Supplementary Material

Methods and Methods

Pharmacological growth inhibition assays.

Cultured cells were seeded into 96-well plates (1-2E^3 cells per well) and twenty-four hours after seeding, serial dilutions of each inhibitor prepared in media were added to cells. Cells were incubated for 72h following addition of drug and cell viability was determined as previously described (1).

ATM shRNA constructs

The ATM shRNA sequences correspond to nucleotides 5101-5119 (NM_000051.3) and were cloned into the *pSIH-H1-EF1-copGFP* lentiviral vector, which co-expresses copGFP (System BioSciences, MountainView, CA, USA). The non-silencing negative control shRNA did not show complete homology to any known human transcript and had the following sequence: 5′ - TTAGAGGCGAGCAAGACTA-3.

Whole genome exome sequencing

Exome sequencing was performed on patient-matched Pre and Prog melanoma tissues. Exonic sequences were enriched using the Illumina TrueSeq technology, targeting 62Mb of sequence encompassing exons. Sequencing was performed with the Illumina HiSeq2000 platform, the read pairs were aligned to the reference human genome (hg19) using BWA and single nucleotide polymorphisms (SNPs) and small insertion/deletions (INDELS) were detected by SAMTOOLS (AXEQ Technologies). To generate a list of somatic variants specific for the Prog-tumor, we removed low coverage variants (SNP Quality <20, read <10) and eliminated variants annotated as common polymorphisms or in the 1000Genomes Project (at least 3%). We used Ingenuity Variant Analysis (http://www.ingenuity.com) to detect Prog-specific cancer driver variants, which included

the amino-acid conservation p-value using PhyloP score.

Figure Legends

Figure S1 Representative Sanger sequencing traces

Traces showing alternate *BRAF* splice junctions and the exon 15 Val600 codon (underlined) in Progs with *BRAF* splice variants. Sequences showing mutations affecting *AKT1*, *MEK1*, *MEK2* and *N-RAS* in Prog biopsies and matched pre-treatment tumors.

Figure S2 Level of V600E BRAF allele relative to the wild type BRAF allele.

The relative amount of the $BRAF^{V600E}$ allele (relative to the wild type BRAF allele) is shown for Prog tumors derived from patient 14 and 29. Graph represents mean ± SD from two independent amplification experiments.

Figure S3 Linear models of MEK1 and MEK2 showing functional domains

Locations of identified *MEK* mutations. NES, nuclear export signal; NRR, negative regulatory region; Pro-rich, Proline-rich region.

Figure S4 ATM^{R337C} does not confer resistance to dabrafenib

A. Sequence trace showing *ATM* codon 337 in Pre and Prog melanoma tumors derived from Patient 18
B. SKMel28 and A375 melanoma cells were stably transduced with the indicated constructs. Cell lysates were analyzed for the indicated proteins 4h after incubation with dabrafenib at the indicated concentrations.
C. Transduced SKMel28 and A375 cells were seeded at low density and 24h after seeding were treated with the indicated concentrations of dabrafenib every 72-96h. Colonies were stained with crystal violet 12 days post transduction.

Figure S5 Transcript expression analyses of additional BRAF inhibitor resistance mechanisms

The log2 fold change (Prog biopsy relative to matched pre-treatment tumor) in expression of the indicated gene transcripts is represented as colored boxes. Grey boxes indicate low signal expression values (detection p value <0.05). A 32-fold increase (relative to the pre-treatment tumor) was observed in the BRAF mRNA transcript of the Prog sample showing *BRAF* copy number gains (Patient 14).

Figure S6 Expression of PDGFRß in melanoma biopsy samples from patient 3

PDGFRß shows increased stromal expression, rather than tumoral expression in Prog tumors compared with the matched pre-treatment tumor.

Figure S7 Differential expression of pAKT in two Prog tumors derived from patient 10 (Prog 1 MAPK-inhibited and Prog 2 MAPK re-activated)

A matched pre-treatment melanoma biopsy was not available for p-AKT staining.

Figure S8 Melanoma cells expressing *BRAF* splice variants are resistant to dabrafenib, but retain sensitivity to trametinib

(A) Sequencing analyses of the *BRAF* RT-PCR product amplified from the SKMel28-derived BR4 subline with acquired resistance to dabrafenib (1) Traces show the alternate exon 3 BRAF splice junction (BRAF exon 4-10Δ) and the Val600Glu substitution in exon 15.

(B) Viability curves of the parental SKMel28 and the dabrafenib-acquired resistant BR4 subline treated with the indicated dabrafenib and trametinib doses for 72 hours (relative to DMSO-treated controls; mean ±SD; n=2).

Table S1 Exome sequencing data summary

Patient	Tumor	Mean read depth of target regions	% coverage of target regions (>10x)
Patient 1	Pre	52.9	90.0
	Prog	54.0	89.0
Patient 17	Pre	63.7	91.8
	Prog	67.7	91.9
Patient 18	Pre	64.9	91.4
	Prog 1	76.4	91.8
Patient 24	Pre	66.6	91.2
	Prog	60.7	91.0

Table S2 Candidate resistant variants identified in Prog melanomas

Patient1

Chr	Position	Ref	Var	Gene Symbol	Transcript ID	Protein Variant	SIFT	Conservation p-value	dbSNP ID
7	50459558	C	T	IKZF1	NM_001220771.1	p.L58F	Tolerated	4.94E-04	
7	116395524	G	A	MET	NM_001127500.1	p.G606E	Damaging	3.39E-05	
7	157997956	C	T	PTPRN2	NM_002847.3	p.W96ter		3.30E-04	
11	2181175	C	G	INS-IGF2	NM_000207.2	p.L80F	Tolerated		
15	66774131	G	A	MAP2K1	NM_002755.3	p.E203K	Damaging	9.77E-07	
17	3631311	C	T	ITGAE	NM_002208.4	p.E996K	Tolerated	2.66E-04	
17	45214527	T	C	CDC27	NM_001114091.1	p.Y641C	Damaging	1.68E-05	62075618
19	42753012	G	A	ERF	NM_006494.2	p.P418S	Activating		

Patient 17

Chr	Position	Ref	Var	Gene Symbol	Transcript ID	Protein Variant	SIFT	Conservation p-value	dbSNP ID
12	120241121	G	A	CIT	NM_001206999.1	p.S395L	Tolerated	1.61E-03	
17	16068377	C	G	NCOR1	NM_001190440.1	p.K69N	Damaging		200020868
19	6702171	C	T	C3	NM_000064.2	p.E803K	Damaging	1.85E-04	
10	89717636	A	T	PTEN*	NM_000314.4	p.K221ter		1.73E-05	
9	21971120	G	A	CDKN2A*	NM_001195132.1	p.R80ter	Damaging		121913388

Patient18 – Prog 1

Chr	Position	Ref	Var	Gene Symbol	Transcript ID	Protein Variant	SIFT	Conservation p-value	dbSNP ID

1	32745298	C	T	LCK	NM_005356.3	p.P331S	Tolerated		
3	12458560	G	A	PPARG	NM_015869.4	p.E365K	Tolerated	4.426E-07	
3	133473462	C	T	TF	NM_001063.3	p.P150L	Damaging	0.0004477	
5	110819804	G	T	CAMK4	NM_001744.4	p.K354N	Tolerated		
5	148904676	G	A	CSNK1A1	NM_001025105.2	p.L97F	Damaging	5.333E-07	
5	159854838	C	T	PTTG1	NM_004219.2	p.P163S	Tolerated		
7	44444152	G	A	NUDCD3	NM_015332.3	p.R225C	Tolerated	0.005781	
7	50467675	G	A	IKZF1	NM_001220771.1	p.E161K	Tolerated	0.00006295	
8	93026809	C	G	RUNX1T1	NM_001198628.1	p.V215L	Tolerated	0.000000507	
10	95930979	C	T	PLCE1	NM_016341.3	p.P512L	Damaging	0.00008472	
11	71946969	C	T	INPPL1	NM_001567.3	p.P940S	Tolerated	0.004375	
11	108117798	C	T	ATM	NM_000051.3	p.R337C	Damaging	0.000113	138398778
11	111782391	G	A	CRYAB	NM_001885.1	p.P20S	Tolerated		
11	118353176	C	T	KMT2A	NM_001197104.1	p.P1351L		0.0052	
12	53682943	C	T	ESPL1	NM_012291.4	p.P1593L	Damaging	0.00003532	
12	70946681	G	A	PTPRB	NM_001109754.2	p.P1447S	Tolerated	0.000002104	
12	101581271	C	T	SLC5A8	NM_145913.3	p.G286R	Damaging	9.661E-07	
12	131359245	A	T	RAN	NM_006325.3	p.K134N	Damaging		
15	88679765	C	T	NTRK3	NM_001007156.2	p.G233D	Tolerated	0.00007638	
15	91308624	C	T	BLM	NM_000057.2	p.Q725ter		0.000002523	
17	39739598	C	T	KRT14	NM_000526.4	p.R388H	Damaging	0.0009727	58645163
18	61170677	C	T	SERPINB5	NM_002639.4	p.P284S	Damaging		
20	6759353	G	A	BMP2	NM_001200.2	p.D270N	Damaging	4.966E-07	
20	9561273	C	T	PAK7	NM_177990.2	p.G170E	Damaging	0.000002388	
22	37524624	C	T	IL2RB	NM_000878.3	p.E390K	Damaging		

Patient 24

Chr	Position	Ref	Var	Gene Symbol	Transcript ID	Protein Variant	SIFT	Conservation p-value	dbSNP ID
1	115258745	C	G	NRAS	NM_002524.4	p.G13R	Damaging	8.17E-07	121434595
1	120572547	T	C	NOTCH2	NM_024408.3	p.N46S	Tolerated	3.03E-03	141882865
2	113539369	T	G	IL1A	NM_000575.3	p.H44P	Tolerated		
3	30715656	T	A	TGFBR2	NM_001024847.2	p.N438K	Tolerated		
8	68864705	T	G	PREX2	NM_025170.4	p.C26G	Tolerated	2.63E-05	
9	139417464	T	G	NOTCH1	NM_017617.3	p.T194P	Tolerated		
10	123846999	C	T	TACC2	NM_206862.2	p.P1662S	Tolerated		
13	32905075	C	T	BRCA2	NM_000059.3	p.S234F	Damaging		
17	16068377	C	G	NCOR1	NM_001190440.1	p.K69N	Damaging		200020868
17	37565448	G	A	MED1	NM_004774.3	p.P1009L	Tolerated	4.47E-04	
17	37565449	G	A	MED1	NM_004774.3	p.P1009S	Tolerated	3.46E-04	
17	45214527	T	C	CDC27	NM_001114091.1	p.Y641C	Damaging	1.68E-05	62075618
17	45214558	G	A	CDC27	NM_001114091.1	p.R631ter			77685276
17	45234417	A	G	CDC27	NM_001114091.1	p.I235T	Tolerated	6.12E-05	193061947
20	8626821	G	A	PLCB1	NM_182734.2	p.E153K	Tolerated	1.19E-05	

******PTEN* and *CDKN2A* variants were identified in Patient 17 Pre and Prog melanoma tumors

Abbreviations SIFT, SIFT Functional prediction; Conservation p-value, Conservation phyloP p-value

Table S3 Median MAPK activity scores of Pre-treatment versus Prog melanoma metastases and associated P-value

MAPK activation gene signature	Pre-treatment	MAPK-reactivated Prog	MAPK-inhibited Prog
Gene set 1			
No.	21	23	6
Median	9.4	9.4	8.7
(LQ,UQ)	(9.2, 9.7)	(9.0, 9.7)	(8.5, 8.8)
P-value*		0.96	<0.001
Gene set 2			
No.	21	23	6
Median	9.6	9.4	8.7
(LQ, UQ)	(9.3, 9.9)	(9.1, 9.7)	(8.3, 8.8)
P-value*		0.28	<0.001

Abbreviations: LQ, lower quartile; UQ, upper quartile

*P-values were determined using Mann-Whitney test by comparing Prog with pre-treatment metastases

MAPK activation gene set 1 derived from (2) and gene set 2 from (3).

Table S4 Gene Set Enrichment Analysis of MAPK-inhibited Prog tumors (n=6) vs. MAPK-re-activated Prog tumors (n=15)

TOP 10 UPREGULATED C2 CURATED GENE SETS	NES	FDR q	FWER p
JAEGER_METASTASIS_DN	3.07	0	0
WINNEPENNINCKX_MELANOMA_METASTASIS_DN	2.63	0	0
SANA_RESPONSE_TO_IFNG_UP	2.62	0	0
LIEN_BREAST_CARCINOMA_METAPLASTIC_VS_DUCTAL_DN	2.54	0	0
FARMER_BREAST_CANCER_CLUSTER_1	2.53	0	0
SENGUPTA_EBNA1_ANTICORRELATED	2.52	0	0
SMID_BREAST_CANCER_NORMAL_LIKE_UP	2.47	0	0
CHARAFE_BREAST_CANCER_LUMINAL_VS_MESENCHYMAL_UP	2.46	0	0
KEGG_ALLOGRAFT_REJECTION	2.39	0	0
CHARAFE_BREAST_CANCER_BASAL_VS_MESENCHYMAL_UP	2.35	0	0
TOP 10 DOWNREGULATED C2 CURATED GENE SETS	**NES**	**FDR q**	**FWER p**
KOBAYASHI_EGFR_SIGNALING_24HR_DN	-3.39	0	0
WINNEPENNINCKX_MELANOMA_METASTASIS_UP	-3.35	0	0
FOURNIER_ACINAR_DEVELOPMENT_LATE_2	-3.25	0	0
CROONQUIST_IL6_DEPRIVATION_DN	-3.14	0	0
BENPORATH_PROLIFERATION	-3.09	0	0
BIDUS_METASTASIS_UP	-3.08	0	0
MISSIAGLIA_REGULATED_BY_METHYLATION_DN	-3.01	0	0
ZHANG_TLX_TARGETS_60HR_DN	-2.99	0	0
SOTIRIOU_BREAST_CANCER_GRADE_1_VS_3_UP	-2.99	0	0
ZHOU_CELL_CYCLE_GENES_IN_IR_RESPONSE_24HR	-2.96	0	0
TOP 10 UPREGULATED C6 ONCOGENIC SIGNATURES	**NES**	**FDR q**	**FWER p**
KRAS.600_UP.V1_DN	2.14	0	0
KRAS.300_UP.V1_DN	2.13	0	0
KRAS.50_UP.V1_DN	2.02	0	0
KRAS.LUNG_UP.V1_DN	1.97	0.003	0.01
KRAS.600.LUNG.BREAST_UP.V1_DN	1.95	0.002	0.01
PTEN_DN.V1_UP	1.86	0.005	0.03

SINGH_KRAS_DEPENDENCY_SIGNATURE	1.86	0.005	0.03
KRAS.BREAST_UP.V1_DN	1.86	0.004	0.03
IL21_UP.V1_UP	1.80	0.004	0.03
CSR_LATE_UP.V1_DN	1.77	0.005	0.04
TOP 10 DOWNREGULATED C6 ONCOGENIC SIGNATURES	**NES**	**FDR q**	**FWER p**
MAPK ACTIVATION GENE SET 1*	-2.86	0	0
MAPK ACTIVATION GENE SET 2*	-2.62	0	0
VEGF_A_UP.V1_DN	-2.33	0	0
CSR_LATE_UP.V1_UP	-2.28	0	0
GCNP_SHH_UP_EARLY.V1_UP	-2.12	0.001	0.01
GCNP_SHH_UP_LATE.V1_UP	-2.06	0.002	0.01
PDGF_UP.V1_UP	-2.01	0.001	0.01
CSR_EARLY_UP.V1_UP	-1.92	0.001	0.01
E2F1_UP.V1_UP	-1.87	0.002	0.02
MOTIF2_UP*	-1.87	0.002	0.02

Abbreviations: NES, normalized enrichment score; FDR q, false discovery rate; FWER p, Familywise-error rate

*MAPK ACTIVATION GENE SET 1 (2), MAPK ACTIVATION GENE SET 2 (3), MOTIF2_UP (4)

Table S5 Inter-tumoral heterogeneity of BRAF inhibitor resistance mechanisms and MAPK activity

Patient	Prog No.	Time from BRAF inhibitor start to biopsy (weeks)	Interval between biopsies (weeks)	Site	Type of Prog lesion	Resistance mechanism	MAPK activity#
3	1	46.9		SQ	new	unknown	+
	2	76.9	30.0	SQ	new	unknown	+
8	1	55.0		SQ	existing - RES	unknown	+
	2	78.0	23.0	SQ	existing - RES	unknown	+
10	1	33.6		SQ	new	IGF-1R	-
	2	52.7	19.1	SQ	new	*BRAF* exon4-8 Δ	+
11	1	19.0		SQ	na	unknown	-
	2	49.3	30.3	brain	new	*AKT1*^{Q79K}	+
18	1	16.3		bowel	existing - RES	unknown	-
	2	26.3	10.0	SQ	new*	*MEK1*^{K57E}	+
28	1	25.1		SQ	existing - RES	*BRAF* exon2-8Δ	+
	2	25.1	0	SQ	existing - RES	*BRAF* exon4-8Δ	+
	3	25.1	0	SQ	existing - RES	*BRAF* exon2-8Δ	+

	4	25.1	0	SQ	existing - RES	*BRAF* exon4-8Δ	+

Abbreviations: SQ, subcutaneous; RES, response; na, data not available

#MAPK activity was determined using GSEA of whole transcriptome data comparing matched pre-treatment (n=21) and Prog (n=29) biopsies. + indicates re-activated, - indicates inhibited

* local recurrence after resection of lesion 1

Table S6 BRAF inhibitor resistance mechanism and response to subsequent MAPK inhibitors.

Patient	Drug	PROG biopsy No.	Site	Type of lesion	Resistance mechanism	MAPK activity#	Subsequent MAPK inhibitor	Best RECIST Response
1	dab	1	SQ	new	*MEK1*^{E203K}	+	LGX818+MEK162	SD
6	dab	1	LN	new	*BRAF* amplification	na	trametinib	SD
10	dab	1	SQ	new	IGF-1R	-	LGX818+MEK162	PD
		2	SQ	new	*BRAF* exon 4-8Δ	+	LGX818+MEK162	PD
16	dab	1	SQ	existing - RES	unknown	na	dab+trametinib	PD
19	dab	1	LN	existing - RES	unknown	na	dab+trametinib	PD
24^	vem	1	SQ	existing - RES	*N-RAS*^{G13R}*	+	trametinib dab+trametinib	SD PD
25	vem	1	SQ	na	unknown	-	dab	SD

Abbreviations: dab, dabrafenib; vem, vemurafenib; SQ, subcutaneous; LN, lymph node; RES, response; SD, stable disease; PD, progressive disease; LGX818=Novartis BRAF inhibitor; MEK162=Novartis MEK inhibitor, na, data not available

* this lesion has previously been shown to display inter-tumoral heterogeneity with *N-RAS* mutant (G13R) and wild-type subpopulations.

^Patient 24 was treated with trametinib and subsequently with dabrafenib and trametinib combined

#MAPK activity was determined using GSEA of whole transcriptome data comparing matched pre-treatment (n=21) and Prog (n=29) biopsies. + indicates re-activated, - indicates inhibited.

Table S7 Amplification and Sequencing Primers

	Forward	Reverse
BRAF	GGCTCTCGGTTATAAGATGGC	ACAGGAAACGCACCATATCC
MEK1	CGTTACCCGGGTCCAAAATG	CTTTGTCACAGGTGAAATGC
	CATGGATGGAGGTTCTCTGG	AGGGCTTGACATCTCTGTGC
MEK2	CTCCCGGCCCGCCCCCTATG	GTGGAGGCGCCAGCCTGTCC
	GTCAGCATCGCGGTTCTCC	TCACCCCGAAGTCACACAG
MEK1/2	TGCAGAAGAAGCTGGAGGAGC	
N-RAS	AGCTTGAGGTTCTTGCTGGT	TCAGGACCAGGGTGTCAGTG
AKT1	AGCGCCAGCCTGAGAGGA	TCTCCATCCCTCCAAGCTAT
		GACAGGTGGAAGAACAGCT

References

1. Gowrishankar K, Snoyman S, Pupo GM, Becker TM, Kefford RF, Rizos H. Acquired Resistance to BRAF Inhibition Can Confer Cross-Resistance to Combined BRAF/MEK Inhibition. J Invest Dermatol. 2012;132:1850-9.
2. Pratilas CA, Taylor BS, Ye Q, Viale A, Sander C, Solit DB, et al. (V600E)BRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway. Proc Natl Acad Sci U S A. 2009;106:4519-24.
3. Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, et al. Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. Nature. 2010;468:973-7. .
4. Hoek KS, Schlegel NC, Brafford P, Sucker A, Ugurel S, Kumar R, et al. Metastatic potential of melanomas defined by specific gene expression profiles with no BRAF signature. Pigment Cell Res. 2006;19:290-302.