downstream signaling. (C) Western blot showing changes in the phosphorylation status of TRKA in *Bcan-Ntrk1*, Trka G598R/G670A double mutant glioma cells treated with increasing concentrations of selitrectinib or cabozantinib for 30 minutes and 24 hours. (D) Proliferation and colony formation (E) assays on the *Bcan-Ntrk1* Trka wild type and mutant glioma cells.

 **Supplementary Figure S8. Type II multikinase inhibitors specifically inhibit TRKA xDFG mutant kinases**

Cell-Titer-Glo-based assays (A) and Western blot analyses (B) performed on the *LMNA-NTRK1*, TRKA G595R and the *LMNA-NTRK1*, TRKA G595R/G667C double mutant primary colorectal cancer (CRC) cell lines showing the effect of type I (i.e., larotrectinib, selitrectinib and repotrectinib), type II (i.e., cabozantinib, foretinib and ponatinib) TRK inhibitors as well as the MET inhibitor crizotinib and the VEGFR inhibitor axatinib on cell proliferation and TrkA-mediated downstream signaling.