BORIS/CTCFL promotes a switch from a proliferative towards an invasive phenotype in melanoma cells

Sanne Marlijn Janssen[1,2,*], Roy Moscona[3], Mounib Elchebly[1], Andreas Ioannis Papadakis[1], Margaret Redpath[1,2,4], Hangjun Wang[1,2,4], Eitan Rubin[3], Léon Cornelis van Kempen[1,2,5], and Alan Spatz[1,2,4,6*]

Affiliations:

[1]Lady Davis Institute for Medical Research, Montréal, QC, Canada.

[2]Department of Pathology, McGill University, Montréal, QC, Canada.

[3]The Shraga Segal Department of Microbiology, Immunology and Genetics, Ben-Gurion University of the Negev, Beer Sheva, Israel.

[4]Division of Pathology, Department of Laboratory medicine, McGill University Health Center, Montreal, QC, Canada

[5]Department of Pathology, Laboratory for Molecular Pathology, University Medical Center Groningen, Groningen, The Netherlands.

[6]Department of Oncology, McGill University, Montréal, QC, Canada.

*To whom correspondence should be addressed:

Sanne M. Janssen (email: sanne.janssen@mail.mcgill.ca)

Alan Spatz (email: alan.spatz@mcgill.ca)

Running Title: BORIS/CTCFL promotes melanoma invasiveness

Abstract

Melanoma is among the most aggressive cancers due to its tendency to metastasize early. Phenotype switching between a proliferative and an invasive state has been suggested as a critical process for metastasis. The mechanisms that regulate these transitions are complex and remain poorly understood. Brother of Regulator of Imprinted Sites (BORIS), also known as CCCTC binding factor-Like (CTCFL), is a transcriptional modulator that becomes aberrantly expressed in melanoma. Here, we show that BORIS is involved in phenotype switching in melanoma. Genetic modification of BORIS expression in melanoma cells combined with whole transcriptome analysis indicated that BORIS expression contributes to an invasion-associated transcriptome. In agreement with this finding, inducible BORIS overexpression in melanoma cells reduced proliferation and increased migration and invasion, demonstrating that the transcriptional switch is accompanied by a phenotypic switch. Overall, our study indicates a pro-invasive role for BORIS in melanoma via transcriptional reprogramming.

Introduction

Primary cutaneous melanoma is one of the most aggressive cancers due to its ability to rapidly disseminate and develop distant metastases. The progression of melanoma from the primary site to regional lymph nodes and distant sites requires cells to migrate and invade into tissue containing lymphovascular channels that allow the tumor cells to enter the circulation. During melanoma progression, cells undergo phenotype switching by transitioning from a proliferative to an invasive state, a process similar to epithelial-mesenchymal transition (EMT) (1, 2). Gene expression profiling of melanoma cell lines and tumor samples has revealed the different transcriptional states that underlie the proliferative and invasive phenotypes of melanoma cells (3-7). While proliferative

cells tend to express high levels of developmental and lineage specification genes such as *MITF*, *MLANA*, and *DCT* as well as *SOX10* and *PAX3* (1, 4, 6), cells with an invasive transcriptional state are marked by expression of amongst others, *ZEB1*, *SNAI1*, *AXL*, *NGFR*, *SPARC*, *WNT5A* and genes involved in TGF-β signaling (4, 6-10). Currently, various transcriptional regulators have been implicated as drivers of the transition towards an invasive phenotype, including BRN2, AP-1, TEAD, NFATC2, and NFIB (4, 11-14). These findings suggest complex transcriptional regulation that requires context-specific involvement of various signaling pathways, transcriptional regulators, and gene networks to achieve the transition towards an invasive state. In line with this idea it was proposed that phenotype switching comprises a gradient of phenotypic states that is accompanied by different transcriptional states (15, 16), similarly to what is reported for EMT in epithelial cancers (17, 18). To gain insight into the transcriptional landscape that underlies these different phenotypic spectra it is important to identify transcriptional regulators of the gene expression changes that confer migratory and invasive properties to melanoma cells.

Brother of Regulator of Imprinted Sites (BORIS) is a DNA binding protein with high similarity to CCCTC binding factor (CTCF) (19), a multifunctional transcription factor that plays an important role in genome organization (20). In contrast to the tumor suppressive functions reported for CTCF (21, 22), multiple studies have indicated an oncogenic role for BORIS (23-27). Like CTCF, BORIS plays a role in transcriptional regulation (24, 25, 28, 29). The mechanisms through which BORIS can alter transcription rely on BORIS' ability to bind the DNA at specific binding motifs, which are often found near promoter regions (30-32). At these binding motifs BORIS can compete with DNA-bound CTCF (32), form a BORIS-CTCF heterodimer, or occupy a so-called BORIS-only binding site (31). Via these mechanisms BORIS can alter transcription by acting as a

transcriptional activator (24, 25, 29, 33), recruiting a transcriptional activator (34), altering DNA methylation (24, 35, 36) or histone modifications (24, 28, 29, 35), or recruiting chromatin modifying proteins (37, 38). In addition, it is believed that BORIS impacts the transcriptional landscape by interfering with CTCF-mediated chromatin loops (31, 39). The *BORIS* gene is located at 20q13 locus, within a chromosomal region that is often amplified in cancer (40). Furthermore, hypomethylation of the *BORIS* promoter is frequently observed in cancer cells (41, 42). Besides regulation of expression via DNA methylation, increased BORIS levels have been observed upon CTCF knockdown (43). *BORIS* is expressed in 60-90% of melanoma cell lines (36, 44), in 16% (4 out of 25) of primary melanomas and in 34% (13 out of 38) of melanoma metastases (44). The higher frequency of BORIS expression in metastatic tumors compared to primary tumors suggests that BORIS plays a role in melanoma progression (44). Currently, little is known about the role of BORIS in melanoma.

In this study, we sought to determine if BORIS plays a role in melanoma progression through its role as transcriptional modulator. We found that BORIS expression in melanoma cells led to altered gene expression as determined by RNA-sequencing (RNA-seq). While the upregulated genes were enriched among invasion-related processes and gene signatures, the downregulated genes were enriched among proliferation-related processes and gene signatures, suggesting that BORIS plays a role in phenotype switching. Phenotypically, we found that BORIS overexpression reduced proliferation and increased the migratory and invasive ability of melanoma cell lines. Overall, we demonstrate that BORIS-mediated transcriptional reprogramming was accompanied by a switch towards an invasive phenotype.

Results

BORIS expression in the MM057 melanoma cell line induces large-scale differential gene expression

First, we sought to confirm that BORIS is expressed in melanoma cell lines as reported previously (36, 43, 44). Our panel of melanoma and non-malignant congenital nevi cell lines was screened for expression of BORIS mRNA and protein. This revealed *BORIS* mRNA in ~60% (11 out of 18, **Fig S1A**) and BORIS protein in ~33% (4 out of 12, **Fig S1B**) of melanoma cell lines. We did not detect *BORIS* expression in the congenital nevi cell lines (**Fig S1A**). In the tested melanoma cell lines *BORIS* mRNA expression was not proportional to BORIS protein level. We observed no correlation between the expression of BORIS and its ubiquitously expressed paralogue CTCF in the tested cell lines (**Fig. S1B and C**). Together, these data indicate that BORIS is expressed in melanoma cells and that this expression is independent of CTCF expression.

Next, we wanted to assess if BORIS, through its role as transcriptional modulator, can alter the transcriptome of melanoma cells. To this end we used the MM057 melanoma cell line, which harbors a proliferative gene signature as reported by Verfaillie *et al.* (4) and expresses no detectable BORIS protein and low BORIS mRNA (**Fig. S1A and B**). The effect of BORIS expression on the transcriptome of MM057 cells was assessed by RNA-seq using three biological replicates. BORIS overexpression was established in this cell line by lentiviral transduction with the doxycycline (dox)-inducible BORIS expression vector and BORIS mRNA and protein levels were compared with those in the control cells for each independent RNA-seq sample (**Fig. 1A**). Since a strong correlation was observed between the biological replicates ($r \geq 0.97$, **Fig. S2A**), all of the data was included for downstream analyses. The unsupervised analysis of gene expression demonstrated

clustering of the samples into two groups, one representing the controls [empty vector without dox (EVneg) and with dox (EVpos), and BORIS without dox (BORneg] and the other representing BORIS expressing cells [BORIS with dox (BORpos)] (**Fig S2B**). Differential gene expression analysis between BORneg and BORpos cells identified 2248 differential expressed genes (DEGs), of which 1361 genes were upregulated and 887 downregulated following BORIS expression (**Fig. 1B and Table S2**). Analysis of the log2 counts per million (CPM) for the top 100 up- and down-regulated genes demonstrated robust differential expression, as well as clustering of all control samples versus BORpos samples (**Fig. 1C**). To validate the RNA-seq dataset, BORIS expression was induced in the MM057 cells and qPCR was performed for genes distributed throughout the list of DEGs. A significant differential expression of upregulated and downregulated genes was observed following BORIS expression compared to the controls (**Fig. 1D**). Together, these data demonstrate that ectopic BORIS expression results in large-scale differential gene expression.

BORIS expression contributes to a switch from a proliferative to invasive transcriptional state

The dataset was further analyzed to investigate if BORIS-regulated genes are involved in processes related to melanoma progression. First, gene set enrichment analysis (GSEA) (45) was performed for biological processes on a pre-ranked gene list. A strong enrichment (false discovery rate (FDR)<0.05) for migration and invasion-related processes was observed, including EMT, cell motility and locomotion, while a strong negative enrichment was found for proliferation-related processes like cell cycle/division, telomere maintenance, and DNA replication/repair (**Fig. 2A**). This analysis provides the first line of evidence that BORIS could promote a pro-invasive transcriptome in melanoma cells. Next, we performed GSEA for the melanoma-specific Hoek (3)

and Verfaillie (4) gene signatures that correspond to either the proliferative or invasive state of melanoma cells. For these gene sets, we identified a strong positive correlation with the Hoek (NES=2.21, FDR=0.001) and Verfaillie (NES=5.99, FDR<10^{-4}) invasive gene signatures (**Fig. 2B**) and a negative correlation with the Hoek (NES=-1.98, FDR=0.006) and Verfaillie (NES=-3.84, FDR<10^{-4}) proliferative gene signatures (**Fig. 2C**). These data support the hypothesis that BORIS expression enables a transcriptional program that promotes a switch from a proliferative to invasive state. To validate the effect of BORIS expression on genes within the gene signatures we measured the expression of four invasion-associated and four proliferation-associated genes from the Hoek and Verfaillie studies in the MM057 cell line following BORIS expression. We observed a significant upregulation of the invasion-associated genes *DKK1, FBN1, INHBA* and *VEGFC* (**Fig. 2D**) and significant downregulation of the proliferation-associated genes *GYG2, LZTS1, MLANA* and *TFAP2A* (**Fig. 2E**) compared to the controls. Together, these results indicate that BORIS promotes a switch from a proliferative to an invasive gene expression signature in melanoma cells.

Transcriptional reprogramming and subsequent phenotype switching have been linked to differences in the expression of transcription factors that are associated with EMT (4, 11, 12). In melanoma, reduced expression of the melanoma-specific transcription factor MITF has been reported in invasive cells compared to proliferative cells (1). MITF is regulated by the switch in expression from ZEB2 in melanocytes to ZEB1 in melanoma cells, with ZEB1 functioning as a transcriptional repressor of E-cadherin (*CDH1*) (46, 47). In the MM057 cells BORIS did not significantly reduce *MITF* expression (**Fig. 3A**). Similarly, BORIS did not significantly alter *ZEB1*, *ZEB2* (**Fig. 3A**) or *CDH1* expression (not expressed). In contrast, expression of the

transcription factor and melanoma EMT inducer SNAIL (*SNAI1*) (48) was increased in BORIS-expressing MM057 cells (**Fig. 3A**). Expression of SNAIL can be upregulated via multiple mechanisms, including elevated levels of the matricellular protein SPARC (49) and TGF-beta (48). Indeed, we observed significantly higher expression of *SPARC* and *TGFB1* in BORIS-induced cells compared to non-induced cells (**Fig. 3B**). Furthermore, the RNA-seq data reveals increased transcription of multiple genes belonging to the TGF-beta family (**Fig. 3C**). These data suggest that the BORIS-induced transcriptional switch may be mediated via TGF-beta signaling and increased *SNAI1* expression.

BORIS expression in melanoma cells results in reduced proliferation

In line with the acquisition of an invasive transcriptional state, the GSEA on BORIS-induced DEGs revealed a negative enrichment for processes involved in proliferation. To investigate the effect of BORIS expression on proliferation we induced BORIS expression with increasing concentration of dox. Expression of BORIS at each time point was confirmed using immunoblot (**Fig. S3A**). We observed a dose-dependent reduction in proliferation over time compared to untreated cells, which was not observed in the control cells (**Fig. 4A**). These findings support the hypothesis that BORIS expression reduces proliferation and promotes a more invasive state in melanoma cells. In addition to an effect of BORIS expression on proliferation, BORIS knockdown in breast cancer and colon cancer cell lines was shown to increased apoptosis (23, 50). To address whether the reduction in proliferation that we observed is due to increased apoptosis, we investigated the effect of BORIS expression on cell death. Compared to control cells, BORIS expression increased the expression of cleaved PARP, a marker for cell death (**Fig. 4B**). In addition, we observed an increase in the number of early apoptotic, apoptotic and necrotic cells

following BORIS expression compared to controls (**Fig. 4C and D**), indicating that BORIS expression in melanoma cells leads to a slight, though significant, increase in apoptosis. Taken together, our results show that expression of BORIS in melanoma cells leads to decreased proliferative activity, which is in part due to increased apoptosis.

BORIS expression promotes an invasive phenotype in melanoma cells.

The contribution of BORIS expression to an invasive transcriptional state and a reduction in proliferation prompted us to ask if BORIS expression promotes an invasive phenotype. First, we assessed the effect of BORIS overexpression on migration using a transwell assay in two melanoma cells lines (MM057 and MM074) that harbor a proliferative transcriptional state (4). Inducible BORIS expression was confirmed by immunoblot (**Fig. S3B**). In both cell lines we observed a significant increase in the percentage of migrating cells compared to the controls (**Fig. 5A and B**). Furthermore, we observed a significant increase in the invasive capacity of both cell lines following BORIS expression in a Matrigel transwell invasion assay (**Fig. 5C and D**). Overall, these results demonstrate that BORIS expression leads to an increase in the migratory and invasive potential of the MM057 and MM074 melanoma cell lines.

Discussion

Phenotype switching is a critical process that allows melanoma cells to acquire an invasive state that fuels disease progression (46). Important for the cellular plasticity required to transition between a proliferative and invasive phenotype is reprogramming of the transcriptional state. Here, we demonstrate that BORIS can alter the gene expression program of melanoma cells in favor of a more invasive phenotype. Transcriptional profiling revealed that genes upregulated in BORIS-

expressing cells are enriched among established invasive gene signatures, while downregulated genes are enriched among the proliferative gene signatures. In line with these findings, we showed that BORIS expression resulted in reduced proliferation, whereas the migratory and invasive abilities of melanoma cells were enhanced. Together, these data indicate that expression of BORIS contributes to the switch from a proliferative towards an invasive state at both the transcriptional and phenotypic level in melanoma cell lines.

In contrast to ubiquitously expressed CTCF, BORIS expression is restricted to the testis (19) and becomes aberrantly re-activated in various types of cancer, including melanoma (42). Here we confirm BORIS expression at the mRNA level (36, 44) and demonstrate BORIS protein expression in melanoma cell lines. Noteworthy, we observe BORIS protein expression in 3 cell lines with low to undetectable *BORIS* mRNA expression, indicating that *BORIS* mRNA expression is not proportional to BORIS protein level. This discordance between BORIS mRNA and protein has been reported previously (51) and is proposed to be due to a short half-life of the BORIS transcript and high protein stability (52).

Our finding that altered BORIS expression leads to large-scale changes in gene expression was expected, as BORIS is known to act as a transcriptional regulator (24, 25, 29, 33). In addition, it reflects the numerous cellular processes that BORIS is involved in (42, 53), many of which are altered during carcinogenesis. Multiple studies found that increased BORIS expression is linked to poor prognosis and more advanced stages of disease, hence BORIS being considered an oncogene (54-57). Nevertheless, one study showed that BORIS overexpression in cancer cell lines results in a similar phenotype as forced expression of the tumor suppressor CTCF (58). Our data

indicates a pro-invasive role for BORIS in melanoma and supports the notion that BORIS acts pro-oncogenic. While an oncogenic phenotype is associated with fast proliferation, one of the characteristics of phenotypic switching towards an invasive state is inhibition of proliferation (1). We indeed demonstrated decreased proliferation upon BORIS overexpression, which is in agreement with previous studies reporting reduced proliferation upon ectopic BORIS expression in both primary cells and various cancer cell lines (58, 59). Though the literature regarding proliferation in the context of BORIS is not uniform, as others have reported increased proliferation upon BORIS overexpression (24, 27, 50). Given the large-scale transcriptional changes, additional effects of BORIS expression on melanoma cell biology are very likely. However, at the moment, other functional roles for BORIS in melanoma have not yet been investigated.

The large number of genes that make up the signatures representing the proliferative and invasive state highlight the complexity that underlies the process of phenotype switching. Multiple transcription factors, signalling pathways and EMT-related genes are important to establish the transitions. Many of these factors are likely involved at different stages of phenotype switching, marked by a transcriptional profile between the proliferative and invasive state (5, 60), and contribute to partial or intermediate phenotype switching (10, 16, 61). Our observation that BORIS expression has no effect on *MITF*, *ZEB1* or *ZEB2* expression, suggest that the MM057 melanoma cells have already undergone a partial switch. This is consistent with their ability to migrate and invade even in the absence of BORIS expression as well as the lack of *CDH1* expression in the control cells, indicating that the equilibrium between ZEB1/2 and E-cadherin might already be in favor of an invasive phenotype in the MM057 cells. Interestingly, we observe increased expression

of the EMT transcription factor SNAIL (*SNAI1*). Ectopic SNAIL expression in human melanoma cells is reported to induce EMT features, which was accompanied by increased expression of TGF-beta, IL-10 and thrombospondin 1 (48). A bidirectional regulation between TGF-beta and SNAIL exists, as TGF-beta can also upregulate SNAIL expression (48). In addition, SNAIL expression can be induced by SPARC (49), a matricellular protein involved in cell-matrix interactions that promotes EMT-like changes (62) and is associated with the metastatic potential of melanoma (63, 64). We showed that in BORIS-expressing melanoma cells *SNAI1*, *SPARC* and *TGFB1* expression is increased. Furthermore, expression of a striking number of TGF-beta family members is increased, indicating activation of TGF-beta driven signaling, which is an important mark of the invasive signature (1, 4). Based on our findings, we propose that BORIS expression induces partial phenotype switching via upregulation of *SPARC* and TGF-beta signaling that leads to *SNAI1* expression. Currently, the mechanisms behind BORIS-mediated transcriptional changes remain elusive, though they likely involve competition with CTCF for DNA binding sites, direct promoter regulation, epigenetic modifications, or chromatin looping.

Our observation that BORIS-expressing melanoma cells have increased migratory and invasive abilities are in agreement with previous studies that demonstrated that BORIS overexpression promoted EMT in liver cancer cell lines (25) as well as the CD44/CD133 positive subpopulation of the IMR-32 neuroblastoma cell line (65). However, in contrast, Alberti *et al.*, revealed that BORIS knockdown promotes EMT in MCF7 breast cancer cells (66). These studies suggest a cell type dependent role for BORIS in cancer progression.

Taken together, our study demonstrates that BORIS contributes to melanoma progression by intrinsically rewiring gene expression to promote an invasive phenotype. Furthermore, these results indicate that BORIS is an important transcriptional regulator during phenotype switching in melanoma and provide a rationale for further studies into BORIS' role as an invasion promoting transcriptional regulator.

Acknowledgements

This work was partially funded by the Israel Cancer Research Fund. SMJ was supported by the Banque National Fellowship, the Dr. Victor K.S. Lui Fellowship and the CIHR/FRSQ training grant in cancer research (FRN53888) of the McGill Integrated Cancer Research Training Program. AIP was supported by the Banque National Fellowship.

Author Contributions

SMJ, LCK and AS designed the study, analyzed the results and wrote the manuscript with input from all other authors. SMJ, ME and AIP performed the tissue culture. SMJ and ME generated the expression vectors. SMJ carried out the qPCRs and immunoblots; ME conducted the *in vitro* experiments with help of MR and HW. Experiment were carried out under the supervision of AS. RM performed the bioinformatics analysis under the supervision of ER.

Competing interests

The authors declare no competing financial interests

References

1. Hoek KS, Eichhoff OM, Schlegel NC, Dobbeling U, Kobert N, Schaerer L, et al. In vivo switching of human melanoma cells between proliferative and invasive states. Cancer Res. 2008;68(3):650-6.

2. Hoek KS, Goding CR. Cancer stem cells versus phenotype-switching in melanoma. Pigment Cell Melanoma Res. 2010;23(6):746-59.

3. Hoek KS, Schlegel NC, Brafford P, Sucker A, Ugurel S, Kumar R, et al. Metastatic potential of melanomas defined by specific gene expression profiles with no BRAF signature. Pigment Cell Res. 2006;19(4):290-302.

4. Verfaillie A, Imrichova H, Atak ZK, Dewaele M, Rambow F, Hulselmans G, et al. Decoding the regulatory landscape of melanoma reveals TEADS as regulators of the invasive cell state. Nat Commun. 2015;6:6683.

5. Widmer DS, Cheng PF, Eichhoff OM, Belloni BC, Zipser MC, Schlegel NC, et al. Systematic classification of melanoma cells by phenotype-specific gene expression mapping. Pigment Cell Melanoma Res. 2012;25(3):343-53.

6. Jeffs AR, Glover AC, Slobbe LJ, Wang L, He S, Hazlett JA, et al. A gene expression signature of invasive potential in metastatic melanoma cells. PLoS One. 2009;4(12):e8461.

7. Tirosh I, Izar B, Prakadan SM, Wadsworth MH, 2nd, Treacy D, Trombetta JJ, et al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. Science (New York, NY). 2016;352(6282):189-96.

8. Weeraratna AT, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M, et al. Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell. 2002;1(3):279-88.

9. Alonso SR, Tracey L, Ortiz P, Perez-Gomez B, Palacios J, Pollan M, et al. A high-throughput study in melanoma identifies epithelial-mesenchymal transition as a major determinant of metastasis. Cancer Res. 2007;67(7):3450-60.

10. Restivo G, Diener J, Cheng PF, Kiowski G, Bonalli M, Biedermann T, et al. low neurotrophin receptor CD271 regulates phenotype switching in melanoma. Nat Commun. 2017;8(1):1988.

11. Aibar S, Gonzalez-Blas CB, Moerman T, Huynh-Thu VA, Imrichova H, Hulselmans G, et al. SCENIC: single-cell regulatory network inference and clustering. Nat Methods. 2017;14(11):1083-6.

12. Fane ME, Chhabra Y, Smith AG, Sturm RA. BRN2, a POUerful driver of melanoma phenotype switching and metastasis. Pigment Cell Melanoma Res. 2018.

13. Pinner S, Jordan P, Sharrock K, Bazley L, Collinson L, Marais R, et al. Intravital imaging reveals transient changes in pigment production and Brn2 expression during metastatic melanoma dissemination. Cancer Res. 2009;69(20):7969-77.

14. Perotti V, Baldassari P, Molla A, Nicolini G, Bersani I, Grazia G, et al. An actionable axis linking NFATc2 to EZH2 controls the EMT-like program of melanoma cells. Oncogene. 2019.

15. Li FZ, Dhillon AS, Anderson RL, McArthur G, Ferrao PT. Phenotype switching in melanoma: implications for progression and therapy. Front Oncol. 2015;5:31.

16. Noguchi K, Dalton AC, Howley BV, McCall BJ, Yoshida A, Diehl JA, et al. Interleukin-like EMT inducer regulates partial phenotype switching in MITF-low melanoma cell lines. PLoS One. 2017;12(5):e0177830.

17. Gopal SK, Greening DW, Mathias RA, Ji H, Rai A, Chen M, et al. YBX1/YB-1 induces partial EMT and tumourigenicity through secretion of angiogenic factors into the extracellular microenvironment. Oncotarget. 2015;6(15):13718-30.

18. Grande MT, Sanchez-Laorden B, Lopez-Blau C, De Frutos CA, Boutet A, Arevalo M, et al. Snail1-induced partial epithelial-to-mesenchymal transition drives renal fibrosis in mice and can be targeted to reverse established disease. Nature medicine. 2015;21(9):989-97.

19. Loukinov DI, Pugacheva E, Vatolin S, Pack SD, Moon H, Chernukhin I, et al. BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. Proc Natl Acad Sci U S A. 2002;99(10):6806-11.

20. Phillips JE, Corces VG. CTCF: master weaver of the genome. Cell. 2009;137(7):1194-211.

21. Kemp CJ, Moore JM, Moser R, Bernard B, Teater M, Smith LE, et al. CTCF haploinsufficiency destabilizes DNA methylation and predisposes to cancer. Cell Rep. 2014;7(4):1020-9.

22. Rasko JE, Klenova EM, Leon J, Filippova GN, Loukinov DI, Vatolin S, et al. Cell growth inhibition by the multifunctional multivalent zinc-finger factor CTCF. Cancer Res. 2001;61(16):6002-7.

23. Dougherty CJ, Ichim TE, Liu L, Reznik G, Min WP, Ghochikyan A, et al. Selective apoptosis of breast cancer cells by siRNA targeting of BORIS. Biochem Biophys Res Commun. 2008;370(1):109-12.

24. Gaykalova D, Vatapalli R, Glazer CA, Bhan S, Shao C, Sidransky D, et al. Dose-dependent activation of putative oncogene SBSN by BORIS. PLoS One. 2012;7(7):e40389.

25. Liu Q, Chen K, Liu Z, Huang Y, Zhao R, Wei L, et al. BORIS up-regulates OCT4 via histone methylation to promote cancer stem cell-like properties in human liver cancer cells. Cancer Lett. 2017;403:165-74.

26. Renaud S, Loukinov D, Alberti L, Vostrov A, Kwon YW, Bosman FT, et al. BORIS/CTCFL-mediated transcriptional regulation of the hTERT telomerase gene in testicular and ovarian tumor cells. Nucleic Acids Res. 2011;39(3):862-73.

27. Smith IM, Glazer CA, Mithani SK, Ochs MF, Sun W, Bhan S, et al. Coordinated activation of candidate proto-oncogenes and cancer testes antigens via promoter demethylation in head and neck cancer and lung cancer. PLoS One. 2009;4(3):e4961.

28. Sun L, Huang L, Nguyen P, Bisht KS, Bar-Sela G, Ho AS, et al. DNA methyltransferase 1 and 3B activate BAG-1 expression via recruitment of CTCFL/BORIS and modulation of promoter histone methylation. Cancer Res. 2008;68(8):2726-35.

29. Zampieri M, Ciccarone F, Palermo R, Cialfi S, Passananti C, Chiaretti S, et al. The epigenetic factor BORIS/CTCFL regulates the NOTCH3 gene expression in cancer cells. Biochim Biophys Acta. 2014;1839(9):813-25.

30. Bergmaier P, Weth O, Dienstbach S, Boettger T, Galjart N, Mernberger M, et al. Choice of binding sites for CTCFL compared to CTCF is driven by chromatin and by sequence preference. Nucleic Acids Res. 2018;46(14):7097-107.

31. Pugacheva EM, Rivero-Hinojosa S, Espinoza CA, Mendez-Catala CF, Kang S, Suzuki T, et al. Comparative analyses of CTCF and BORIS occupancies uncover two distinct classes of CTCF binding genomic regions. Genome Biol. 2015;16:161.

32. Sleutels F, Soochit W, Bartkuhn M, Heath H, Dienstbach S, Bergmaier P, et al. The male germ cell gene regulator CTCFL is functionally different from CTCF and binds CTCF-like

consensus sites in a nucleosome composition-dependent manner. Epigenetics Chromatin. 2012;5(1):8.

33. Hong JA, Kang Y, Abdullaev Z, Flanagan PT, Pack SD, Fischette MR, et al. Reciprocal binding of CTCF and BORIS to the NY-ESO-1 promoter coincides with derepression of this cancer-testis gene in lung cancer cells. Cancer Res. 2005;65(17):7763-74.

34. Kang Y, Hong JA, Chen GA, Nguyen DM, Schrump DS. Dynamic transcriptional regulatory complexes including BORIS, CTCF and Sp1 modulate NY-ESO-1 expression in lung cancer cells. Oncogene. 2007;26(30):4394-403.

35. Bhan S, Negi SS, Shao C, Glazer CA, Chuang A, Gaykalova DA, et al. BORIS binding to the promoters of cancer testis antigens, MAGEA2, MAGEA3, and MAGEA4, is associated with their transcriptional activation in lung cancer. Clin Cancer Res. 2011;17(13):4267-76.

36. Vatolin S, Abdullaev Z, Pack SD, Flanagan PT, Custer M, Loukinov DI, et al. Conditional expression of the CTCF-paralogous transcriptional factor BORIS in normal cells results in demethylation and derepression of MAGE-A1 and reactivation of other cancer-testis genes. Cancer Res. 2005;65(17):7751-62.

37. Jelinic P, Stehle JC, Shaw P. The testis-specific factor CTCFL cooperates with the protein methyltransferase PRMT7 in H19 imprinting control region methylation. PLoS Biol. 2006;4(11):e355.

38. Nguyen P, Bar-Sela G, Sun L, Bisht KS, Cui H, Kohn E, et al. BAT3 and SET1A form a complex with CTCFL/BORIS to modulate H3K4 histone dimethylation and gene expression. Mol Cell Biol. 2008;28(21):6720-9.

39. Lobanenkov VV, Zentner GE. Discovering a binary CTCF code with a little help from BORIS. Nucleus. 2018;9(1):33-41.

40. Klenova EM, Morse HC, Ohlsson R, Lobanenkov VV. The novel BORIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer. Seminars in Cancer Biology. 2002;12(5):399-414.

41. Martin-Kleiner I. BORIS in human cancers -- a review. Eur J Cancer. 2012;48(6):929-35.

42. Soltanian S, Dehghani H. BORIS: a key regulator of cancer stemness. Cancer Cell Int. 2018;18:154.

43. Renaud S, Pugacheva EM, Delgado MD, Braunschweig R, Abdullaev Z, Loukinov D, et al. Expression of the CTCF-paralogous cancer-testis gene, brother of the regulator of imprinted sites (BORIS), is regulated by three alternative promoters modulated by CpG methylation and by CTCF and p53 transcription factors. Nucleic Acids Res. 2007;35(21):7372-88.

44. Kholmanskikh O, Loriot A, Brasseur F, De Plaen E, De Smet C. Expression of BORIS in melanoma: lack of association with MAGE-A1 activation. Int J Cancer. 2008;122(4):777-84.

45. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 2005;102(43):15545-50.

46. Caramel J, Papadogeorgakis E, Hill L, Browne GJ, Richard G, Wierinckx A, et al. A switch in the expression of embryonic EMT-inducers drives the development of malignant melanoma. Cancer Cell. 2013;24(4):466-80.

47. Denecker G, Vandamme N, Akay O, Koludrovic D, Taminau J, Lemeire K, et al. Identification of a ZEB2-MITF-ZEB1 transcriptional network that controls melanogenesis and melanoma progression. Cell death and differentiation. 2014;21(8):1250-61.

48. Kudo-Saito C, Shirako H, Takeuchi T, Kawakami Y. Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. Cancer Cell. 2009;15(3):195-206.

49. Robert G, Gaggioli C, Bailet O, Chavey C, Abbe P, Aberdam E, et al. SPARC represses E-cadherin and induces mesenchymal transition during melanoma development. Cancer Res. 2006;66(15):7516-23.

50. Zhang Y, Fang M, Song Y, Ren J, Fang J, Wang X. Brother of Regulator of Imprinted Sites (BORIS) suppresses apoptosis in colorectal cancer. Sci Rep. 2017;7:40786.

51. Jones TA, Ogunkolade BW, Szary J, Aarum J, Mumin MA, Patel S, et al. Widespread expression of BORIS/CTCFL in normal and cancer cells. PLoS One. 2011;6(7):e22399.

52. Ogunkolade BW, Jones TA, Aarum J, Szary J, Owen N, Ottaviani D, et al. BORIS/CTCFL is an RNA-binding protein that associates with polysomes. BMC Cell Biol. 2013;14:52.

53. Marshall AD, Bailey CG, Rasko JE. CTCF and BORIS in genome regulation and cancer. Curr Opin Genet Dev. 2014;24:8-15.

54. Cheema Z, Hari-Gupta Y, Kita GX, Farrar D, Seddon I, Corr J, et al. Expression of the cancer-testis antigen BORIS correlates with prostate cancer. Prostate. 2014;74(2):164-76.

55. Chen K, Huang W, Huang B, Wei Y, Li B, Ge Y, et al. BORIS, brother of the regulator of imprinted sites, is aberrantly expressed in hepatocellular carcinoma. Genet Test Mol Biomarkers. 2013;17(2):160-5.

56. D'Arcy V, Abdullaev ZK, Pore N, Docquier F, Torrano V, Chernukhin I, et al. The potential of BORIS detected in the leukocytes of breast cancer patients as an early marker of tumorigenesis. Clin Cancer Res. 2006;12(20 Pt 1):5978-86.

57. Woloszynska-Read A, Zhang W, Yu J, Link PA, Mhawech-Fauceglia P, Collamat G, et al. Coordinated cancer germline antigen promoter and global DNA hypomethylation in ovarian cancer: association with the BORIS/CTCF expression ratio and advanced stage. Clin Cancer Res. 2011;17(8):2170-80.

58. Tiffen JC, Bailey CG, Marshall AD, Metierre C, Feng Y, Wang Q, et al. The cancer-testis antigen BORIS phenocopies the tumor suppressor CTCF in normal and neoplastic cells. Int J Cancer. 2013;133(7):1603-13.

59. Rosa-Garrido M, Ceballos L, Alonso-Lecue P, Abraira C, Delgado MD, Gandarillas A. A cell cycle role for the epigenetic factor CTCF-L/BORIS. PLoS One. 2012;7(6):e39371.

60. Rambow F, Rogiers A, Marin-Bejar O, Aibar S, Femel J, Dewaele M, et al. Toward Minimal Residual Disease-Directed Therapy in Melanoma. Cell. 2018;174(4):843-55 e19.

61. Leclerc J, Garandeau D, Pandiani C, Gaudel C, Bille K, Nottet N, et al. Lysosomal acid ceramidase ASAH1 controls the transition between invasive and proliferative phenotype in melanoma cells. Oncogene. 2019;38(8):1282-95.

62. Brekken RA, Sage EH. SPARC, a matricellular protein: at the crossroads of cell-matrix communication. Matrix biology : journal of the International Society for Matrix Biology. 2001;19(8):816-27.

63. Ledda F, Bravo AI, Adris S, Bover L, Mordoh J, Podhajcer OL. The expression of the secreted protein acidic and rich in cysteine (SPARC) is associated with the neoplastic progression of human melanoma. The Journal of investigative dermatology. 1997;108(2):210-4.

64. Salvatierra E, Alvarez MJ, Leishman CC, Rivas Baquero E, Lutzky VP, Chuluyan HE, et al. SPARC Controls Melanoma Cell Plasticity through Rac1. PLoS One. 2015;10(8):e0134714.

65. Garikapati KR, Patel N, Makani VKK, Cilamkoti P, Bhadra U, Bhadra MP. Down-regulation of BORIS/CTCFL efficiently regulates cancer stemness and metastasis in MYCN amplified neuroblastoma cell line by modulating Wnt/beta-catenin signaling pathway. Biochem Biophys Res Commun. 2017;484(1):93-9.

66. Alberti L, Losi L, Leyvraz S, Benhattar J. Different Effects of BORIS/CTCFL on Stemness Gene Expression, Sphere Formation and Cell Survival in Epithelial Cancer Stem Cells. PLoS One. 2015;10(7):e0132977.

Figures legends

Figure 1. BORIS expression in the MM057 melanoma cell line leads to large-scale differential gene expression.

a Expression of BORIS mRNA and protein as assessed by qPCR and immunoblot. Anti-Tubulin was used as a loading control for immunoblot. **b** MA plot representing the RNA-seq data of all BORneg versus BORpos samples. Significantly upregulated DEGs are depicted in red and downregulated DEGs in blue (FDR < 0.05). The blue horizontal lines mark $\log_2$FC = 1. **c** Heat map representing the $\log_2$CPM for the top 100 up- and down-regulated genes across all RNA-seq samples. **d** BORIS expression was induced in the MM057 cell line using 50ng/ml dox for 5 days followed by qPCR for upregulated and downregulated genes. For all qPCR experiments, a technical triplicate was performed for two biological replicates. Expression was normalized to *HPRT1* and *TBP*. Error bars represent the standard error of the mean. R: replicate, dox: doxycycline, FDR: false discovery rate.

Figure 2. BORIS promotes a switch from a proliferative to invasive gene signature in the MM057 melanoma cell line.

a-c GSEA on the RNA-seq data for **a** biological processes and the hallmark EMT gene set from the Molecular Signatures Database, **b** the invasive, and **c** proliferative gene signatures. **d, e** BORIS expression was induced in the MM057 cell line using 50ng/ml doxycycline for 5 days followed by qPCR for **d** invasive and **e** proliferative genes. For each sample, a technical triplicate was performed for two biological replicates. Expression was normalized to *HPRT1* and *TBP*. Error bars represent the standard error of mean. GO: gene ontology (biological process), H: Hallmark gene set, NES: normalized enrichment score, FDR: false discovery rate.

Figure 3. BORIS expression in the MM057 melanoma cell line leads to increased expression of *SNAI1*, *SPARC* and members of the TGF-beta family.

a, c Bar graph representing the $\log_2$ fold change between BORneg and BORpos RNA-seq samples. **b** BORIS expression was induced in the MM057 cell line using 50ng/ml doxycycline for 5 days followed by qPCR for *SPARC* and *TGFB1*. For each sample, a technical triplicate was performed for two biological replicates. Expression was normalized to *HPRT1* and *TBP*. Error bars represent the standard error of mean. * indicates significantly different from BORneg (*p<0.05. **p<0.01, ***p<0.001).

Figure 4. BORIS expression in the MM057 melanoma cell line results in reduced proliferation.

a Cell proliferation upon expression of BORIS protein with an increasing dose of dox for 3, 5 or 7 days of cell culture. **b-d** BORIS expression was induced in the MM057 cell line with 50ng/ml dox for 5 days. **b** Whole cell lysate was used for immunoblotting with anti-BORIS, anti-cleaved PARP (c-PARP) and anti-PARP antibodies. Anti-Tubulin was used as a loading control. **c** Bar

graph representing the mean percentage of Annexin V and PI positive cells as determined by flow cytometry. **d** Representative image of the percentage of apoptotic and necrotic cells as determined by flow cytometry analysis of Annexin V and PI staining. Error bars represent the standard error of mean. * indicates significantly different from **A-** untreated, **C-** the control (*p<0.05. **p<0.01, ***p<0.001). Dox: doxycycline

Figure 5. BORIS expression results in increased migration and invasion of melanoma cells with a proliferative gene signature.

a-d BORIS expression was induced with 100ng/ml doxycycline for 5 days. *In vitro* transwell cell **a, b** migration and **c, d** invasion. Bar graphs display the percentage of cells per field that **a, b** migrated and **c, d** invaded through the membrane and are accompanied by a representative image. For each assay, images of six fields were captured and counted using the Cell Counter option in ImageJ. Three independent experiments were performed, each consisting of two technical replicates. * indicates significantly different from the controls (*p<0.05. **p<0.01, ***p<0.001).

Figure 1

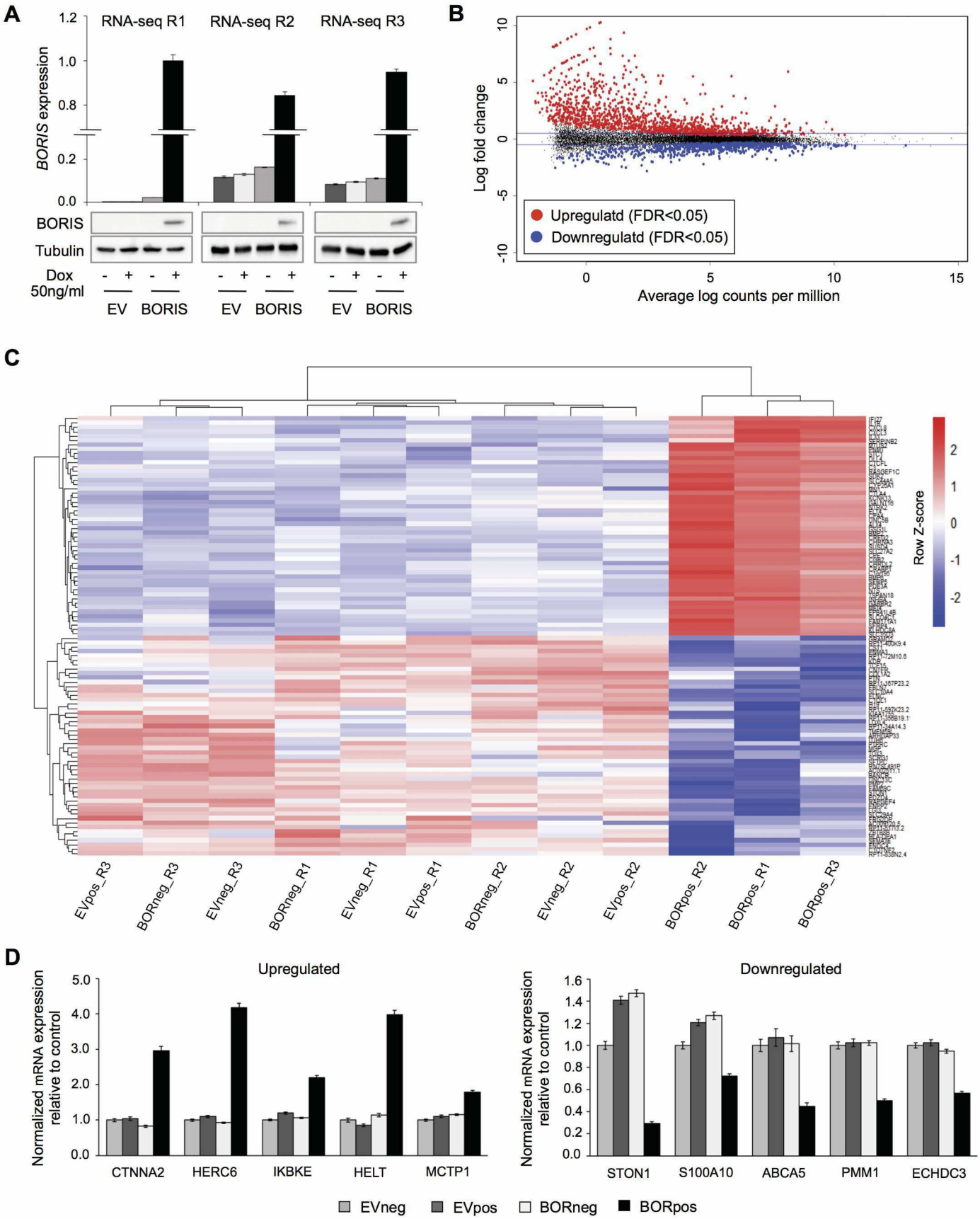

A
RNA-seq R1
RNA-seq R2
RNA-seq R3
BORIS expression
1.2
1.0
0.8
0.2
0.0
BORIS
Tubulin
Dox
50ng/ml
- + - +
EV BORIS
B
Log fold change
Average log counts per million
Upregulatd (FDR<0.05)
Downregulatd (FDR<0.05)
C
Row Z-score
EVpos_R3
BORneg_R3
EVneg_R3
BORneg_R1
EVneg_R1
EVpos_R1
BORneg_R2
EVneg_R2
EVpos_R2
BORpos_R2
BORpos_R1
BORpos_R3
D
Upregulated
Downregulated
Normalized mRNA expression relative to control
CTNNA2
HERC6
IKBKE
HELT
MCTP1
STON1
S100A10
ABCA5
PMM1
ECHDC3
EVneg
EVpos
BORneg
BORpos

Figure 2

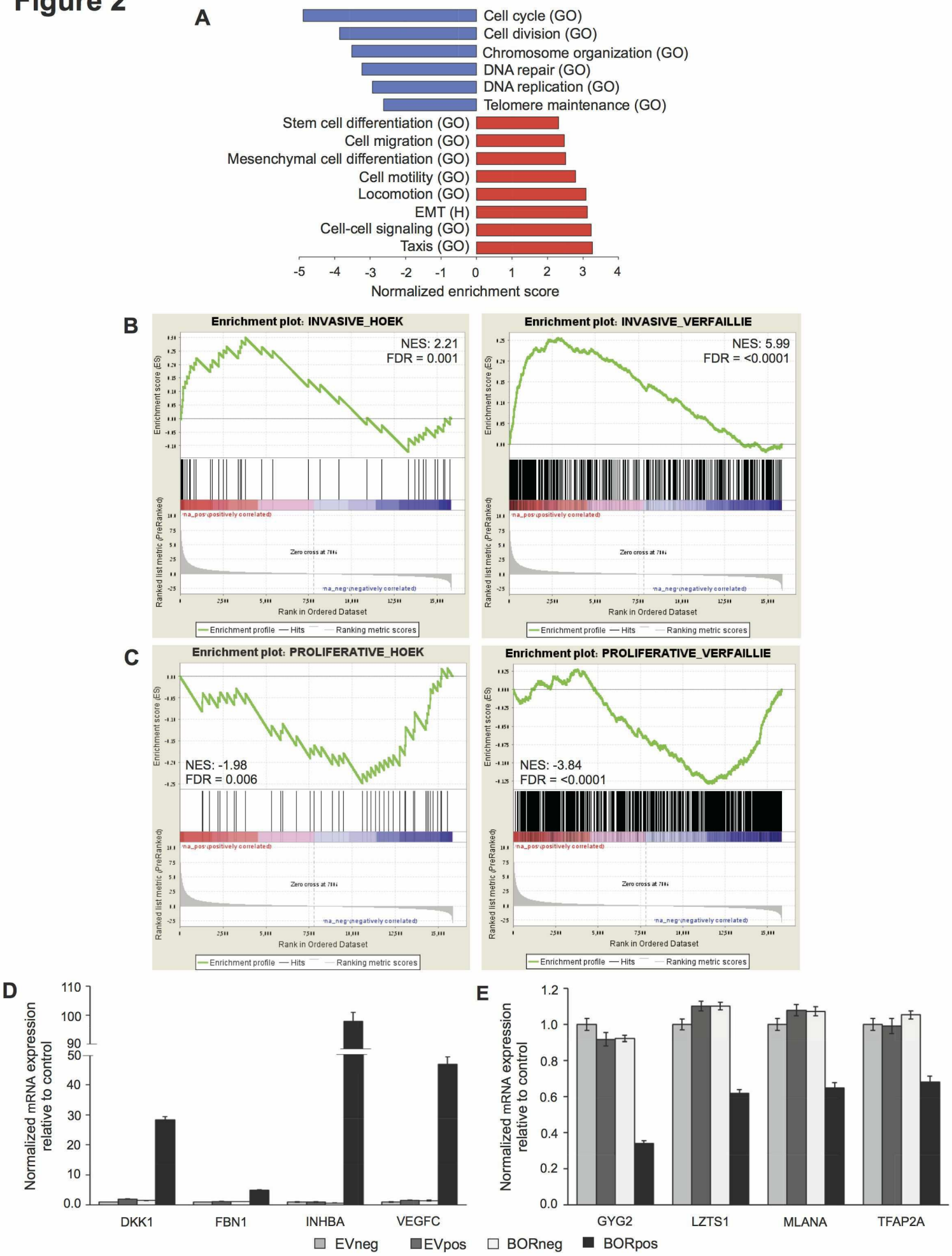

A
Cell cycle (GO)
Cell division (GO)
Chromosome organization (GO)
DNA repair (GO)
DNA replication (GO)
Telomere maintenance (GO)
Stem cell differentiation (GO)
Cell migration (GO)
Mesenchymal cell differentiation (GO)
Cell motility (GO)
Locomotion (GO)
EMT (H)
Cell-cell signaling (GO)
Taxis (GO)
-5 -4 -3 -2 -1 0 1 2 3 4
Normalized enrichment score
B
Enrichment plot: INVASIVE_HOEK
NES: 2.21
FDR = 0.001
Enrichment plot: INVASIVE_VERFAILLIE
NES: 5.99
FDR = <0.0001
C
Enrichment plot: PROLIFERATIVE_HOEK
NES: -1.98
FDR = 0.006
Enrichment plot: PROLIFERATIVE_VERFAILLIE
NES: -3.84
FDR = <0.0001
Enrichment score (ES)
Ranked list metric (PreRanked)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
D
Normalized mRNA expression relative to control
DKK1
FBN1
INHBA
VEGFC
E
GYG2
LZTS1
MLANA
TFAP2A
EVneg
EVpos
BORneg
BORpos

Figure 3

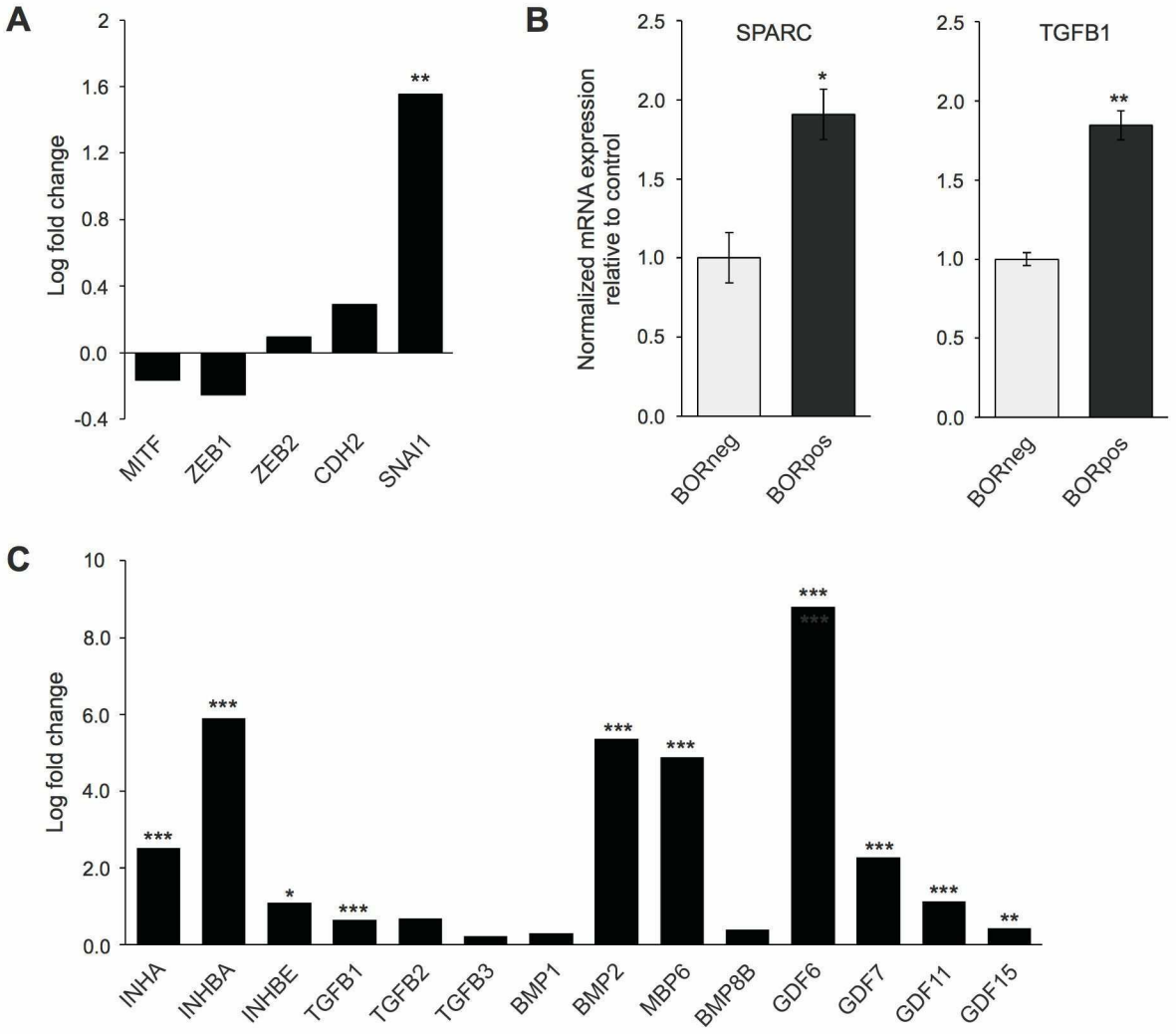

A
Log fold change
2
1.6
1.2
0.8
0.4
0.0
-0.4
**
MITF
ZEB1
ZEB2
CDH2
SNAI1
B
Normalized mRNA expression relative to control
SPARC
TGFB1
2.5
2.0
1.5
1.0
0.5
0.0
*
**
BORneg
BORpos
C
Log fold change
10
8.0
6.0
4.0
2.0
0.0
***
*
**
INHA
INHBA
INHBE
TGFB1
TGFB2
TGFB3
BMP1
BMP2
MBP6
BMP8B
GDF6
GDF7
GDF11
GDF15

Figure 4

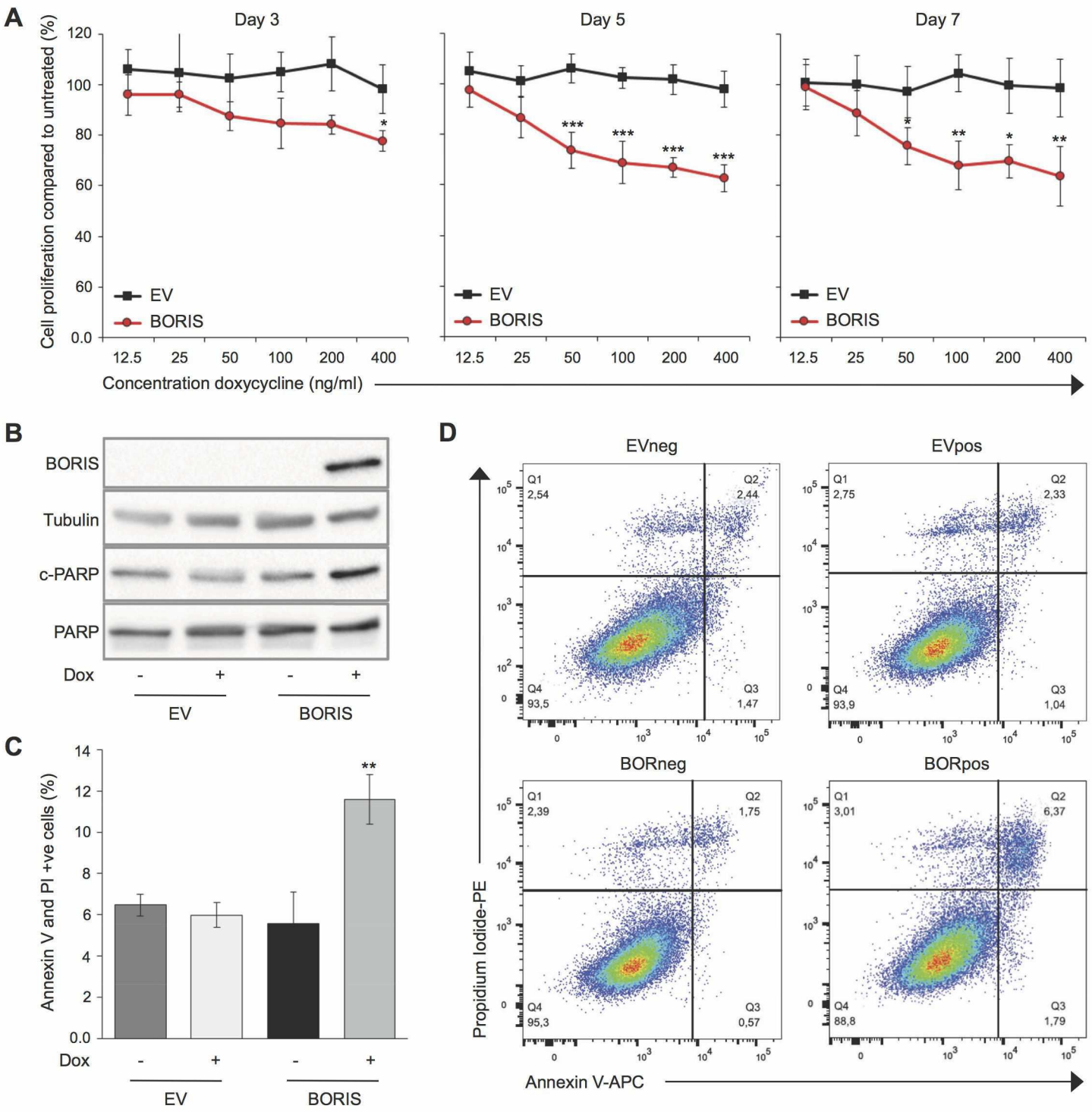
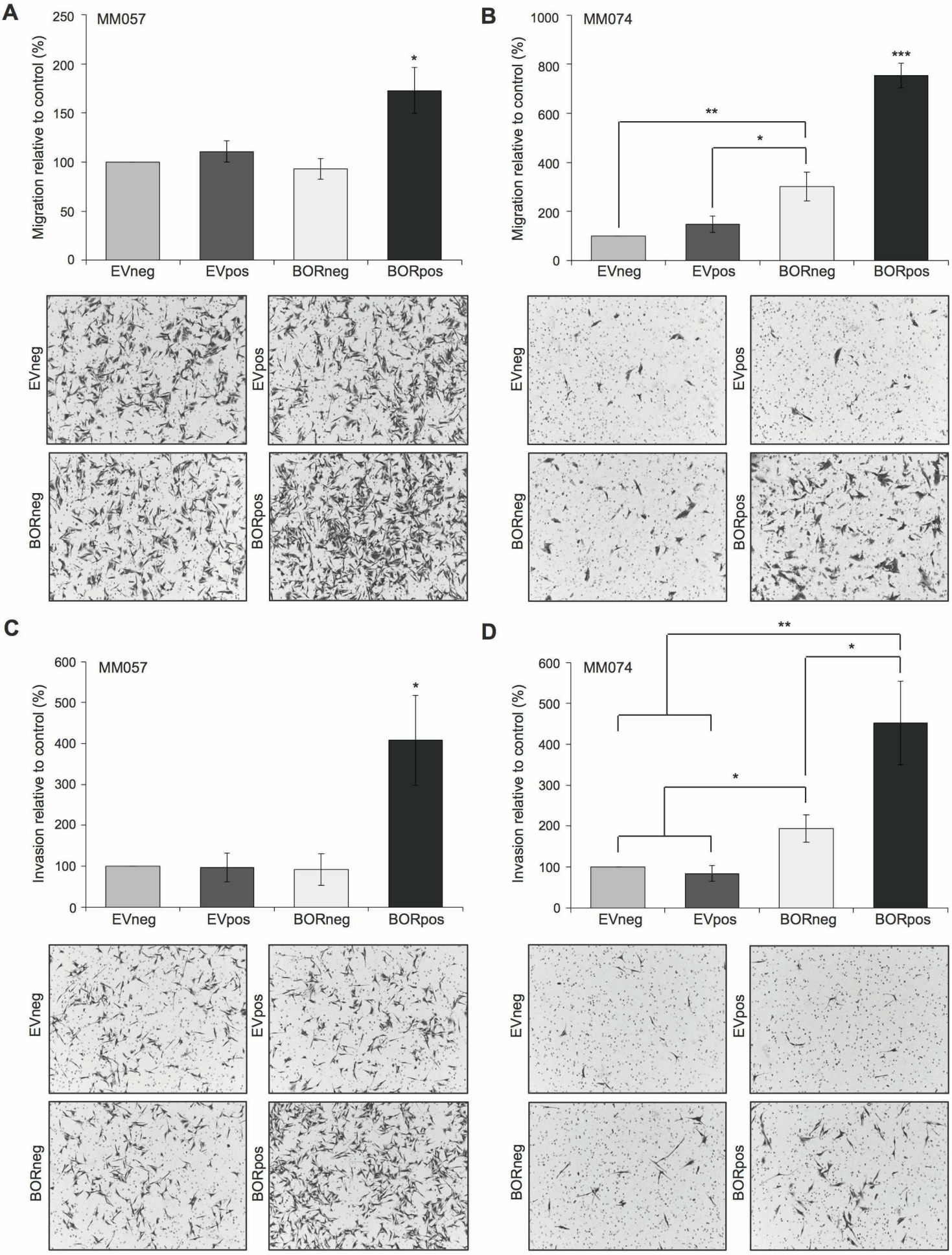

A
Day 3
Day 5
Day 7
Cell proliferation compared to untreated (%)
120
100
80
60
40
60
0.0
EV
BORIS
12.5
25
50
100
200
400
Concentration doxycycline (ng/ml)
B
BORIS
Tubulin
c-PARP
PARP
Dox
-
+
EV
BORIS
C
Annexin V and PI +ve cells (%)
14
12
10
8
6
4
2
0.0
D
EVneg
EVpos
BORneg
BORpos
Q1
2,54
Q2
2,44
Q4
93,5
Q3
1,47
Q1
2,75
Q2
2,33
Q4
93,9
Q3
1,04
Q1
2,39
Q2
1,75
Q4
95,3
Q3
0,57
Q1
3,01
Q2
6,37
Q4
88,8
Q3
1,79
Propidium Iodide-PE
Annexin V-APC

Figure 5


A
MM057
Migration relative to control (%)
250
200
150
100
50
0
*
EVneg
EVpos
BORneg
BORpos
B
MM074
Migration relative to control (%)
1000
800
600
400
200
0
**
*
***
EVneg
EVpos
BORneg
BORpos
C
MM057
Invasion relative to control (%)
600
500
400
300
200
100
0
*
EVneg
EVpos
BORneg
BORpos
D
MM074
Invasion relative to control (%)
600
500
400
300
200
100
0
**
*
*
EVneg
EVpos
BORneg
BORpos