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Small Molecule Inhibition of Cytoskeletal Dynamics in Melanoma Tumors Results in Altered Transcriptional Expression Patterns of Key Genes Involved in Tumor Initiation and Progression

CARRIE SPENCER[*], **JOHN MONTALVO**[*], **SARAH R. MCLAUGHLIN**, and **BRAD A. BRYAN**
Ghosh Science and Technology Center, Department of Biology, Worcester State University, Worcester, MA, U.S.A

Abstract

Background—Rho kinase signaling plays an important role in the oncogenic process largely through its regulation of F-actin dynamics, and inhibition of this pathway results in reduction in tumor volume and metastasis across a number of tumor types. While the cytoskeletal-regulatory role of Rho kinase has been a topic of in-depth study, the mechanisms linking Rho kinase to altered gene expression are largely unknown.

Materials and Methods—Global gene expression analysis was performed on melanoma tumors treated with sham or the small molecule inhibitor Y27632.

Results—Inhibition of Rho kinase activity in melanoma tumors results in a statistically significant change in gene transcription of 94 genes, many of which are critically involved in tumor initiation and progression.

Conclusion—In addition to regulating tumorigenesis through modulation of the phosphoproteome, Rho kinase signaling also contributes to the regulation of the tumor transcriptome.

Keywords

Melanoma; chorioallantoic membrane tumor assay; Rho kinase; cytoskeleton; microarray; tumor initiation; tumor progression

Rho-associated protein kinases 1 and 2 (ROCK1/2, collectively known as Rho kinase) belong to a family of serine/threonine kinases which serve as key regulators of actin cytoskeletal dynamics and thus control cell migration and motility (1). Rho kinase phosphorylates many protein targets including the catalytic subunit of myosin phosphatase, myosin light chain, intermediate filaments, ezrin/radixin/ moesin family proteins, Lin11/ Isl1/Mec3 domain kinase (LIMK), collapsin response mediator protein-2, calponin, and adducin (2). Deregulation of Rho kinase signaling contributes to the metastatic behavior of many tumor types (3–6), and several preclinical and clinical studies have targeted this pathway for anticancer therapeutics in prostate, lung, melanoma, and glioblastoma tumors with good efficacy (7–10).

While the role of Rho kinase in regulating the phosphoproteome is well understood, considerably less attention has been given to the function of Rho kinase signaling in

Correspondence to: Brad A. Bryan, Ghosh Science and Technology Center, Worcester State University, 486 Chandler Street, Worcester, MA 01602 U.S.A. Tel: 15089298721, brad.bryan@worcester.edu.
[*]Both Authors contributed equally to this project.

regulating gene transcription. A handful of studies have identified global transcription alterations when Rho kinase is inhibited in epithelial and mesenchymal cell types grown in monolayers on plastic (11–13); however no reports examine global transcription regulation by Rho kinase using *in vivo* systems such as solid tumors. In this study, we utilized an *in ovo* melanoma cell xenograft system to perform whole-genome microarray analysis specifically on the melanoma tumor cells, while selectively excluding gene expression changes in cells of non-tumor origin such as endothelial cells, fibroblasts, stromal cells, and immune cells.

Materials and Methods

Cell culture

Mouse B16F1 melanoma (ATCC, Manassas, VA, USA), human NGP neuroblastoma (a generous gift from Dr. Rani George, Harvard Medical School), and human 4T1 breast cancer cells (a generous gift from Dr. Gary Sahagian, Tufts Medical Center) were cultured using standard tissue culture procedures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 80 U/ml penicillin, and 50 μg/ml streptomycin C.

Melanoma tumors

Tumors were grown using the gelatin sponge-chorioallantoic membrane (CAM) assay according to previously published methods (14). Briefly, a false air-sac was generated using needle aspiration directly over the CAM of fertilized chicken eggs (Charles River Laboratories, North Franklin, CT, USA) at day 8 post-fertilization. Using dissecting scissors, a 10×10 mm window was cut, revealing the underlying embryo and CAM vessels. A hand-cut 1 mm^3 gelatin sponge (Harvard Apparatus, Holliston, MA, USA) containing 20,000 dissociated tumor cells was placed onto the CAM and the window was sealed with sterile parafilm. A sham solution of isotonic saline solution or 10 μM solution of *trans*-4-[(1R)-1-aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide dihydrochloride (Y27632) (Enzo Life Sciences, Plymouth Meeting, PA, USA) was added daily directly onto the CAM tumor. At the indicated timepoint, tumors were collected, weighed, photographed on a lightbox, and stored in RNAlater (Ambion, Austin, TX, USA) according to the manufacturer's directions. In total, greater than 20 tumors per time point were collected for each condition over three independent experiments.

Hematoxylin and eosin staining of tumors

Tumors collected at each timepoint were processed, cryosectioned, and hematoxylin and eosin stained by Excalibur Pathology (Moore, Oklahoma, USA). Images were collected at ×50 magnification using a Carl Zeis Axioscope.

Microarray analysis

Two sham- and two Y27632-treated samples, consisting of at least four tumors pooled per sample, were collected at four days' treatment and subjected to triplicate microarray analysis per sample. DNA microarray analysis was performed using the Mouse v2 Whole Genome OneArray (Phalanx Biotech, Belmont, CA, USA). RNA quality and integrity were determined utilizing an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Only high quality RNA, having a RNA integrity number (RIN) of >7.0, and absorbance ratios A260/A280 >1.8 and A260/A230 >1.6, was utilized for further experimentation. RNA was converted to double-stranded cDNA and amplified using *in vitro* transcription that included amino-allyl UTP, and the cDNA product was subsequently conjugated with Cy5 NHS ester

(GEH Lifesciences, Piscataway, NJ, USA). Fragmented cDNA was hybridized at 42°C overnight using the HybBag mixing system with 1× OneArray Hybridization Buffer (Phalanx Biotech, Belmont, CA, USA), 0.01 mg/ml sheared salmon sperm DNA (Promega, Madison, WI, USA), at a concentration of 0.025 mg/ml labeled target. After hybridization, the arrays were washed according to the OneArray protocol. Raw intensity signals for each microarray were captured using a Molecular Dynamics Axon 4100A scanner, measured using GenePixPro Software, and stored in GPR format. The data from all microarrays in each experimental set was then passed to Rosetta Resolver (Microsoft, Redmond, WA, USA) for analysis. Testing was performed by combining technical replicates and performing statistical analyses using Rosetta Resolver's proprietary modeling techniques.

Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was extracted using Trireagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. RNA was converted to cDNA using Verso cDNA kit (Thermo Scientific) according to the manufacturer's instructions. PCR amplification of specific cDNAs was performed using primers designed by Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels were used as a control.

Results

Small molecule inhibition of Rho kinase signaling in CAM tumor assays

Inhibition of Rho kinase signaling has been shown to decrease tumor progression and metastasis in a number of *in vivo* models. Indeed our lab has previously demonstrated that systemic treatment of mice harboring subcutaneous B16F1 melanoma tumors with Y27632, a competitive inhibitor of ATP binding to the catalytic site of Rho kinase proteins, resulted in decreased tumor volume and inhibition of oncogenic properties such as tumor cell survival and migration (9). Similarly, B16F1 tumors grown on gelatin sponges using *in ovo* xenograft CAM tumor assays exhibited reduced tumor size following eight days of Y27632 treatment (Figure 1A), suggesting similar endpoint phenotypes between CAM and mouse tumor assays. Upon visual observation, Y27632-treated tumors appeared spongier and less defined than sham-treated tumors – an observation we previously observed in Y27632-treated B16F1 tumors grown in mice (9). Hematoxylin and eosin staining of cryosections confirmed this observation, revealing significant tissue disorganization and poorly defined tumor edges in Rho kinase inhibited tumors (Figure 1B). While later time points (8 days post-impantation) suggested that Y27632-treated tumors were at a growth disadvantage compared to sham treatments, observations of earlier time points (particularly four days' post-implantation) revealed Y27632-treated tumors were initially larger in size and weight, albeit greatly disorganized, compared to sham-treated tumors. Similar results were obtained from NGP human neuroblastoma and 4T1 human breast cancer CAM tumors collected after four days of treatment (Figure 2A and B).

Gene expression analysis

To understand the role of Rho kinase signaling in modulating gene expression in very early stages of tumor formation, we collected sham- and Y27632-treated CAM melanoma tumors four days after tumor inoculation onto the CAM and subjected them to microarray analysis. Out of a total of approximately 26,000 murine genes tested, 94 genes demonstrated a statistically significant fold change of >1.8 in the Y27632-treated melanoma tumors over the sham-treated tumors. Specifically, there were 9 genes that exhibited elevated mean expression levels (1.8- to 3.1-fold) and 85 genes that exhibited reduced mean expression levels (1.8- to 7.1-fold) in Y27632 melanoma tumors compared to sham-treated tumors (Tables I and II).

The molecular and cellular functions of both up- and down-regulated genes included cell cycle regulation (*Strada, Cdk2, Smad7, Tob1, Cdkl3*), DNA replication (*Lig3, Dscc1*), extracellular matrix/cell adhesion (*Fcgbp, Itgb5, Cyr61, Pcdh17*), cytoskeletal dynamics (*Capzb, Dmd, Mzt1, Lbr, Mylip*), cell death (*Atg4a*), transcriptional regulation (*Foxn3, Pknox1, Zfp618, Tcf12, Med1, Maf, Atf3*), growth factors and hormones (*Stc1*, *Pgf*), cell signaling (*Smad7, Rock1, Tec, Pde4d*), biogenesis and metabolism (*Gmps, Agxt2l1, Prps1l1, Cyp20a1, Idh1, Tars2, Lta4h, Acox2, Dimt1, Enpp2, Glrx2, Decr2, Ggps1, Serinc5, Tyr, Tnks2, Etnk1*), post-translational protein modification (*Ube2d3, Ppp2r3c, Usp31, Stt3a, Trim23, Thop1, Zdhhc2*), vesicle transport (*Dync1li1*), receptor proteins and receptor regulators (*Grk4, Klhl24, Erbb3, Plxnc1*), transport proteins (*Slc26a9, Mtch2, Slc25a36, Slc38a9, Unc93b1, Slc44a1*), nuclear localization (*Osbp, Mdfi*), mRNA splicing (*Srsf2, Rnu12, Mbnl2*), translational regulation (Mrps10, Etf1) and of unknown/presumed function (*Gm10876, Fam161a, Gm4769, Ankrd26, Zfp385b, Prosc, Asnsd1, 1110018J18Rik, Bc030336, Sypl, Fam46a, Slmo1, Gm10786, 4930579G24Rik, 2310035C23Rik, 1700066M21Rik, 5031439G07Rik, Heatr3, 4632427E13Rik, Fam84b, Mospd2, Gm10825, Loc100503583*). The genes whose expression was most significantly altered following Y27632 treatment were independently verified by semi-quantitative RT-PCR on cDNA synthesized from sham-and Y27632-CAM melanoma tumor samples collected four days-post-implantation (Figure 3).

Discussion

Rho kinase regulation of the phosphoproteome has been extensively studied over the past decade, yet how these cellular changes regulate the global transcriptome is poorly understood. An extensive literature search primarily reveals reports mentioning singular instances of gene regulation by altering Rho kinase signaling, but only a handful of large scale genomic approaches have addressed global Rho kinase mediated transcriptional changes. Global analysis of gene regulation in Rho kinase-inhibited fibroblast monolayers revealed that 2.3% of genes exhibited significantly altered expression levels to greater or less than 20% those of the sham (11). Pharmacological ROCK2 inhibition in endothelial cells, smooth muscle cells, and fibroblasts using the small molecule inhibitor SLx-2119 revealed changes in the low hundreds of genes per cell type, with only a very minimal overlap of common gene changes between cell types (12), suggesting that transcriptional alterations in response to Rho kinase inhibition are uniquely cell type specific. Lastly, a total of 66 genes were altered by Y27632 treatment in cultured human corneal stromal cells (13). These three studies utilized *in vitro* cell culture systems where cells were grown in monolayers on plastic – a system that is convenient, but hardly represents the complex microenvironment of an *in vivo* tissue. To date, no genomics-based study has been performed on Rho kinase-inhibited cancer lines or solid tumors. The findings reported in this manuscript are the first of their kind to describe the global transcriptome regulation by Rho kinase signaling in solid tumors.

Distinct disadvantages often exist when performing microarray analysis on tumors. Namely, in addition to the cancer cells, a mixture of various host cell types including immune cells, stromal cells, endothelial cells, and fibroblasts, are present which contaminate and complicate the analysis. Therefore gene expression profiles using mRNA isolated from total tumor tissue are very unlikely to reflect the genomic changes that occur specifically in the cancer cells themselves. To overcome this issue, we utilized an *in ovo* xenograph system whereby melanoma cancer cells were solely of mouse origin, while all other associated infiltrating cells were of chicken origin. Thus we could address the global gene changes specific for the melanoma cells in response to Rho kinase inhibition without non-tumor cell contamination. We established a cutoff for significant gene changes at greater or less than 1.8-fold, with a stringent *p*-value of less than 0.01. Under these conditions, a total of 94

genes were identified, with 9 genes exhibiting elevated mean expression levels and 85 genes exhibiting reduced mean expression levels in Y27632-treated melanoma tumors.

Among the many genes identified in this study, approximately 20% of these have been suggested to have roles as tumor suppressors/oncogenes, angiogenesis regulators, and/or serve as molecular markers of cancer. Plxnc1 (−2.4-fold) is a receptor for the GPI-anchored semaphorin Sema7A and serves as a tumor suppressor by opposing melanocyte dendricity and melanoma progression, as well as inactivating cofilin and R-Ras GAP-mediated cell migration (15, 16). Moreover, Plxnc1 expression is lost during melanoma metastasis and is down-regulated across a panel of melanomas (15–17). A deficiency in the *Pknox1* gene (−1.9-fold), encoding an atypical homeobox transcription factor that binds DNA cooperatively with E2A/myogenic transcription factors, leads to decreased levels of the anti-apoptotic protein Bcl-Xl and spontaneous tumor formation in mice (18, 19). Additionally, 70% of tumors have been reported to exhibit reduced or a lack of expression of Pknox1 (18). *Maf* (−2.5-fold) is considered a potent oncogene with strong transforming activity, and functions as a transcriptional activator of cyclin D2 and other proliferative genes (20). Maf is translocated or up-regulated in multiple myelomas and T-cell lymphomas (21, 22). Erbb3 (−2.1-fold) is a member of the epidermal growth factor receptor family of proteins. This strong oncogene regulates the phosphoinositol 3-kinase/AKT pathway to promote survival and proliferation, and recent results link high ERBB3 activity with escape from chemotherapy targeting other ERBBs in lung and breast cancer (23). Erbb3 has been implicated in the initiation and progression of tumors in a very large number of tissue types (23). Smad7 (−1.9-fold) is an inhibitor of the TGFbeta signaling pathway, and under normoxic conditions functions as a strong tumor suppressor; however, its expression is greatly up regulated in response to hypoxia and contributes to the resistance of TGFbeta growth suppression in hypoxic tumors (24, 25). Moreover, particular polymorphisms in the *Smad7* gene have been shown to increase an individual's risk to colon cancer (26). Cyr61 (−1.9-fold) is a strongly pro-angiogenic secreted extracellular matrix (ECM) protein that regulates cell survival, apoptosis, inflammation, cell adhesion, migration, and metastasis, and is believed to influence the disease course of many cancers through binding with integrins (27). Not only does Cyr61 promote *in vitro* and *in vivo* angiogenesis, but it also serves a role in the recruitment of endothelial progenitor cells to tumors (28). Expression of Cyr61 is regulated in response to hypoxia (29) and is up-regulated in a variety of cancer types (29–31). Interestingly, Rho kinase inhibition down-regulates the expression of Rho kinase 1 (ROCK1; −1.8-fold). ROCK1 plays a major role in the regulation of tumor cell invasion and metastasis (32). Moreover, pharmacological inhibition of Rho kinase activity effectively inhibits solid tumor growth and spreading across numerous cancer types (32). Unfortunately, small molecule inhibition of Rho kinase in cisplatin treated neuroblastomas leads to acquisition of a chemoresistant phenotype through altered cell cycle regulation, up regulation of cisplatin influx/efflux genes, and up regulation of nucleotide excision repair genes (33), suggesting that Rho kinase may also play a role in chemotherapy resistance. Rho kinase is also an essential regulator of endothelial cell fate determination and *in vitro* and *in vivo* blood vessel formation (34). Other interesting genes identified in this study are the tumor suppressors *Pcdh17* (35), *Zdhhc2* (36), and *Tob1* (37), the cell type variable tumor suppressor/oncogene *Atf3* (38, 39), the angiogenic regulators *Pgf* (40) and *Itgb5* (41), and the tumor biomarkers *Stc1* (42, 43), *Fcgbp* (44), and *Med1* (45–47).

The data presented in this study examine for the first time the global transcriptional regulation controlled by Rho kinase in melanoma tumors. These findings identified many Rho kinase-responsive genes involved directly in the tumorigenic process, and further suggest that targeting Rho kinase activity may not only disrupt the tumor phosphoproteome, but also disturb the tumor transcriptome. Future experiments should address temporal

regulation of global gene expression during tumor progression and identify commonalities and differences in genes regulated by Rho kinase between different tumor cell types.

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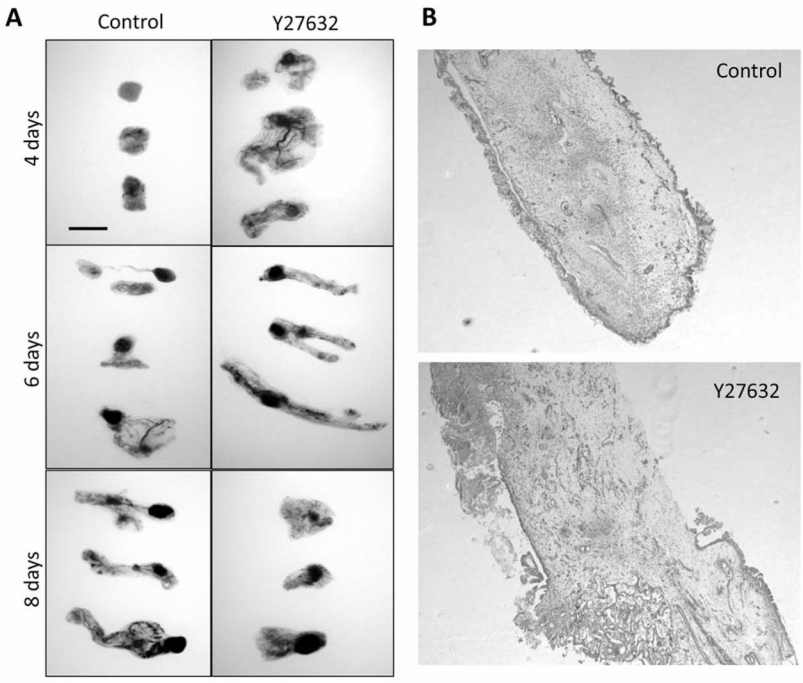

Figure 1.

Pharmacological inhibition of Rho kinase disrupts CAM melanoma tumor progression. A: Melanoma tumors were grown using the gelatin sponge-chorioallantoic membrane (CAM) assay according to previously published methods (14). A sham solution of isotonic saline solution or 10 μM solution of Y27632 was added daily directly onto the CAM tumor. At the indicated timepoint after tumor implantation on the CAM, tumors were collected and photographed on a lightbox. At least 20 tumors per time point were collected for each condition over three independent experiments. Scale bar=200 μm. B: Sham and Y27632 treated tumors were cryosectioned, hematoxylin and eosin stained, and photographed at ×50 magnification (data shown is for tumors collected at 6 days post-implantation).

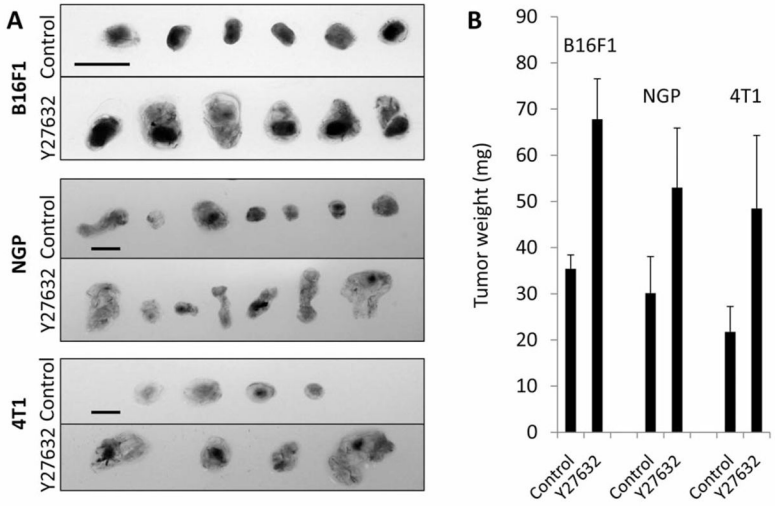

Figure 2.

Y27632 treatment of newly formed CAM tumors results in enlarged initial tumor size and weight. A: B16F1 mouse melanoma, NGP human neuroblastoma, and 4T1 human breast cancer tumors were grown using the gelatin sponge-chorioallantoic membrane (CAM) assay according to previously published methods (14). A sham solution of isotonic saline solution or 10 μM solution of Y27632 was added daily directly onto the CAM tumor. At four days' treatment, tumors were collected and photographed on a lightbox. B: Average tumor weights at four days' treatment. At least 20 tumors were collected per condition over three independent experiments. Scale bar=200 μm.

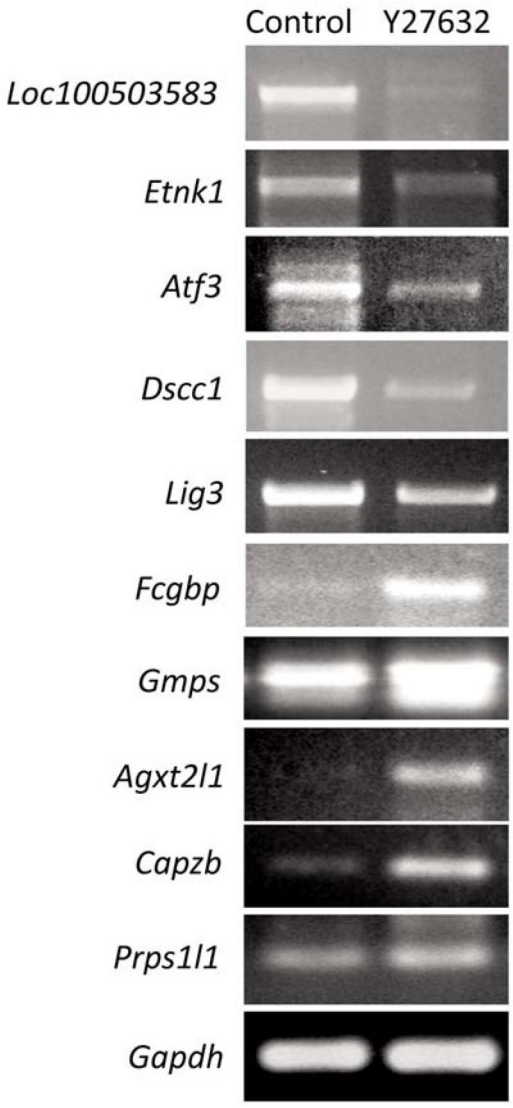

Figure 3.

Semi-quantitative RT-PCR confirmation of microarray results. cDNA collected from sham- and Y27632-treated tumors at four days' treatment were subjected to semi-quantitative RT-PCR analysis using primers specific for the five most up- and down-regulated protein coding genes identified in the microarray analysis.

Table I

Genes up-regulated by more than 1.8-fold in Y27632-treated melanoma tumors.

Gene symbol	Gene name	Accession number	Fold change	*P*-value
Fcgbp	Fc fragment of IgG binding protein	NM_001122603.1	3.1	0.002
Gmps	Guanine monophosphate synthetase	NM_001033300.2	2.6	0.002
Agxt2l1	Alanine glyoxylate-aminotransferase 2-like 1	NM_001163587.1	2.3	0.009
Capzb	Capping protein (actin filament) muscle Z-line beta	NM_001037761.1	2.1	0.008
Prps1l1	Phosphoribosyl pyrophosphate synthetase 1-like 1	NM_029294.2	2.1	0.006
Dmd	Dystrophin, muscular dystrophy	NM_007868.5	2.0	0.007
Gm10876	Predicted gene 10876	XM_003085005.1	1.9	0.0003
Grk4	G-Protein-coupled receptor kinase 4	NM_001080743.1	1.9	0.004
Slc26a9	Solute carrier family 26, member 9	NM_177243.4	1.8	0.01

Table II

Genes down-regulated by more than 1.8-fold in Y27632-treated melanoma tumors.

Gene symbol	Gene name	Accession number	Fold change	*P*-value
Rnu12	RNA U12, small nuclear	NR_004432.2	−7.1	0.00001
LOC100503583	Fibrinogen silencer-binding protein-like	XM_003084562.1	−3.7	0.006
Etnk1	Ethanolamine kinase 1	NM_029250.2	−3.2	0.001
Atf3	Activating transcription factor 3	NM_007498.3	−2.9	0.0002
Dscc1	Defective in sister chromatid cohesion 1	NM_183089.2	−2.9	0.005
Lig3	Ligase III, DNA, Atp-depedent	NM_010716.2	−2.9	0.002
Gm10825	Predicted gene 10825	NR_028580.1	−2.7	0.008
Mospd2	Motile sperm domain containing 2	NM_029730.3	−2.7	0.0004
Maf	Musculoaponeurotic fibrosarcoma	NM_001025577.2	−2.5	0.003
Mylip	Myosin regulatory light chain interactin protein	NM_153789.3	−2.5	0.005
Slc44a1	Solute carrier family 44, member 1	NM_001159633.1	−2.5	0.0009
Lbr	Lamin B receptor	NM_133815.2	−2.4	0.003
Tnks2	TRF1-interacting ankyrin-related ADP ribose	NM_001163635.1	−2.4	0.002
Fam84b	Family with sequence similarity 84, member B	NM_001162926.1	−2.4	0.0007
Plxnc1	Plexin C1	NM_018797.2	−2.4	0.0002
Pde4d	Phosphodiesterase 4D cAMP specific	NM_011056.2	−2.3	0.0003
Med1	Mediator complex subunit 1	NM_134027.2	−2.3	0.009
4632427E13Rik	RIKEN cDNA 4632427E13 gene	NR_015510.1	−2.3	0.001
Cdkl3	Cyclin dependent kinase-like 3	NM_001166657.1	−2.3	0.003
Unc93b1	Unc-93 homolog B1	NM_019449.2	−2.3	0.0004
Tcf12	Transcription factor 12	NM_011544.3	−2.3	0.0003
Heatr3	HEAT repeat containing 3	NM_172757.3	−2.2	0.009
Slc38a9	Solute carrier family 38, member 9	NM_178746.4	−2.2	0.0001
Mbnl2	Muscleblind-like 2	NM_207515.2	−2.2	0.003
Tyr	Tyrosinase	NM_011661.4	−2.1	0.0005
5031439G07Rik	RIKEN cDNA 5031439G07 gene	NM_001033273.2	−2.1	0.0001
Serinc5	Serine incorporator 5	NM_172588.2	−2.1	0.006
Ggps1	Geranylgeranyl diphosphate synthase 1	NM_010282.2	−2.1	0.001
Pcdh17	Protocadherin 17	NM_001013753.2	−2.1	0.007
Zfp618	Zing finger protein 618	NM_028326.1	−2.1	0.007
Erbb3	Erythroblastic leukemia viral oncogene homolog 3	NM_010153.1	−2.1	0.002
Zdhhc2	Zinc finger, DHHC domain containing 20	NM_029492.4	−2.1	0.001
Thop1	Thimet oligopeptidase 1	NM_022653.4	−2.0	0.0005
1700066M21Rik	RIKEN cDNA 1700066M21 gene	NM_028546.1	−2.0	0.001
Tob1	Transducer of ErbB-2.1	NM_009427.2	−2.0	0.0002
Decr2	2-4-Dienoyl-coenzyme A reductase 2, peroxisomal	NM_011933.2	−2.0	0.004
Trim23	Tripartite motif-containing 23	NM_030731.3	−2.0	0.002
Stt3a	Subunit oligosaccharyltransferase complex homolog-A	NM_008408.4	−2.0	0.006
Glrx2	Glutaredoxin 2 (thioltransferase)	NM_001038593.1	−1.9	0.009

Gene symbol	Gene name	Accession number	Fold change	*P*-value
Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	NM_015744.2	−1.9	0.003
Pknox1	Pbx/knotted 1 homeobox	NR_027493.1	−1.9	0.001
Tec	Tec protein tyrosine kinase	NM_001113464.1	−1.9	0.005
2310035C23Rik	RIKEN cDNA 2310035C23 gene	NM_029349.1	−1.9	0.0002
Osbp	Oxysterol binding protein	NM_001033174.1	−1.9	0.006
Cyr61	Cysteine rich protein 61	NM_010516.2	−1.9	0.0009
Pgf	Placental growth factor	NM_008827.2	−1.9	0.002
4930579G24Rik	RIKEN cDNA 4930579G24 gene	NM_029482.1	−1.9	0.0001
Itgb5	Integrin beta 5	NM_001145884.1	−1.9	0.0008
Atg4a	Autophagy-related 4A	NM_174875.3	−1.9	0.0007
Gm10786	Predicted gene 10786	XM_003084921.1	−1.9	0.0001
Slmo1	Slowmo homolog 1	NM_144867.2	−1.9	0.009
Etf1	Eukaryotic translation termination factor 1	NM_144866.3	−1.9	0.0003
Usp31	Ubiquitin specific peptidase 31	NM_001033173.1	−1.9	0.0001
Srsf2	Serine/arginine-rich splicing factor 2	NM_011358.2	−1.9	0.0001
Mdfi	MyoD family inhibitor	NM_001109973.1	−1.9	0.000005
Foxn3	Forkhead box N3	NM_183186.2	−1.9	0.0008
Fam46a	Family with sequence similarity 46, member A	NM_001160379.1	−1.9	0.00009
Smad7	MAD homolog 7	NM_001042660.1	−1.9	0.001
Dimt1	DIM1 dimethyladenosine transferase 1-like	NM_025447.4	−1.8	0.002
Rock1	Rho-associated coiled-coil containing protein kinase 1	NM_009071.2	−1.8	0.0002
Acox2	Acyl-coenzyme A oxidase 2, branched chain	NM_001161667.1	−1.8	0.0007
Lta4h	Leukotriene A4 hydrolase	NM_008517.2	−1.8	0.001
Dync1li1	Dynein cytoplasmic 1 light intermediate chain 1	NM_146229.2	−1.8	0.00005
Fam161a	Family with sequence similarity 161, member A	NM_028672.2	−1.8	0.008
Ube2d3	Ubiquitin-conjugating enzyme E2D 3	NM_025356.4	−1.8	0.008
Mrps10	Mitochondrial ribosomal protein S10	NM_001146211.1	−1.8	0.001
Ppp2r3c	Protein phosphatase 2, regulatory subunit B, gamma	NM_021529.3	−1.8	0.0005
Mtch2	Mitochondrial carrier homolog 2	NM_019758.2	−1.8	0.001
Gm4769	Predicted gene 4769	NM_177596.3	−1.8	0.004
Klhl24	Kelch-like 24	NM_029436.3	−1.8	0.002
Cyp20a1	Cytochrome P450, family 20, subfamily A, polypeptide 1	NM_030013.2	−1.8	0.000009
Ankrd26	Ankyrin repeat domain 26	NM_001081112.1	−1.8	0.00007
Zfp385b	Zinc finger protein 385B	NM_178723.6	−1.8	0.00007
Prosc	Proline synthetase co-transcribed	NM_001039078.2	−1.8	0.00008
Idh1	Isocitrate dehydrogenase 1 (NADP+), soluble	NM_001111320.1	−1.8	0.0003
Slc25a36	Solute carrier family 25, member 36	NM_138756.4	−1.8	0.00009
Asnsd1	Asparagine synthetase domain containing 1	NM_133728.2	−1.8	0.0001
1110018J18Rik	RIKEN cDNA 1110018J18 gene	NM_025370.2	−1.8	0.0007
Bc030336	cDNA sequence BC030336	NM_001164580.1	−1.8	0.0001
Strada	STE20-related kinase adaptor alpha	NM_028126.2	−1.8	0.0001
Cdk2	Cyclin-dependent kinase 2	NM_016756.4	−1.8	0.001

Gene symbol	Gene name	Accession number	Fold change	*P*-value
Sypl	Synaptophysin-like protein	NM_013635.3	−1.8	0.0008
Stc1	Stanniocalcin 1	NM_009285.3	−1.8	0.002
Mzt1	Mitotic spindle organizing protein 1	NM_175245.4	−1.8	0.0004
Tars2	Threonyl-tRNA synthetase 2, mitochondrial (putative)	NM_001163617.1	−1.8	0.00003