Somatic Mutation of GRIN2A in Malignant Melanoma Results in Loss of Tumor Suppressor Activity via Aberrant NMDAR Complex Formation

Todd D. Prickett[1], Brad J. Zerlanko[1], Victoria K. Hill[1], Jared J. Gartner[1], Nouar Qutob[2], Jiji Jiang[1], May Simaan[3], John Wunderlich[4], J. Silvio Gutkind[3], Steven A. Rosenberg[4] and Yardena Samuels[1,2]

The ionotropic glutamate receptors (*N*-methyl-D-aspartate receptors (NMDARs)) are composed of large complexes of multi-protein subunits creating ion channels in the cell plasma membranes that allow for influx or efflux of mono- or divalent cations (e.g., Ca^{2+}) important for synaptic transmissions, cellular migration, and survival. Recently, we discovered the high prevalence of somatic mutations within one of the ionotropic glutamate receptors, GRIN2A, in malignant melanoma. Functional characterization of a subset of GRIN2A mutants demonstrated a loss of NMDAR complex formation between GRIN1 and GRIN2A, increased anchorage-independent growth in soft agar, and increased migration. Somatic mutation of GRIN2A results in a dominant negative effect inhibiting the tumor-suppressive phenotype of wild-type (WT) GRIN2A in melanoma. Depletion of endogenous GRIN2A in melanoma cells expressing WT GRIN2A resulted in increased proliferation compared with control. In contrast, short-hairpin RNA depletion of GRIN2A in mutant cell lines slightly reduced proliferation. Our data show that somatic mutation of GRIN2A results in increased survival, and we demonstrate the functional importance of GRIN2A mutations in melanoma and the significance that ionotropic glutamate receptor signaling has in malignant melanoma.

Journal of Investigative Dermatology (2014) **134,** 2390–2398; doi:10.1038/jid.2014.190; published online 15 May 2014

INTRODUCTION

Glutamate receptors are involved in cell homeostasis, cell growth, neurotransmission, cell survival, and programmed cell death (Komuro and Rakic, 1993; Kaderlik *et al.*, 1994; Nacher and McEwen, 2006; Schlett, 2006; Wang *et al.*, 2007). Glutamate receptors are composed of two different major types; the ionotropic and the metabotropic family (Stepulak *et al.*, 2009). Ionotropic glutamate receptors (e.g., *N*-methyl-D-aspartate (NMDA) receptors) are composed of large complexes of multi-protein subunits creating ion channels in the cell plasma membranes allowing influx or efflux of mono- or divalent cations (e.g., Ca^{2+}) (Monyer *et al.*, 1992). Upon binding of glutamate and glycine, these ligand-gated ion channels change their conformation resulting in ion permeability and intracellular oscillations that, in many neuronal cells (cerebellar granule cells, neurons, astrocytes, and glial cells), are important for synaptic transmissions, cellular migration, and survival (Komuro and Rakic, 1993; Lau and Tymianski, 2010; Traynelis *et al.*, 2010).

The NMDA receptor has a major role in many neuronal processes and disease etiologies such as learning, memory, and neurodegeneration (i.e., Schizophrenia; Lang *et al.*, 2007). The NMDARs are heterotetrameric complexes consisting of two NR1 (GRIN1) subunits and two NR2 (GRIN2 (A–D)), or a mixture of GRIN2 and GRIN3 subunits predominately expressed in neuronal cells. Upon binding of its cognate ligands, NMDAR permits Ca^{2+} influx resulting in increased intracellular calcium levels leading to activation of calcium-dependent signal transduction (Traynelis *et al.*, 2010). However, hyperactivation of NMDAR by glutamate leads to calcium-dependent cell death (excitotoxicity), a phenomenon observed in neurodegenerative diseases and brain tumors (Ye and Sontheimer, 1999; Takano *et al.*, 2001). Recent studies have demonstrated the importance of functional NMDA receptors in neurological systems. GRIN2A and GRIN2B were found to be mutated in Schizophrenia patients and patients with epilepsy causing decreased channel permeation (Endele *et al.*, 2010; Carvill *et al.*, 2013; Lemke *et al.*, 2013; Lesca *et al.*, 2013). The implication that the NMDA receptor functions as a conveyor of cellular signals important for cognitive learning and apoptosis has been well

[1]*Cancer Genetics Branch, National Human Genetics Research Institute, National Institutes of Health, Bethesda, Maryland, USA;* [2]*Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel;* [3]*Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland, USA and* [4]*Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA*

Correspondence: Yardena Samuels, Cancer Genetics Branch, National Human Genetics Research Institute, National Institutes of Health, Bethesda, Maryland 20892, USA. E-mail: Yardena.samuels@weizmann.ac.il

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NMDAR, N-methyl-D-aspartate receptor; shRNA, short-hairpin RNA; WT, wild type

Received 3 July 2013; revised 14 January 2014; accepted 27 January 2014; accepted article preview online 16 April 2014; published online 15 May 2014

established in neuronal diseases and brain tumors (Rzeski *et al.*, 2001; Hahn *et al.*, 2006; Lau and Tymianski, 2010). Interestingly, glioma cells (either in culture or in patients) secrete excess glutamate to the extracellular milieu (composed of many cell types including glial and neuronal) (Ye and Sontheimer, 1999) causing overstimulation of glutamate receptors on neurons leading to increased Ca^{2+}-mediated apoptosis, allowing glioma cells room to grow and spread (Takano *et al.*, 2001).

Recently, our lab demonstrated through whole-exome sequencing a high prevalence of somatic mutations in GRIN2A in malignant melanoma (Wei *et al.*, 2011). Whole-exome sequencing of our matched samples revealed the unexpected discovery that *GRIN2A* was somatically mutated in ~25% of the melanoma cases. The mutations were distributed throughout the gene, with clustering of mutations at amino acids within important functional domains (e.g., the ligand binding domain). We also observed three recurrent alterations at S278F, E371K, and E1175K as well as five nonsense mutations. Recently, another group recently published a whole-exome screen of eight melanoma samples and found two additional somatic mutations in GRIN2A, suggesting that genetic alteration of this gene is important (Stark *et al.*, 2011). Furthermore, seven somatic mutations found in our screen have been discovered by others as well as some listed in the COSMIC database (D252N, S278F, W343X, G449E, M653I, R920K, S929F, and E1073K; Berger *et al.*, 2012; Hodis *et al.*, 2012) (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). Taken together with the recent publication of mutations found in Schizophrenic patients and our data we decided to analyze how these mutations affected GRIN2As function giving us a better understanding of how NMDA receptors work in melanomagenesis.

RESULTS

To determine how somatic mutations in the NMDA receptor subunit *GRIN2A* effect the functioning of NMDARs (e.g., complex formation or cation influx), we cloned specific mutations based on location within important functional domains or if they truncated the protein product (see schematic in Supplementary Figure S1 online). We used wild-type (WT) GRIN2A to insert mutations and first examined complex formation between GRIN1 and GRIN2A using a transient expression assay. HEK293T cells were transiently co-transfected with WT GRIN1 with GRIN2A (WT, E371K, W372X, E373K, G889E, Q891X, R920K, E1172K, or W1271X) or empty vector control and further tested for complex formation via co-immunoprecipitation using anti-GRIN1 (Figure 1a). As can be seen, WT GRIN1 precipitated WT GRIN2A and to a lesser extent GRIN2A (W1271X). However, the rest of the mutations in GRIN2A had very little to no association with GRIN1 *in vitro*. To rule out a lack of protein expression in these cells we probed cell lysates from the same experiment with anti-GRIN2A and found equal expression (Figure 1a bottom panel). We probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Furthermore, we tested complex formation now focusing on protein–protein binding between GRIN1 and GRIN2A precipitating with GRIN2A and probing for GRIN1. HEK293T cells were transiently co-transfected with GRIN1 and GRIN2A (WT, W372X, Q891X, R920K, W1271X), or empty vector control followed by co-immunoprecipitation using anti-GRIN2A (Figure 1b). We observed that WT GRIN2A and to lesser extent GRIN2A (R920K) precipitated GRIN1. However, GRIN2A (W372X, Q891X, or W1271X) mutants showed a lack of binding to GRIN1 corroborating our previous results. The differences observed between binding of GRIN1 to GRIN2A (W1271X) may be a result of the use of antibodies directed toward GRIN1 or GRIN2A and their efficiencies or epitope-binding domains. These results suggest that GRIN2A somatic mutations resulting in truncated proteins reduce proper complex formation with GRIN1. As GRIN2A ultimately forms a cation channel, loss of such a complex formation would result in decreased influx and ultimately attenuation of the cation-dependent signaling mechanism.

NMDA receptors require proper complex formation between GRIN1 and one of the many different GRIN2/3 subunits to allow for calcium influx into cells. We used a co-expression assay in HEK293T cells to test whether calcium influx into cells is affected by these mutations. Transiently transfected HEK293T cells were analyzed for influx of calcium upon stimulation with NMDA, a specific ligand for the NMDA receptor. Cells expressing WT GRIN1 and WT GRIN2A, upon stimulation with 200 μM of NMDA, show proper NMDA receptor function with an increase in calcium permeability in the cells as a measure of fold change over time (Figure 1c, top left panel). However, expression of GRIN1 with GRIN2A (Q891X or R920K) results in nonfunctional NMDA receptors (Figure 1c), confirming the previous results showing reduced binding between GRIN1 and these mutants. Interestingly, expression of GRIN1 with GRIN2A (W1271X) shows diminished calcium influx in transiently transfected cells (Figure 1c). Our transient results demonstrate that proper complex formation is required between GRIN1 and GRIN2A for the calcium channel to function appropriately. We hypothesize that functional NMDA receptor signaling in melanoma cells containing GRIN2A truncation mutations is lost resulting in diminished activation of Ca^{2+}-dependent pathways, potentially leading to increased survival through suppression of cell death (excitotoxicity).

To determine the nature of the loss of functioning of these mutations, we examined the ability of these mutations to inhibit complex formation via a dominant negative effect. We co-expressed WT GRIN1 and WT GRIN2A in the absence or presence of mutated GRIN2A in a transient system. Figure 1d shows that increased expression of mutant GRIN2A results in loss of complex formation between WT GRIN1 and WT GRIN2A compared with increased amounts of empty vector transfected into cells. These results suggest that somatic mutations of GRIN2A cause a loss of functioning potentially through a dominant negative effect inhibiting normal NMDAR signaling downstream to known effector molecules (e.g., p38 MAPK (mitogen-activated protein kinase); Doronzo *et al.*, 2010).

Hyperactivation of NMDA receptors via glutamate or NMDA stimulation leads to prolonged influx of Ca^{2+} ions

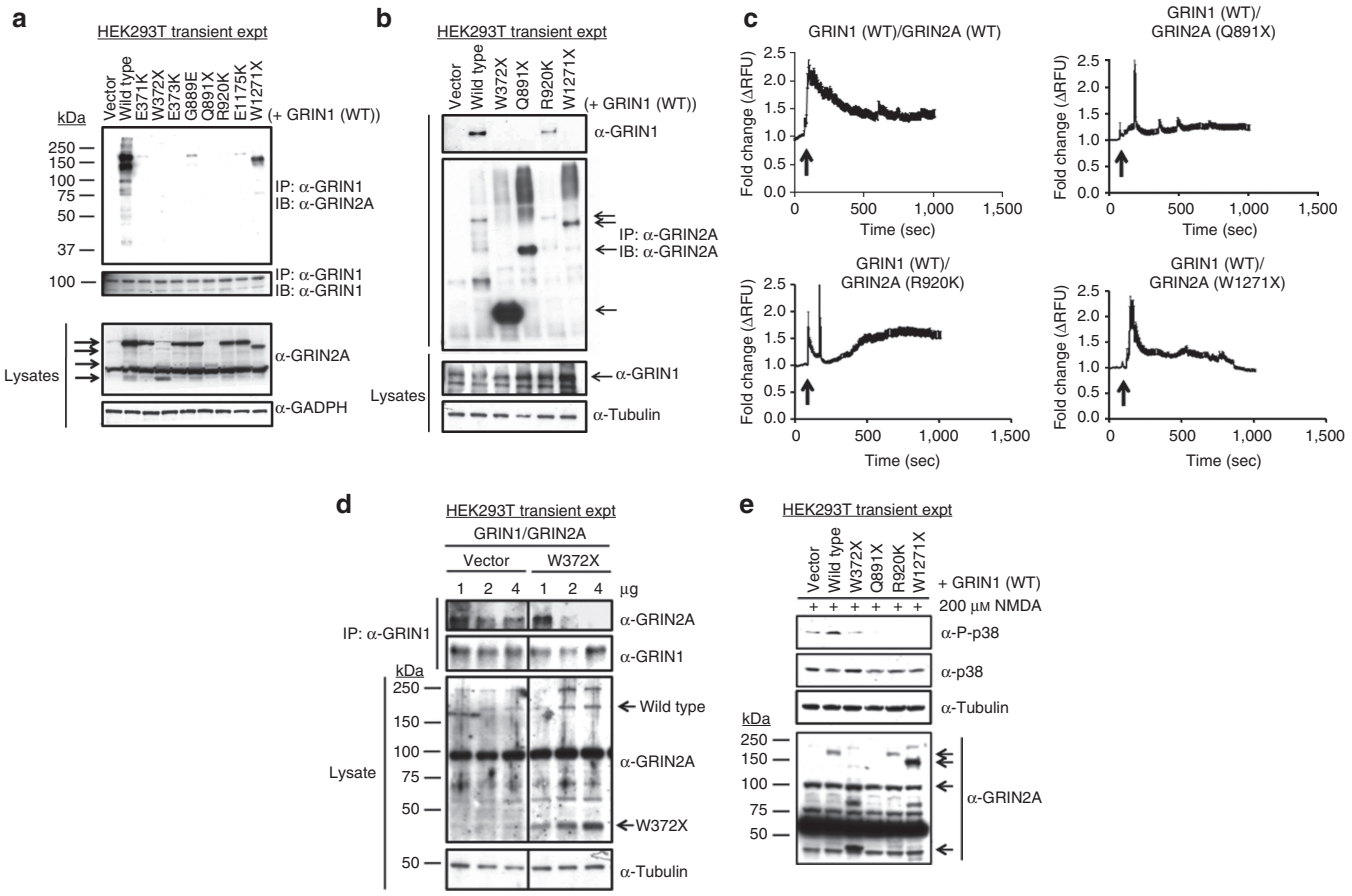

Figure 1. Somatic mutations in GRIN2A have adverse effects on receptor function and formation. Mutant forms of GRIN2A-binding GRIN1 with reduced affinity thus causing decreased NMDAR complex formation. (**a**) HEK293T cells were transiently transfected with wild-type (WT) GRIN1 and GRIN2A (WT or mutants) or empty vectors as control and immunoprecipitated with anti-GRIN1. Immunoprecipitates (IPs) were probed with anti-GRIN2A and anti-GRIN1 to confirm binding. Lysates were probed with anti-GRIN2A, and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) was used as a loading control. (**b**) HEK293T cells were transiently transfected with WT GRIN1 and GRIN2A (WT or mutants) or empty vectors as control and immunoprecipitated with anti-GRIN2A. Immunoprecipitates were probed with anti-GRIN1 and anti-GRIN2A to confirm binding. Lysates were probed with anti-GRIN1 and anti-GAPDH as a loading control. (**c**) Influx of calcium upon *N*-methyl-D-aspartate (NMDA) stimulation of transient transfected HEK293T cells shows decreased calcium permeability in cells expressing mutant forms of GRIN2A. Cells were pretreated with the Fluo-3/AM probe prior to imaging on Zeiss confocal microscope. Images were taken every 5 seconds over a period of 15 minutes. NMDA (200 μM) was added to the dishes at the 100 second mark. Graphs are fold increase in fluorescent signal vs. time (seconds). Values are representative of 8–10 individual cells from the image view. (**d**) HEK293T cells were transiently co-transfected with WT GRIN1 and WT GRIN2A and increasing amounts of empty vector or W372X and immunoprecipitated with anti-GRIN1. Immunoprecipitates were probed with anti-GRIN1 and anti-GRIN2A to confirm binding. Lysates were probed with anti-GRIN1 and anti-GAPDH as a loading control (samples are on one gel but adjusted to be next to each other in figure). (**e**) HEK293T cells were transiently co-transfected with WT GRIN1 and GRIN2A (WT or mutants) or empty vectors as control and stimulated with 200 μM NMDA in the presence of Ringer's solution for 60 minutes. Lysates were probed with anti-P-p38 mitogen-activated protein kinase (MAPK), anti-p38 MAPK, and anti-GRIN2A to confirm expression. Lysates were probed with anti-tubulin as a loading control. Figures were adjusted to remove unwanted overall background due to western blotting and film development. IB, immunoblot.

and hyperactivation of pro-apoptotic signaling mechanisms (e.g., p38 MAPK activation) leading to reduced cell proliferation, migration, invasion, and even cell death in certain cellular contexts (e.g., excitotoxicity; Wang *et al.*, 2013). To determine how somatic mutation of GRIN2A effects its ability to activate Ca^{2+}-mediated signaling to pro-apoptotic pathways, we co-expressed GRIN2A (WT or mutants) or empty vector with GRIN1 (WT) in the presence of Ringer's solution and NMDA. Cells expressing WT GRIN1 and WT GRIN2A had a significant increase in p38 MAPK activation when stimulated with NMDA (Figure 1e). However, co-expression of WT GRIN1 with any of the GRIN2A mutant constructs or empty vectors resulted in little to no p38 MAPK activation in the presence of NMDA (Figure 1e). These results further corroborate our hypothesis that GRIN2A may function as a tumor suppressor in melanoma cells whereby certain somatic mutations cause attenuation of NMDAR-dependent Ca^{2+}-mediated pro-apoptotic signaling.

To better understand how somatic mutations of GRIN2A in melanoma affect its ability to potentiate tumorigenesis, we created stable pooled clones expressing a subset of the mutations initially tested. Two independent cell lines, 31T and SK-Mel-2, were used to co-express GRIN1 and GRIN2A (WT, W372X, Q891X, R920K, or W1271X) or empty vector

control via sequential transduction and dual selection. We tested for expression and the ability to form proper NMDAR complexes in both cell lines (31T or SK-Mel-2) by immunoprecipitating using either anti-GRIN2A or anti-GRIN1 and analyzing via western blot analysis (Figure 2a and b, respectively). GRIN2A expression was similar for GRIN2A, W372X, Q891X, and W1271X in both cell lines; however, there were some differences of expression for WT GRIN2A and GRIN2A (R920K) between 31T and SK-Mel-2 cells. GRIN1 was relatively equally expressed in both stable cell

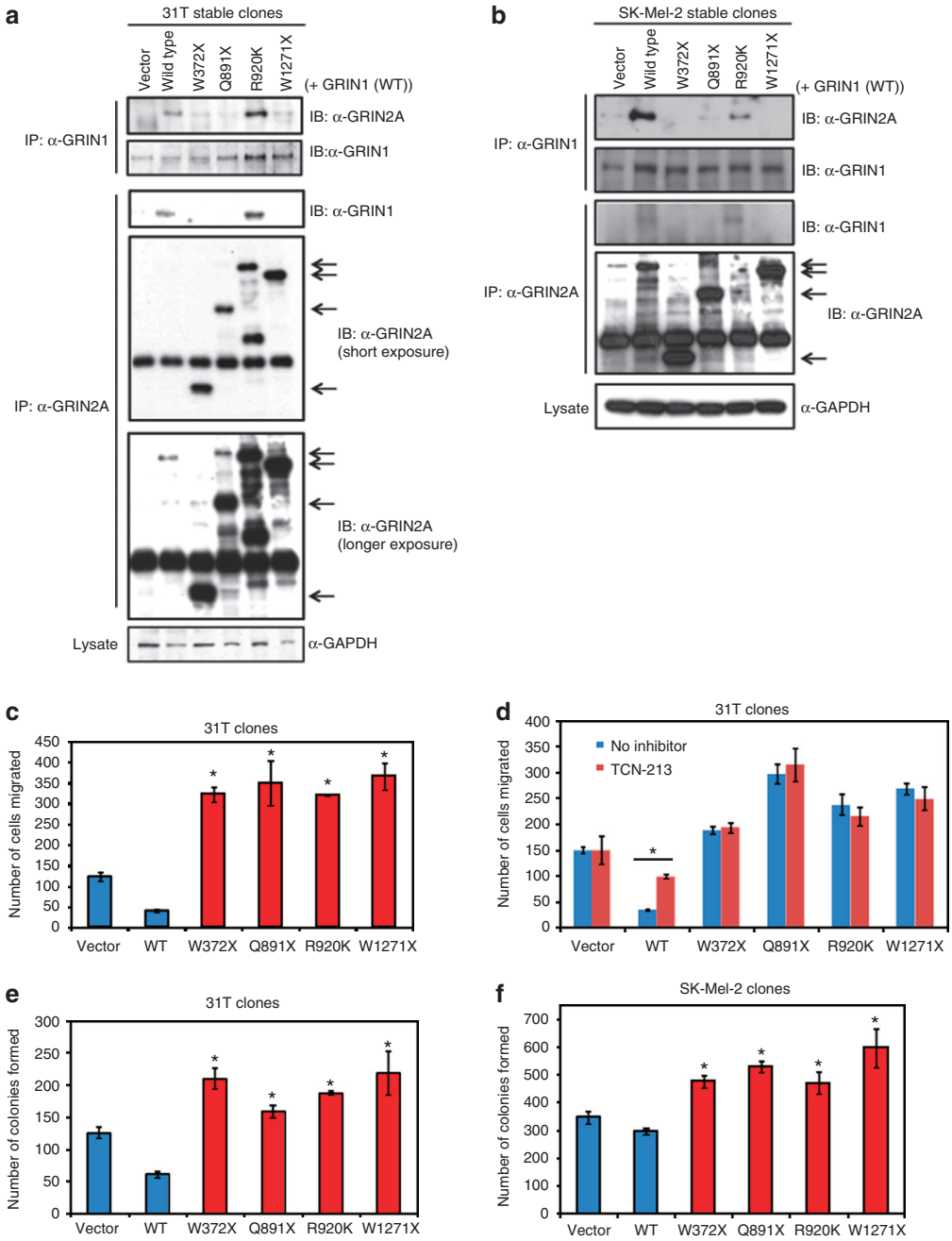

Figure 2. Melanoma cells expressing mutant forms of GRIN2A have increased ability for anchorage-independent growth. Stable melanoma cell lines 31T and SK-Mel-2 expressing wild-type (WT) GRIN1 and GRIN2A (WT or mutants) or empty vector control and immunoprecipitated with anti-GRIN1 and anti-GRIN2A. Immunoprecipitates (IP) were probed with anti-GRIN2A and anti-GRIN1 to examine *N*-methyl-D-aspartate receptor (NMDAR) complexes in 31T (**a**) or SK-Mel-2 (**b**). Melanoma cell line 31T cells (**c**) stably expressing GRIN1 and GRIN2A mutants or empty vector were seeded into Boyden chambers in 0.5% serum and grown for 48 hours before staining and counting. Melanoma cell line 31T cells (**d**) stably expressing GRIN1 and GRIN2A mutants or empty vector were seeded into Boyden chambers plus and minus 10 μM TCN-213 for 48 hours before staining and counting. Panels **e** and **f** are quantitative graphs of colony formation of 31T and SK-Mel-2 cells in soft agar, respectively. Error bars are representative of $n = 3$ (SD) (*$P < 0.01$, Student's *t*-test). Figures were adjusted to remove unwanted overall background due to western blotting and film development. IB, immunoblot.

lines tested (Figure 2a and b). We observed normal complex formation between WT GRIN1 and WT GRIN2A and to lesser extent GRIN2A (R920K) in both stable cell lines (Figure 2a and b). Conversely, expression of GRIN2A (W372X, Q891X, or W1271X) resulted in loss of complex formation to WT GRIN1, again observed in both stable cell lines. Furthermore, we tested expression of WT or mutant forms of GRIN2A on proliferation. Interestingly, little to no effect on cellular proliferation in either the 2359 (31T) or SK-Mel-2 stable cell lines was observed (Supplementary Figure S2 online).

To determine how expression of GRIN1 and GRIN2A (WT or mutant) in melanoma affects melanoma biology, we used cell migration and anchor-independent growth assays. Stable pooled clones (31T or SK-Mel-2) expressing WT GRIN1 and GRIN2A (WT or mutants) or empty vector were seeded into Boyden chamber assays and grown for 24–48 hours prior to analysis of a migratory phenotype. Expression of WT GRIN1 and WT GRIN2A in 31T caused reduced migration compared with empty vector (Figure 2c). However, expression of mutant GRIN2A (W372X, Q891X, R920K, and W1271X) showed increased migration in 31T and SK-Mel-2 compared with both WT GRIN2A and empty vector. Migration of 2359 cells expressing WT GRIN2A was reversed in the presence of a specific NMDAR2A (GRIN2A) antagonist, TCN-213. As seen in Figure 2d, stable clones expressing WT GRIN2A migrated significantly better in the presence of 10 μM TCN-213 compared with migration in the absence of antagonists. However, vector- or mutant-expressing clones were unaffected by TCN-213. SK-Mel-2 cells were tested for the ability to migrate using multiple time points and cell numbers with no migration phenotype observed at any point. Furthermore, we analyzed the tumorigenic effect of the mutants in melanoma using an anchorage-independent growth assay. Expression of WT GRIN1 and WT GRIN2A in 31T and SK-Mel-2 caused diminished colony formation compared with empty vector (Figure 2e and f). Expression of GRIN2A mutants showed increased soft agar colony formation, again in both 31T and SK-Mel-2 stable clones. These results demonstrate that expression of somatically mutated forms of GRIN2A in melanoma cells leads to an increased migration and colony formation through the loss of tumor-suppressive activity via a NMDAR-dependent Ca^{2+} influx.

To further delineate the role GRIN2A has in melanoma cell biology, we stably depleted endogenous GRIN2A using short-hairpin RNA (shRNA) and further functionally analyzed the effects caused by knockdown. Using melanoma cell lines that express WT GRIN2A (31T and 39T) or mutant GRIN2A (E1175K) (501Mel and 125T) we stably transduced GRIN2A-specific shRNAs: shRNA#1, #2, and #3) into each cell line and a vector control (pLKO.1). As seen in Figure 3, GRIN2A is knocked down at the message level by 20–70% as determined by quantitative reverse transcriptase–PCR analysis in all cell lines tested. We examined growth potential of cells depleted of GRIN2A in WT or mutant backgrounds. Strikingly, shRNA knockdown of WT GRIN2A resulted in increased proliferation of cells stably transduced with shRNAs #1–3 compared with cells transduced with pLKO.1 alone (Figure 3c and d). In contrast, depletion of mutant GRIN2A caused a slight reduction in proliferation compared with vector control cells (Figure 3e and f); however, the level of knockdown in the two mutant lines is notably less than the WT lines, possibly indicating a higher level of cell dependency on this mutated protein. These results support our hypothesis that WT GRIN2A functions as a tumor suppressor in melanoma cells given the increase in growth potential in cells devoid of only 50–60% of total GRIN2A messenger RNA. Although further analyses of the signaling mechanisms downstream of glutamate receptors are needed, our study provides an insight into the biological relevance of GRIN2A somatic mutations in melanoma and reveals a possible node of clinical intervention for the treatment of malignant melanoma.

DISCUSSION

Here we show in malignant melanoma the functional analysis of the ionotropic glutamate receptor subunit, GRIN2A. On the basis of previous whole-exome sequencing results, our lab revealed GRIN2A to be highly mutated (Wei *et al.*, 2011) in melanoma demonstrating the importance that glutamate signaling has in tumorigenesis. Recent work demonstrated GRIN2A promoter methylation present in human colorectal carcinoma (Kim *et al.*, 2008). These researchers further demonstrated that expression of WT GRIN2A with WT GRIN1 resulted in suppressed cell growth in soft agar. In our present study, we showed that somatic mutation of GRIN2A resulted in decreased complex formation of the NMDAR leading to increased migration and colony formation in soft agar, whereas expression of WT GRIN2A acted as a tumor suppressor in melanoma. Our results suggest that expression of mutant forms of GRIN2A may have a protective role in melanoma, potentially acting as a dominant negative suppressing WT GRIN2A normal activity.

NMDAR signaling has opposing roles depending on the partners that are found within the larger complex (Xiao *et al.*, 2011; Choo *et al.*, 2012). Neurological cells use different signaling mechanisms that function in a protective manner and when overstimulated via glutamate (excitotoxic) result in programmed cell death. NMDA receptor signaling uses divalent cation influx (e.g., Ca^{2+}) via glutamate/glycine binding to mediate signaling to downstream elements involved in survival and/or cell death pathways (Xiao *et al.*, 2011; Choo *et al.*, 2012; Ru *et al.*, 2012). Recently, it was demonstrated that hippocampal cells, found in the central nervous system, use differential signaling for either the p38 MAPK pathway or the Erk1/2 pathway depending on which GRIN2 subunit is present in the NMDAR complex with GRIN1 (Xiao *et al.*, 2011). Immature hippocampi preferentially express GRIN2B and upon stimulation of the NMDAR activate the Erk1/2. Interestingly, these authors show that mature hippocampi from rats mainly express GRIN2A compared with GRIN2B and upon NMDAR stimulation activate p38 MAPK signaling causing a more pronounced neuronal cell death. The fact that we observed so many somatic mutations causing truncated or nonfunctional versions of GRIN2A may be one way that melanoma cells use to potentiate a more proliferative and pro-survival phenotype; via subunit switching to GRIN2B or a dominant negative effect.

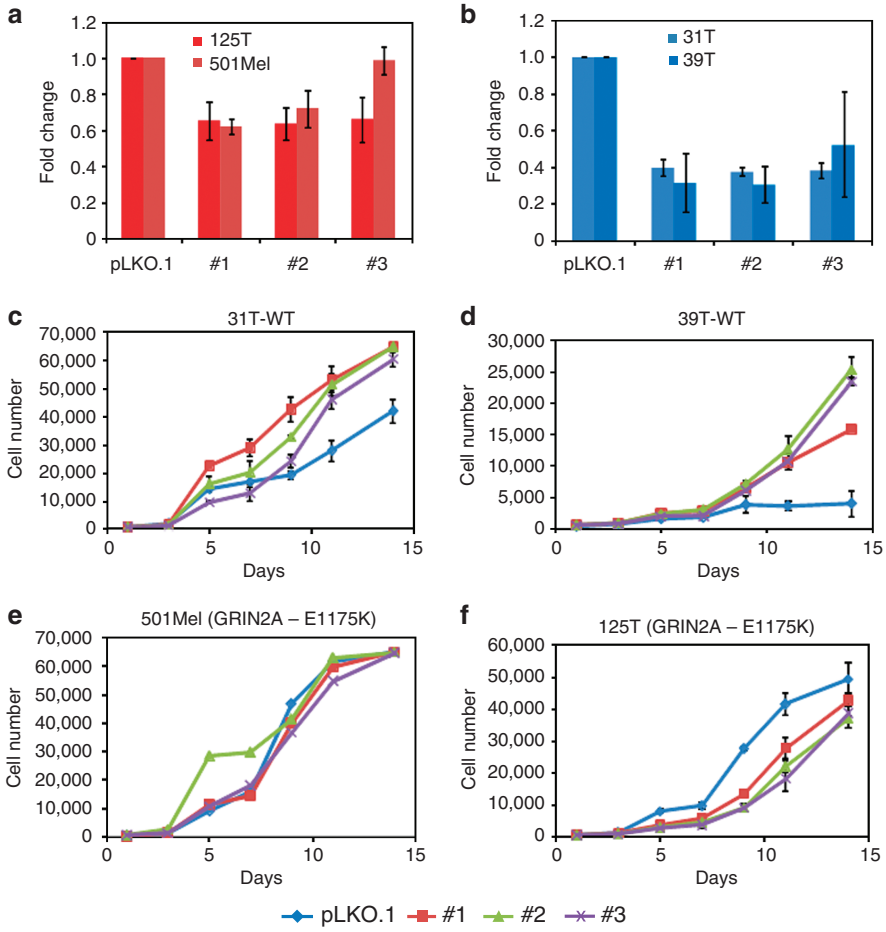

Figure 3. GRIN2A functions as a tumor suppressor in melanoma cells. Stable depletion of endogenous GRIN2A in melanoma cells. Melanoma cells stably depleted of endogenous GRIN2A were confirmed via quantitative reverse transcriptase–PCR of all clones. (**a**, **b**) Analysis of knockdown used specific GRIN2A primers and efficiency was compared with glyceraldehyde-3-phosphate dehydrogenase levels in each sample, thus generating a fold change. Proliferation assay of WT 31T (**c**) or 39T (**d**) and mutant 501Mel (**e**) or 125T (**f**) expressing GRIN2A cell lines depleted of GRIN2A. Knockdown resulted in little to no change in proliferation for 125T or 501Mel melanoma cell lines expressing mutant GRIN2A but increased proliferation for both 31T and 39T cell lines expressing WT GRIN2A. Each cell line used pLKO.1 as an empty vector control.

The functional domains found in GRIN2A have major roles in Mg^{2+}-gated calcium channel influx of NMDA receptors. Reports by Endele *et al.* (2010), Maki *et al.* (2013), and others show that mutations found in both the GRIN2A or GRIN2B result in reduced Mg^{2+} gating of the NMDAR causing nonfunctional calcium signaling. Recent studies identified that patients with epilepsy or encephalopoathies harbor germline mutations in NMDAR2A (*GRIN2A*) (Carvill *et al.*, 2013; Lemke *et al.*, 2013; Lesca *et al.*, 2013). Interestingly, the spectrum of the identified mutations was very similar to the ones we observed with multiple non-synonymous mutations resulting in missense and nonsense alterations. Mutations observed in these patients also resulted in decreased or aberrant calcium (ion) influx. Another study by the Kutsche group examined patients with neurological disorders ranging from mental retardation to epilepsy. They discovered that mutations found in GRIN2A (W218X or N615K) resulted in truncated or nonfunctional subunits of the NMDA receptor leading ultimately to reduced calcium channel permeability. GRIN2B was also shown to carry several mutations that disrupted its functionality, resulting in reduced NMDA receptor signaling. Maki *et al.* (2013) showed that alanine screening for potentially important residues in the C-terminal domain of GRIN2A revealed two important sites of phosphorylation, Ser^{900} and Ser^{929}. Mutation of GRIN2A at either site resulted in reduced calcium channel conductance potentially causing reduced signaling. Interestingly, we found one of the same sites to be somatically mutated (Ser929Phe) in our malignant melanoma samples (Wei *et al.*, 2011; Supplementary Figure S1 online). These results not only corroborate our findings that somatic mutations in GRIN2A result in loss of function in calcium channel signaling but also reveal the importance of proper NMDAR signaling in brain (neuronal) and melanocyte development and survival. Future functional analyses are required to further examine the role that GRIN2A may have in the etiology of the disease. This would allow a better understanding of the already established importance that NMDA receptor signaling has in melanoma.

MATERIALS AND METHODS

Construction of WT and mutant GRIN2A expression vectors

Mouse *GRIN1* (BC039157) and mouse *GRIN2A* (BC148800) were cloned by PCR as previously described (Palavalli *et al.*, 2009) using clones (MMM4769-202766680 (GRIN1) or OMM5895-202524480 (GRIN2A)) purchased from Open Biosystems (Huntsville, AL) with primers listed in Supplementary Table S1 online. The PCR products were cloned into the mammalian expression vectors pCDF-MCS2-EF1-Puro or pCDH1-MCS-CMV-Neo (Systems Biosciences, Mountain View, CA) via the *Xba*I and *Not*I restriction sites. Point mutations were introduced as previously described (Prickett *et al.*, 2009) using the primers found in Supplementary Table S1 online.

Cell culture and transient expression

HEK293T cells were purchased from ATCC (Manassas, VA) and maintained in complete RPMI-1640 medium supplemented with 10% FBS. HEK293T cells were transfected with Arrest-IN reagent (Open Biosystems) at a 6:1 ratio with DNA (μl:μg) using 2–6 μg of plasmid DNA. Ringer's solution was obtained from the veterinary pharmacy at the National Institutes of Health. All experiments were conducted in accordance with institutional guidelines.

Immunoprecipitation and western blotting

Transfected cells were gently washed two times in phosphate-buffered saline and then lysed using 1.0 ml 1% NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, complete protease inhibitor tablet, EDTA-free (Roche, Indianapolis, IN), 1 μM sodium orthovanadate, 1 mM sodium fluoride, and 0.1% β-mercapto-ethanol) per T75 flask for 20 minutes on ice. Lysed cells were scraped and transferred into a 1.5 ml microcentrifuge tube. Extracts were centrifuged for 10 minutes at 14,000 r.p.m. at 4 °C. Next, 800 μl of supernatant was immunoprecipitated overnight using 20 μl of anti-GRIN1 and 30 μl of 50% slurry of protein A/G beads (Roche). The immunoprecipitates were washed and subjected to SDS-PAGE and western blotting as previously described (Palavalli *et al.*, 2009). Primary antibodies used in our signal transduction pathway analysis were anti-GRIN1 (sc162902), anti-GRIN2A (sc1468), or anti-GRIN2A (sc-136004) (Santa Cruz Biotechnology, Dallas, TX), anti-P-p38 MAPK (#9211), anti-p38 (#9212) (Cell Signaling), and anti-GAPDH (#CB1001) (Calbiochem-EMD Biosciences, San Diego, CA).

Pooled stable expression

To make lentiviruses, *GRIN1* or *GRIN2A* constructs were co-transfected into HEK293T cells seeded at 1.5×10^6 per T75 flask with pVSV-G and pFIV-34N (kind gifts from Todd Waldman, Georgetown University) helper plasmids for pCDF1 based or pPACKH1 viral production mixture from SBI (Mountain View, CA) for pCDH1 based using Arrest-IN as described by the manufacturer. Virus-containing medium was harvested 60 hours after transfection, filtered, aliquoted, and stored at −80 °C. 31T cells (kind gift from Dr Rosenberg) were grown in RPMI-1640 (Lonza, Walkersville, MD) and supplemented with 10% FBS (HyClone, Logan, UT). A375 cells were purchased from National Cancer Institute, Division of Cancer Treatment, Developmental Therapeutics Program, Frederick, MD and maintained in RPMI-1640 and supplemented with 10% FBS. 31T or SK-Mel-2 cells were seeded at 1.5×10^6 cells per T75 flask 24 hours prior to infection. Lentivirus for *GRIN1* and *GRIN2A* (WT or mutants) and empty vector control were used to sequentially infect 31T or SK-Mel-2 cells as previously described (Prickett *et al.*, 2009). Stable expression of GRIN1 proteins (WT) was determined by immunoprecipitation and SDS-PAGE analysis followed by immunoblotting with anti-GRIN1 and anti-GAPDH to show equivalent expression among pools. Stable expression of GRIN2A (WT or mutants) was determined by reverse transcriptase–PCR analysis of messenger RNA from 31T or SK-Mel-2 stable pooled clones using *GRIN2A*-specific primers and *GAPDH* primers as a loading control.

Proliferation assays

To examine growth potential, pooled 31T or SK-Mel-2 pooled clones were seeded into 96-well plates at 300 cells per well in either 1, 2.5, or 10% serum-containing medium and incubated for 13–17 days. Samples were analyzed every 48 hours by lysing cells in 50 μl of 0.2% SDS per well and incubating for 2 hours at 37 °C prior to addition of 150 μl per well of SYBR Green I solution (1:750 SYBR Green I (Invitrogen, Molecular Probes, Carlsbad, CA) diluted in dH_20). Plates were analyzed using a BMG Labtech (Cary, NC) FLOUstar Optima.

Migration assays

31T or SK-Mel-2 pooled clones were seeded into preconditioned migration wells (8.0 μm—BD Biocoat, BD Biosciences, San Jose, CA) at 30,000–100,000 cells per well in serum-free medium in the top chamber and incubated for 24–48 hours with complete serum-containing medium in the bottom chamber before harvesting. Antagonist studies used 10 μM of TCN-213 (Tocris, Minneapolis, MN) dissolved in DMSO in the top and bottom chambers of the Boyden chamber. DMSO was used as a negative control for this assay using $<0.02\%$ (vol/vol). Inserts were fixed and stained using Hema 3 Stat Pack (Fisher Scientific, Pittsburgh, PA) as per the manufacturer's protocol. Inserts were analyzed and counted for cells migrated per field view and quantitated using ImageJ (National Institutes of Health, Bethesda, MD).

Soft agar assay

31T or SK-Mel-2 pooled clones were plated in triplicate at 1000 cells per well and in top plugs consisting of sterile 0.33% Bacto-Agar (BD, Sparks, MD) and 10% FBS (HyClone, Logan, UT) in a 24-well plate. The lower plug contained sterile 0.5% Bacto-Agar and 10% FBS. After 2 weeks, the colonies were photographed and quantitated using ImageJ (NIH software).

Lentiviral shRNA

Constructs for stable depletion of *GRIN2A* (cat#RHS4533-EG2903) were obtained from Open Biosystems and were confirmed to efficiently knockdown GRIN2A at the message level. Lentiviral stocks were prepared as previously described (Prickett *et al.*, 2009). Melanoma cell lines (31T, 39T, 501Mel, or 125T) were infected with shRNA lentiviruses for each condition (vector and 2–3 different *GRIN2A*-specific shRNAs). Selection of stable pooled clones was carried out in the presence of $3\,\mu g\,ml^{-1}$ puromycin-containing normal medium for 3–5 days prior to determining knockdown efficiency. Stably infected pooled clones were tested in functional assays.

Reverse transcription PCR

Total RNA was extracted from pooled clones of melanoma cells stably knocked down for endogenous *GRIN2A* following the manufacturer's protocol for the RNeasy Mini Kit (QIAGEN #74101,

Gaithersburgh, MD). Total RNA was eluted in 30 µl diethylpyrocarbonate-treated distilled H_2O. A total of 1 µg of total RNA was used for single-strand complementary DNA synthesis using a SuperScript III First Strand kit (Invitrogen #18080-051). Complementary DNA was amplified using the olido dT20 primer supplied in the kit. To test for loss of *GRIN2A* message, we used 1 µl of complementary DNA in the PCR reaction with either *GRIN2A* primers or *GAPDH* primers (Supplementary Table S2 online).

Proliferation assays of stable knockdown cells

To examine growth potential, stable pooled knockdown clones for *GRIN2A* were seeded into 96-well plates at 500 cells per well in either 1, 2.5, or 10% serum-containing medium and incubated for 6–8 days. Samples were analyzed every 2–3 days by lysing cells in 50 µl of 0.2% SDS per well and incubating for 2 hours at 37 °C prior to addition of 150 µl per well of SYBR Green I solution (1:750 SYBR Green I (Invitrogen-Molecular Probes) diluted in dH_2O). Plates were analyzed using a BMG Labtech FLOUstar Optima.

Calcium assays

HEK293T cells seeded in 35 mm glass-bottomed culture dishes (MatTek, Ashland, MA) were transiently transfected with GRIN1 and either GRIN2A WT or mutants (Q891X, R920K, W1271X). Thirty-six hours post-transfection cells were washed with Tyrode solution (5 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.9 mM $CaCl_2$, 5.6 mM glucose, buffered to pH 7.4 with Tris base). Cells were loaded with 10 µM Fluo-3AM diluted in Tyrode supplemented with 0.1% BSA for 45 minutes in the dark, followed by two washes with Tyrode-BSA and two washes with Tyrode alone. Loaded cells were then left for 15 minutes to ensure complete hydrolysis of the acetoxymethyl ester groups. Images were taken every 5 seconds over a period of 15 minutes and collected on a Zeiss LSM-700 laser scanning microscope (Thornwood, NY) with a ×63 oil immersion lens in a single track mode using excitation 488 nm and emission BP 505-530 filter sets.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank UR for acquiring tumor specimens, CS and PP for establishment of the majority of melanoma cell lines, and VM, HA, and PC for generating the sequence data analyzed here. We thank VG Prieto for pathologic review of the biospecimens from the Melanoma Informatics, Tissue Resource, and Pathology Core (MelCore) at MD Anderson. We thank TW for bioinformatics help and JF and DL for graphical assistance. This work was supported by the Intramural Research Programs of the National Human Genome Research Institute, the National Institute of Dental and Craniofacial Research, the National Cancer Institute, by the Henry Chanoch Krenter Institute for Biomedical Imaging and Genomics, the estate of Alice Schwarz-Gardos, the estate of John Hunter, and the Knell Family. YS is supported by the Israel Science Foundation grant numbers 1604/13 and 877/13 and the ERC (StG-335377).

AUTHOR CONTRIBUTIONS

TDP, JSG, and YS designed the study; JJG, JW, and SAR collected and analyzed the melanoma samples; NQ performed the bioinformatics analyses, TDP, BJZ, VKH, and JJ performed the functional analyses. All authors contributed to the final version of the paper.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Berger MF, Hodis E, Heffernan TP *et al.* (2012) Melanoma genome sequencing reveals frequent PREX2 mutations. *Nature* 485:502–6

Carvill GL, Regan BM, Yendle SC *et al.* (2013) GRIN2A mutations cause epilepsy-aphasia spectrum disorders. *Nat Genet* 45:1073–6

Choo AM, Geddes-Klein DM, Hockenberry A *et al.* (2012) NR2A and NR2B subunits differentially mediate MAP kinase signaling and mitochondrial morphology following excitotoxic insult. *Neurochem Int* 60:506–16

Doronzo G, Russo I, Del Mese P *et al.* (2010) Role of NMDA receptor in homocysteine-induced activation of mitogen-activated protein kinase and phosphatidyl inositol 3-kinase pathways in cultured human vascular smooth muscle cells. *Thromb Res* 125:e23–32

Endele S, Rosenberger G, Geider K *et al.* (2010) Mutations in GRIN2A and GRIN2B encoding regulatory subunits of NMDA receptors cause variable neurodevelopmental phenotypes. *Nat Genet* 42:1021–6

Hahn CG, Wang HY, Cho DS *et al.* (2006) Altered neuregulin 1-erbB4 signaling contributes to NMDA receptor hypofunction in schizophrenia. *Nat Med* 12:824–8

Hodis E, Watson IR, Kryukov GV *et al.* (2012) A landscape of driver mutations in melanoma. *Cell* 150:251–63

Kaderlik KR, Minchin RF, Mulder GJ *et al.* (1994) Metabolic activation pathway for the formation of DNA adducts of the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in rat extrahepatic tissues. *Carcinogenesis* 15:1703–9

Kim MS, Chang X, Nagpal JK *et al.* (2008) The *N*-methyl-D-aspartate receptor type 2A is frequently methylated in human colorectal carcinoma and suppresses cell growth. *Oncogene* 27:2045–54

Komuro H, Rakic P (1993) Modulation of neuronal migration by NMDA receptors. *Science* 260:95–7

Lang UE, Puls I, Muller DJ *et al.* (2007) Molecular mechanisms of schizophrenia. *Cell Physiol Biochem* 20:687–702

Lau A, Tymianski M (2010) Glutamate receptors, neurotoxicity and neurodegeneration. *Pflugers Arch* 460:525–42

Lemke JR, Lal D, Reinthaler EM *et al.* (2013) Mutations in GRIN2A cause idiopathic focal epilepsy with rolandic spikes. *Nat Genet* 45:1067–72

Lesca G, Rudolf G, Bruneau N *et al.* (2013) GRIN2A mutations in acquired epileptic aphasia and related childhood focal epilepsies and encephalopathies with speech and language dysfunction. *Nat Genet* 45:1061–6

Maki BA, Cole R, Popescu GK (2013) Two serine residues on GluN2A C-terminal tails control NMDA receptor current decay times. *Channels (Austin)* 7:126–32

Monyer H, Sprengel R, Schoepfer R *et al.* (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science* 256:1217–21

Nacher J, McEwen BS (2006) The role of *N*-methyl-D-asparate receptors in neurogenesis. *Hippocampus* 16:267–70

Palavalli LH, Prickett TD, Wunderlich JR *et al.* (2009) Analysis of the matrix metalloproteinase family reveals that MMP8 is often mutated in melanoma. *Nat Genet* 41:518–20

Prickett TD, Agrawal NS, Wei X *et al.* (2009) Analysis of the tyrosine kinome in melanoma reveals recurrent mutations in ERBB4. *Nat Genet* 41:1127–32

Ru W, Peng Y, Zhong L *et al.* (2012) A role of the mammalian target of rapamycin (mTOR) in glutamate-induced down-regulation of tuberous sclerosis complex proteins 2 (TSC2). *J Mol Neurosci* 47:340–5

Rzeski W, Turski L, Ikonomidou C (2001) Glutamate antagonists limit tumor growth. *Proc Natl Acad Sci USA* 98:6372–7

Schlett K (2006) Glutamate as a modulator of embryonic and adult neurogenesis. *Curr Top Med Chem* 6:949–60

Stark MS, Woods SL, Gartside MG *et al.* (2011) Frequent somatic mutations in MAP3K5 and MAP3K9 in metastatic melanoma identified by exome sequencing. *Nat Genet* 44:165–9

Stepulak A, Luksch H, Gebhardt C *et al.* (2009) Expression of glutamate receptor subunits in human cancers. *Histochem Cell Biol* 132:435–45

Takano T, Lin JH, Arcuino G *et al.* (2001) Glutamate release promotes growth of malignant gliomas. *Nat Med* 7:1010–5

Traynelis SF, Wollmuth LP, McBain CJ *et al.* (2010) Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev* 62: 405–96

Wang JQ, Fibuch EE, Mao L (2007) Regulation of mitogen-activated protein kinases by glutamate receptors. *J Neurochem* 100:1–11

Wang XF, Zhou QM, Du J *et al.* (2013) Baicalin suppresses migration, invasion and metastasis of breast cancer via p38MAPK signaling pathway. *Anticancer Agents Med Chem* 13:923–31

Wei X, Walia V, Lin JC *et al.* (2011) Exome sequencing identifies GRIN2A as frequently mutated in melanoma. *Nat Genet* 43:442–6

Xiao L, Hu C, Feng C *et al.* (2011) Switching of *N*-methyl-D-aspartate (NMDA) receptor-favorite intracellular signal pathways from ERK1/2 protein to p38 mitogen-activated protein kinase leads to developmental changes in NMDA neurotoxicity. *J Biol Chem* 286:20175–93

Ye ZC, Sontheimer H (1999) Glioma cells release excitotoxic concentrations of glutamate. *Cancer Res* 59:4383–91