Supplemental

Results

Comparison of the highly metastatic (either brain or visceral) variants (131/4-5B1, 131/4-5B2 and 113/6-4L) versus the poorly metastatic parental WM239 cells showed differential expression of 60 genes (36 upregulated and 24 downregulated), as described in supplemental table 1. Among these, 26 showed an upregulation ≥1.5 fold and 14 showed a downregulation greater than 0.67 fold. Prominent among these genes is the up-regulation of a number of members of the G-antigen (*GAGE 1, 4, 5, 6, 7, 7b, 8, GAGEE3*, *X-antigen 1*, *P-antigen 5*), Thymosin-like (*TMSL1, 2, 3, 4, 6, b4x)* and human leukocyte antigen (*HLA B*, *D* and *H*) families. The commonly shared increased expression of these genes suggests that they may enhance metastatic potential of malignant melanoma. In this regard, the expression of *gage* genes in melanomas (and other cancer types) and their correlation with poor survival have been noted previously ([1]. While no similar association has been noted for *TMSL* genes in melanoma, *TMSL4BX* has been shown to be upregulated in lymphoma and colorectal cancer [2]. Additional upregulations have been noted for *HLA* genes (B and C) in a number of cancer types [3-5].

Materials and methods

Microarray analysis.

Data has been deposited in NCBI's Gene Expression Omnibus and are accessible through http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE23601.

Confirmation of clinical relevance:

In order to confirm that the expression of these gene plays a relevant role with respect to metastatic disease, cells from the visceral multi-organ metastatic variant 113/6-4L cell line (which was derived from the WM239 parental cell line) were transduced with the *EDNRB* or *BCL2A1* cDNA (6-4*EDNRB and 6-4BCL2A1 respectively*) containing vector (the methodology used to induce stable upregulation or downregulation of relevant genes is described in further detail in “supplemental materials and methods”) and implanted orthotopically ($1x10^6$ cells) in mice (CB17/SCID, n=10) in order to induce primary tumors. Primary tumors were resected when they reached a volume of 400 mm^3. The lineage relationship of WM239, 113/6-4L and 131/4-5B1 and B2 brain metastatic variants derived *in vivo* from the 113/6-4L cell line is described in supplemental Figure S1. Control group consisted of mice that were implanted with cells transduced with empty vector (6-4vector). Mice in the control and experimental groups were monitored regularly in accordance with the institutional guidelines stipulated by the Sunnybrook Health Sciences Centre Animal Care Committee and sacrificed when they showed signs of distress as a result of metastatic disease (*e.g.*

breathing difficulty and/or weight loss). Tissues were excised, sectioned and immunostained with HMB-45 antibody (Novacastra) to detect the presence of melanoma metastases. The incidence of brain metastases in all groups was noted. A parallel experiment was conducted using intravenous injection of these cell lines ($1x10^5$ cells in a volume of 250 µl).

Confirmation of functional relevance:

cDNAs encopding *EDNRB* were obtained following RT-PCR from cDNA derived from mRNA harvested from 131/4-5B2 cells. EDNRB was stably upregulated in the parental 113/6-4L cell line by means of transduction with lentiviral vectors. cDNA encoding *EDNRB* was subcloned into a bicistonic dsRED expressing lentiviral expression vector downstream of a CMV5 promoter . Similarly, lentiviral contructs were obtained containing hp sequences chosen to specifically silence EDNRB (available in the GIPZ Lentiviral shRNAmir vector from Open Lab BioSystems) or by design (sublconed into in the pLKO vector lentiviral transfer vector, Addgene plasmid 10878), Production of concentrated viral stocks was accomplished using the VSVG packaging cell line 293SFPacLV29-6 in a manner adapted from the procedure disclosed in [6]. Following transduction of the cDNA overexpressing lentivirus into cells of the visceral metastatic variant 113/6-4L cell line or the hp-expressing lentivirus into brain metastatic B1 or B2 cell lines, pools of stably expressing cells were obtained and used for all functional studies following evaluation of target gene mRNA levels by Q-RTPCR. Where appropriate, melanoma cell lines generated from lentivruses harboring appropriate empty lentivirus vector were used as negative controls.

Examination of gene expression in melanoma cell lines and clinical samples:

RNA was isolated from samples of clinical CNS melanoma metastases (Brain Tumor Bank-London, Ontario and Ontario Cancer Institute), as well as primary malignant melanoma (Ontario Tumor Bank and Tumor Tissue Repository-British Columbia). Complementary DNA was generated and the expression of *EDNRB* and *GAPDH* in melanoma cell lines, brain metastases and primary melanoma was examined by means of QRTPCR using primers specific for human *EDNRB, BCL2A1* and *GAPDH*:

EDNRB forward: 5'TGAACTTTTGAGCTTTCTGTTGGTA3'

EDNRB reverse 5'ATACAGAGCAATTGGGTTAATGCA3'

BCL2A1 forward: 5' ACAGGCTGGCTCAGGACTATCT 3'

BCL2A1 reverse 5'TGTAGCACTCTGGACGTTTTGC3'

GAPDH forward: 5'ACCCACTCCTCCACCTTTGA3'

GAPDH reverse 5'CATACCAGGAAATGAGCTTGACAA3'.

RNA was isolated from subconfluent cultures of each cell line using RNeasy mini kit (Qiagen) using manufacturer's instructions and cDNA generated using Omniscript RT kit (Qiagen). Q-RTPCR was performed using an ABI 700

Sequence Detection System (Applied Biosystems) in the presence of SYBR-green (QuantiTect SYBR Green PCR kit-Qiagen).

Immunostaining for EDNRB was performed in order to compare the expression of this gene in independent clinical samples of primary melanoma and melanoma metastases to lymph node, lung and brain. Tissue was harvested, fixed in 10% neutral buffered formalin for 24 hours, processed, and embedded in paraffin. Immunohistochemistry was performed using a rabbit affinity purified polyclonal antibody against endothelin Breceptor (Novus Biologicals). The antibody was diluted to 1:65 and stained on a Discovery XT Automated Immunostainer (Ventana Medical Systems) using DAB detection and counter stained with hematoxylin. Adrenal tissue was used as a positive control, which was conducted with every IHC run. Representative images showing staining for positive control, negative control, primary melanoma, lymph node met, brain met and lung met are shown in supplemental figure 5 E-J. Scoring for immunostaining intensity was conducted blindly.

Proliferation in the presence of brain-conditioned media.

Brain conditioned media (CM) was prepared as described previously (12). Briefly, brains from 8 week-old CB17/SCID mice were resected, minced and incubated overnight in serum-free RPMI 1640 media. The resulting media was then centrifuged, filtered and store at -70°C. Melanoma cells (2000 cell/well) were cultured for in 96-well plates for 6 days in the presence of brain-CM, and in the presence or absence of EDNRB inhibitor A192621 (Abbott Laboratories). The

number of viable cells was assessed at the end of 6 days of culture in the presence of brain-CM by means of MTS assay (Promega) as well as tritiated-thymidine uptake. Values are expressed as proliferation relative to the respective cell line cultured in the absence of brain CM. Similar experiments were conducted by culturing melanoma cells in the presence of purified recombinant endothelin-3 (Tocris; concentrations used 1, 10 and 100nM) in the presence or absence A192621. In order to induce stable knock-down of EDNRB expression, lentiviral vectors (PLKO) were used to deliver shRNA sequences for this gene. The level of downregulation was determined by means of QRTPCR as above. Among the various clones tested, 131/4-5B1 3119/989 and 131/4-5B2 3119/172 showed the highest levels of downregultation and were selected for subsequent studies. As above, values are expressed relative to proliferation in the absence of brain CM.

References

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Supplemental figure legends

Supplemental figure S1: Schematic representation of the protocol used for generating the highly 'visceral' metastatic variants, *i.e.* to lung and liver (113/6-4L, 113/7-4L and 113/8-2L) and brain metastatic (131/4-5B1 and 131/4-5B2) cell lines using the poorly metastatic parental WM239 human melanoma cell line (originally generated from a clinical sample of lymph node metastases) as a starting point.

Supplemental figure S2: A) Clustering analysis of gene expression in brain-metastatic cell lines (131/4-5B1 and B2) compared to the highly visceral metastatic parental cell line 113/6-4L as well as poorly metastatic parental WM239 cell line. Column 1: 113/6-4L expression profile using WM239 as baseline, column 2 and 3: comparison of 131/4-5B1 or B2 expression profiles, respectively, for which WM239 was used as baseline, column 4 and 5: comparison of 131/4-5B1 and B2 expression profiles, respectively, using 113/6-4L as baseline. Four replicates were used per comparison. **B and C)** QRTPCR confirmation of upregulation of *EDNRB* and *BCL2A1*respectively in the 131/4-5B1 and B2 brain metastatic cell lines but not in visceral metastatic variants 113/7-4L and 113/8-2L. Values are expressed as relative to visceral metastatic parental 113/6-4L cells which were used to derive all these cell lines (One-way ANOVA, $P<0.05$).

Supplemental figure 3: QRT-PCR examination of levels of expression of additional genes highlighted by microarray analysis. Values are expressed as relative to visceral metastatic parental 113/6-4L cells which were used to derive all these cell lines.

Supplemental figure S4: A) The expression of *EDNRA* was examined in the visceral and brain metastatic variants relative to parental 113/6-4L showed no significant upregulation. **B)** EDNRB expression in clinical samples of brain metastases was significantly higher than ednra. Values are expressed as mean ± SEM.**C)** Positive immunostaning control for EDNRB in adrenals. **D)** Negative immunostaining control for EDNRB. Representative images showing EDNRB immunostaining in clinical samples of primary melanoma, lymph node metastases, brain metastases and lung metastases are shown in **E, F, G and H** respectively.

Supplemental figure S5: A) QRTPCR confirmation of stable upregulation of *EDNRB* induced in parental 113/6-4L cell line. Values are expressed relative to expression in 113/6-4L. **B)** Western blot analysis was used to confirm the upregulation of EDNRB in the 113/6-4L cell line, as well as, **(C)** the stable knockdown of EDNRB in 131/4-5B2 achieved by means of shRNA lentiviral vectors. Similar amounts of protein were loaded for each of the samples examined.

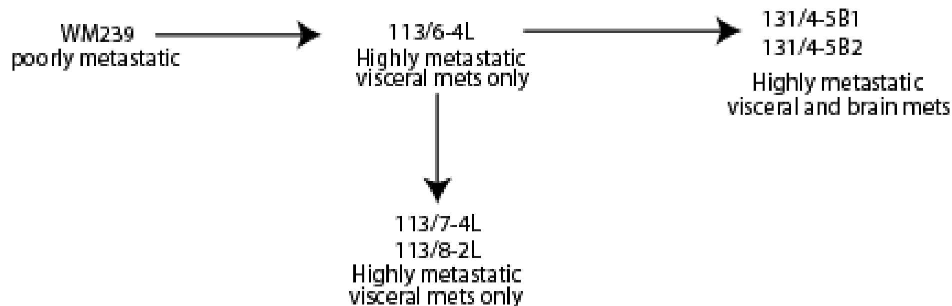
Supplemental figure S6: Confirmation of ET1 and ET3 expression in brain and lung. A) Significant expression of *ET3* was noted in brain of SCID mice with lower levels for *ET1*. The expression for both of these genes was significantly higher in lungs. **B, C and D)** Immunostaining for ET3 in mice brains of SCID mice showed the presence of the molecule in neurons in the cortex and cerebellum.

Supplemental figure S7: Role of EDNRB in melanoma cell proliferation and intracranial melanoma growth. A) In the absence of ET3, the presence of A192621 failed to induce a significant inhibitory effect. Cell viability is expressed relative to control (cells cultured in the absence of A192621). **B and C)** Stable knockdown of EDNRB in brain-metastatic cell lines (131/4-5B2 hp2136 and hp3119) resulted in decreased proliferation in the presence of brain-CM when compared to empty vector control (6-4 PLKO) (One-way ANOVA, $P<0.05$). Values are expressed as proliferation relative to the respective cell line cultured in the absence of brain CM. **D)** Additional overexpression cell lines were used to further confirm the effect of BCL2A1 and EDNRB upregulation in intracranial tumor growth. Intracranial implantation resulted in significantly larger melanoma tumors for 6-4*BCL2A1*-2 and 6-4*EDNRB*-2 when compared to empty vector control (6-4vector). **E)** Induced overexpression of EDNRB did not affect in vitro proliferation relative to cells transduced with empty vector. **F)** In vitro examination of the effect of the combination of A192621 and cyclosporin A on cell viability. Brain metastatic variant cell line 131/4-5B2 was cultured in vitro in the presence of A192621 alone, cyclosporin A alone, or the combination of the two

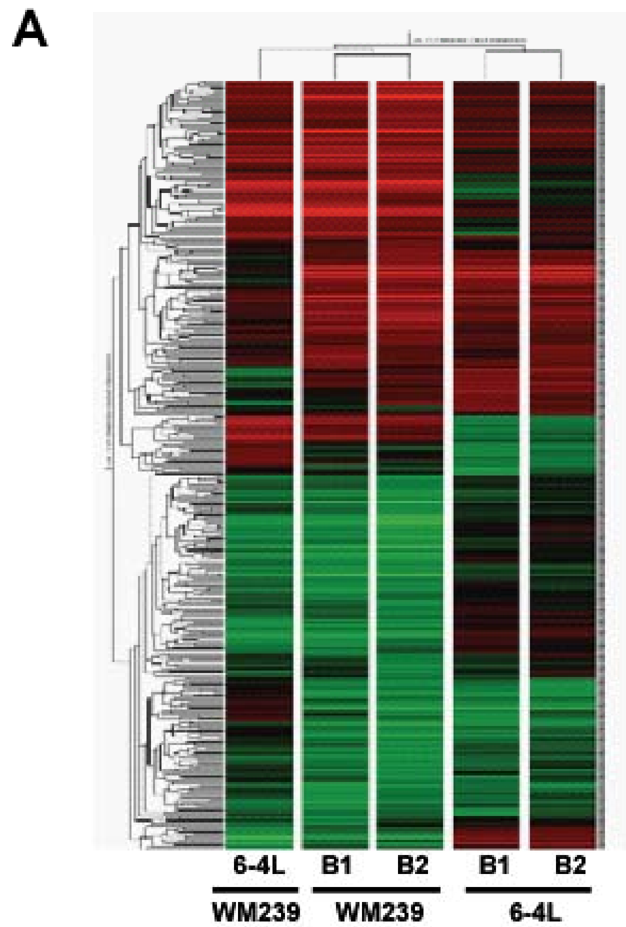
compounds. The addition of 100 nM A192621 did not enhance the cytoxicity associated with cyclosporin A at any of the doses used (10, 1 or 0.25 µg/ml).

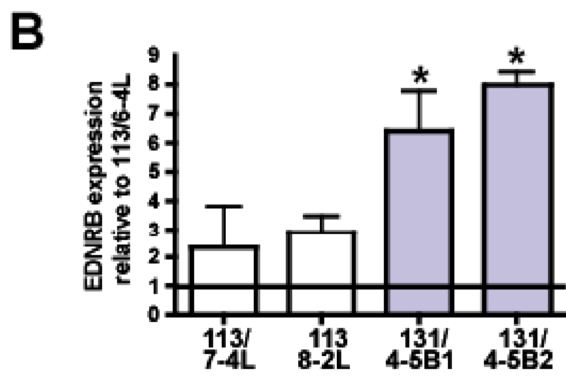
Supplemental figure S8: Examination of the role of BCL2A1 in intracranial tumor growth. A) Brain-metastatic variants show increase resistance to TNF-alpha when compared to parental 113/6-4L cell line (Two-way Anova, $P<0.05$). Values are expressed as proliferation relative to the respective cell line cultured in the absence of TNF-alpha. **B)** The induced and stable overexpression of BCL2A1 in the parental 113/6-4L cell line results in increased cell viability in the presence of 10 ng/ml of TNF-alpha. Conversely**,** stable downregulation of BCL2A1 in the brain-metastatic variants results in decreased cell viability (One-way ANOVA, $P<0.05$). Values are expressed as proliferation relative to the respective cell line cultured in the absence of TNF-alpha.

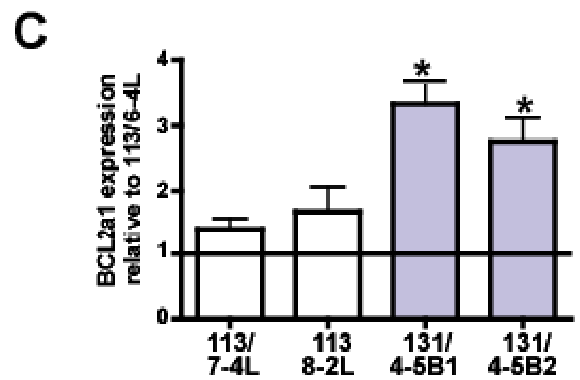
Vbl/CTX
Vbl/CTX
brain metastases detected
all cell lines established from individual brain mets
113/7-4L
113/8-2L
131/4-5B1
131/4-5B2
4 weeks
4 weeks
primary tumor resection
primary tumor resection
4 weeks
4 weeks
visceral mets only
Visceral mets
Brain mets

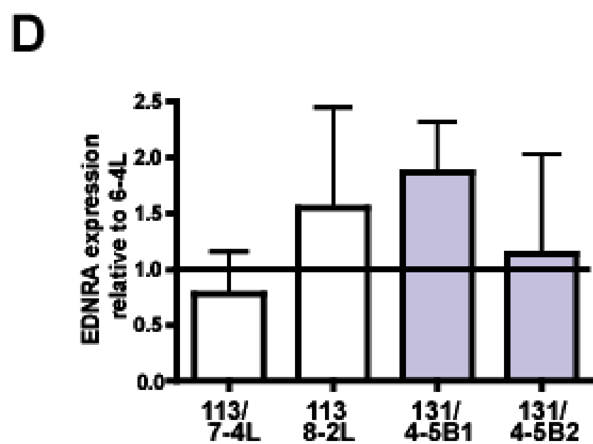


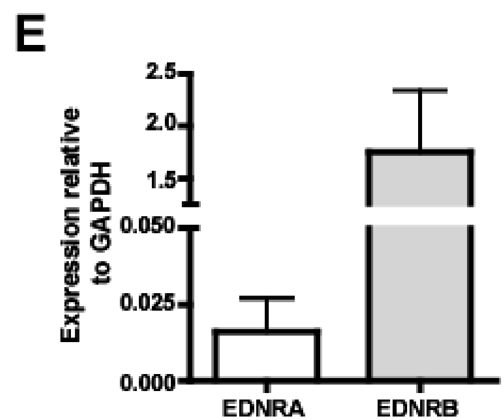
Supplemental Figure S1











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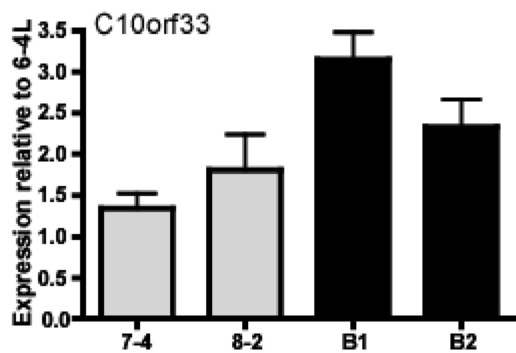
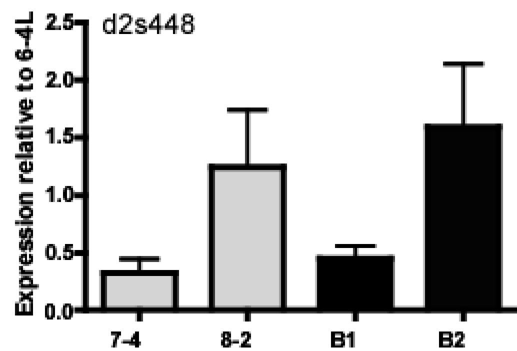
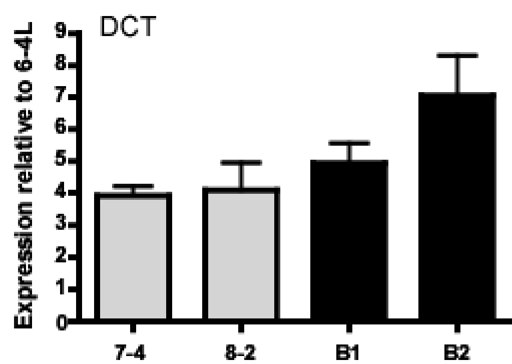
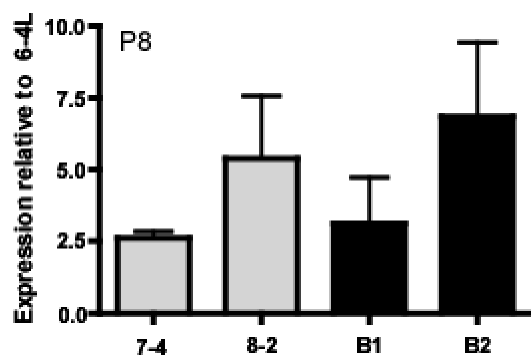
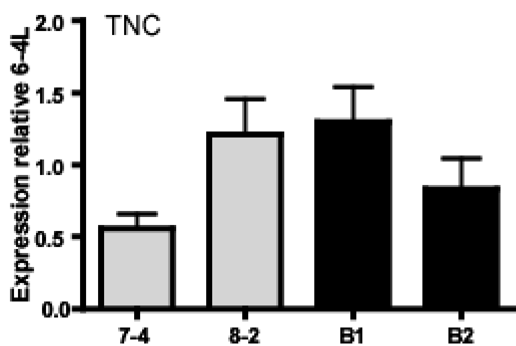
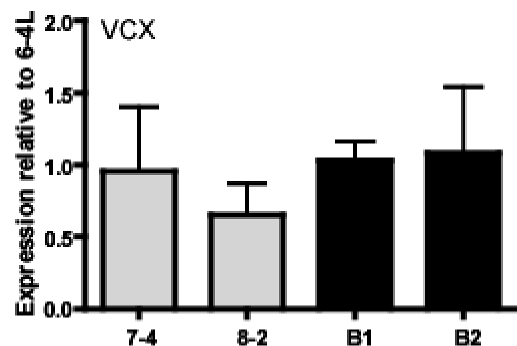






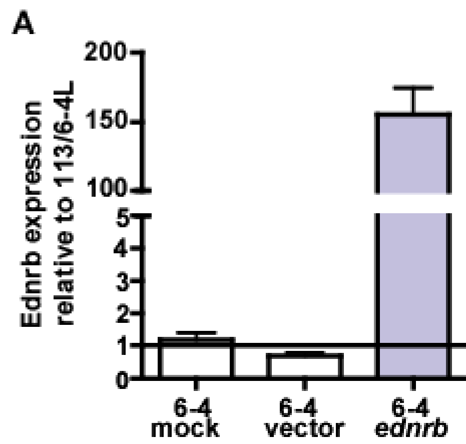






Supplemental Figure 3

A



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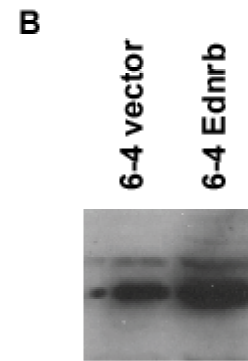

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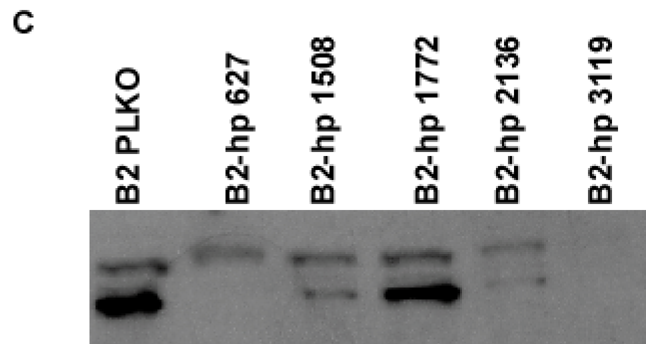
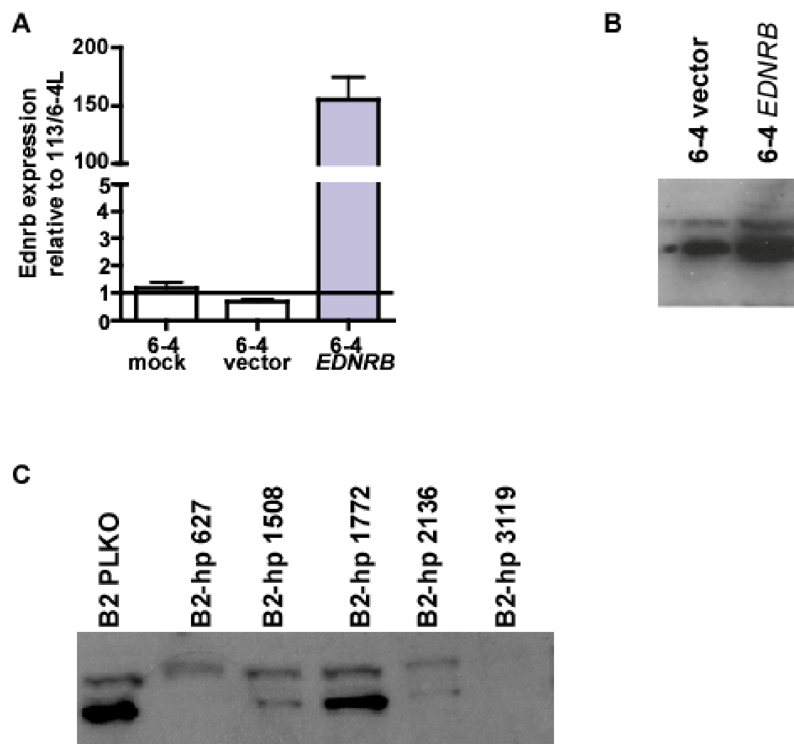
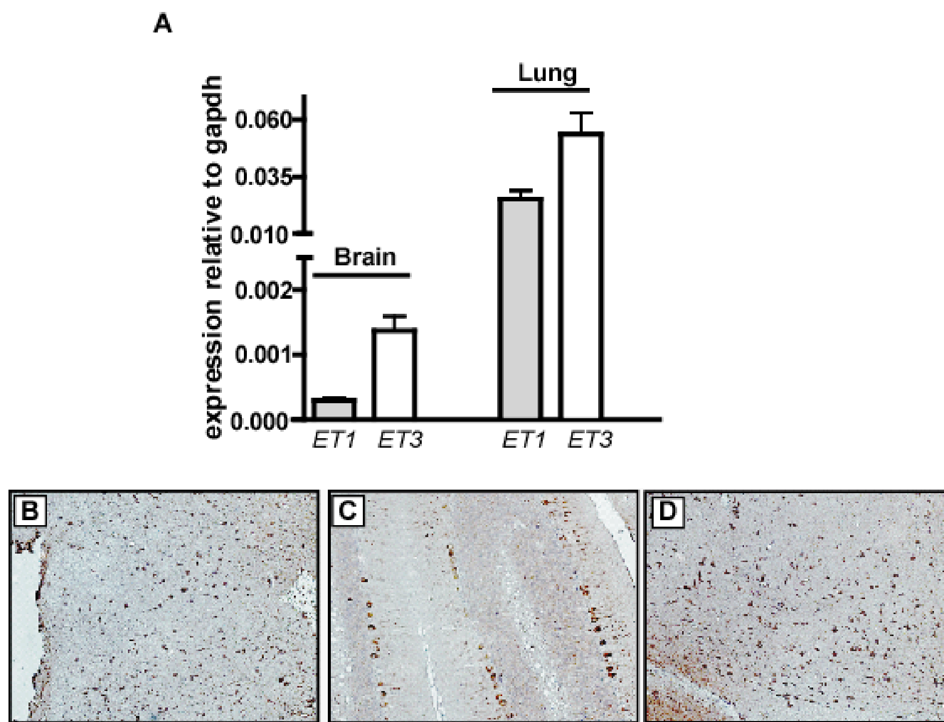


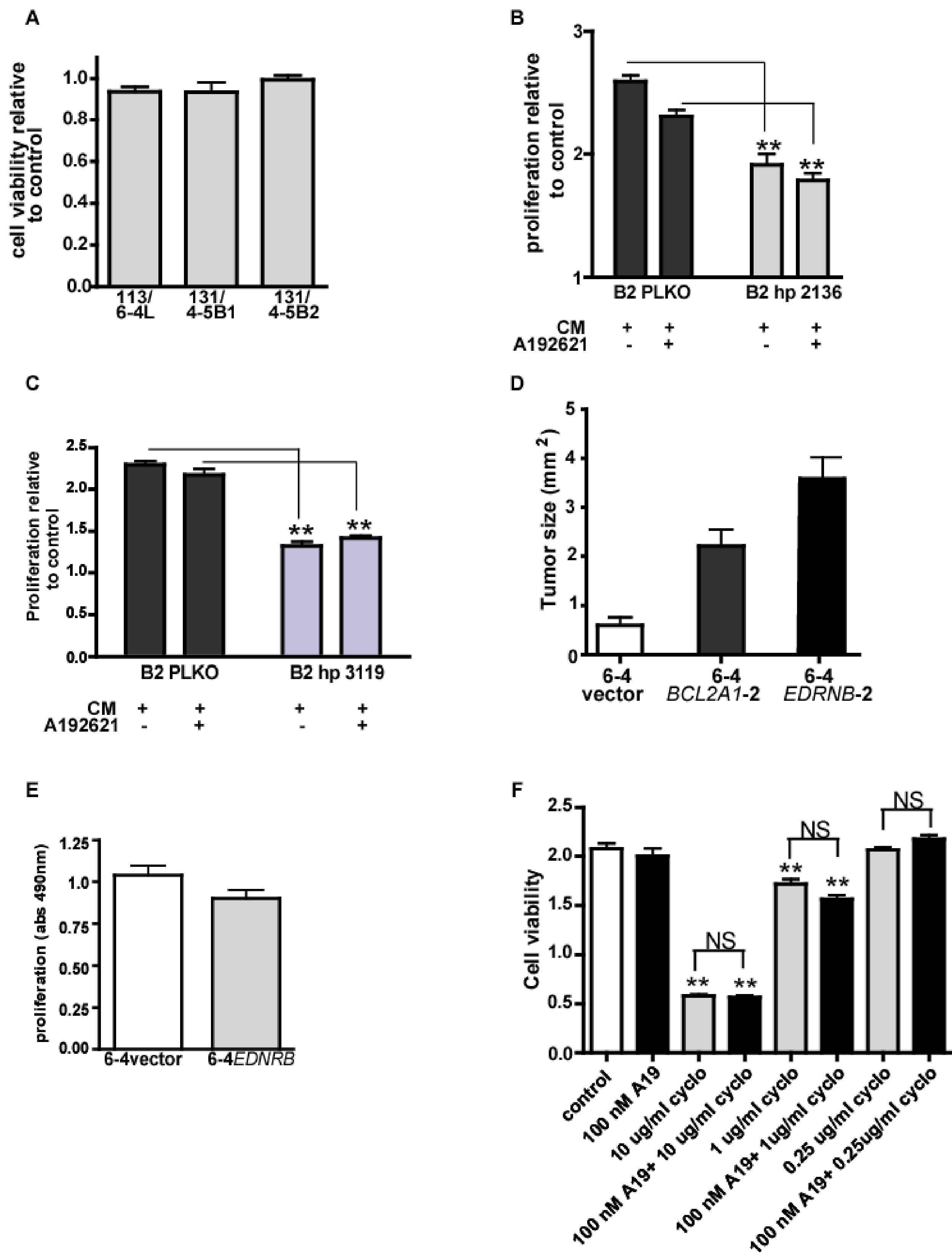
Supplemental Figure 4



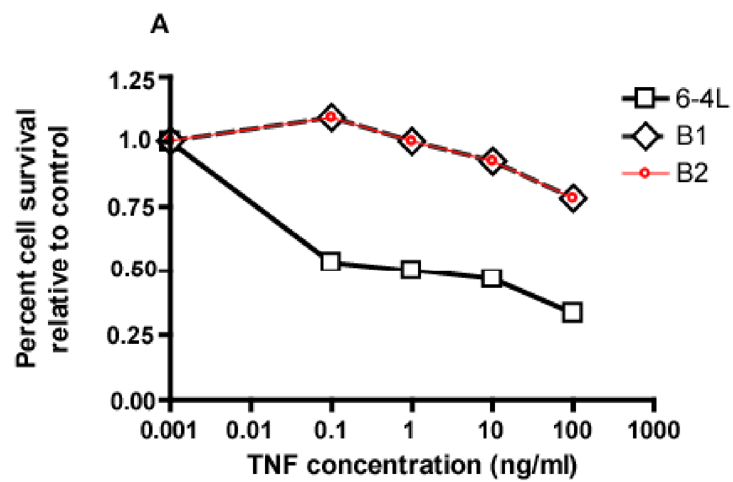
Supplemental Figure 5

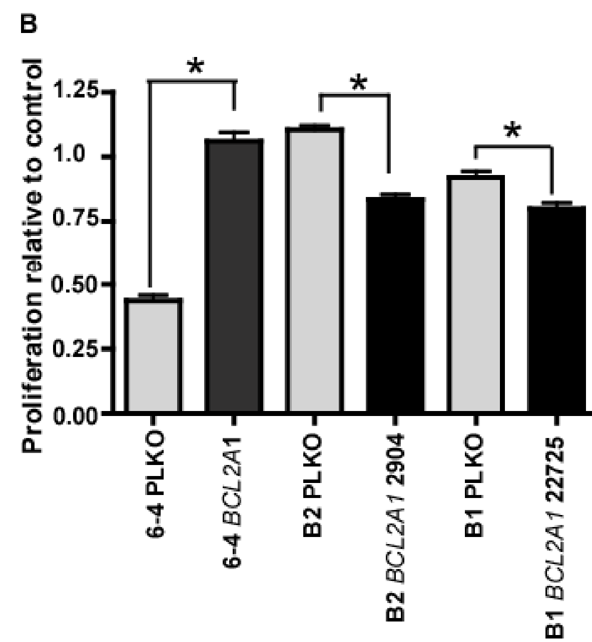


Supplemental figure S6



Supplemental figure S7





Supplemental figure S8