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Supplemental Data

Oncogenic BRAF Induces Senescence and Apoptosis through Pathways Mediated by the Secreted Protein IGFBP7

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Supplemental Experimental Procedures

Antibodies

Additional antibodies used in supplemental figures and not mentioned in the Experimental Procedures accompanying the main text include: α-JUN (Upstate); and α-phospho-MEK, α-MEK and α-RKIP, which were all obtained from Cell Signaling Technology.

Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assays

Paraffin sections of 5 μm thickness were tested for DNA fragmentation by the non-isotopic in situ TUNEL method using the ApopTag Peroxidase in situ Apoptosis Detection Kit Apo-Direct^{TM} TUNEL Assay Kit (Chemicon International).

Figure S1. Characterization of the PFF and Melanocyte KD Cell Lines.
(A) Quantitative proliferation assays of the 17 BRAFV600E/PFF KD cell lines shown in Figure 1B. PFFs stably expressing the indicated shRNA were infected with the BRAFV600E-expressing retrovirus and after 14 days analyzed by the trypan blue exclusion assay. Growth of PFFs expressing a non-silencing shRNA (PFF-NS) relative to growth of untreated PFFs is shown. For the BRAFV600E/PFF KD cell lines, values were normalized to the growth of the corresponding PFF KD cell line in the absence of BRAFV600E expression. Error bars represent standard error.
(B) Analysis of target gene expression in the PFF and melanocyte KD cell lines. Quantitative real-time RT-PCR was used to analyze target gene expression in each of the 17 PFF (left) or melanocyte (right) KD cell lines.
(C) Confirmation of all 17 candidate genes using a second, unrelated shRNA directed against the target gene. (Left) Quantitative proliferation assays. For each of the 17 candidate genes, a second, unrelated shRNA directed against the same target gene was used to derive an independent PFF KD cell line. Each PFF KD cell line was infected with the BRAFV600E-expressing retrovirus and proliferation monitored after 14 days. Growth of BRAFV600E/PFFs is expressed relative to the growth of normal PFFs. For the BRAFV600E/PFF KD cell lines, values were normalized to the growth of the corresponding PFF KD cell line in the absence of BRAFV600E expression. (Right) Quantitative real-time RT-PCR was used to analyze target gene expression in each of the 17 PFF KD cell lines.

Figure S2. Analysis of the BRAFV600E/Melanocyte KD Cell Lines After an Additional 15 Population Doublings, and Further Characterization of the BRAFV600E/PFF KD Cell Lines.
(A) (Top) DNA replication assays. The BRAFV600E/melanocyte KD cell lines were allowed to undergo an additional 15 population doublings (+15 PD) and then analyzed for BrdU incorporation. Error bars represent standard error. (Bottom) Immunoblot analysis monitoring acetylation of histone 3 lysine 9 (H3K9) after an additional 15 population doublings. β-ACTIN (ACTB) was monitored as a loading control.
(B) Apoptosis assays (top) and DNA replication assays (bottom) of the BRAFV600E/PFF KD cell lines. Apoptosis was monitored by Annexin V-PE staining 4 days after BRAFV600E expression; DNA replication was monitored by BrdU incorporation 4 days after BRAFV600E expression.

Figure S3. IGFBP7 Upregulation Occurs Downstream of BRAF-MEK-ERK Signaling.
(A) IGFBP7 protein levels in CM from BRAFV600E-expressing melanocytes (top) or mRNA expression in BRAFV600E-expressing melanocytes (bottom) was monitored in the presence of increasing amounts of a MEK inhibitor. The results show that BRAFV600E-mediated induction of IGFBP7 was blocked by addition of a MEK inhibitor. Error bars represent standard error.
(B) IGFBP7 protein levels in CM from melanocytes (top) or mRNA expression in melanocytes (bottom) was monitored in the presence of BRAFV600E or a constitutively activated ERK mutant (ERK2Q103A or ERK2L73P,S151D). The results show that expression of a constitutively activated ERK mutant was sufficient to activate *IGFBP7* transcription. Collectively, the results of parts A and B demonstrate that IGFBP7 induction is downstream of BRAF-MEK-ERK signaling.

Figure S4. A Role for the AP-1 Transcription Factor in BRAFV600E-Mediated Upregulation of IGFBP7 Expression.

(Top) Schematic of the *IGFBP7* promoter showing a consensus binding site for the dimeric AP-1 (JUN/FOS) transcription factor located ~1 kb upstream of the transcription start-site.

(A) Chromatin immunoprecipitation (ChIP) analysis. ChIP assays were performed using extracts prepared 4 days following BRAFV600E retroviral infection. Error bars represent standard error. The results show that JUN binds to the *IGFBP7* promoter in response to BRAFV600E expression in melanocytes.

(B) AP-1 is required for BRAFV600E-mediated upregulation of IGFBP7 expression. (Left) Quantitation of *IGFBP7* mRNA levels in BRAFV600E-expressing melanocytes following treatment with a non-silencing (NS) or JUN siRNA. The results demonstrate that JUN is required for BRAFV600E-mediated induction of *IGFBP7* transcription. (Right) Quantitative real-time RT-PCR was used to analyze *JUN* mRNA levels following siRNA treatment. The results of parts A and B, in conjunction with our other results, indicate that BRAFV600E induces expression of *IGFBP7*, at least in part, through activation of ERK, which in turn activates AP-1, resulting in binding of AP-1 to the *IGFBP7* promoter and stimulation of transcription.

Figure S5. Induction of Gene Expression by BRAFV600E and IGFBP7.

(A) Quantitation of gene expression in BRAFV600E/melanocytes compared to melanocytes (left), rIGFBP7-treated melanocytes compared to untreated melanocytes (middle), and rIGFBP7-treated SK-MEL-28 cells compared to untreated cells (right). Gene expression was monitored by qRT-PCR in untreated and treated cells, and expressed as fold-upregulation.

(B) Melanocytes expressing either a non-silencing or IGFBP7 shRNA were monitored for BRAFV600E-induced expression of seven genes by quantitative real-time RT-PCR. Error bars represent standard error. The results show that BRAFV600E-mediated induction of all seven genes did not occur following knockdown of IGFBP7.

Figure S6. BRAFV600E-Mediated Induction of SMARCB1 and BNIP3L is Dependent Upon and Downstream of IGFBP7.

(A) Immunoblot analysis showing SMARCB1 and BNIP3L induction in BRAFV600E-expressing melanocytes in the presence of a non-silencing (NS) or IGFBP7shRNA. β-ACTIN (ACTB) was monitored as a loading control. The results show that induction of SMARCB1 and BNIP3L was blocked following IGFBP7 knockdown.

(B) Immunoblot analysis monitoring SMARCB1, BNIP3L and $p16^{INK4a}$ in naïve melanocytes following addition of CM from normal melanocytes, BRAFV600E/melanocytes, BRAFV600E/melanocytes stably expressing an IGFBP7 shRNA or in BRAFV600E/melanocyte CM immunodepleted of IGFBP7. The results show that addition of CM from BRAFV600E-expressing melanocytes to naïve melanoctyes substantially upregulated SMARCB1 and BNIP3L, which did not occur with CM from BRAFV600E/melanocytes expressing an IGFBP7 shRNA or with CM from BRAFV600E/melanocytes following immunodepletion of IGFBP7. Collectively, the results of parts A and B demonstrate that BRAFV600E-mediated induction of SMARCB1 and BNIP3L is dependent upon and downstream of IGFBP7.

Figure S7. IGFBP7-Dependent Inhibition of BRAF-MEK-ERK Signaling in Melanocytes Following BRAFV600E Expression or Growth Factor Stimulation.
(A) Immunoblot of phospho-ERK and total ERK in BRAFV600E-expressing melanocytes compared to BRAFV600E-expressing melanocytes containing a non-silencing (NS) or IGFBP7 shRNA. The results confirm that phospho-ERK levels are markedly decreased in BRAFV600E/melanocytes, which are senescent. This decrease in phospho-ERK levels did not occur in BRAFV600E/melanocytes expressing an IGFBP7 shRNA.
(B) Immunoblot analysis of phospho-ERK and total ERK in naïve melanocytes following addition of CM from normal melanocytes, BRAFV600E/melanocytes, BRAFV600E/melanocytes stably expressing an IGFBP7 shRNA or in BRAFV600E/melanocyte CM immunodepleted of IGFBP7. The results show that addition of CM from BRAFV600E-expressing melanocytes to naïve melanocytes substantially decreased the level of phospho-ERK, which did not occur with the various control CMs that lacked IGFBP7.
(C) Immunoblot analysis of phospho-ERK and total ERK levels in melanocytes treated in the presence or absence of rIGFBP7 (10 μg/ml), or the presence or absence of melanocyte growth factors (1X Human Melanocyte Growth Supplement (Cascade Biologics)). Cells were treated for 48 hrs prior to harvesting cells. The results demonstrate that rIGFBP7 also blocked growth factor-induced ERK activation.

Figure S8. Mechanism of IGFBP7-Mediated Inhibition of BRAF-MEK-ERK Signaling.
(A) (Top) Immunoblot analysis monitoring expression of RAF inhibitory protein (RKIP), phospho-MEK1/2, total MEK1/2, phospho-ERK and total ERK in BRAFV600E-positive SK-MEL-28 cells following addition of rIGFBP7 for 0, 3, 6, 12 or 24 hours. (Bottom) Apoptosis of rIGFBP7-treated SK-MEL-28 cells was monitored by Annexin V-PE staining. Error bars represent standard error. The results show that addition of rIGFBP7 to SK-MEL-28 cells resulted in induction of RKIP and decreased levels of activated MEK1/2, corresponding with the reduced phospho-ERK levels and apoptosis.
(B) Immunblot analysis of phospho-ERK and total ERK levels in SK-MEL-28 cells treated in the presence or absence of rIGFBP7, and following expression of a constitutively activated MEK1 mutant. The results show that ectopic expression of a constitutively activated MEK1 mutant prevented the ability of IGFBP7 to block ERK activation.
(C) Immunoblot analysis monitoring expression of RKIP, phospho-MEK1/2, total MEK1/2, phospho-ERK and total ERK in SK-MEL-28 cells treated in the presence or absence of rIGFBP7, and with a non-silencing (NS) or RKIP siRNA. The results show that following knockdown of RKIP in SK-MEL-28 cells, rIGFBP7 failed to block activation of MEK or ERK. Collectively, the results of parts A, B and C indicate that IGFBP7 inhibits ERK activation by inducing RKIP, which prevents BRAF from phosphorylating MEK.

Figure S9. Inhibition of BRAF-MEK-ERK Signaling is Required for BRAFV600E- and IGFBP7-Induction of Senescence in Melanocytes.
Quantitative proliferation assays showing that constitutively activated ERK2 mutants block BRAFV600E-induced (left) or IGFBP7-induced (right) senescence in melanocytes. Error bars represent standard error.

Figure S10. Systemic Administration of rIGFBP7 Induces Apoptosis in BRAFV600E-Positive Mouse Xenografts.
Tumors derived from the rIGFBP7 systemic administration experiment shown in Figure 5B were analyzed in a deoxyuridine triphosphate nick-end labeling (TUNEL) assay. The results show that following treatment with rIGFBP7, BRAFV600E-positive tumors were TUNEL-positive, indicating that suppression of tumor growth resulted from apoptosis.

Figure S1

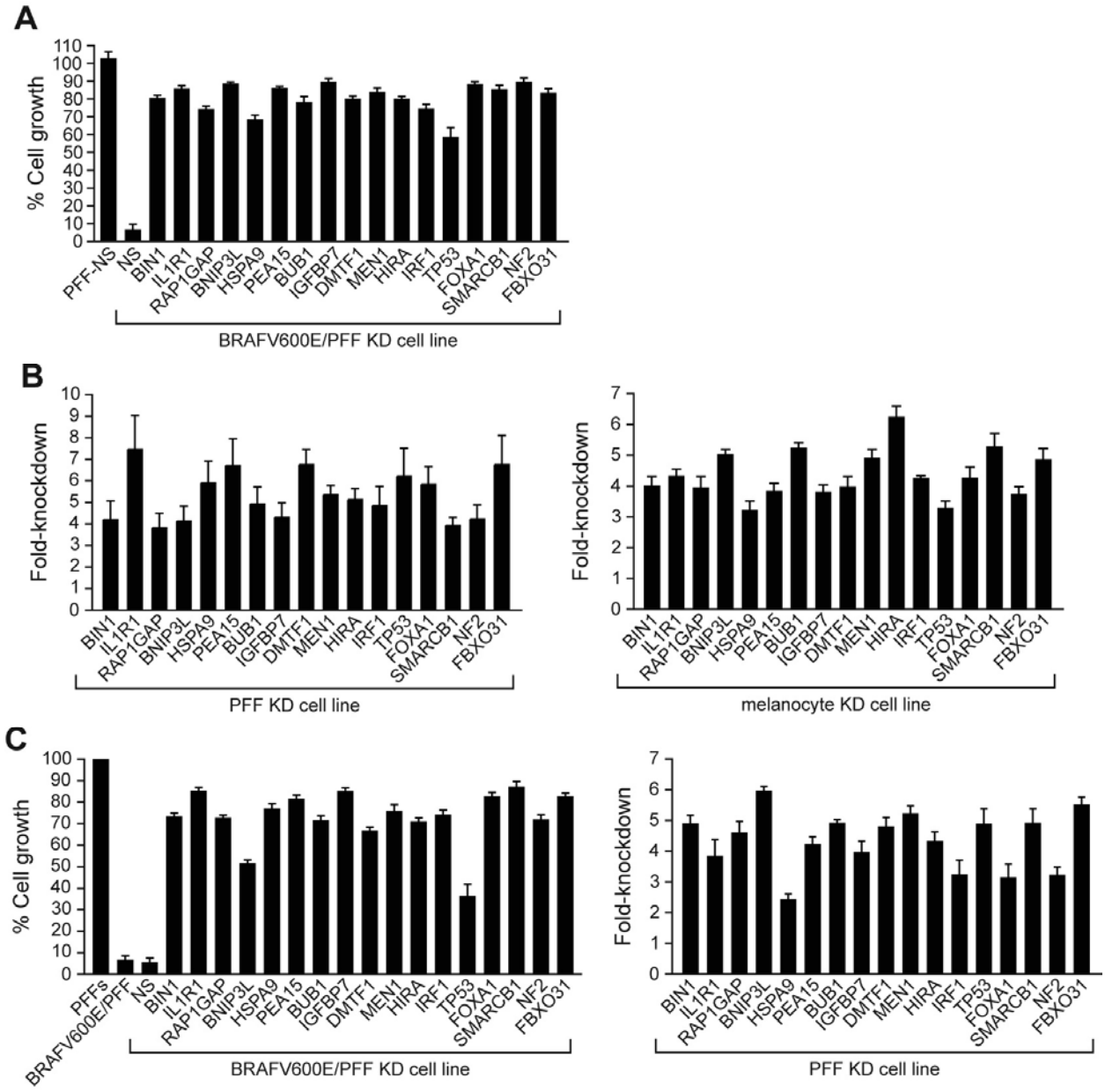
A
% Cell growth
PFF-NS
NS
BIN1
IL1R1
RAP1GAP
BNIP3L
HSPA9
PEA15
BUB1
IGFBP7
DMTF1
MEN1
HIRA
IRF1
TP53
FOXA1
SMARCB1
NF2
FBXO31
BRAFV600E/PFF KD cell line
B
Fold-knockdown
PFF KD cell line
melanocyte KD cell line
C
% Cell growth
PFFs
BRAFV600E/PFF
BRAFV600E/PFF KD cell line
Fold-knockdown
PFF KD cell line

Figure S2

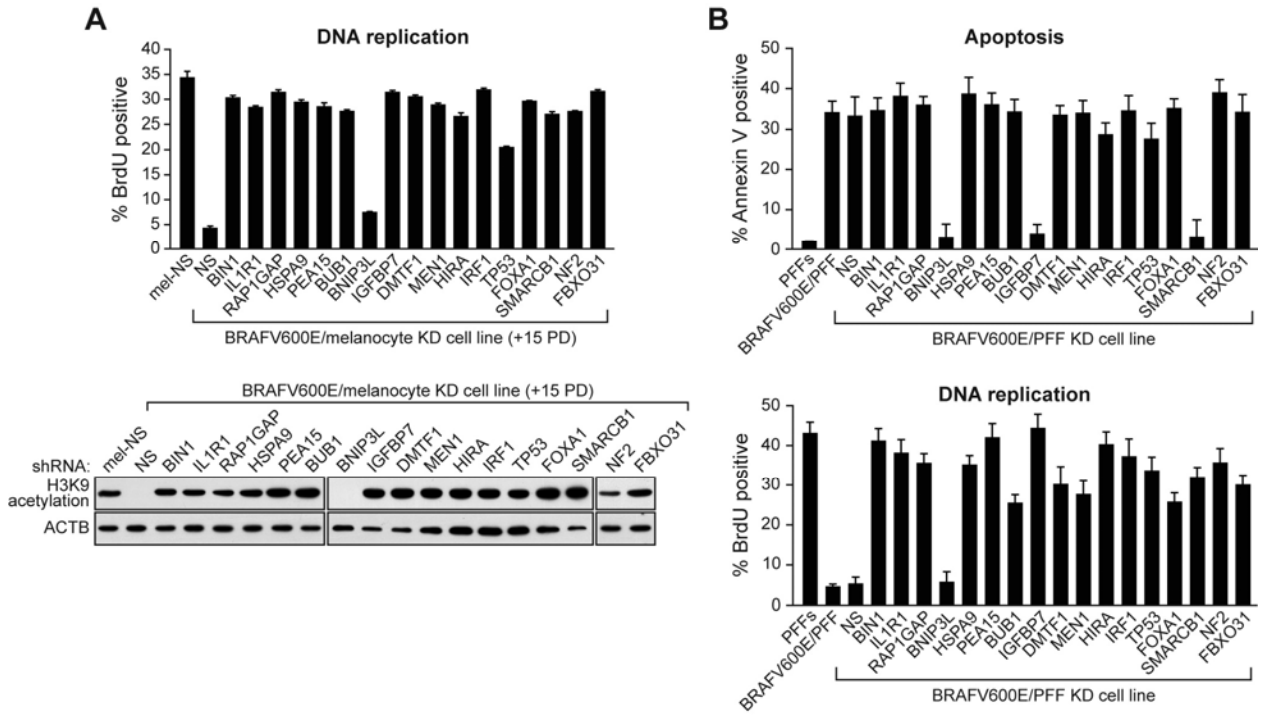
A
DNA replication
% BrdU positive
mel-NS
NS
BIN1
IL1R1
RAP1GAP
HSPA9
PEA15
BUB1
BNIP3L
IGFBP7
DMTF1
MEN1
HIRA
IRF1
TP53
FOXA1
SMARCB1
NF2
FBXO31
BRAFV600E/melanocyte KD cell line (+15 PD)
shRNA:
H3K9 acetylation
ACTB
B
Apoptosis
% Annexin V positive
PFFs
BRAFV600E/PFF
BRAFV600E/PFF KD cell line
DNA replication
% BrdU positive

Figure S3

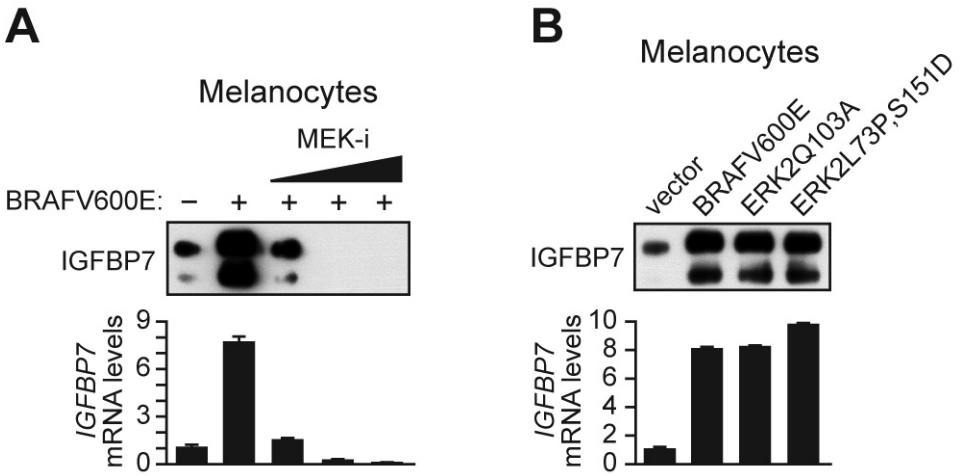
A
Melanocytes
MEK-i
BRAFV600E: – + + + +
IGFBP7
IGFBP7 mRNA levels
9
6
3
0
B
Melanocytes
vector
BRAFV600E
ERK2Q103A
ERK2L73P,S151D
IGFBP7
IGFBP7 mRNA levels
10
8
6
4
2
0

Figure S4

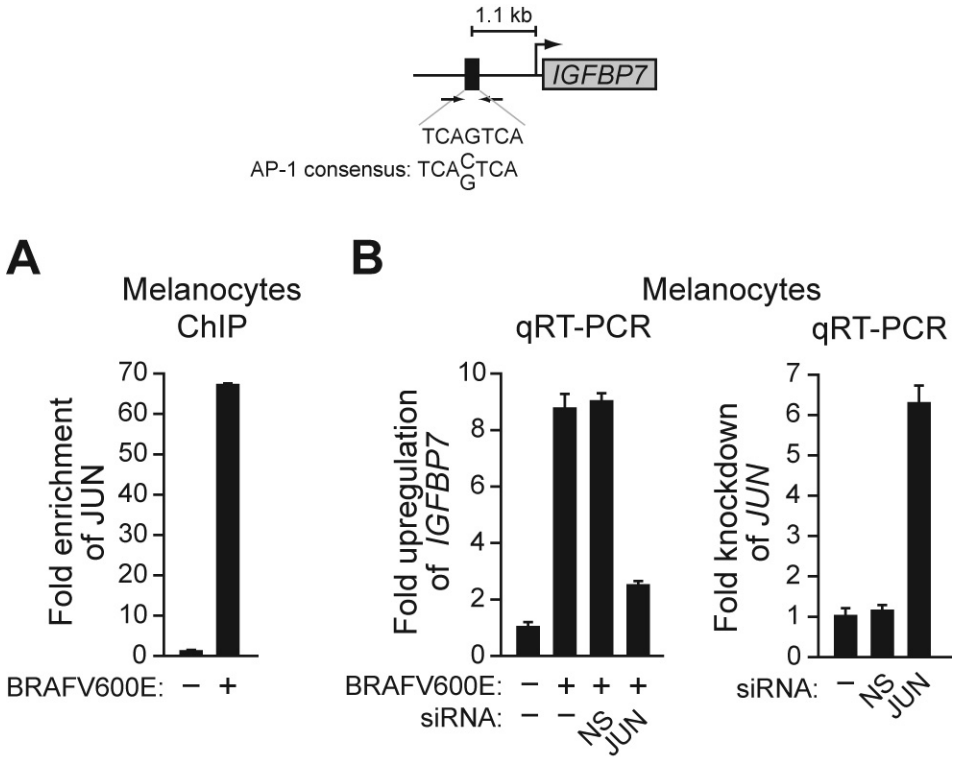
1.1 kb
IGFBP7
TCAGTCA
AP-1 consensus: TCA C/G TCA
A
Melanocytes
ChIP
Fold enrichment of JUN
70
60
50
40
30
20
10
0
BRAFV600E: – +
B
Melanocytes
qRT-PCR
Fold upregulation of IGFBP7
10
8
6
4
2
0
BRAFV600E: – + + +
siRNA: – – NS JUN
Melanocytes
qRT-PCR
Fold knockdown of JUN
7
6
5
4
3
2
1
0
siRNA: – NS JUN

Figure S5

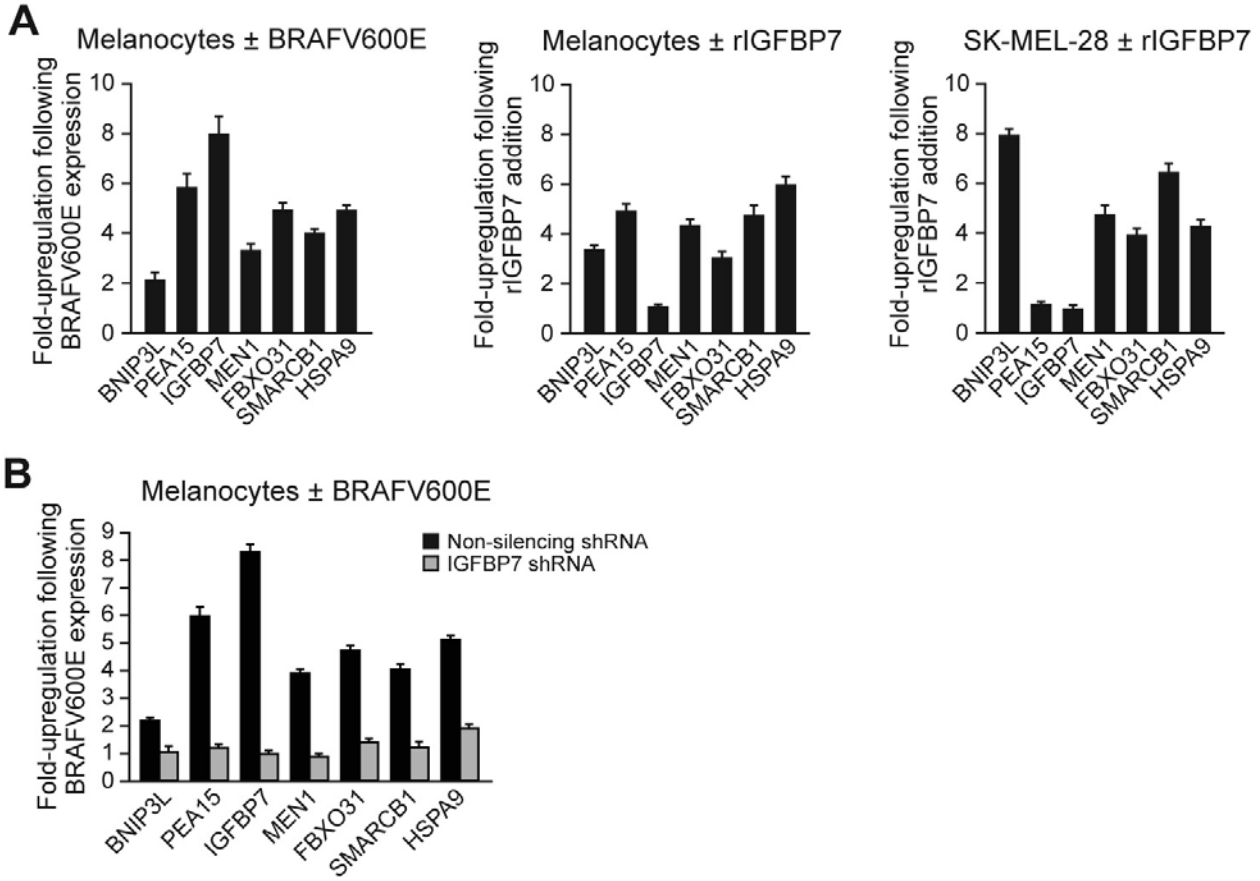
A
Melanocytes ± BRAFV600E
Fold-upregulation following BRAFV600E expression
Melanocytes ± rIGFBP7
Fold-upregulation following rIGFBP7 addition
SK-MEL-28 ± rIGFBP7
Fold-upregulation following rIGFBP7 addition
BNIP3L
PEA15
IGFBP7
MEN1
FBXO31
SMARCB1
HSPA9
B
Melanocytes ± BRAFV600E
Fold-upregulation following BRAFV600E expression
Non-silencing shRNA
IGFBP7 shRNA

Figure S6

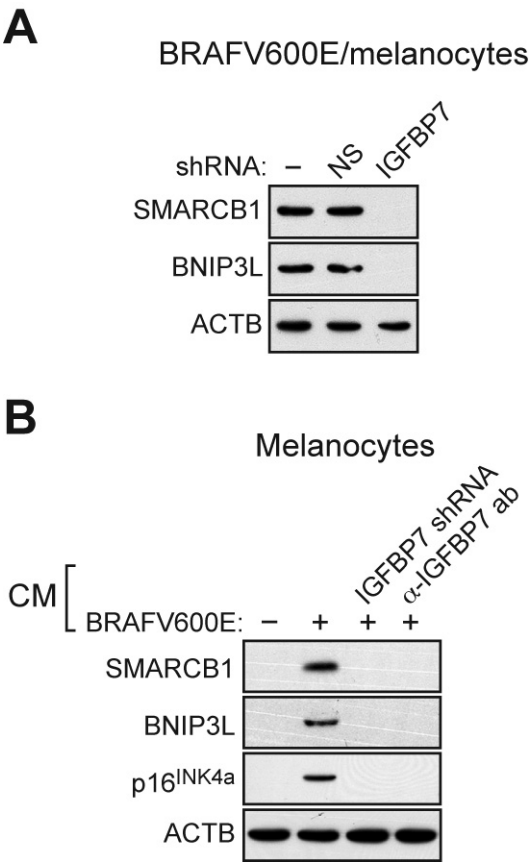
A
BRAFV600E/melanocytes
shRNA: – NS IGFBP7
SMARCB1
BNIP3L
ACTB
B
Melanocytes
IGFBP7 shRNA
α-IGFBP7 ab
CM
BRAFV600E: – + + +
SMARCB1
BNIP3L
p16INK4a
ACTB

Figure S7

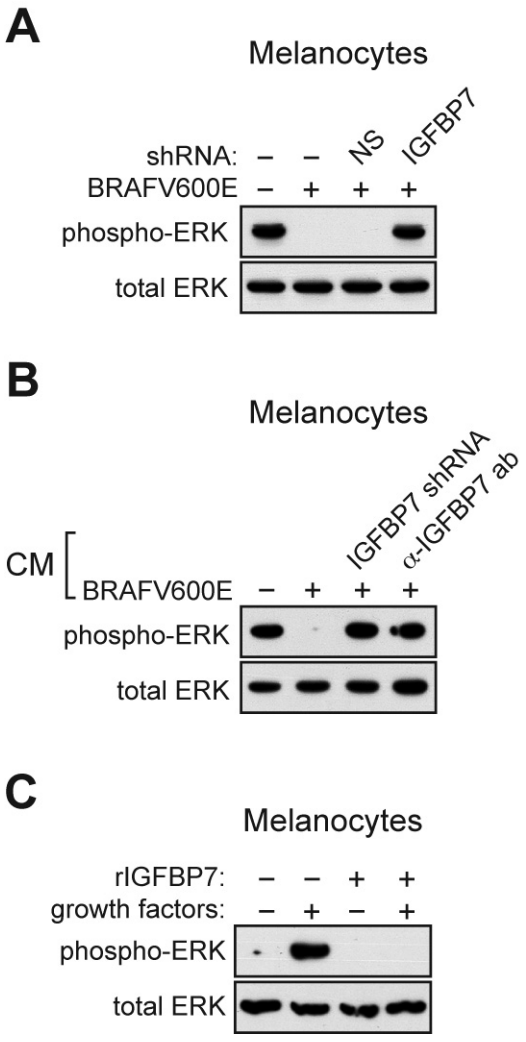
A
Melanocytes
shRNA: – – NS IGFBP7
BRAFV600E – + + +
phospho-ERK
total ERK
B
Melanocytes
IGFBP7 shRNA
α-IGFBP7 ab
CM
BRAFV600E – + + +
phospho-ERK
total ERK
C
Melanocytes
rIGFBP7: – – + +
growth factors: – + – +
phospho-ERK
total ERK

Figure S8

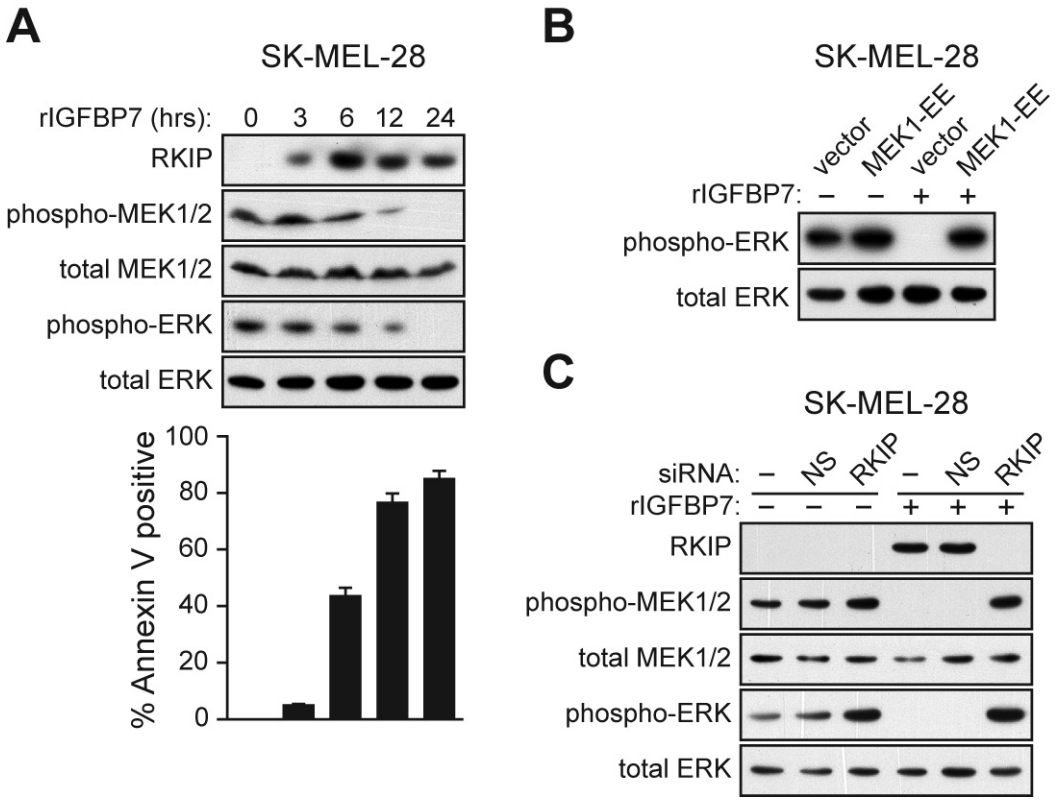
A
SK-MEL-28
rIGFBP7 (hrs): 0 3 6 12 24
RKIP
phospho-MEK1/2
total MEK1/2
phospho-ERK
total ERK
% Annexin V positive
100
80
60
40
20
0
B
SK-MEL-28
vector
MEK1-EE
vector
MEK1-EE
rIGFBP7: – – + +
phospho-ERK
total ERK
C
SK-MEL-28
siRNA: – NS RKIP – NS RKIP
rIGFBP7: – – – + + +
RKIP
phospho-MEK1/2
total MEK1/2
phospho-ERK
total ERK

Figure S9

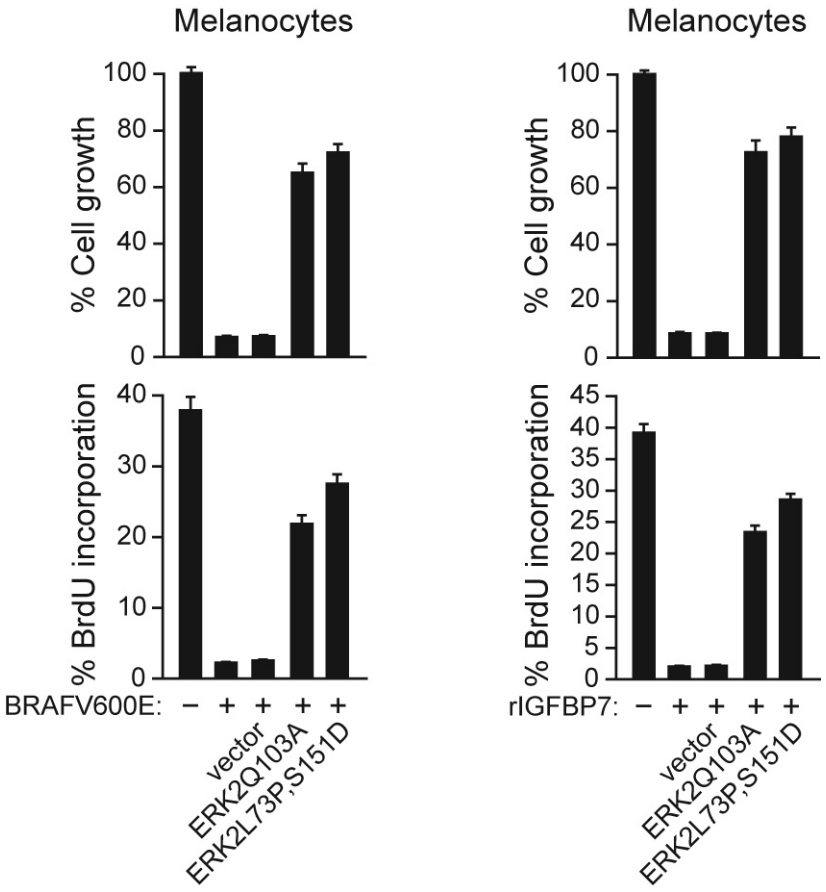
Melanocytes
% Cell growth
100
80
60
40
20
0
% BrdU incorporation
40
30
20
10
0
BRAFV600E: – + + + +
vector
ERK2Q103A
ERK2L73P,S151D
Melanocytes
% Cell growth
100
80
60
40
20
0
% BrdU incorporation
45
40
35
30
25
20
15
10
5
0
rIGFBP7: – + + + +
vector
ERK2Q103A
ERK2L73P,S151D

Figure S10

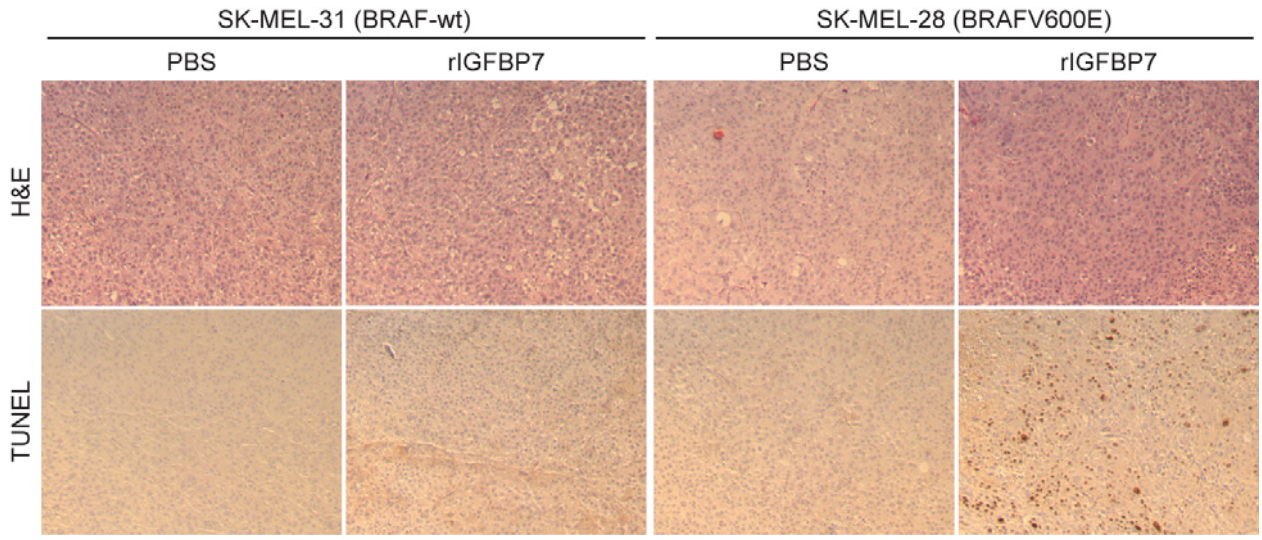
SK-MEL-31 (BRAF-wt)
SK-MEL-28 (BRAFV600E)
PBS
rIGFBP7
PBS
rIGFBP7
H&E
TUNEL

Table S1. Genes required for BRAFV600E to block proliferation of human PFFs and melanocytes

Biological process	Accession number	Gene symbol	Gene name
Apoptosis	NM_004331	*BNIP3L*	BCL2/adenovirus E1B 19kDa interacting protein 3-like
Cell cycle regulation	NM_004336	*BUB1*	budding uninhibited by benzimidazoles 1 homolog (yeast)
Signal transduction	NM_004305	*BIN1*	bridging integrator 1
	NM_004134	*HSPA9*	heat shock 70 kDa protein 9 (mortalin)
	NM_001553	*IGFBP7*	insulin-like growth factor binding protein 7
	NM_000877	*IL1R1*	interleukin 1 receptor, type I
	NM_003768	*PEA15*	phosphoprotein enriched in astrocytes 15
	NM_002885	*RAP1GAP*	RAP1 GTPase activating protein
Transcription regulation	NM_021145	*DMTF1*	cyclin D binding myb-like transcription factor 1
	NM_004496	*FOXA1*	forkhead box A1
	NM_002198	*IRF1*	interferon regulatory factor 1
	NM_000244	*MEN1*	multiple endocrine neoplasia 1
	NM_000546	*TP53*	tumor protein p53 (Li-Fraumeni syndrome)
Chromatin remodeling	NM_003325	*HIRA*	HIR histone cell cycle regulation defective homolog A (*S. cerevisiae*)
	NM_001007468	*SMARCB1*	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1
Genome stability	NM_000268	*NF2*	neurofibromin 2 (bilateral acoustic neuroma)
Unknown	NM_024735	*FBXO31*	F-box protein 31

Table S2. IGFBP7 expression in human skin, nevi and melanoma samples

Tissue sample	Number	BRAFV600E status	IGFBP7 status
Normal skin	5	–	+
Benign nevi	20	+	+
Melanoma	13	+	–
Melanoma*	7	–	+

* Genotyping showed that all BRAF-wild type melanomas were also wild type for RAS.

Table S3. Source ID numbers and clone locations for shRNAs obtained from Open Biosystems, and sequences of synthesized shRNAs and siRNAs

Open Biosystems shRNAs

Gene	Source ID	Clone Location
BIN1	V2HS_192552	SH2136-G-11
	V2HS_238893	SH2490-D-11
BNIP3L	V2HS_15157	SH2240-F-2
	V2HS_15153	SH2117-G-8
BUB1	V2HS_15109	SH2335-D-10
	V2HS_15112	SH2123-B-6
CDKN2A (p16)	V2HS_195839	SH2517-C-6
DMTF1	V2HS_18765	SH2249-A-4
	V2HS_18766	SH2114-C-10
FBXO31	V2HS_15701	SH2119-C-4
	V2HS_25023	SH2497-B-9
FOXA1	V2HS_16780	SH2118-F-5
	V2HS_16813	SH2260-H-2
HIRA	V2HS_179296	SH2205-H-3
	V2HS_171670	SH2526-G-6
HSPA9	V2HS_173169	SH2210-F-7
	V2HS_263034	SH2661-D-2
IGFBP7	V2HS_132291	SH2710-D-9
	V2HS_132289	SH2094-C-3
IRF1	V2HS_133394	SH2096-C-1
	V2HS_133391	SH2095-E-6
IL1R1	V2HS_131082	SH2445-F-2
	V2HS_131085	SH2517-E-6
MEN1	V2HS_76534	SH2523-E-5
	V2HS_76605	SH2638-A-12
NF2	V2HS_115769	SH2336-C-2
	V2HS_115768	SH2563-F-9
PEA15	V2HS_23701	SH2137-E-11
	V2HS_23702	SH2320-C-5
RAP1GAP	V2HS_175368	SH2458-H-11
SMARCB1	V2HS_153159	SH2218-F-3
TP53	V2HS_93615	SH2423-C-10

Synthesized shRNAs

Gene	Sequence (5' -> 3')
RAP1GAP	TGCTGTTGACAGTGAGCGCGCCACCAGTGTGACCCTGTTATAGTGAAGC CACAGATGTATAACAGGGTCACACTGGTGGCTTGCCTACTGCCTCGGA
SMARCB1	TGCTGTTGACAGTGAGCGCTCACTGATTGTCCCTGGGAAGTAGTGAAGC CACAGATGTACTTCCCAGGGACAATCAGTGATTGCCTACTGCCTCGGA
TP53	TGCTGTTGACAGTGAGCGACCTGTGCAGCTGTGGGTTGATTAGTGAAGC CACAGATGTAATCAACCCACAGCTGCACAGGGTGCCTACTGCCTCGGA

Synthesized siRNAs

Gene	Sequence (5’ -> 3’)
JUN	GGAACAGGTGGCACAGCTT
RKIP	CACTCACTCTGATTTATGT
STAT1	CCCAGAATGCCCTGATTAA
Non-silencing	AACACCGAACGAGACACGA

Table S4. Primers used for quantitative real time RT-PCR, ChIP, *BRAF* genotyping and bisulfite sequencing

RT-PCR

Gene	Forward or reverse primer	Sequence (5' → 3')
BIN1	forward	TCTCCAGAAGCTGGGGAAGG
	reverse	TCCATCCACAGCAGGTCGTT
BNIP3L	forward	CGGACTCGGCTTGTTGTGTT
	reverse	ATGGGTAGCTCCACCCAGGA
BUB1	forward	GATGCTTGAAGCCCACATGC
	reverse	AGGGGATGACAGGGTTCCAA
JUN	forward	GGTAGCAGATAAGTGTTGAG
	reverse	GGCGCTAGCTCTGGGCAGTT
DMTF1	forward	AGCCAAGAGGCCAACCTGTC
	reverse	CCTGGCTGGAGCTGTTCTCA
FBXO31	forward	AATCCGGCCTTTTGACCAGA
	reverse	TCCGCTCACAGGAAGAGCAC
FOXA1	forward	GGAGCCGGCGTACTACCAAG
	reverse	TTTTGCACTGGGGGAAAGGT
HIRA	forward	ACACTCTCGCCGCTGATGAC
	reverse	GGGGCTAGTGTCCACCTTGG
HSPA9	forward	ACGGGAAGCTGCTGAAAAGG
	reverse	TGGGGCTCTGCCAAAAAGAT
IGFBP7	forward	GGCATGGAGTGCGTGAAGAG
	reverse	CTTGCTGACCTGGGTGATGG
IRF1	forward	CCGTTCTTGCCCTCCTGAGT
	reverse	TCCATGGCCTCTGCCTTACA
IL1R1	forward	TCTTGCCTCGACCCTTCCTC
	reverse	TGCTCCTGGAAGGCAGTGAG
MEN1	forward	CAGGGGCCAGACAGTCAATG
	reverse	GGTGGGCTCCAGCTCCTCTA
NF2	forward	TCGGGCCCTGAATTTTCTGT
	reverse	GAGGTTGCAGTGAGCCGAGA
PEA15	forward	CTAGGGGAGGGGGCTGAGTT
	reverse	GGTGGGGGTTGAGTGGTCTC
RAP1GAP	forward	GGCAGACCTCCGAGGAAGAA
	reverse	GGAAGACCACAGCCACGATG
SMARCB1	forward	TCTGGATTTGAACCCGCTGA
	reverse	TGCTGTATGCGATGGTGGTG
STAT1	forward	GTTAGAAAAGCAAGACTG
	reverse	CATCTGGATTGGGTCTTCC
TP53	forward	AGCTCCCAGAATGCCAGAGG
	reverse	AAGCCCAGACGGAAACCGTA

ChIP

Gene	Forward or reverse primer	Sequence (5' → 3')
BNIP3L	forward	CCTCATTCCGTTTCTCCTCA
	reverse	GCCCAGGCCACGTGATGA
IGFBP7	forward	GATTGGAGGATGTTTCCC
	reverse	CATGTCACATTGTGGTTCTT
SMARCB1	forward	TGCTACAGTGGCTTCTTAAC
	reverse	TTCTACCCGAGGTTAGT

BRAF Genotyping
Forward primer: 5'-TCATAATGCTTGCTCTGATAGGA-3'
Reverse primer: 5'-GGCCAAAATTTAATCAGTGGA-3'

Bisulfite Sequencing
Forward primer: 5'-GAGAAATTAGAGGGTGGAAG-3'
Reverse primer: 5'-AACAAAAACAACAACCCAAC-3'