

New type of drug-resistant isogenic cell model created by CRISPR genome editing

Lysa-Anne Volpe, M.S., Metewo Selase Enuameh, Ph.D., Michael Jackson, Ph.D., Luping Chen, B.S., John Foulke, M.S., Elizabeth Turner, Ph.D., Stephen King, M.S., and Fang Tian, Ph.D.

American Type Culture Collection, ATCC Cell Systems, Manassas, VA 20110

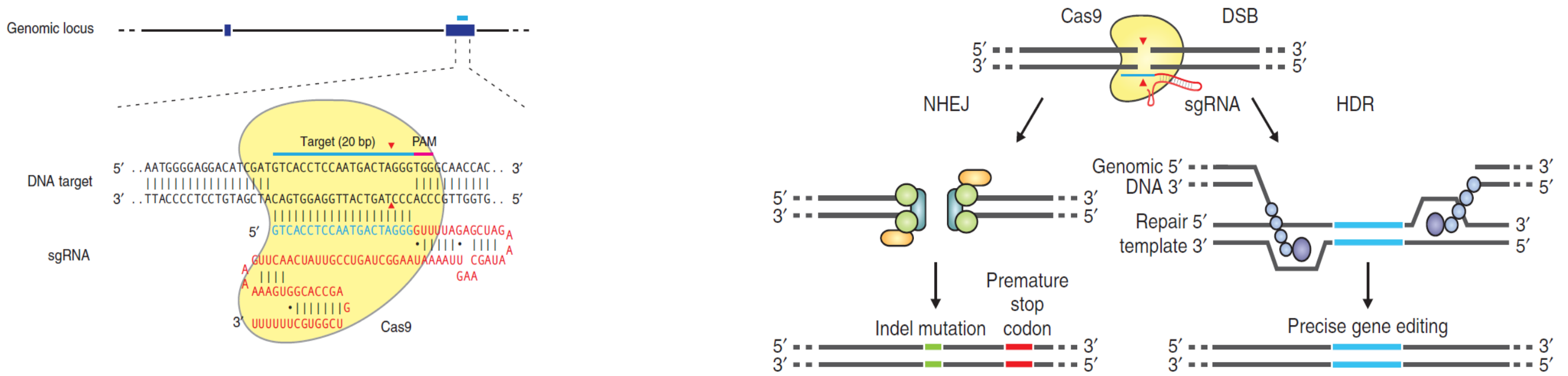
AACR Abstract # 3836

Introduction

A mutant BRAF gene can lead to uncontrolled cell growth through overactivation of the RAS-RAF-MAPK signaling pathway. The BRAF^{V600E} mutation occurs in approximately 40% to 50% of melanomas. Although current BRAF inhibitors have been used to successfully treat melanomas containing the BRAF^{V600E} mutation, patients often become resistant to BRAF inhibitors within a few months. A number of clinical studies have indicated that secondary mutations in RAS or NF1 are associated with BRAF resistance. However, due to the genetic heterogeneity commonly observed in tumors, it is unclear if those secondary mutations already existed within low percentage subclones, or if they were acquired through drug treatment. Further, it has yet to be determined whether such genetic variants are only associated with resistance, or whether they actually cause the BRAF inhibitor resistance. In this study, we used genome editing CRISPR technology to generate two drug-resistant melanoma cell lines (ATCC® CRL-1619IG-2™ and CRL-1619IG-1™) that contain NRAS^{Q61K} or KRAS^{G13D} mutations. These isogenic lines were derived from the parental A375 (ATCC® CRL-1619™) melanoma cell line, which naturally contains BRAF^{V600E}. When compared to the parental line, the isogenic cell models demonstrated that genetically modified NRAS or KRAS genes at the endogenous level directly leads to significant resistance to BRAF inhibitors. Single guide RNAs (sgRNAs) were designed and built to guide Cas9 to bind and cut desired regions in the NRAS or KRAS gene targets. The parental cell line A375 was co-transfected with the single guide and CRISPR all-in-one plasmid alongside a donor plasmid. Transfected cells were sorted into single cells and expanded for subsequent screening of desired gene mutation events. The introduction of the NRAS^{Q61K} or KRAS^{G13D} mutation in the cells was then confirmed via Sanger sequencing and NGS at the genetic and transcriptional levels. Drug responses to BRAF-specific inhibitors and non-specific chemotherapy drugs were compared between RAS isogenic A375 cell lines and parental A375 cell line in 2D and 3D culture environments. Testing results demonstrated that the isogenic cell lines created by CRISPR showed significant resistance to BRAF inhibitors in comparison to the parental control in both 2D and 3D culture environments. These two novel *in vitro* cell models with endogenous level RAS mutants provide direct biofunctional evidence that acquiring a drug-resistant gene drives tumor cell survival under targeted therapeutic treatment.

Background

CRISPR genome editing technology¹



RAS-mediated resistance to BRAF inhibitor in melanoma²

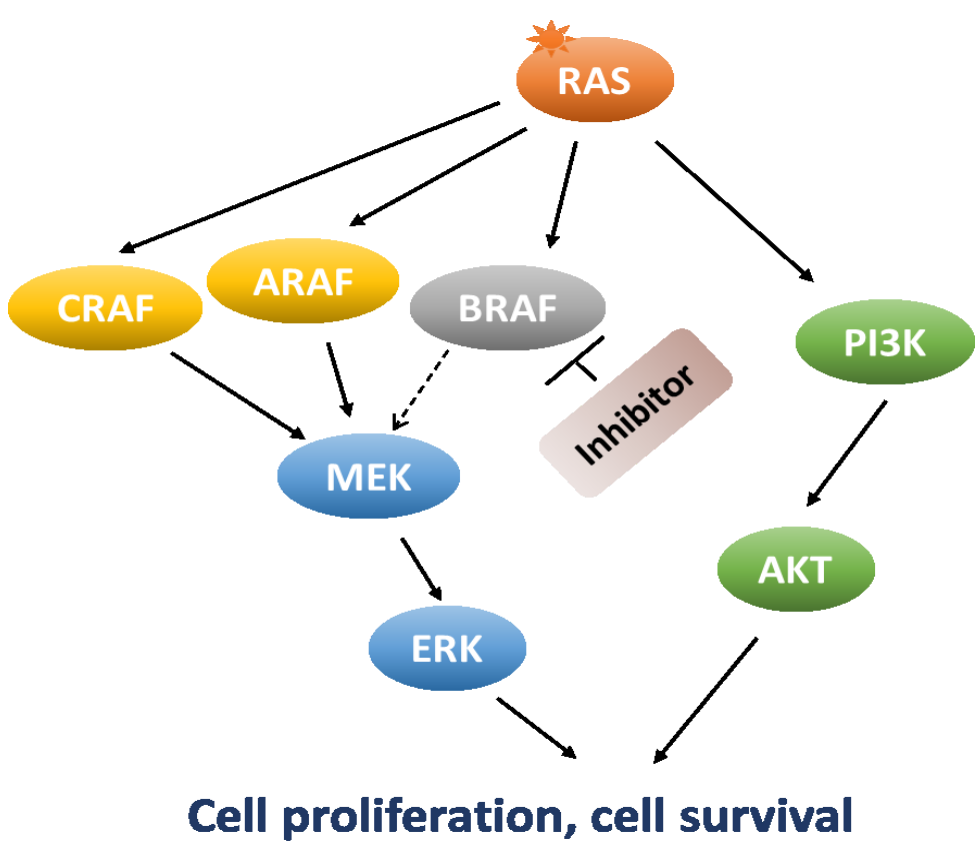


Figure 1. BRAF^{V600E} melanoma cells chronically treated with BRAF inhibitors acquire drug resistance by switching between the three isoforms of RAF (ARAF, BRAF, CRAF) to activate the MAPK pathway. Increased growth factor receptor signaling or reactivation of the MAPK pathway may allow for resistance. This can occur following the acquisition of activating mutations in NRAS (Q61K), MEK (C121S), and elevated expression of other key components of the pathway.

Results

Design and generation of NRAS^{Q61K} mutant cell clones

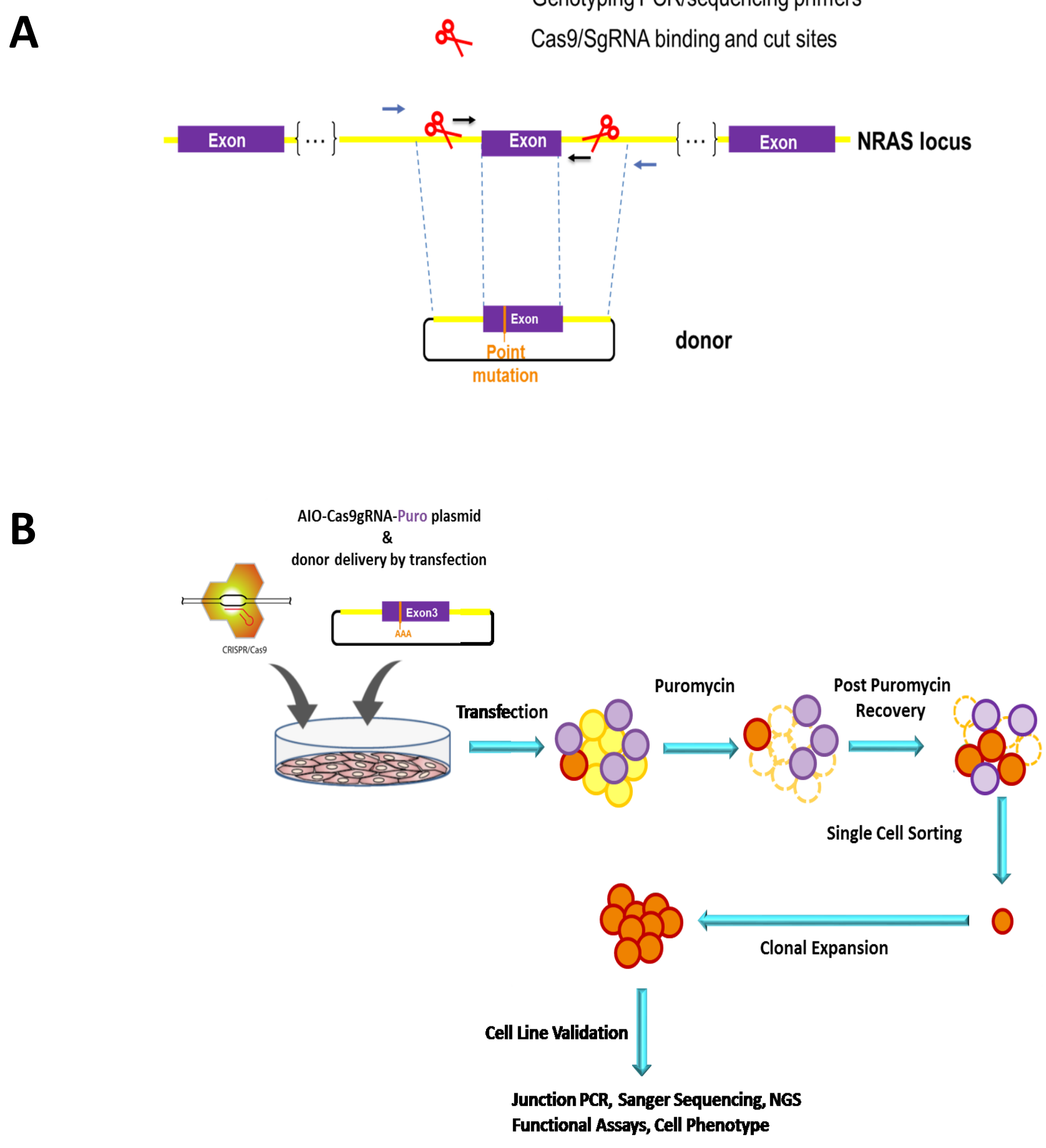


Figure 2. A) Schematic of the target region for integration of the knock-in DNA bearing the NRAS^{Q61K} point mutation. B) Cell clone generation workflow: Transfected cells were enriched by puromycin selection for 48 hrs. After puromycin recovery, the cells were sorted into single cells. Clonal expansion and validation was then performed at the genomic, transcriptional, translational, and functional levels on the cells.

PCR and sequencing to confirm NRAS^{Q61K} knock-in mutation

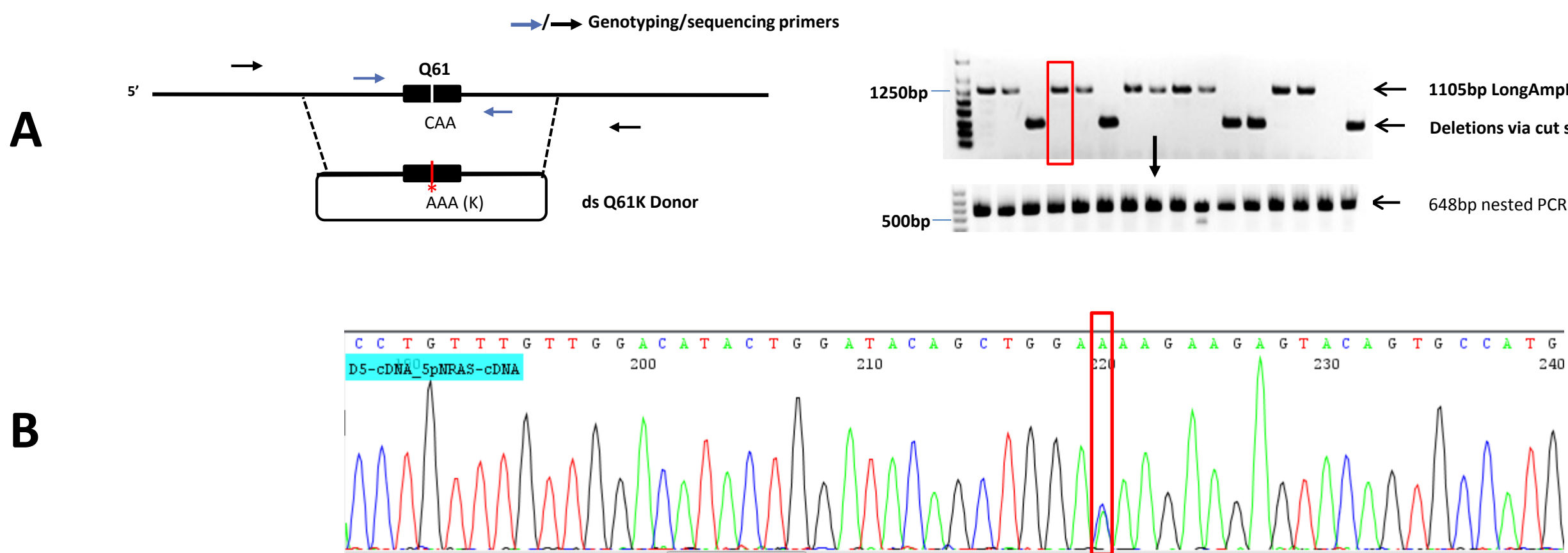


Figure 3. A) Screening for the NRAS^{Q61K} point mutation integration in recovered clones was carried out using PCR primers as shown left, with the gel image of PCR products on the right. B) Introduction of the NRAS^{Q61K} point mutation in the cell line was confirmed via sequencing primers shown in A. Boxed in red is the expected C>A mutation.

Confirmation of NRAS^{Q61K} mRNA expression in single cell clones

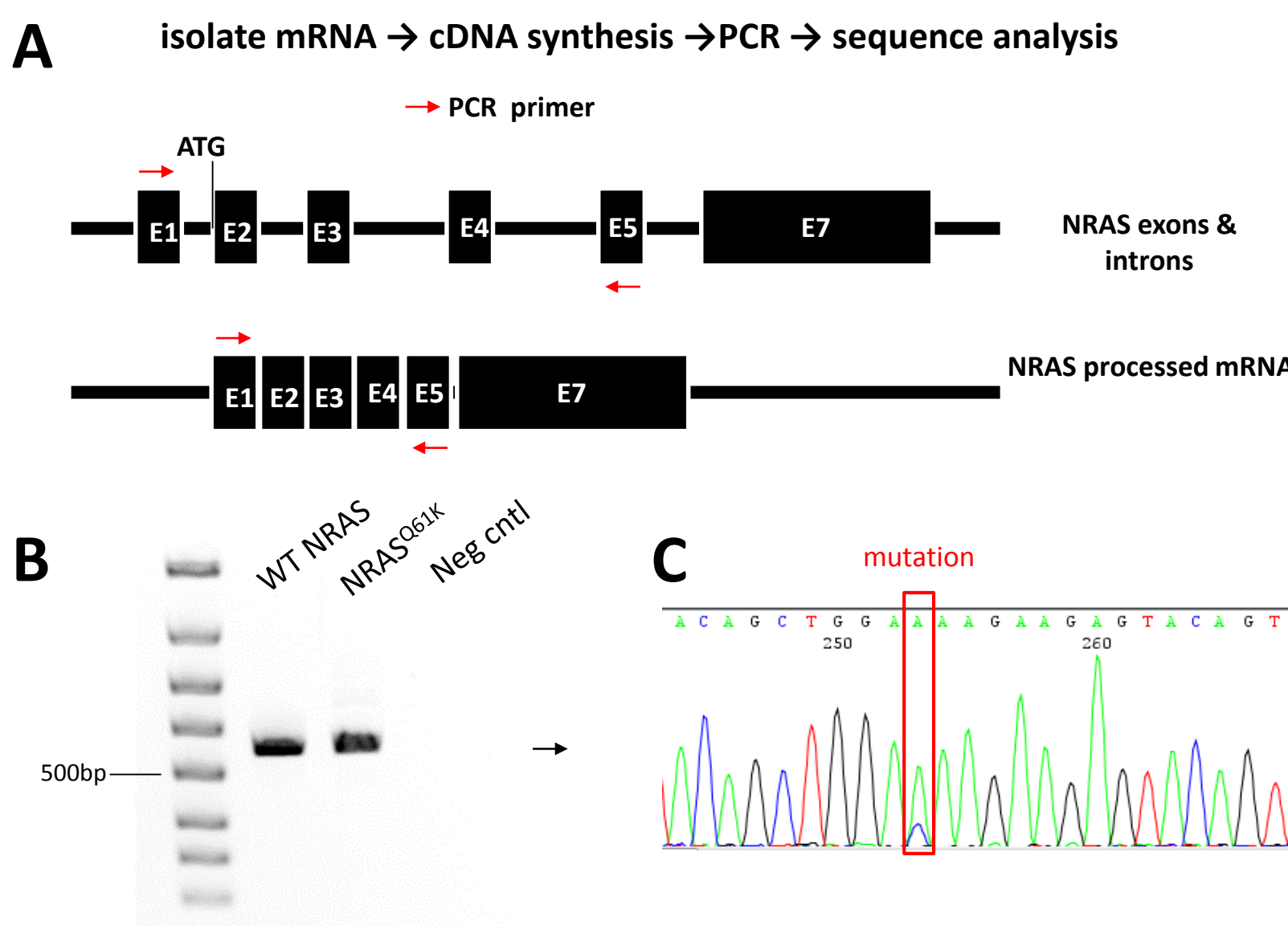


Figure 4. A) NRAS mRNA in cell clones carrying the Q61K mutations. Screening for NRAS^{Q61K} point mutation in recovered clones was carried out via cDNA generation from cells and performing PCR (red arrows). B) Gel image of the PCR products. C) The introduction of the NRAS^{Q61K} point mutation in the cell line was confirmed via Sanger sequencing on the right. Boxed in red is expected point mutation.

NRAS^{Q61K} isogenic line is resistant to BRAF inhibitors

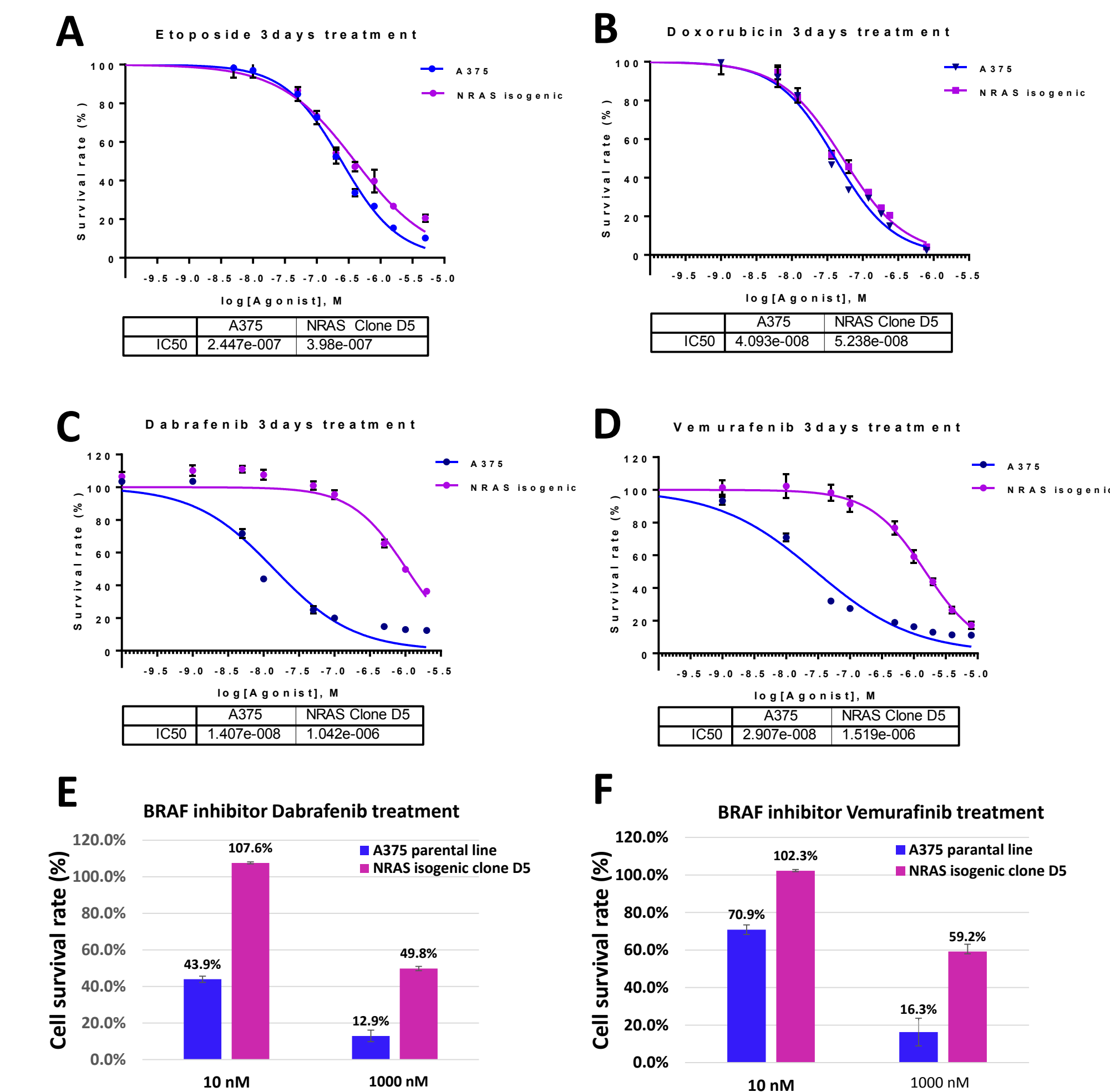


Figure 5. Drug response of the A375 parental line and NRAS^{Q61K} isogenic lines. CellTiter-Glo luminescent cell viability assay was used to evaluate cell line drug response to the BRAF inhibitors vemurafenib and dabrafenib, as well as the chemotherapy drugs doxorubicin and etoposide. Data are expressed as mean ± SD (n=4). NRAS^{Q61K} isogenic clone D5 (ATCC® CRL-1619IG-2™) exhibits drug resistance to the BRAF inhibitors relative to the parental A375 cell line (C, D, E, and F). The responses of the A375 and NRAS^{Q61K} isogenic lines to non-specific chemotherapy drugs were similar (A and B).

ERK and AKT activation is reduced in the NRAS isogenic line

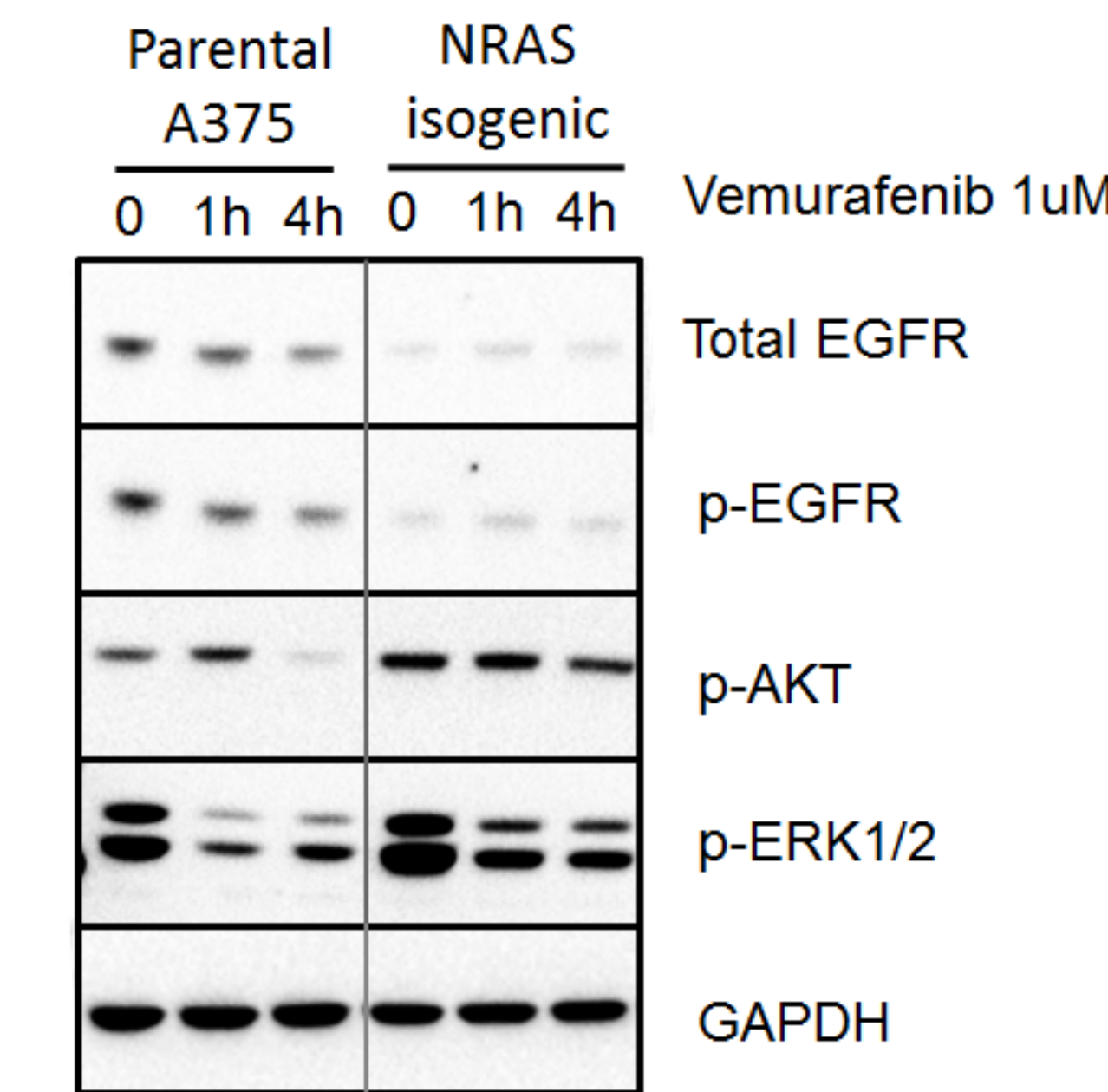


Figure 6. Activation of AKT and ERK pathways mediates the NRAS^{Q61K} isogenic line's resistance to BRAF inhibitor. Parental A375 cells and NRAS^{Q61K} isogenic cells were treated with 1 uM Vemurafenib or DMSO for 1 hour and 4 hours. Cells were then harvested, lysed, and analyzed by immunoblotting with antibodies against phospho-EGFR, total EGFR, phospho-AKT, phospho-ERK and GAPDH (loading control).

NRAS^{Q61K} isogenic line is resistant to BRAF inhibitors in 3D culture

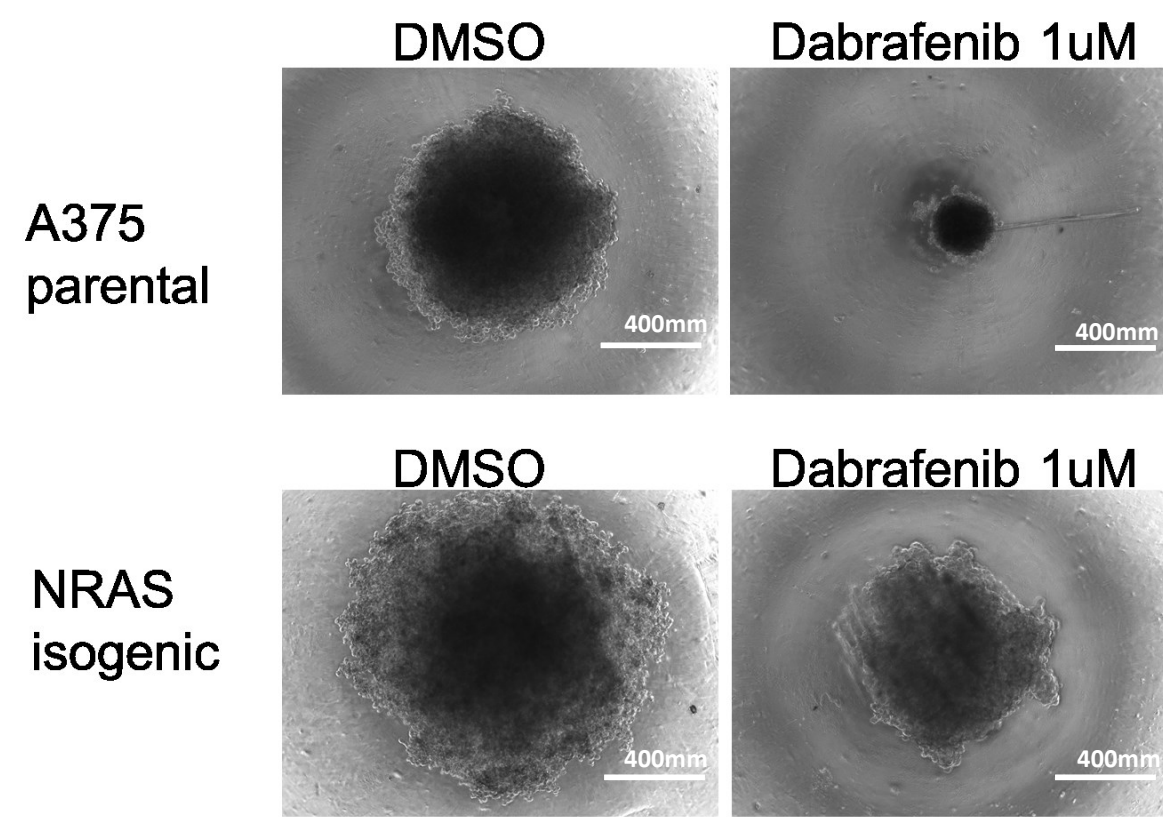


Figure 7. Spheroid 3D cultures were generated by seeding cells in triplicate on a low attachment plate for 4 days. Cultures were examined under the microscope after treatment with the BRAF inhibitor dabrafenib for 10 days. NRAS^{Q61K} isogenic line showed resistance to BRAF inhibitor, which was consistent with the 2D culture results.

The KRAS^{G13D} isogenic line is also resistant to BRAF inhibitors

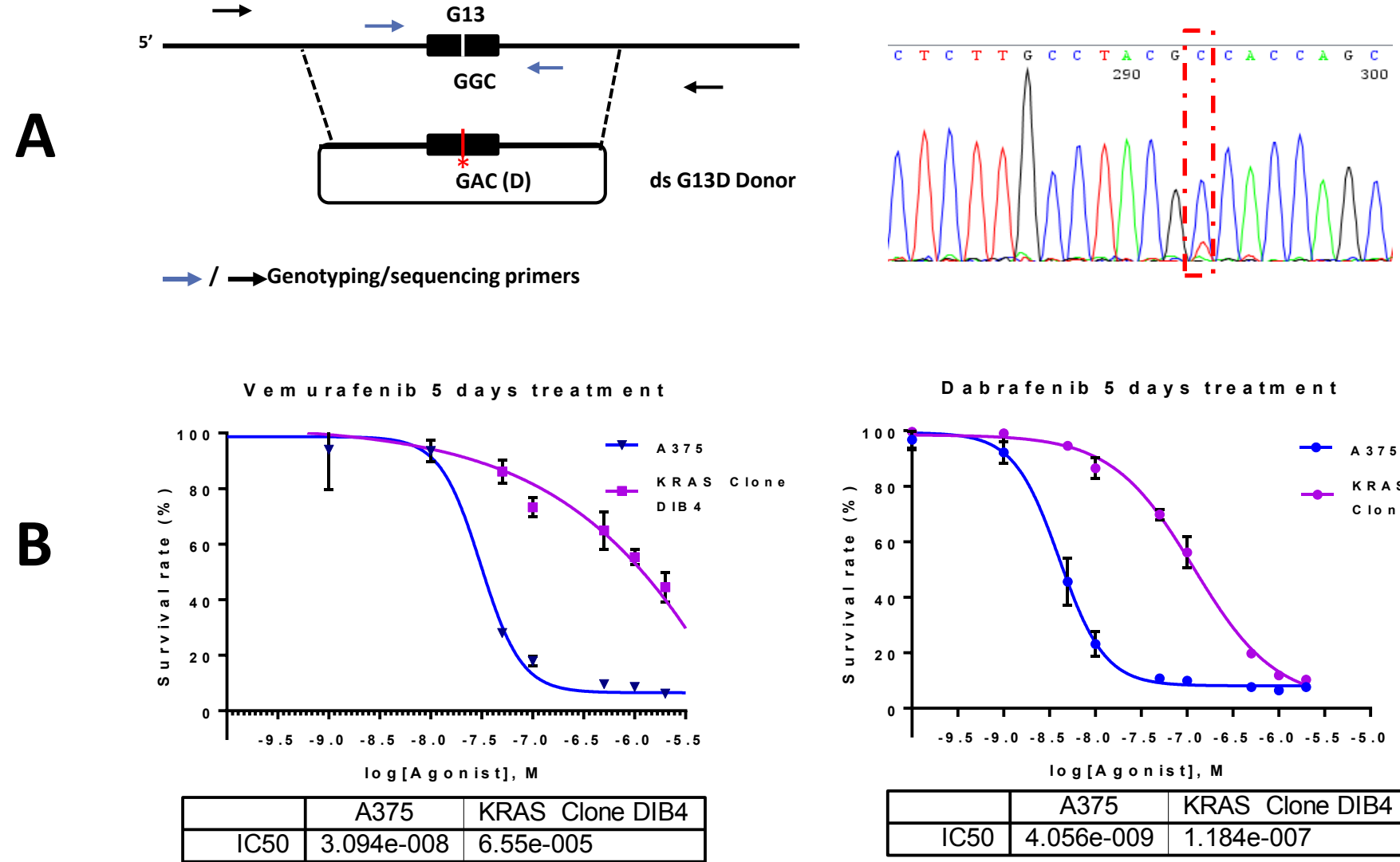


Figure 8. The same method that was used to create the NRAS isogenic line was employed to generate the KRAS^{G13D} isogenic line (ATCC® CRL-1619IG-1™). A) The KRAS^{G13D} knock in was validated by sequencing. B) The KRAS^{G13D} isogenic line demonstrated drug resistance to BRAF inhibitors relative to the parental A375 cell line. Data are expressed as mean ± SD (n=4).

Discussion

We used CRISPR/Cas9 genome editing technology to target endogenous loci in human cells and create precise genetic point mutations. Unlike conventional drug resistance cell models that have been developed through anti-cancer therapy clonal drug resistance selection procedures, the A375 NRAS^{Q61K} and KRAS^{G13D} isogenic cell lines represent advanced *in vitro* cell models that contain defined genetic drug resistance mechanisms. The RAS-mutant isogenic cell lines sustain the permanent and genetically stable drug resistance characteristics without being maintained in anti-cancer therapy selection culture environments. Therefore, they provide valuable tools to facilitate the development of next-generation therapeutics that can overcome BRAF drug resistance in melanoma.

References

- Ran F, *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 8(11): 2281-308, 2013.
- Sanchez-Laorden B, *et al.* BRAF inhibitors induce metastasis in RAS mutant or inhibitor-resistant melanoma cells by reactivating MEK and ERK signaling. *Sci Signal* 7(318): ra30, 2014.

© 2017 American Type Culture Collection. The ATCC trademark and trade name, and any other trademarks listed in this publication are trademarks owned by the American Type Culture Collection unless indicated otherwise.