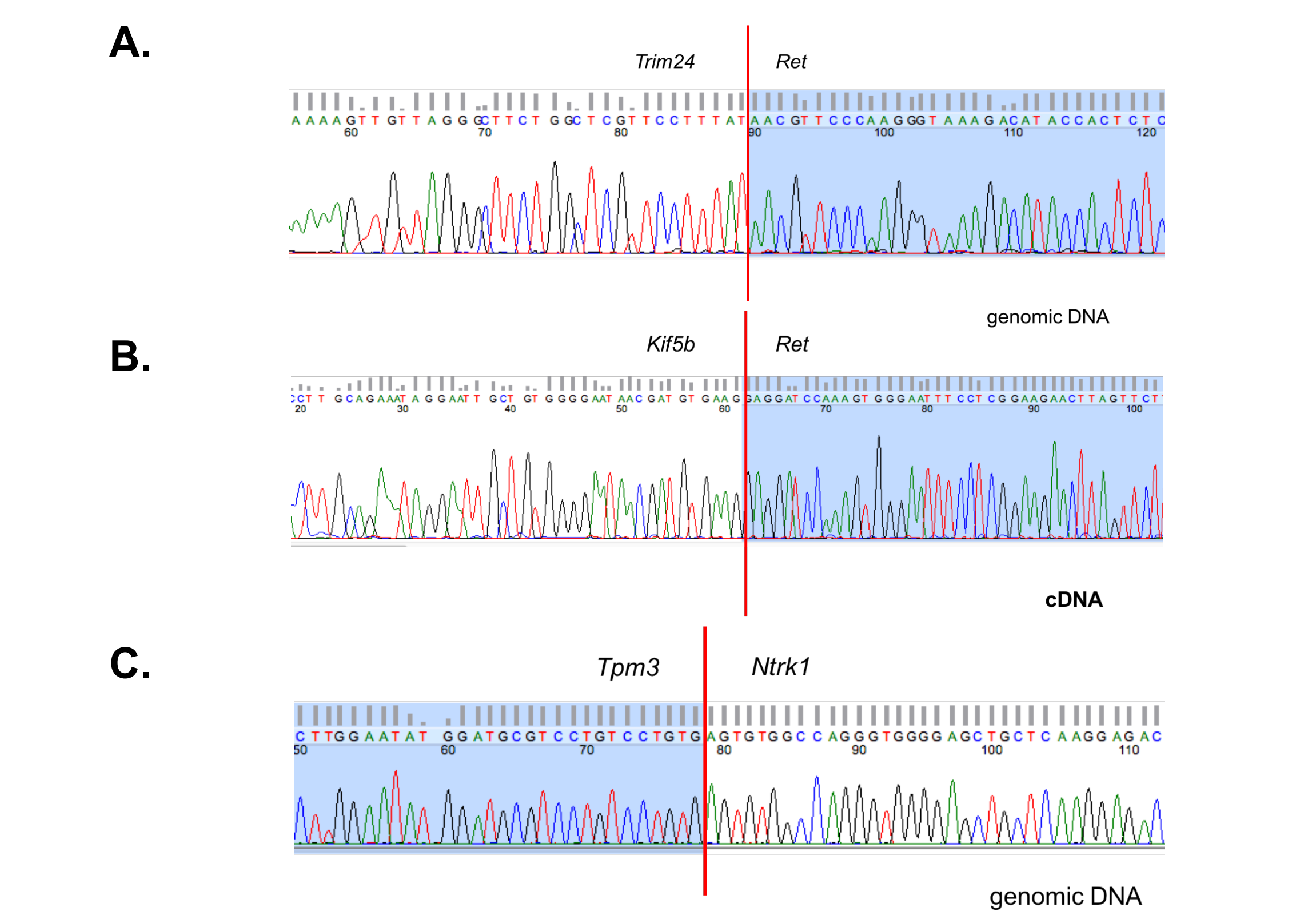
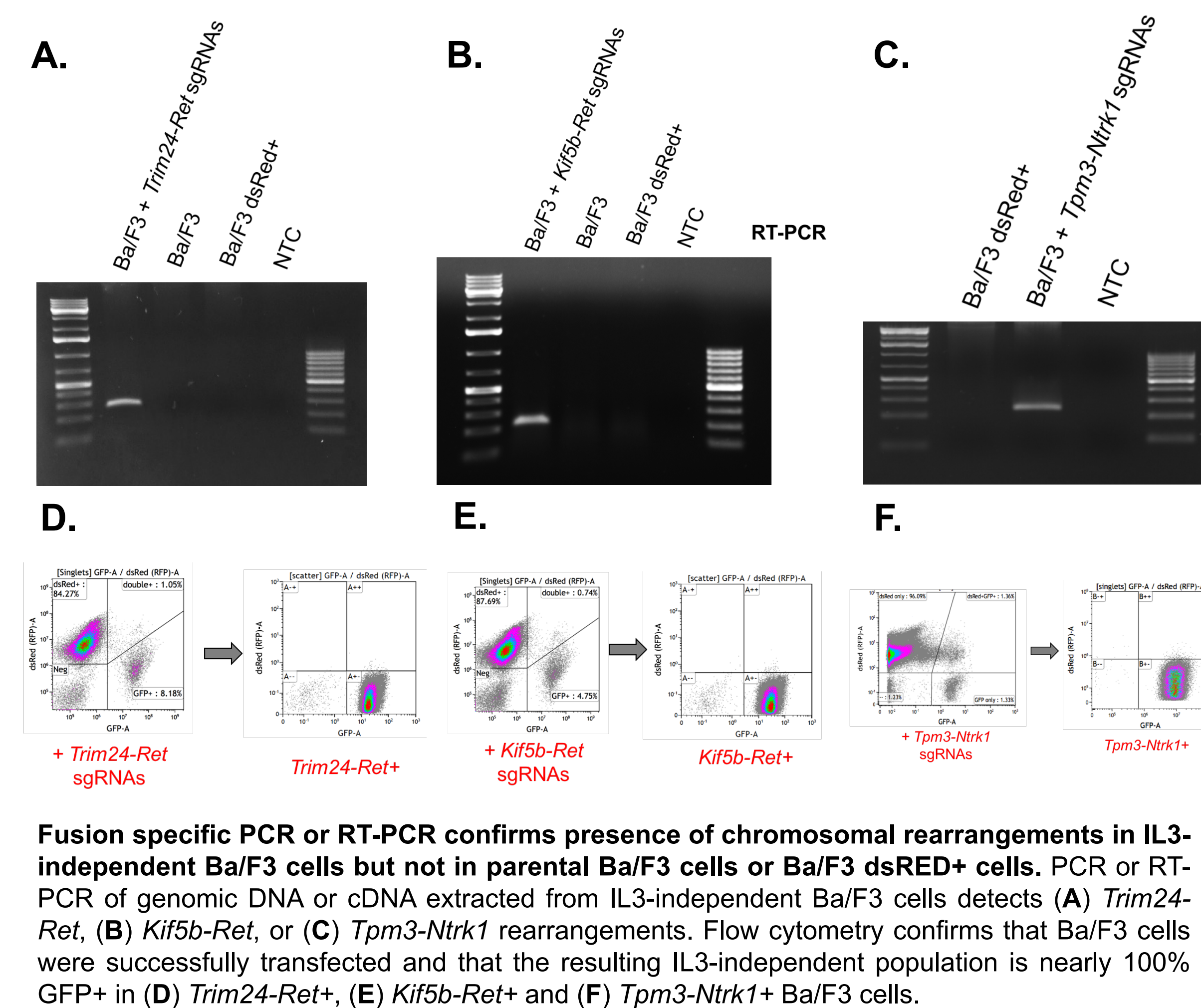


Laura Schubert, Anh T. Le, Stephen P. Malkoski, Raphael Nemenoff, Robert C. Doebele
Department of Medicine University of Colorado - Anschutz Medical Campus, Aurora, CO

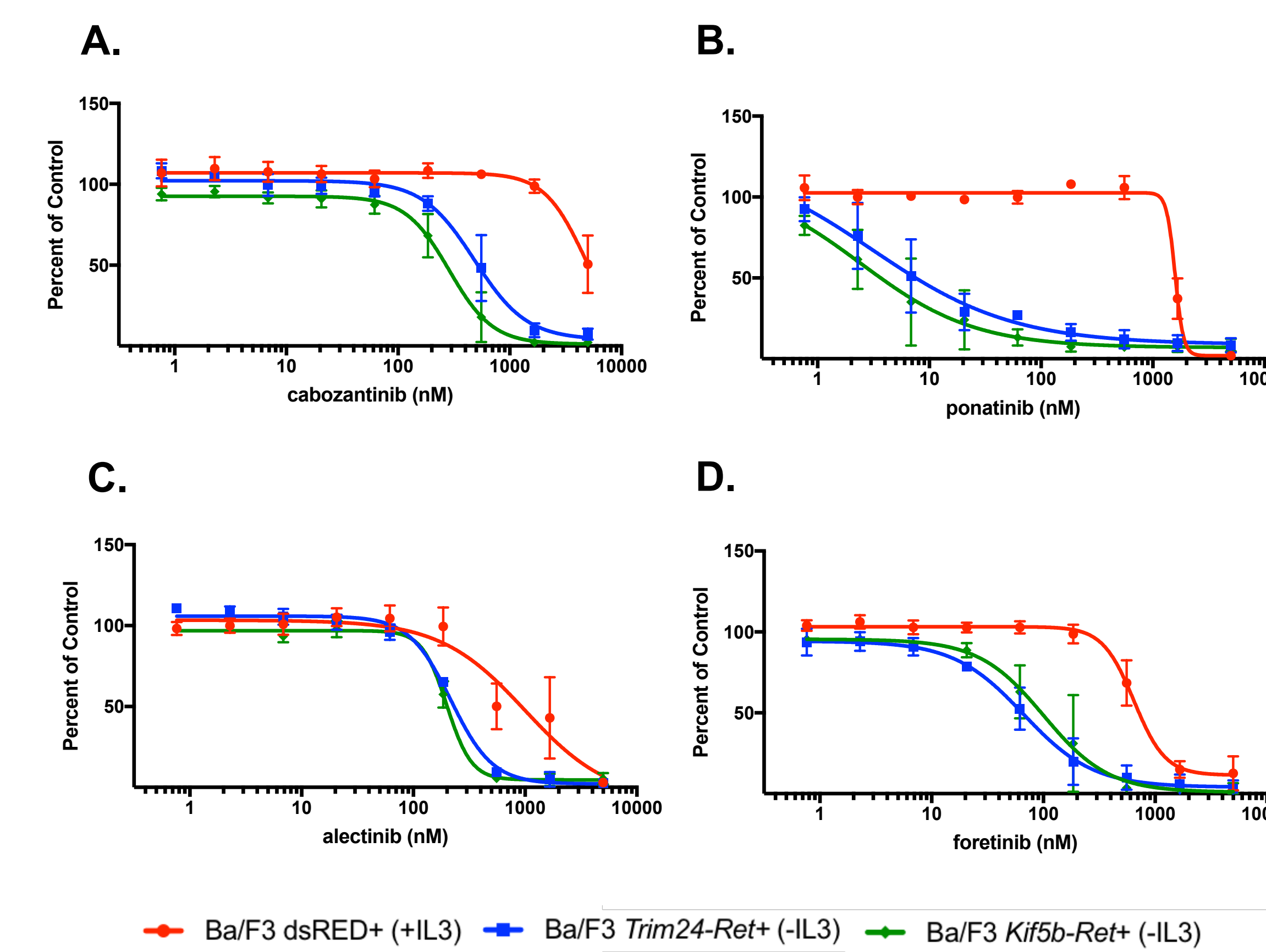
INTRODUCTION

- Chromosomal rearrangements of *ALK*, *RET*, *ROS1* and *NTRK1* collectively represent approximately 10% of oncogenic drivers in lung adenocarcinoma.
- These rearrangements result in the aberrant expression and constitutive activation of a chimeric fusion kinase, that promotes growth, proliferation and survival of the cancer cell.
- CRISPR/Cas9 technology can now be employed to generate these chromosomal rearrangements both *in vitro* and *in vivo*.
- We have developed a novel *in vitro* screening strategy to identify sgRNAs that successfully generate the intended rearrangements.
- Ba/F3 cells are a murine B-cell cell line whose proliferation is normally dependent on the exogenous addition of the cytokine interleukin 3 (IL3), but can be rendered IL3-independent if an oncogenic alteration is introduced.
- We hypothesized that IL3-independence in Ba/F3 cells could be used as a method to select for cells that had successfully created chromosomal rearrangements leading to oncogenic fusions.
- Using this screening system, we successfully generated *Kif5b-Ret*, *Trim24-Ret* and *Tpm3-Ntrk1* rearrangements in Ba/F3 cells.

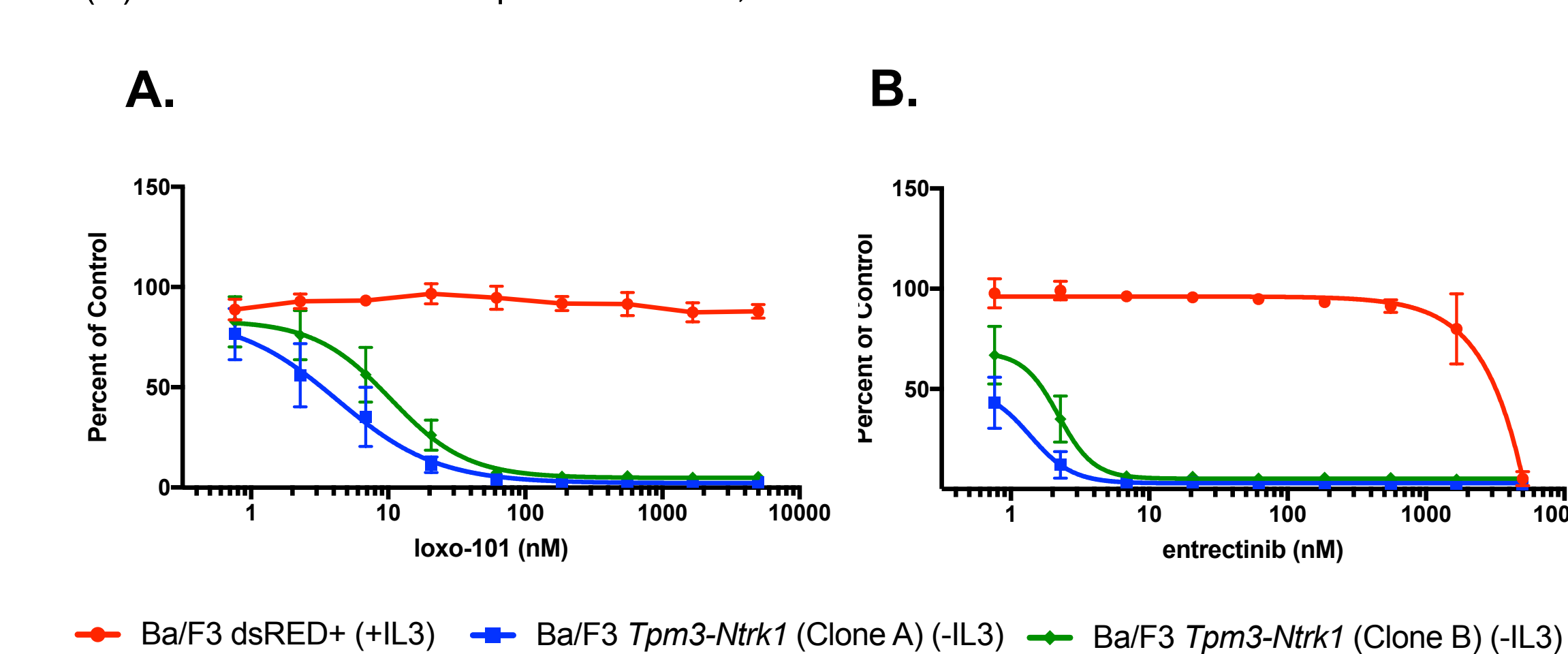
RESULTS



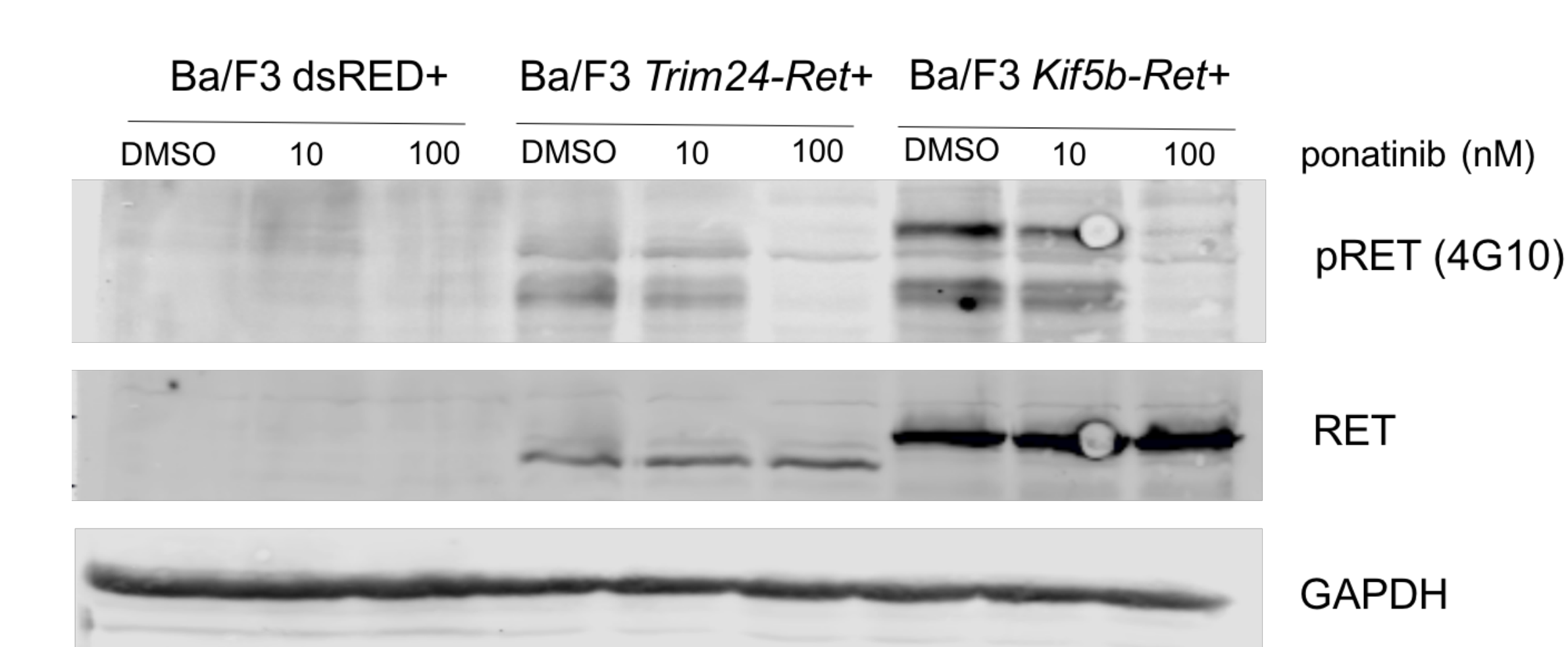
Sequencing of IL3-independent Ba/F3 cells confirms presence of *Trim24-Ret*, *Kif5b-Ret* or *Tpm3-Ntrk1* fusion chromosomes. Sequencing of genomic DNA or cDNA from IL3-independent Ba/F3 cells confirms the presence of (A) *Trim24-Ret* (B) *Kif5b-Ret* or (C) *Tpm3-Ntrk1* fusions.



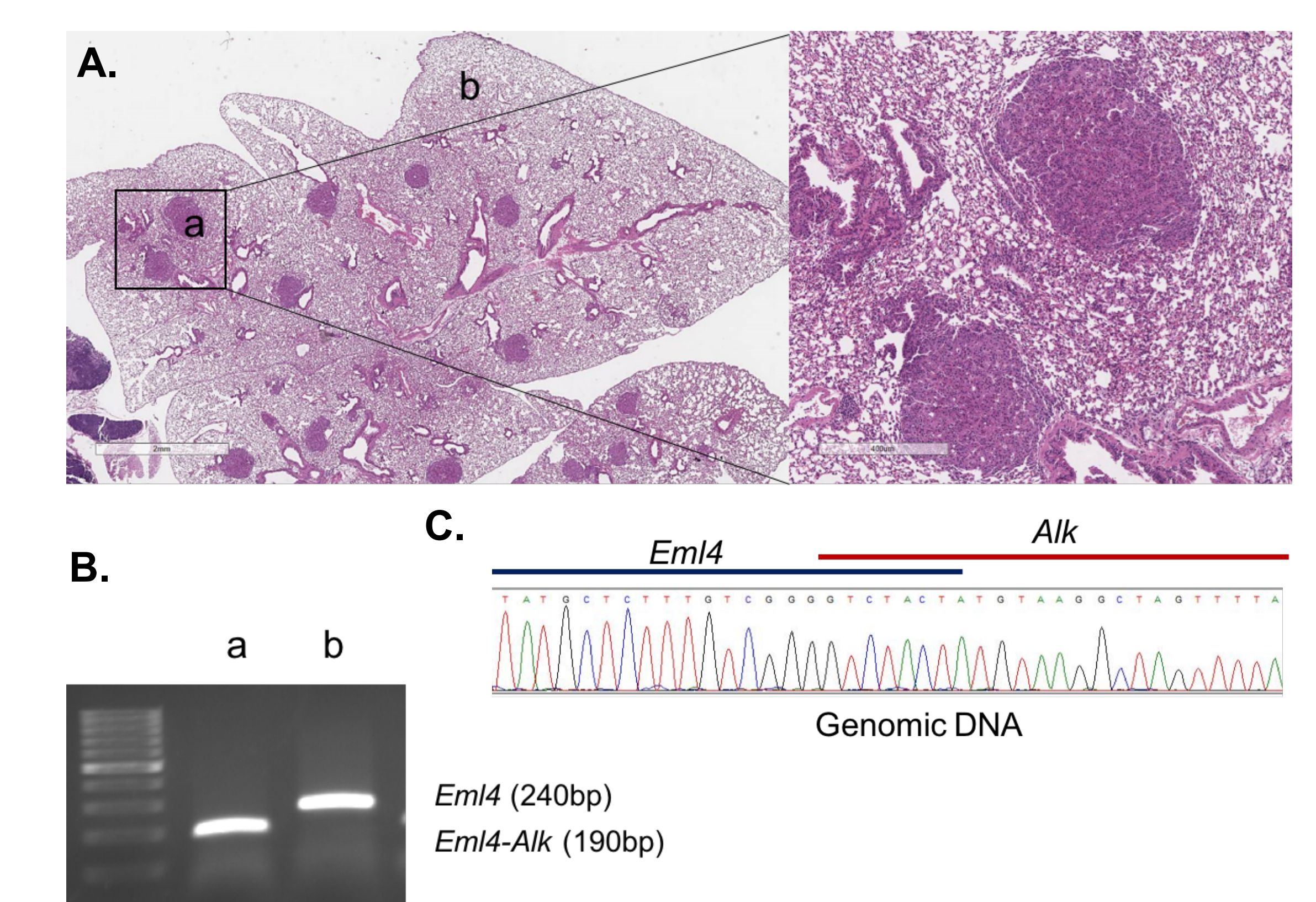
Ba/F3 cells harboring *Trim24-Ret* or *Kif5b-Ret* fusions are sensitive to RET inhibition. MTS proliferation assays of Ba/F3 dsRED+, Ba/F3 *Trim24-Ret*+, or Ba/F3 *Kif5b-Ret*+ cells treated with increasing doses of the RET inhibitors (A) cabozantinib, (B) ponatinib, (C) alectinib or (D) foretinib. Error bars represent \pm SEM, N=3.



Ba/F3 cells harboring *Tpm3-Ntrk1* fusions are sensitive to TRK inhibition. MTS proliferation assays of Ba/F3 dsRED+, Ba/F3 *Tpm3-Ntrk1*+ (clone A) or Ba/F3 *Tpm3-Ntrk1*+ (clone B) cells treated with increasing doses of the TRK inhibitors (A) loxo-101 or (B) entrectinib. Error bars represent \pm SEM, N=3. Clone A and Clone B were generated with different pairs of sgRNAs.



RET protein expression can be detected in Ba/F3 cells that have CRISPR/Cas9 generated *Ret* rearrangements. Western Blot analysis of Ba/F3 dsRED+, Ba/F3 *Trim24-Ret*+ or Ba/F3 *Kif5b-Ret*+ cells treated with 0, 10 or 100nM ponatinib for two hours.



CRISPR/Cas9 generation of *Eml4-Alk* yields adenomas in lungs. (A) (left) Low and (right) high-power micrographs of H&E from a mouse lung following intratracheal injection of 5×10^7 adenovirus (pacAd5-ALK-EML4-Cas9). (B) qRT-PCR confirms presence of *Eml4-Alk* fusion in adenomas (a) but not in normal tissue (b). (C) Genomic sequencing additionally confirms the presence of *Eml4-Alk* fusion in adenomas.

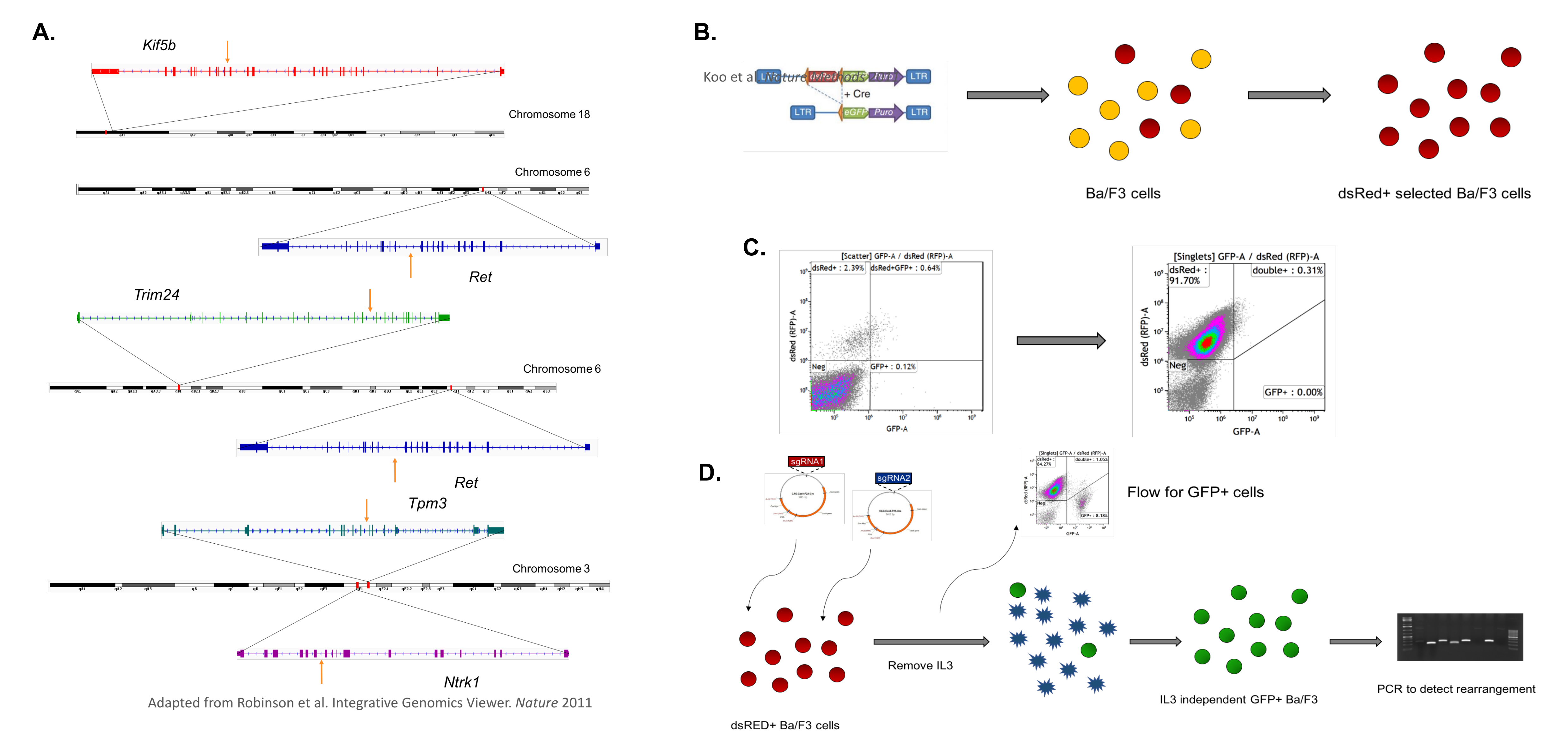
CONCLUSIONS

- IL3 independence in Ba/F3 cells can be used to screen and select for sgRNAs that result in oncogenic rearrangements.
- Trim24-Ret*, *Kif5b-Ret* and *Tpm3-Ntrk1* rearrangements were successfully generated in Ba/F3 cells using CRISPR/Cas9.
- Ba/F3 cells with CRISPR/Cas9 generated rearrangements are sensitive to their cognate tyrosine kinase inhibitor.
- Ret protein expression can be detected in Ba/F3 cells with *Ret* rearrangements.
- Tumors bearing *Eml4-Alk* rearrangements can be introduced into 10/10 C57/BL6 mice using adenoviral vectors.

Grant Support
DOD LCRP LC140748 (RCD)
Lung Cancer SP0RE Pilot Grant P50 CA058187 (RCD)
CCTSI TL1 5TL1TR001081-03 (LS)
NIH NRSA T32CA190216-01A1 (LS)

Acknowledgments
We kindly thank Dr. Andrea Ventura for providing pacAd5-ALK-EML4-Cas9 adenovirus.

In Vitro sgRNA SCREENING METHOD



In vitro sgRNA screening method. (A) Schematic of *Ret* and *Ntrk1* breakpoints and rearrangements. Yellow arrows indicate regions that sgRNAs target. (B) Ba/F3 cells were first transduced with pMSCV-loxp-dsRed-loxp-eGFP-Puro-WPRE retrovirus. This construct contains a floxed dsRed gene, which is excised when a Cre recombinase is expressed which allows GFP to be expressed. We have engineered Cre into our pX330 plasmid to allow us to monitor transfection efficiency. (C) Flow cytometry confirms that Ba/F3 cells were successfully transduced with pMSCV-loxp-dsRed-loxp-eGFP-Puro-WPRE retrovirus. Transduced cells were sorted based on dsRED expression. Flow cytometry confirms ~97% of cells are dsRED+ after FACS. (D) dsRED+ Ba/F3 cells are then transfected with two pX330 plasmids, one containing an sgRNA targeting the 5' fusion partner and the other targeting the 3' partner. Successful transfection is confirmed by flow cytometry for GFP positivity. Cells are then withdrawn from IL3 and monitored for IL3-independence. After outgrowth of IL3-independent cells they are screened for their intended rearrangement with PCR or RT-PCR, sequencing and flow cytometry.