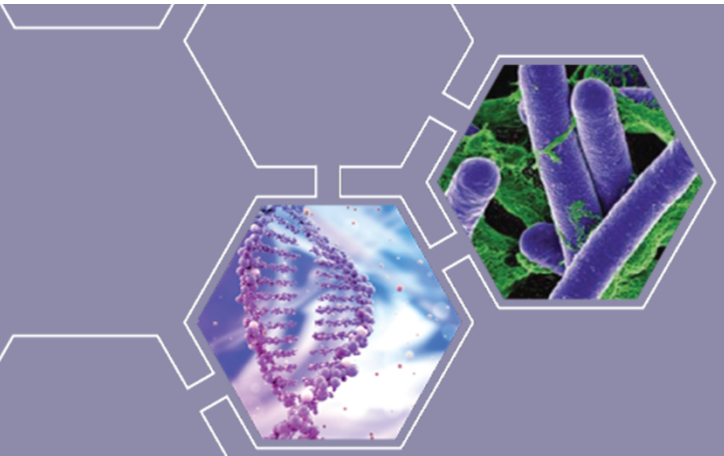
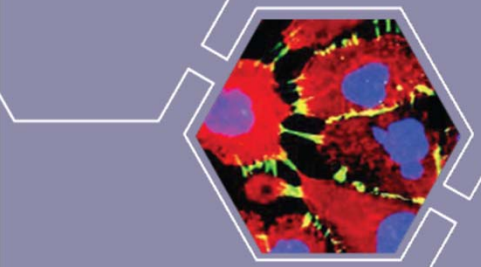




CRISPR/Cas9-Engineered 3D Tissue Culture Models of Drug-Resistant Melanoma

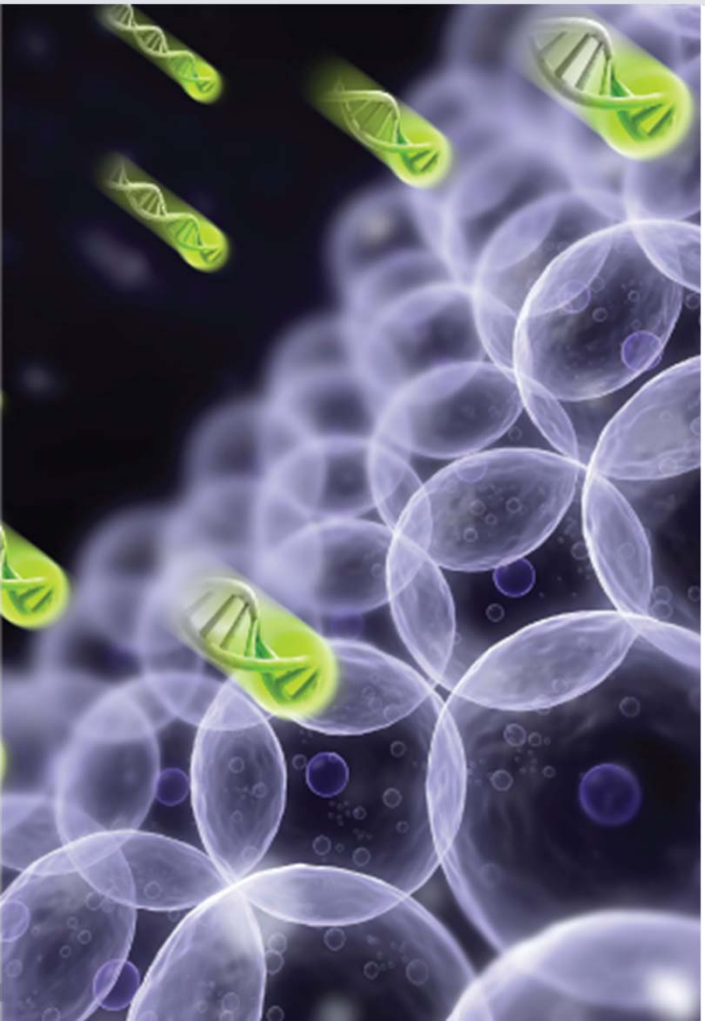
Elizabeth Gillies, Ph.D.
Scientist, ATCC Cell Biology

ATCC®

ATCC overview

- Founded in 1925, ATCC is a non-profit organization with HQ in Manassas, VA, and an R&D & Services center in Gaithersburg, MD
- Worldwide brand name and quality recognition
- World's premiere biological materials resource and standards development organization
  - 4,000 cell lines
  - 70,000 microbes
- ATCC collaborates with and supports the scientific community with industry-standard and innovative biological solutions
  - Growing portfolio of products and services
  - Sales and distribution over 140 countries, 15 International distributors
- Talented team of 475+ employees; > one third with advanced degrees
- Multiple accreditations including ISO 9001 and ISO 17025

Outline



I. Precision genome engineering of new cell-based models for drug discovery

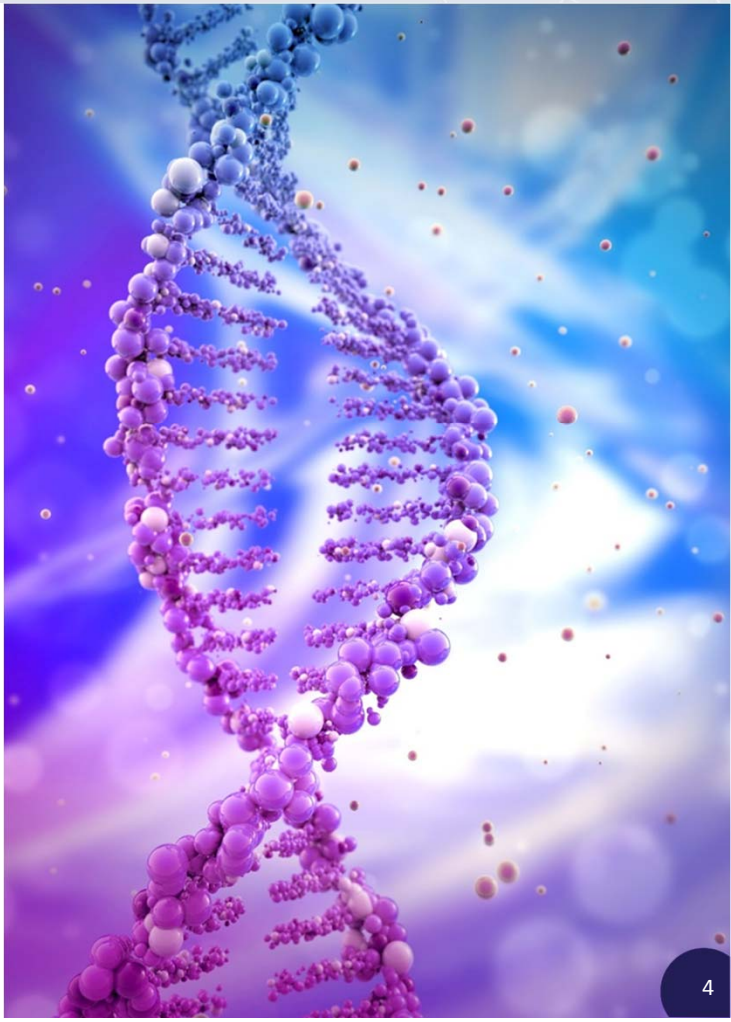
II. A375 drug-resistant melanoma model cell lines – ATCC quality and reproducibility

III. Melanoma model lines 2D/3D tissue culture system

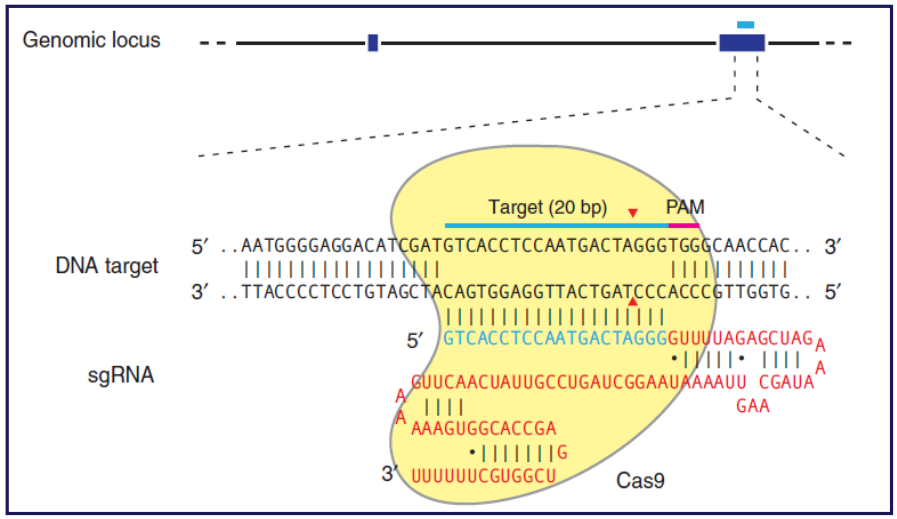
Precision genome engineering of new cell-based models for drug discovery

This section covers:

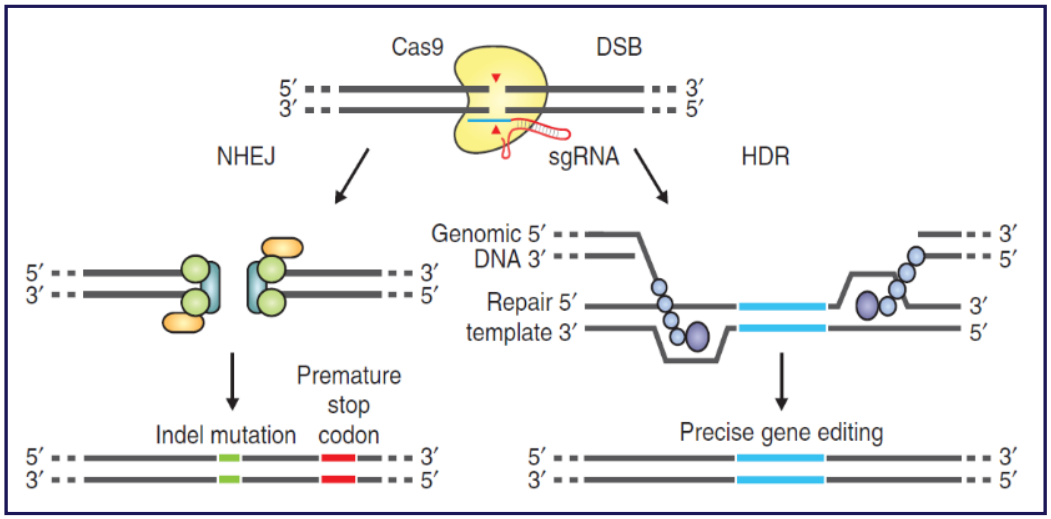
- Precision genome engineering with CRISPR/Cas9
- Applications of CRISPR/Cas9 in drug discovery
- ATCC CRISPR/Cas9 genome editing platform
- Cell-based models of acquired drug resistance
- BRAF mutation in melanoma
- Mechanisms of acquired BRAF inhibitor resistance



Precision gene editing with CRISPR/Cas9



RNA-guided Cas9 endonuclease cuts genomic DNA at a precise genomic locus

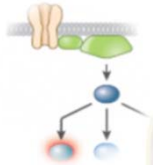


Cellular DNA repair mechanisms repair this damage using Non-Homologous End Joining or Homology Directed Repair

With CRISPR/Cas9, it is now feasible and cost-effective to use human cells as genetically engineered disease model systems

Ran F, *et al*. Nat Protoc 8(11): 2281-308, 2013.

Application of CRISPR/Cas9 in drug discovery



Functional Screening

Generate precision cell models

Generate animal models of human disease

Generate therapeutics

Genome-scale knockout, inhibition, and activation screens

Isogenic cell lines for drug target validation, mechanistic analysis, and drug screening

CRISPR/Cas9 shows promise as a therapeutic agent

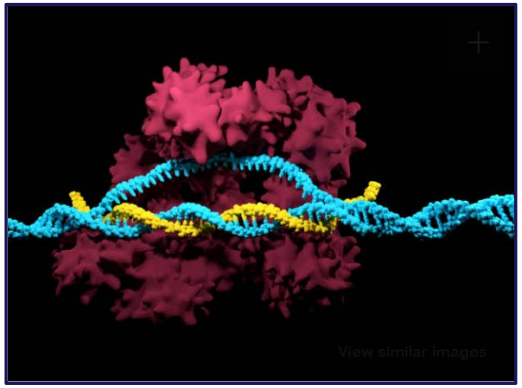
ATCC CRISPR/Cas9 gene-editing platform

Cell Biology

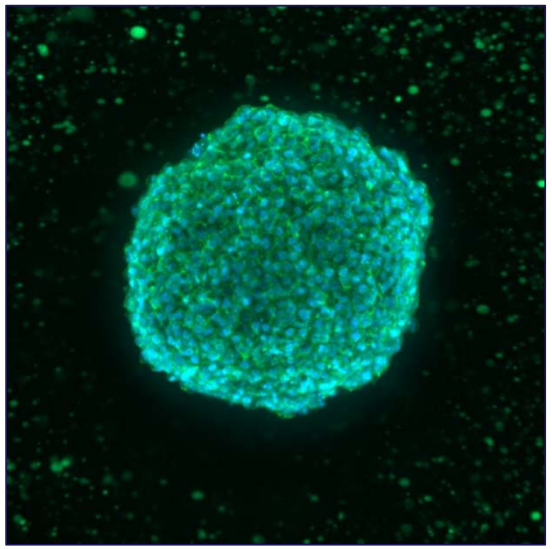
- ✓ Cell banking
- ✓ Cell line authentication
- ✓ Modification of extant lines
- ✓ Single cell cloning
- ✓ Phenotype validation

Molecular Biology

- ✓ CRISPR reagent design
- ✓ Expression vector toolbox
- ✓ Molecular cloning
- ✓ ddPCR™ and qPCR
- ✓ Sanger and next-gen sequencing

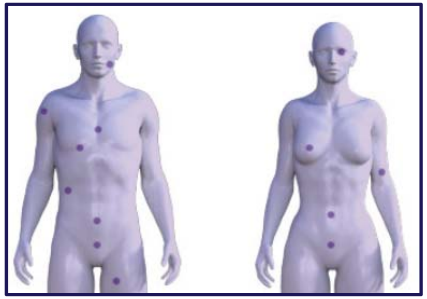


ATCC® CRISPR Engineering Platform



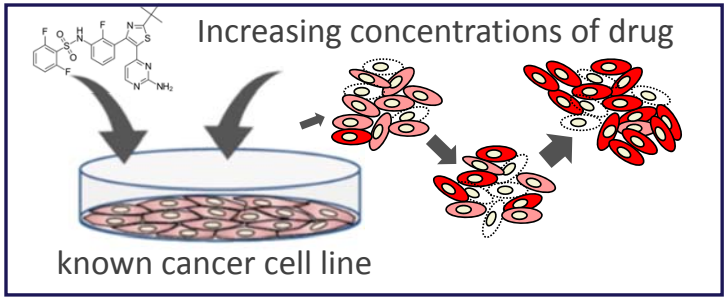
New cell-based models of human disease

Cell-based models of acquired drug resistance



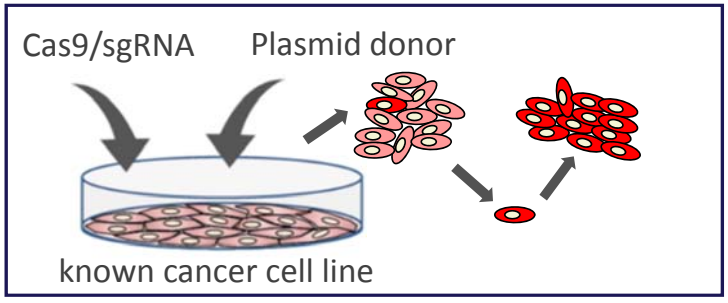
Isolation of resistant cells from clinical tumor samples

- Relatively easy to isolate
- Not time intensive
- New line is uncharacterized
- Heterogeneous mix of cells
- No control cell line



Progressive dosing of known cancer cell line

- Can take up to 18 months
- Long-term drug pressure causes spurious mutations
- Accumulation of spurious mutations makes parental line a poor control
- Heterogeneous mix of cells
- Constant drug pressure required to maintain drug-resistance phenotype

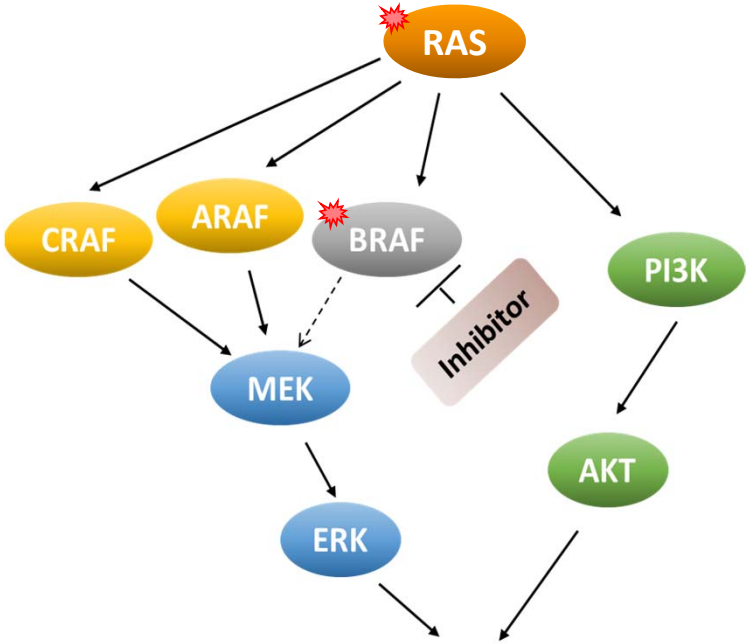


CRISPR/Cas9 genome editing

- Precise gene-editing method
- Homogeneous cell population
- Parental cell line is an excellent control
- Defined drug-resistance mechanism
- No drug pressure required during routine cell culture
- Stable resistance phenotype
- Highly reproducible results

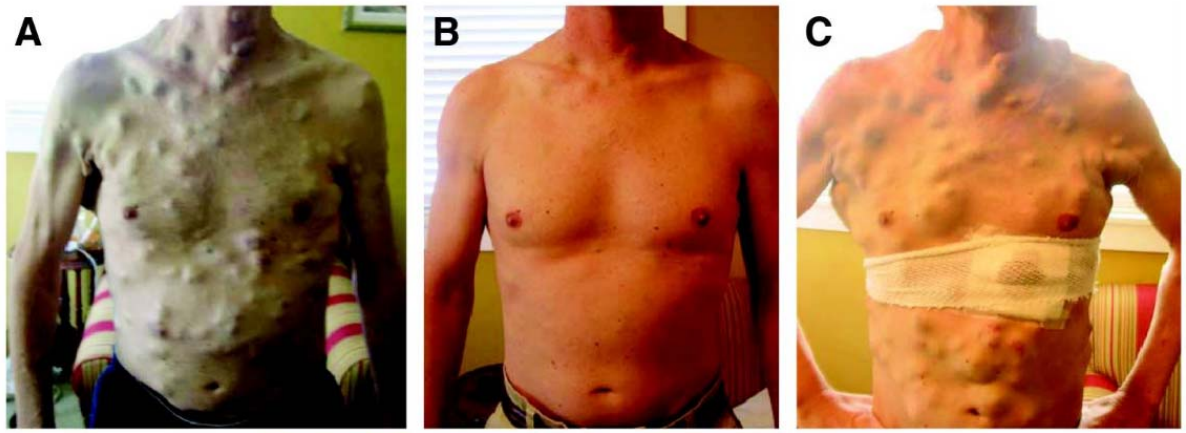
BRAF mutation in melanoma

Ras/Raf/MEK/ERK MAP kinase signaling pathway



Cell survival and proliferation

50% of melanomas carry an activating BRAF mutation and are sensitive to BRAF inhibitors



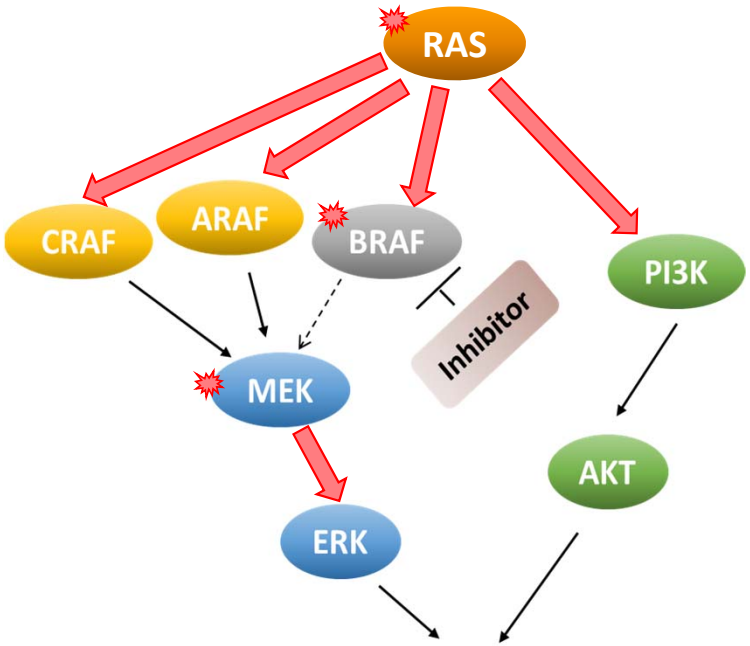
Week 1 **Week 15** **Week 23**

However, BRAF inhibitor resistance can develop after several months of treatment, resulting in tumor regrowth

Reproduced from Wagle N, *et al.* J Clin Oncol 29(22): 3085-3096, 2011

Mechanisms of acquired BRAF inhibitor resistance

Secondary mutations bypass BRAF Inhibition



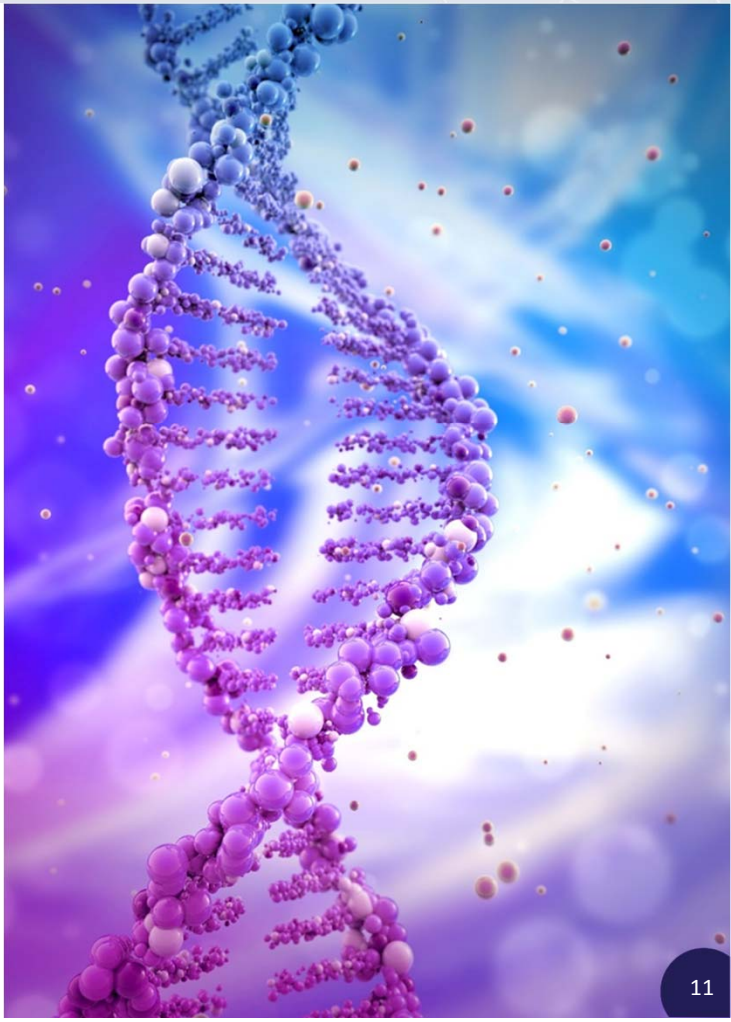
Treatment with BRAF inhibitors drives acquired BRAF inhibitor resistance

- Continued BRAF-inhibitor treatment frequently leads to secondary activating mutations in the Ras/Raf/MEK/ERK MAP kinase signaling pathway
- Secondary mutations bypass BRAF inhibition, resulting in:
  - BRAF inhibitor resistance
  - Cancer progression
  - Poor clinical outcomes
- Chemotherapeutics and treatment regimens do not address melanomas with acquired-inhibitor resistance
- Development of new drugs and combination therapies is hindered by the lack of well-controlled and physiologically relevant cell-based models

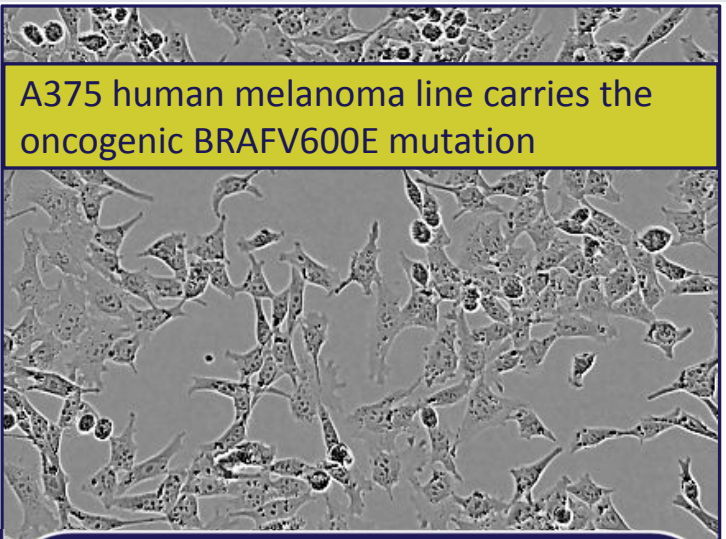
A375 drug-resistant melanoma model cell lines – ATCC quality and reproducibility

This section covers:

- Use of CRISPR/Cas9 to create isogenic drug-resistant melanoma model cell lines
- ATCC drug-resistant isogenic melanoma model cell system
- Genome- and transcript-level validation of melanoma model lines
- Off-target cut and Cas9 integration of melanoma model lines
- Functional validation of isogenic melanoma model drug resistance



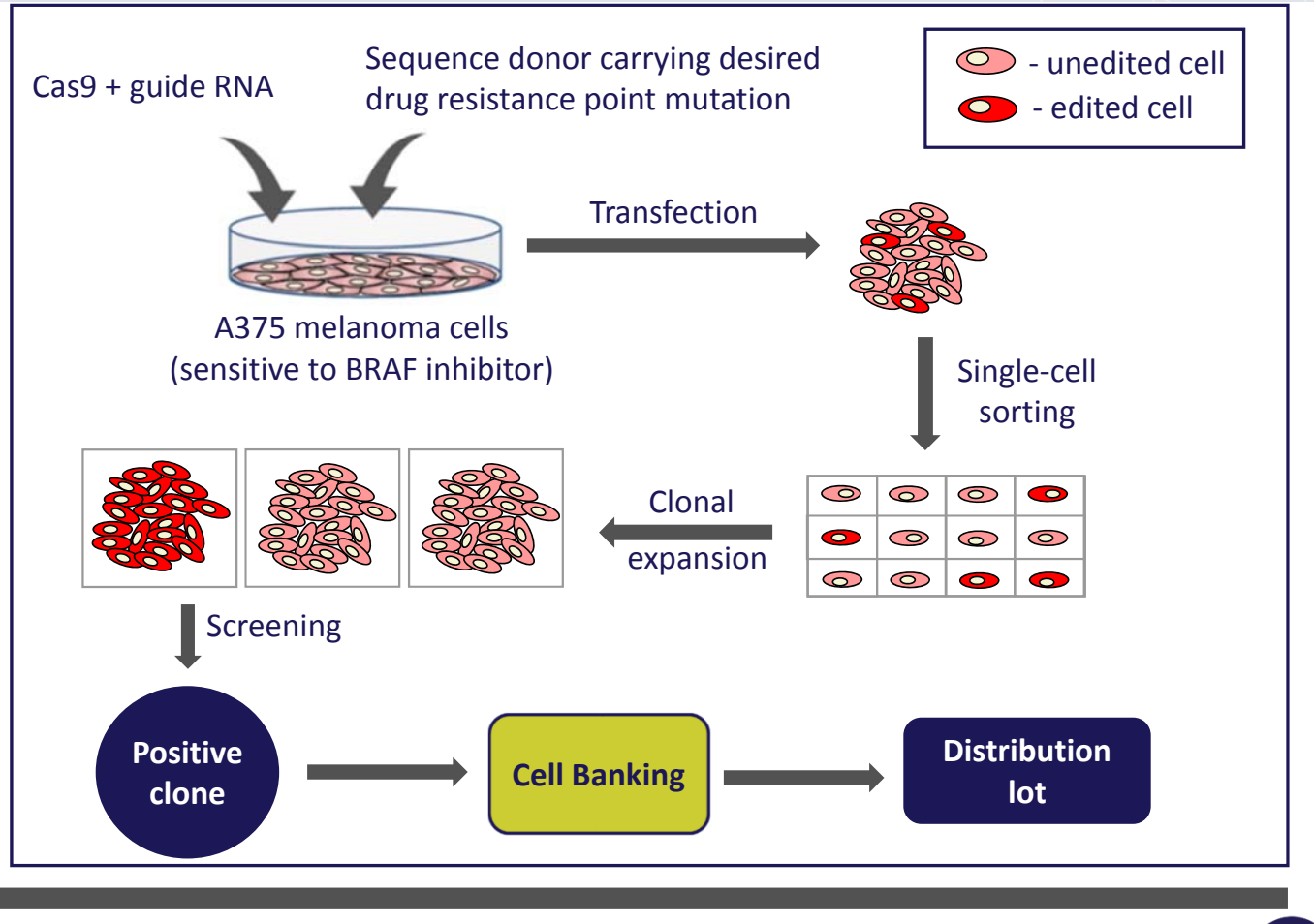
Use of CRISPR/Cas9 to create isogenic melanoma model cell lines



A375 human melanoma line carries the oncogenic BRAFV600E mutation

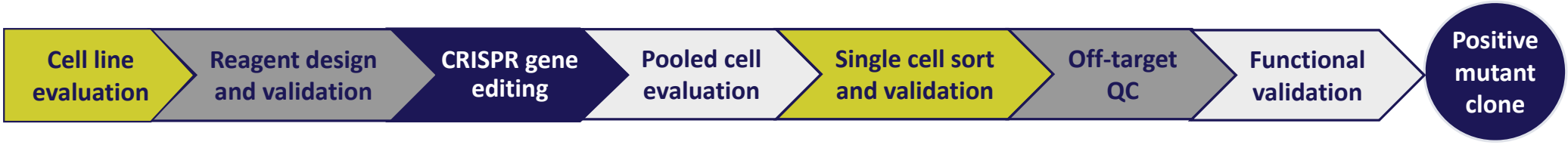
ATCC Quality and Standards

- Functionally validated
- Cell line purity and sterility confirmed
- Species identity verified
- Post-freeze viability verified
- Target site genotyped

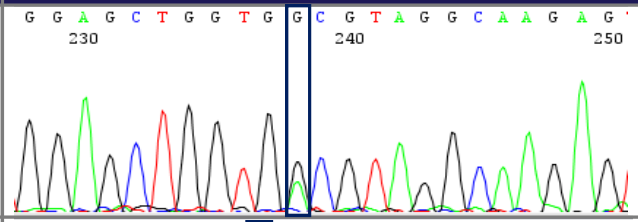
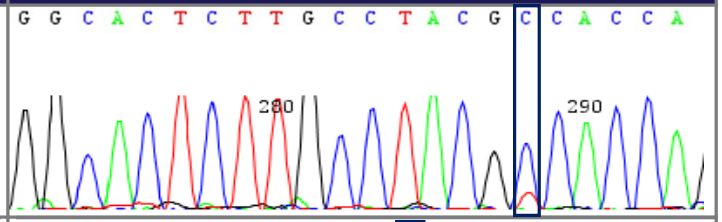
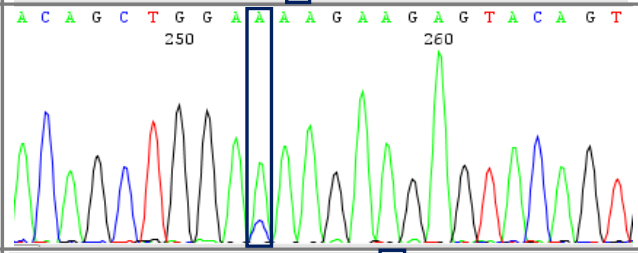
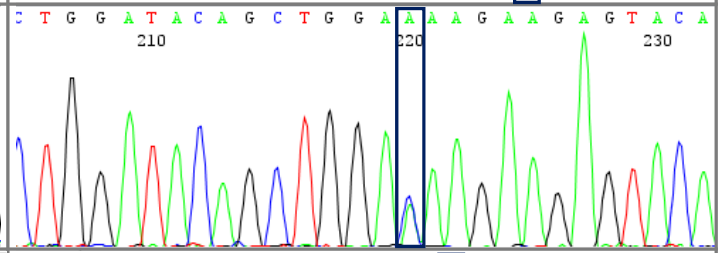
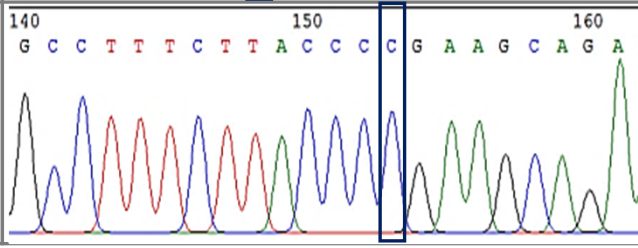
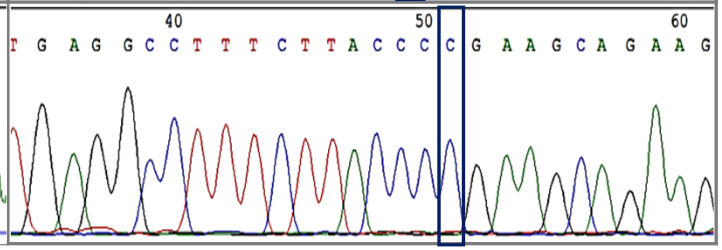


Drug-resistant isogenic melanoma model cell system

Cell Line Name	ATCC® No.	BRAF V600E	Engineered Mutation	Engineered Genotype	BRAF Inhibitor Resistance	MEK Inhibitor Resistance	3D Functional Validation
Unedited A375	CRL-1619™	+	N/A	N/A	−	−	+
KRAS Mutant-A375 Isogenic	CRL-1619IG-1™	+	KRAS G13D	heterozygous	+	−	+
NRAS Mutant-A375 Isogenic	CRL-1619IG-2™	+	NRAS Q61K	heterozygous	+	−	+
MEK1 Mutant-A375 Isogenic	CRL-1619IG-3™	+	MEK1 Q56P	homozygous	+	+	+



Genome and transcript-level validation of melanoma model lines

Cell Line Name	Engineered Genotype	Target Site Genome Sequence	Transcript Sequence of Target Gene
KRAS Mutant-A375 Isogenic	KRAS G13D heterozygous		
NRAS Mutant-A375 Isogenic	NRAS Q61K heterozygous		
MEK1 Mutant-A375 Isogenic	MEK1 Q56P homozygous		

Isogenic lines are screened for off-target cut and Cas9 integration

MEK1 mutant A375 isogenic off-target cut screening results

Genomic Coordinates	Strand	MM	Target Sequence	PAM	Nearest Gene	Location	MEK1 Mutant A375 Isogenic
chr15:66434832-66434854	+	0	CATGTTGG[TGATAGTCATCC]	CGG	MAP2K1	target site	N/A
chr1:10853864-10853886	-	3	AATGATGG[TGACAGTCATCC]	TGG	HSPE1P24	intergenic	PASSED
chr11:108532803-108532825	-	4	CAAGTATG[AGATAGTCATCC]	AGG	EXPH5	intronic	PASSED
chr19:5663907-5663929	+	4	ACTCTTGG[TGAAAGTCATCC]	TGG	SAFB	intronic	PASSED
chr3:185058989-185059011	-	4	CTTTTTGA[TCATAGTCATCC]	TGG	VPS8	intergenic	PASSED
chr22:29816431-29816453	+	3	CAAGTTGG[AGTTAGTCATCC]	AGG	ASCC2	intronic	PASSED
chr8:137426391-137426413	-	4	GATAATGG[TGACAGTCATCC]	AGG	ZYXP1	intergenic	PASSED
chr15:29974505-29974527	-	4	CATTTTCT[TAATAGTCATCC]	CGG	TJP1	intergenic	PASSED
chr2:86435176-86435198	-	3	CATGTTTT[TGAGAGTCATCC]	AGG	KDM3A	intergenic	PASSED
chr20:4745349-4745371	-	3	AATGTTGG[TGTCAGTCATCC]	TGG	PRNT	intergenic	PASSED
chr3:112605446-112605468	+	4	CATGATGA[CGGTAGTCATCC]	TGG	CCDC80	exonic	PASSED

```
>Reference genome sequence
>Unmodified A375 OT1 HSPE1P24 – intergenic
>MEK1 Mutant A375 OT1 HSPE1P24 – intergenic
>OT1 HSPE1P24 – intergenic
                *         *         *         *         *         *         *         *         *         *
185>ACCATCACTACCATCACCAGGATGACTGTCACCATCATTATCACTGCCATCATTACCACCATAATCATCACCATCTATCACTACCACTATTGTTATCACC>284
600>ACCATCACTACCATCACCAGGATGACTGTCACCATCATTATCACTGCCATCATTACCACCATAATCATCACCATCTATCACTACCACTATTGTTATCACC>699
601>ACCATCACTACCATCACCAGGATGACTGTCACCATCATTATCACTGCCATCATTACCACCATAATCATCACCATCTATCACTACCACTATTGTTATCACC>700
  1>~~~~~~~~~~~~~~~~~~~~~GGATGACTGTCACCATCATT~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>20
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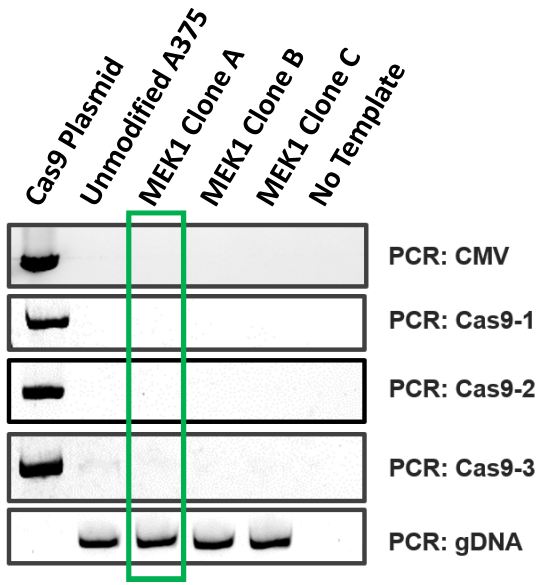
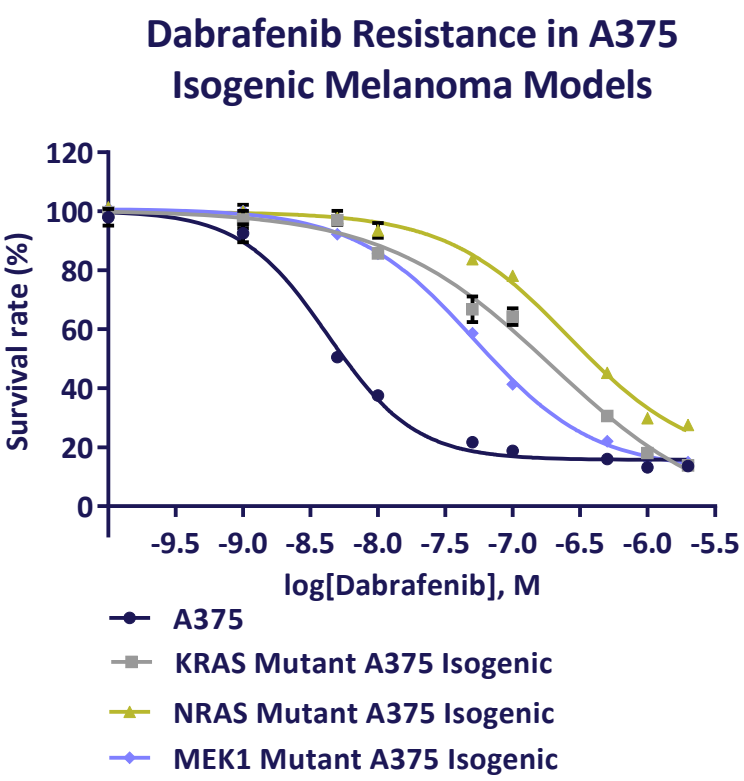
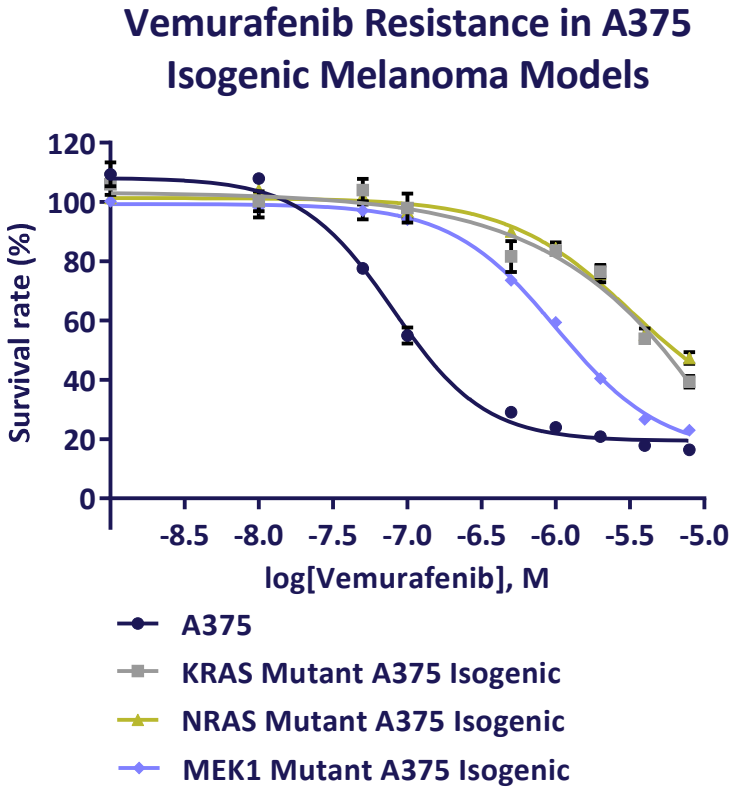


MEK1 mutant A375 isogenic line plasmid integration screening

BRAF inhibitor resistance in melanoma model lines

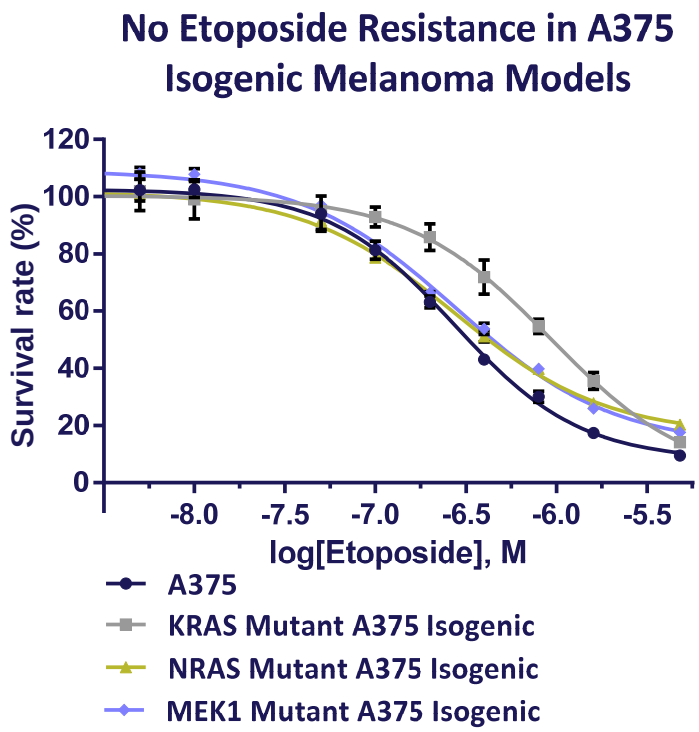
2D functional validation

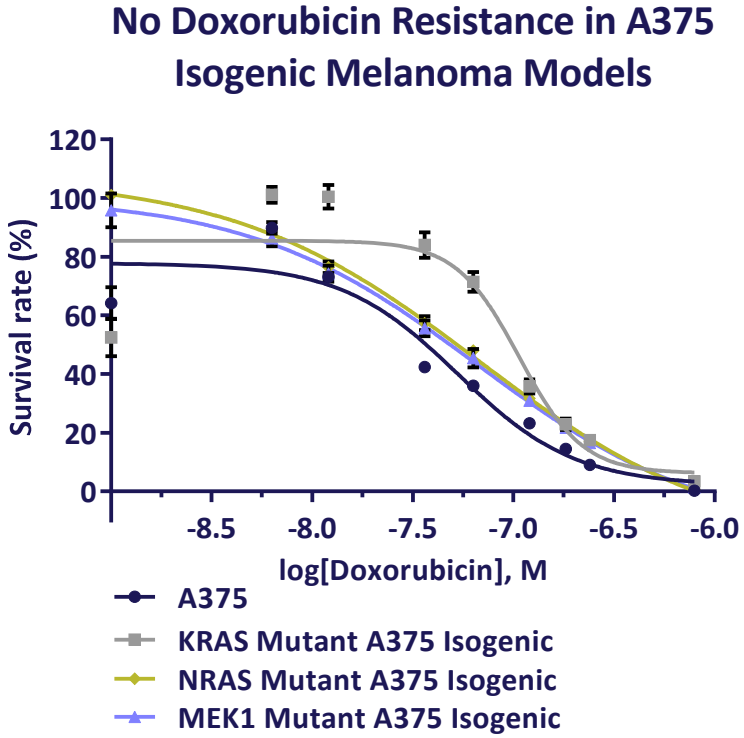




No resistance to nonspecific chemotherapeutics in melanoma model lines

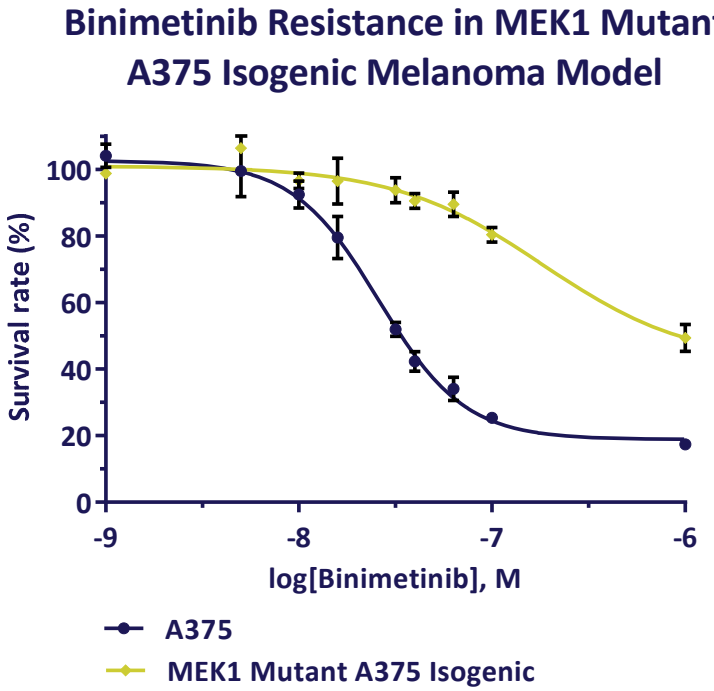
2D functional validation

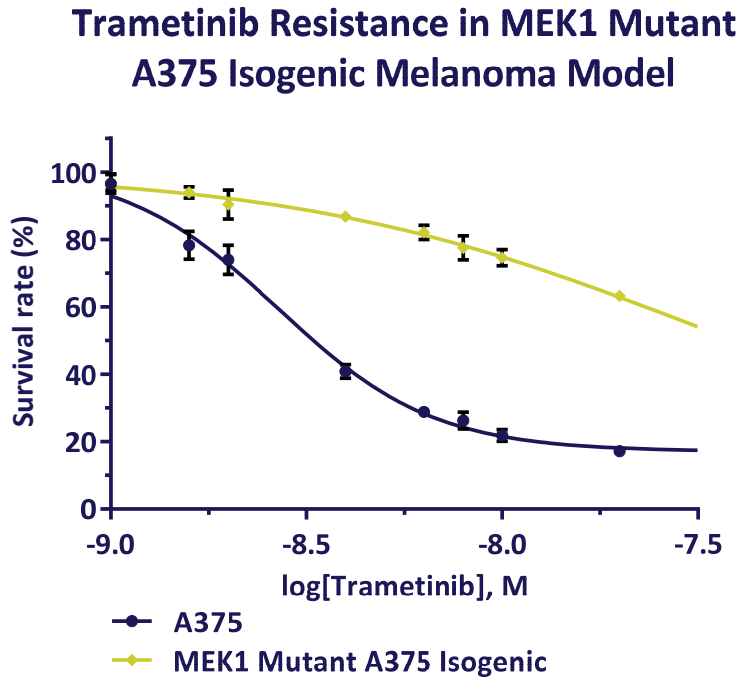




MEK inhibitor resistance in the MEK1 isogenic melanoma model line

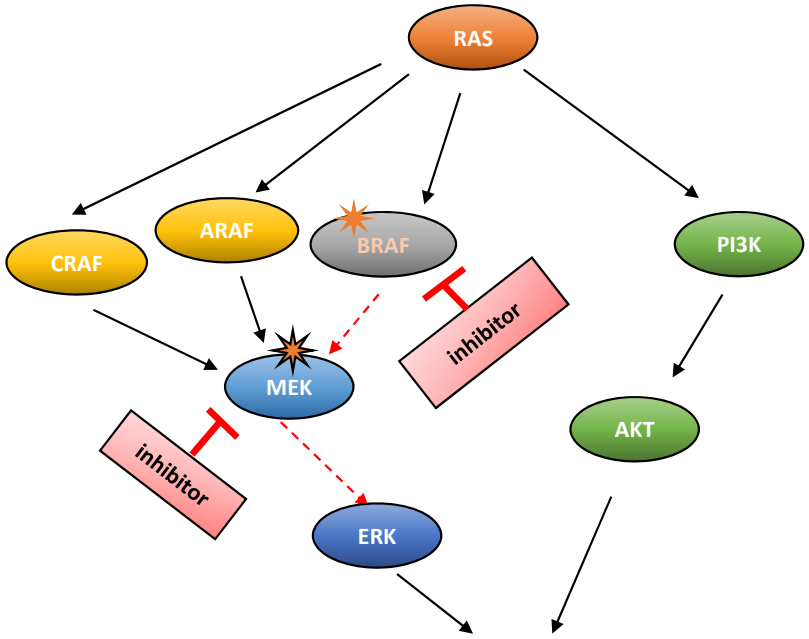
2D functional validation





Combination inhibitor treatment in drug-resistant MEK1 mutant-A375 isogenic cell line

Two-target MAP kinase pathway inhibition in multidrug-resistant MEK1 melanoma model



Cell proliferation, cell survival

Primary Mutation BRAF V600E

Secondary Mutation MEK1 Q56P

Normal signaling

Inhibited signaling

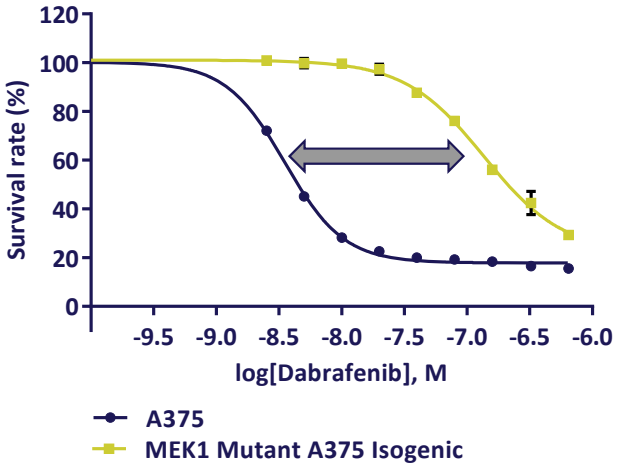
Benefits of Combination Drug Treatment

- Lower doses required
- Reduced side effects
- Improved clinical outcomes

½ dose BRAF inhibitor + ½ dose MEK inhibitor = synergistic pathway inhibition

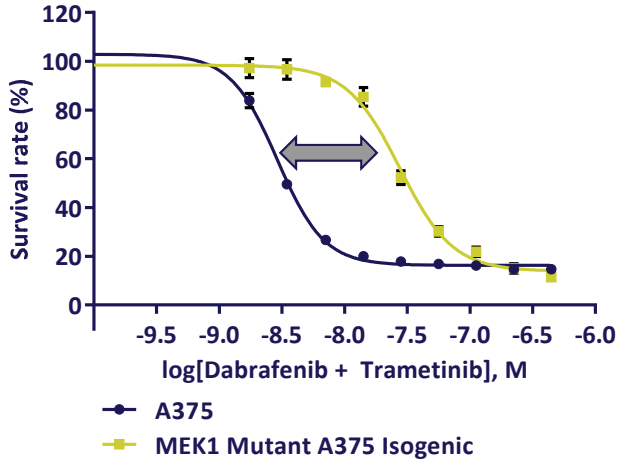
MEK1 mutant-A375 isogenic cell line is sensitive to combination MEK/BRAF inhibitors

Dabrafenib Resistance in MEK1 Mutant A375 Isogenic Melanoma Model



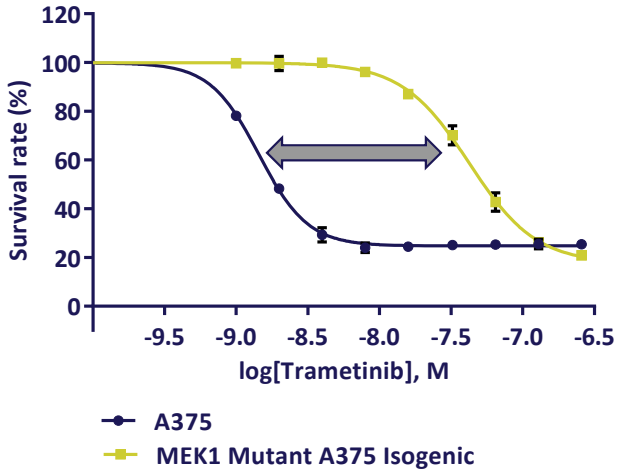
BRAF inhibitor dose-response

Combination MEK + BRAF Inhibitor Treatment Sensitivity in MEK Mutant A375 Isogenic Line



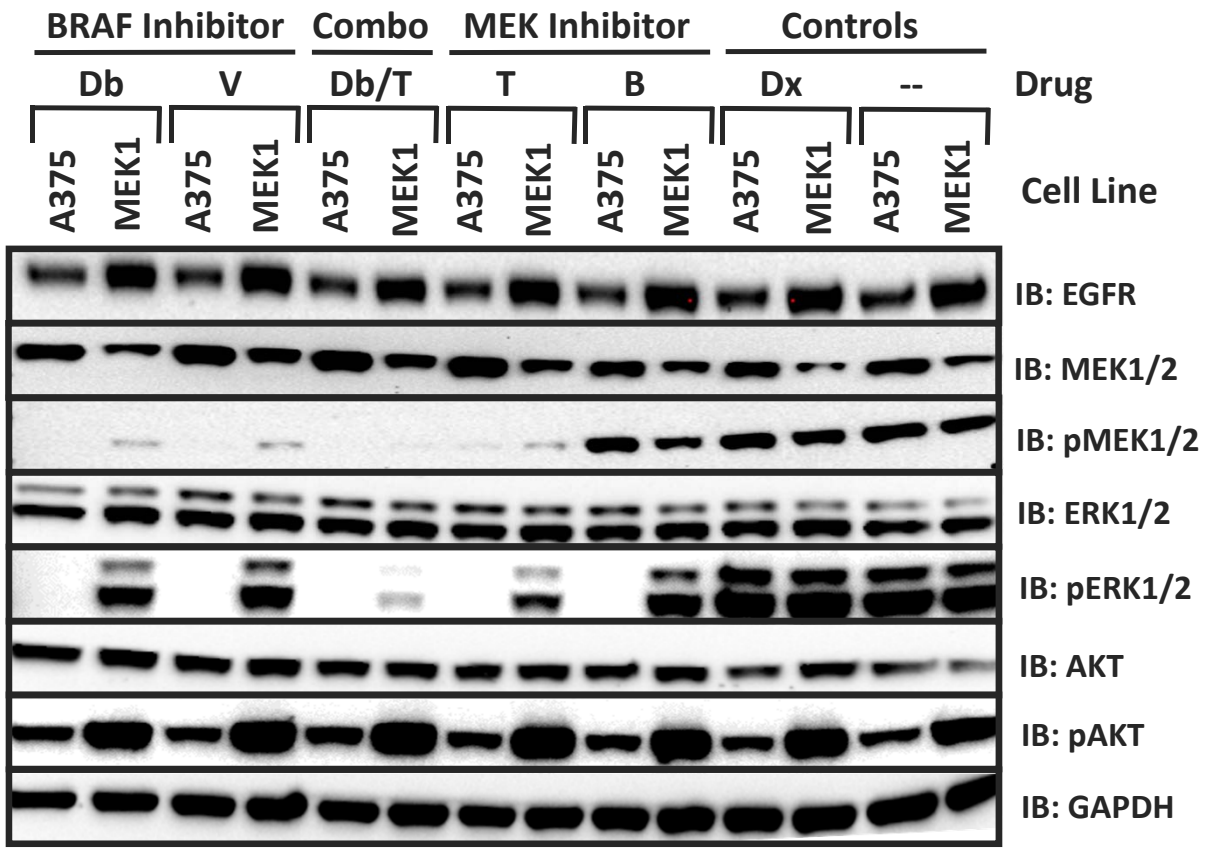
Combination inhibitor dose-response

Trametinib Resistance in MEK1 Mutant A375 Isogenic Melanoma Model



MEK inhibitor dose-response

MAP kinase signaling in MEK1 mutant-A375 isogenic cell line



Cell Lines Tested

A375 - original A375 melanoma cell line

MEK1 - MEK1 Mutant-A375 Isogenic Line

Drug Key

Db - Dabrafenib 1uM

V - Vemurafenib 2uM

Db/T - 0.5uM each

T - Trametinib 1uM

B - Binimetinib 2uM

Dx - Doxorubicin 2uM

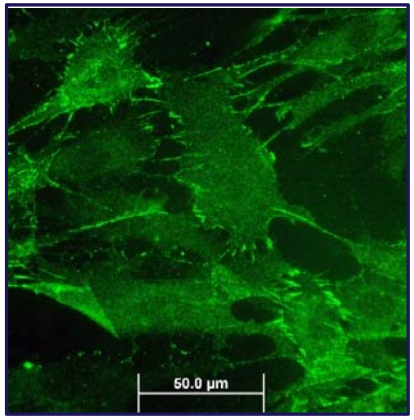
-- - DMSO control

Melanoma model lines 2D/3D tissue culture system

This section covers:

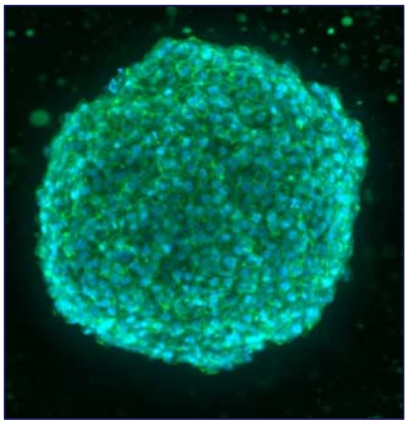
- Model systems for drug screening and validation
- Drug resistant 2D/3D melanoma model cell system
- Melanoma model lines 3D spheroid formation
- Functional validation of 3D tissue culture drug-resistant model melanoma models
- Automated analysis of 3D melanoma model drug response

Model systems for drug screening and validation



2D Tissue Culture

- Human host system
- Least time intensive, lowest cost
- Simple automated assay readout
- Lowest system complexity
- Highest clinical trial failure rate



3D Tissue Culture

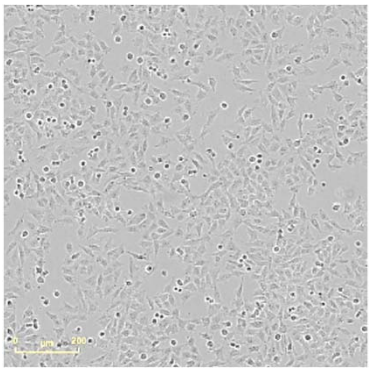
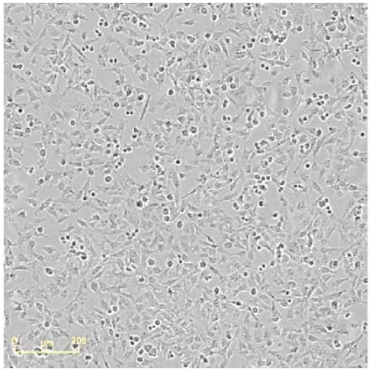
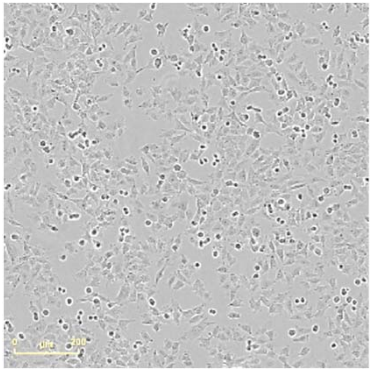
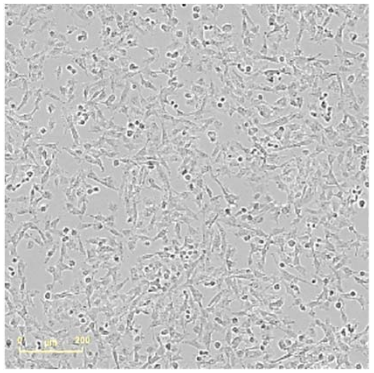
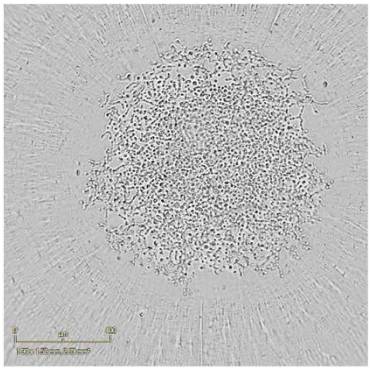
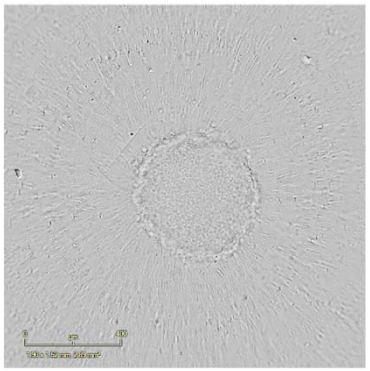
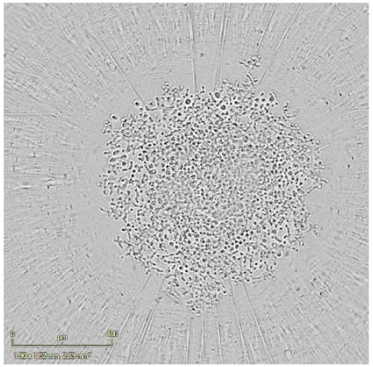
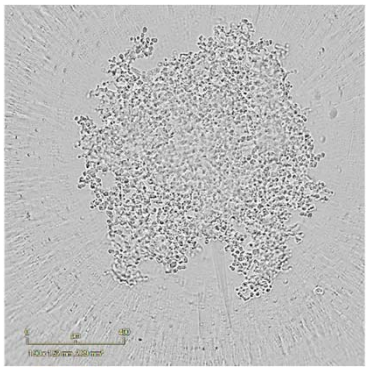
- Human host system
- Higher system complexity
- Potential for lower clinical trial failure rate
- Slightly increased time and cost
- More complex automated assay readout

Animal Model

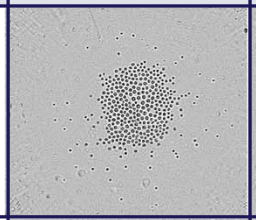
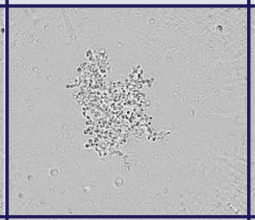
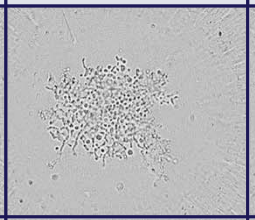
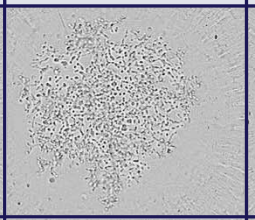
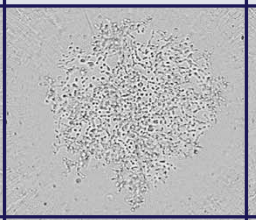
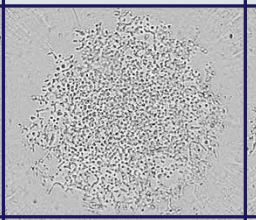
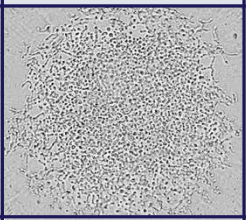
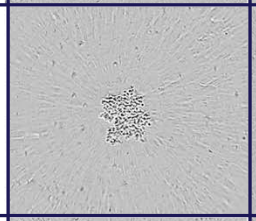
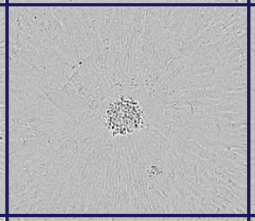
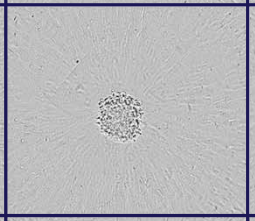
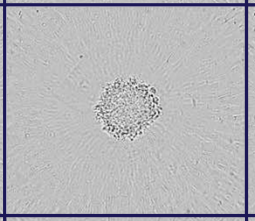
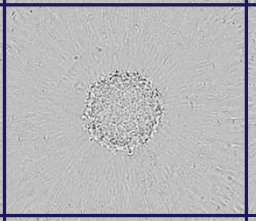
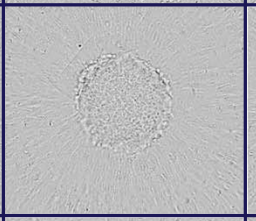
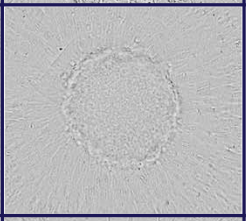
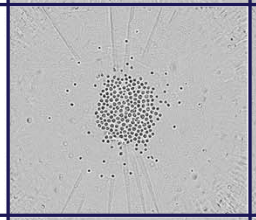
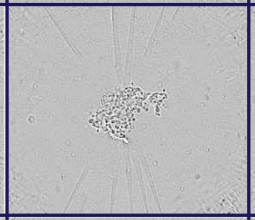
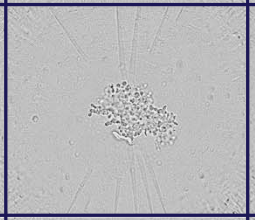
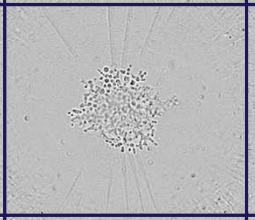
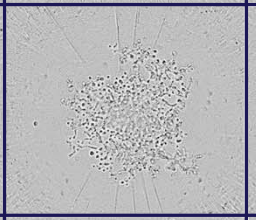
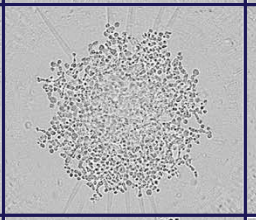
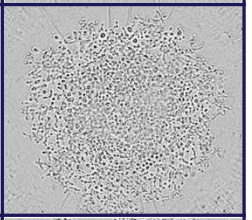
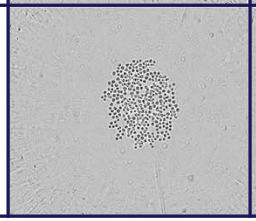
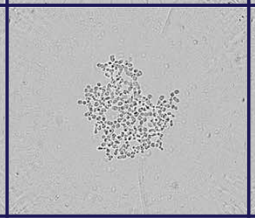
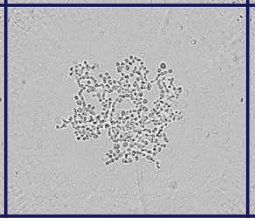
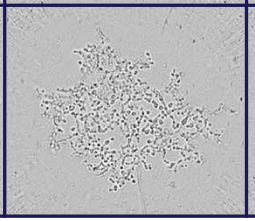
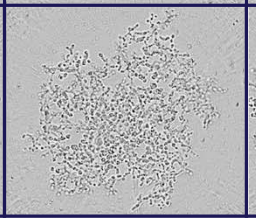
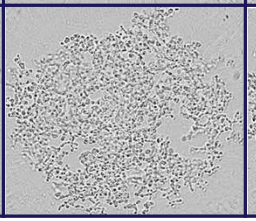
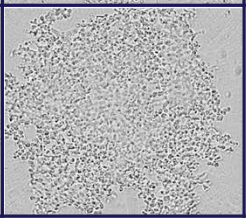
- Highest system complexity
- Lowest clinical trial failure rate
- Most time intensive, highest cost
- Results can be difficult to interpret
- Non-human model system

All of our isogenic melanoma model cell lines have been functionally validated in both 2D and 3D tissue culture

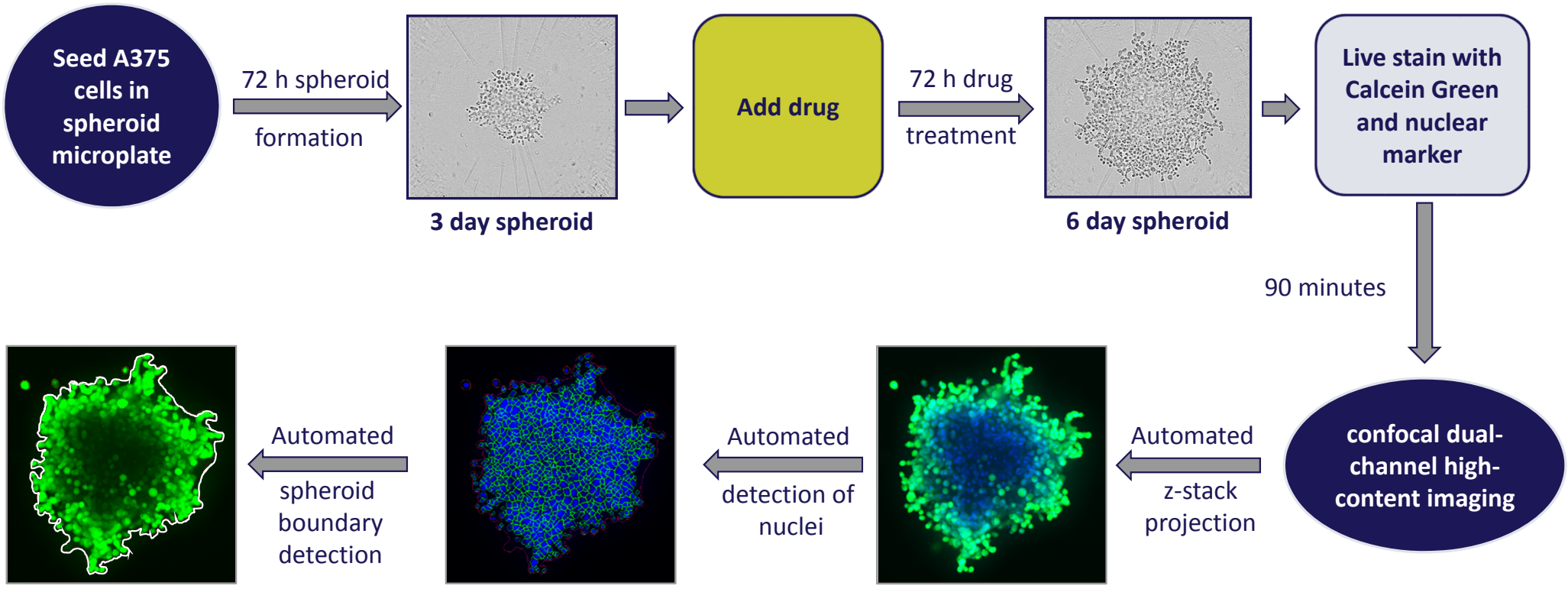
Drug-resistant isogenic 2D/3D melanoma model cell system

	Unmodified A375	KRAS Mutant-A375 Isogenic	NRAS Mutant-A375 Isogenic	MEK1 Mutant-A375 Isogenic
2D Monolayer				
3D Spheroid				

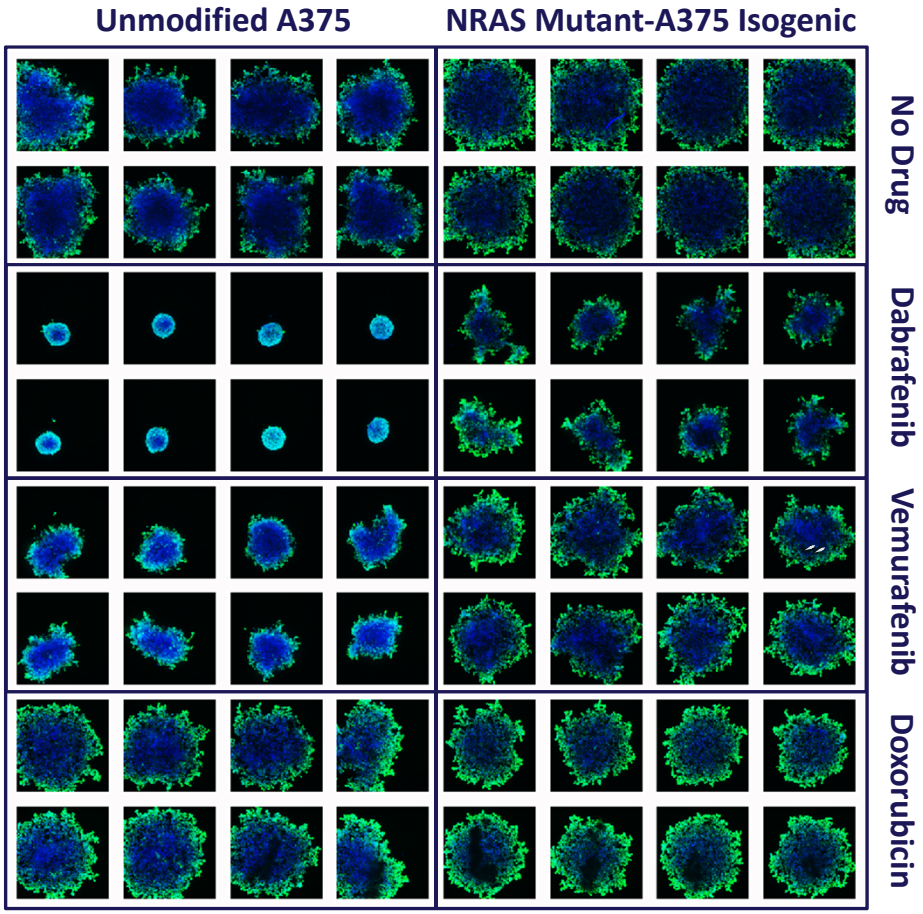
3D spheroid formation in melanoma model lines

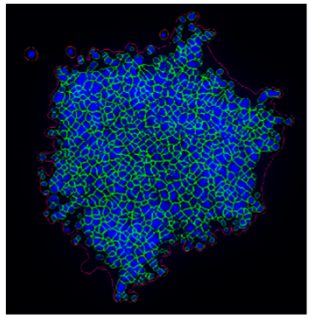
Cell Line	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Unmodified A375							
KRAS Mutant-A375 Isogenic							
NRAS Mutant-A375 Isogenic							
MEK1 Mutant-A375 Isogenic							

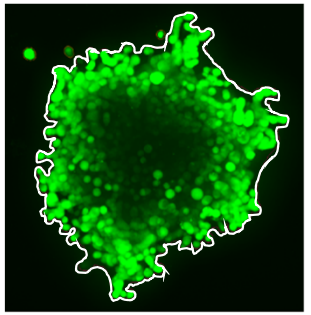
3D functional validation of melanoma model lines



Automated analysis of 3D spheroid drug response







Nuclei count

Spheroid size + stain intensity

Raw data

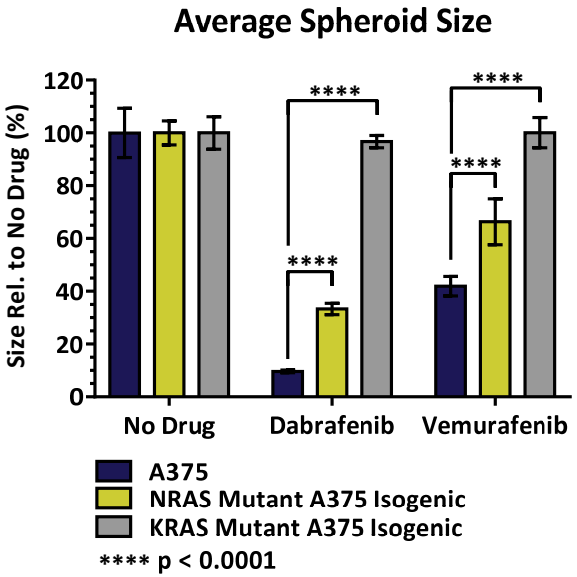
Normalize to un-drugged condition

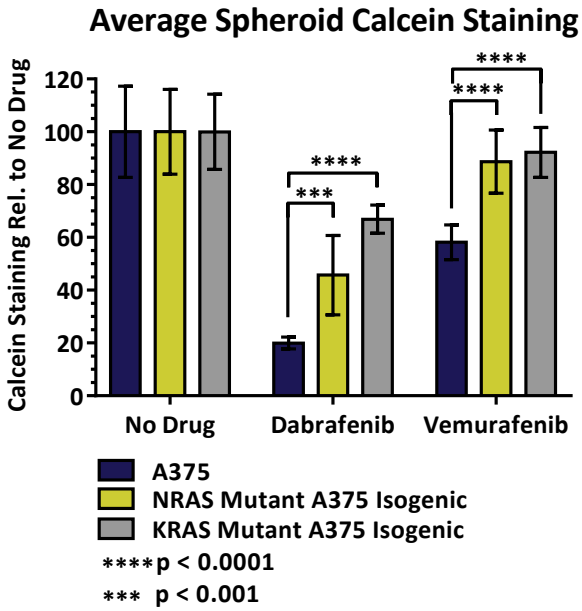
Average replicates

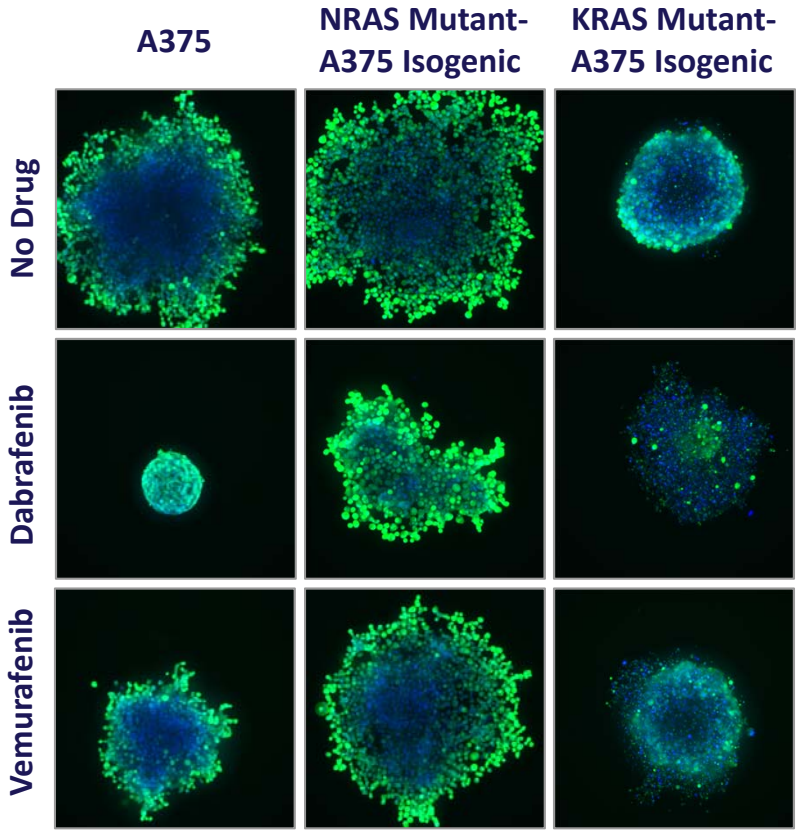
Final dataset

BRAF inhibitor resistance in NRAS- and KRAS-mutant A375 isogenic melanoma model lines

3D functional validation

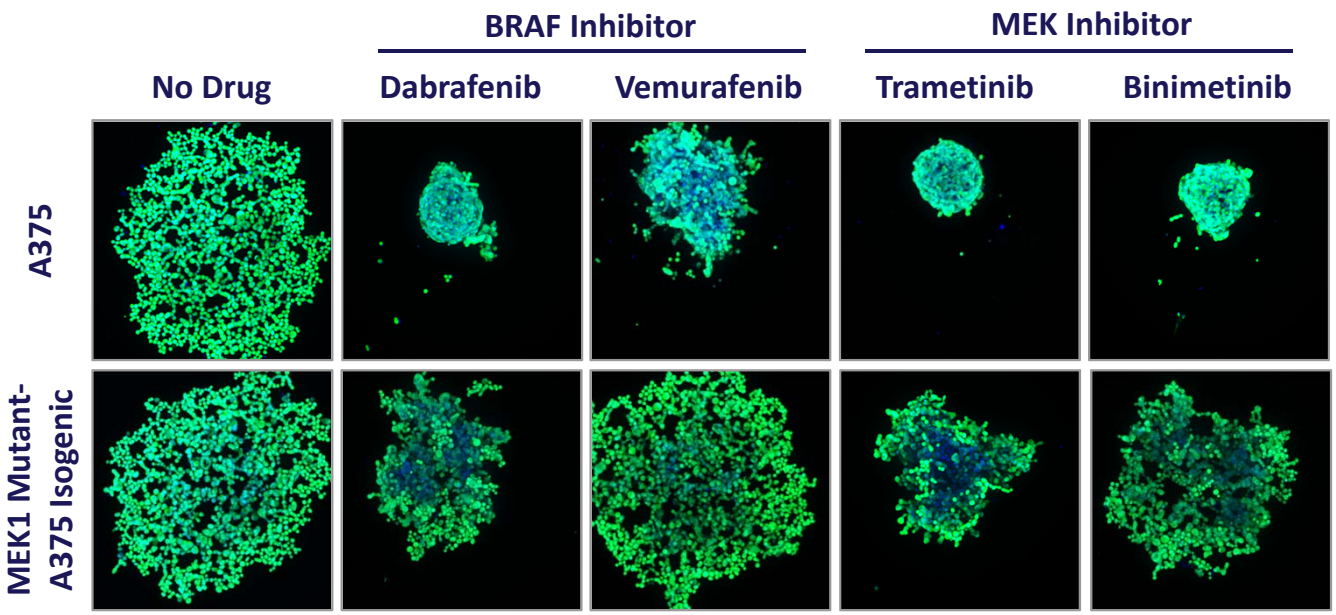


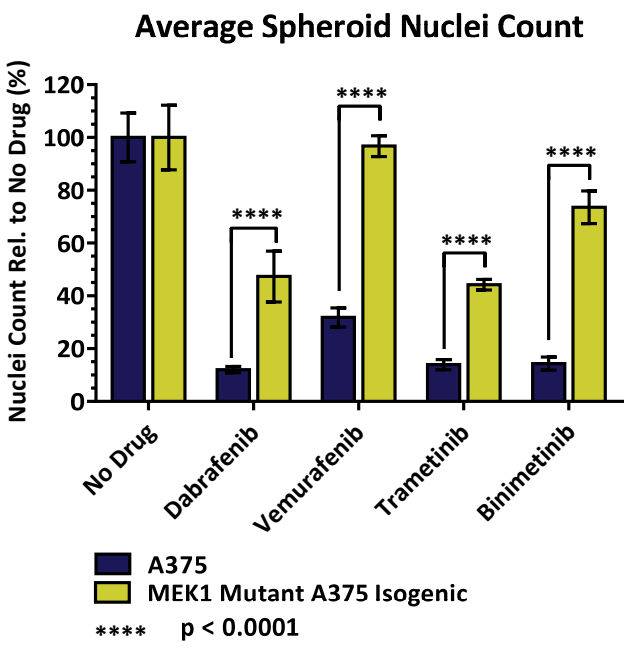




MEK and BRAF inhibitor resistance in MEK1-mutant A375 isogenic cell line

3D functional validation





Key points

CRISPR/Cas9 engineering of cell-based models for drug discovery	▪ Applications of CRISPR/Cas9 in drug discovery ▪ ATCC CRISPR/Cas9 genome editing platform ▪ Cell-based models of acquired drug resistance ▪ Mechanisms of acquired inhibitor resistance
Precision engineered models of inhibitor-resistant melanoma	▪ Use of CRISPR/Cas9 to create isogenic drug-resistant melanoma model cell lines ▪ ATCC drug-resistant 2D/3D isogenic melanoma model cell system
Screening and functional validation of isogenic A375 melanoma models	▪ Genome and transcript level validation of melanoma model lines ▪ Off-target cut and Cas9 integration of melanoma model lines ▪ Functional validation of isogenic melanoma model drug resistance
Automated analysis of drug response in 3D A375 isogenic melanoma models	▪ Model systems for drug screening and validation ▪ Melanoma model lines 3D spheroid formation ▪ 3D tissue culture drug resistant model functional validation ▪ Automated analysis of 3D melanoma model drug response

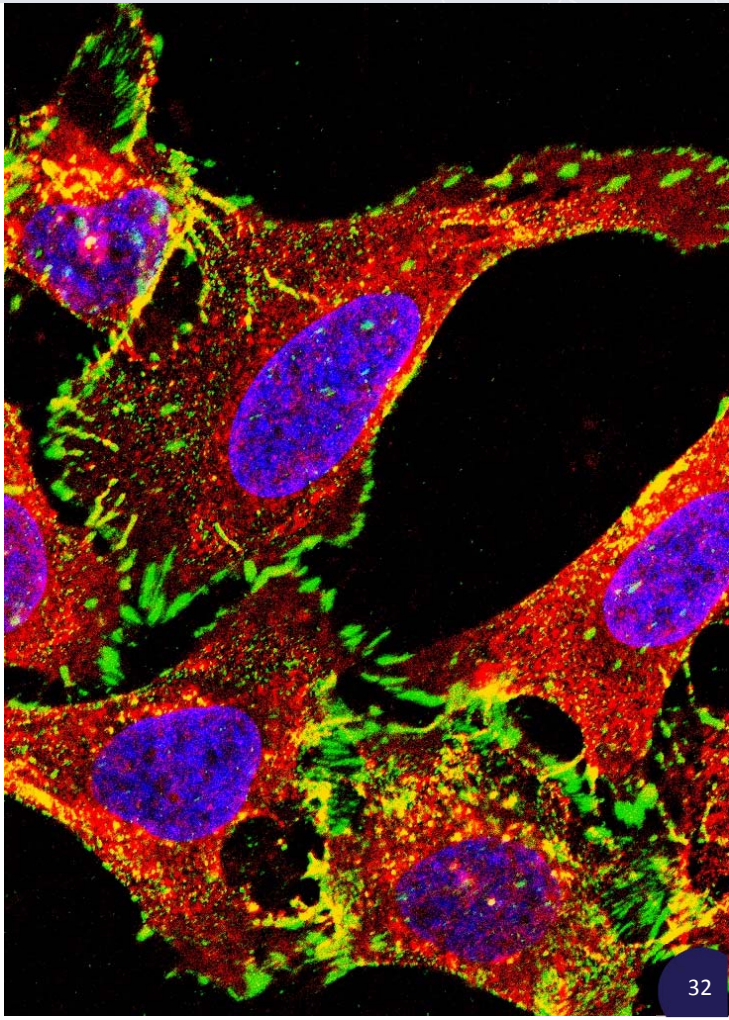
Conclusions: Key Features of Engineered Melanoma Models

Clinically relevant cancer cell models are critical both for studies of molecular and cellular mechanisms of tumorigenesis and for the design and screening of novel cancer therapeutics. With new genome editing tools such as CRISPR/Cas9, ATCC can now use its extensive cell-banking resources to generate novel isogenic disease model cell lines. We have engineered isogenic lines with mutations in key oncogenes that are ideally suited for the identification of novel, personalized treatment regimens.

Key Features of ATCC CRISPR/Cas9 engineered isogenic melanoma model cell lines:

- Parental line is carefully selected for disease and drug-target relevance. Parental line is well characterized.
- Precisely edited isogenic cell lines have been thoroughly validated at genomic, transcript, protein, and cellular bio-functional levels.
- Additional bio-functional characterization with specific inhibitors has been performed for isogenic melanoma model lines in both 2D and 3D tissue culture.
- When used together with authenticated parental line, CRISPR/Cas9-edited isogenic melanoma model lines provide useful *in vitro* models for both basic and translational research

Disclaimer



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