Methylomic Signatures of High Grade Serous Ovarian Cancer

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Keywords: ovarian cancer, epigenetics, methylation, platinum, resistance

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This is the author's manuscript of the article published in final edited form as:

Cardenas, H., Fang, F., Jiang, G., Perkins, S. M., Zhang, C., Emerson, R. E., Hutchins, G., Keer, H. N., Liu, Y., Matei, D., & Nephew, K. (2021). Methylomic Signatures of High Grade Serous Ovarian Cancer. Epigenetics, 1–16. https://doi.org/10.1080/15592294.2020.1853402

Abstract

High-grade serous ovarian cancer (HGSOC) harbors aberrant epigenetic features, including DNA methylation. The overall objective of this study was to delineate pathways and networks altered by DNA methylation and associated with HGSOC initiation and progression to a platinum resistant state. By including tumors from patients who had been treated with the hypomethylating agent (HMA) guadecitabine, we also addressed the role of HMAs in treatment of HGSOC. Tumors from patients with primary (platinum-naïve) HGSOC (n=20) were compared to patients with recurrent platinum-resistant HGSOC and enrolled in a recently completed epigenetic therapy clinical trial (NCT01696032). Human ovarian surface epithelial cells (HOSE; n=5 samples) served as normal controls. Genome-wide methylation levels were determined by using the Infinium Human Methylation450 Bead Chip (Illumina). DNA methyltransferase (DNMT) expression levels were determined by immunohistochemistry and correlated with clinical outcomes. Among differentially methylated genes (DMGs) in primary OC when compared to HOSE, cancer-related and tumorigenesis networks were enriched. In the comparison between platinum-resistant and primary tumors, 452 CpG island (CGI)-containing gene promoters acquired DNA methylation; of those loci, decreased ($P<0.01$) methylation after HMA treatment was observed in 42% (n=189 CGI). DMGs representing networks related to stem cell pluripotency and cytokine metabolism were observed in recurrent platinum resistant OC tumors, while drug metabolism and transport-related networks were significantly downregulated in tumors from patient treated with HMA compared to HOSE. A functional role for key cancer genes was validated using epithelial OC cell lines. Furthermore, lower DNMT1 and 3B protein levels in pre-treatment tumors were

associated with improved progression free survival. The findings provide important insight into the DNA methylation landscape of HGSOC tumorigenesis, platinum resistance and epigenetic resensitization. We conclude that epigenetic reprogramming plays an important role in the etiology of HGSOC and contributes to clinical outcomes.

Introduction

Epithelial ovarian cancer is the most lethal gynecologic malignancy and with no adequate early detection screening, the majority of patients are diagnosed with advanced stage disease [1]. High grade serous ovarian cancer (HGSOC), the most common OC histological type, initially responds to platinum-based therapy [2]. However, up to 75% of responding patients relapse and eventually develop platinum-resistant disease. HGSOC survival rates have remained essentially unchanged for decades [2].

It has been hypothesized that aberrant epigenetic processes, such as DNA methylation and histone modifications, play an important role in HGSOC initiation and progression to a platinum-resistant state [3-5]. Those epigenetic changes contribute to broad changes across the epigenome, including transcriptional inactivation of tumor suppressor genes (TSGs) [6]. A large number of TSGs have been identified as being hypermethylated and epigenetically silenced in OC [7-10]. Development-associated genes [11, 12] have also been shown to be epigenetically silenced in advanced HGSOC. Furthermore, bench-to-clinic therapeutic interventions using tumors biopsied before and after hypomethylating agent (HMA) treatment identified demethylated TSGs whose increased expression was functionally linked to resensitization of ovarian tumors to platinum and associated with improved clinical outcomes [8, 9, 13-23]. Collectively, pre-clinical and clinical studies support the broad scale of epigenetic complexity in OC and a role for DNA methylation in platinum-resistant disease.

The overall objective of this study was to delineate pathways and networks altered by DNA methylation and associated with HGSOC initiation and progression to a platinum resistant state. The effects of a HMA on methylated genes in platinum resistant HGSOC were also measured. Toward this goal, we made multiple comparisons using bioinformatic analyses of differentially methylated genes (DMGs) generated from normal human ovarian surface epithelium cells (HOSE), primary (untreated) tumors, recurrent (platinum-resistant) tumors, and tumors from patients who had been treated with a HMA and platinum as part of a clinical trial (NCT01696032) [19]. By including tumors of HMA-treated-patients, we addressed the emerging role of HMAs in treatment of HGSOC. Pathways and networks associated with OC tumorigenesis and acquisition of platinum resistance were identified, including regulation of epithelial mesenchymal transition, cancer metastatsis signaling, and DNA damage-induced 14-3-3σ signaling. Functional validation of significant DMGs was performed. Further, expression of specific DNMT isoforms, the enzymes responsible for *de novo* and maintenance methylation in tumor samples was measured and correlated with clinical outcomes. The findings provide new insight into epigenetic reprogramming in HGSOC in general, and the DNA methylation landscape of platinum resistance and epigenetic resensitization in particular.

Materials and Methods

Study samples/Patient. For this study, four groups of samples were analyzed 1) **Group 1**: primary HGSOC (n=20) obtained from the Indiana University Simon Comprehensive Cancer Center Tissue Bank, according to institutional guidelines; 2) **Group 2:** recurrent platinum resistant baseline HGSOC tumors from patients (n= 42) enrolled in a clinical trial

(NCT01696032; study design and patient information were previously described [8]); 3) **Group 3:** HGSOC tumors from patients after treatment with the HMA guadecitabine + carboplatin (G+C) for 2 cycles (Cycle 2, Day 8, prior to the carboplatin infusion, n= 11; NCT01696032; [19]); 4) **Group 4:** human ovarian surface epithelial cells, HOSE (n=5), served as normal epithelium controls for this study. Samples were tumor fragments (Group 1) or needle biopsies (Gropus 2 and 3) and were stored frozen in liquid nitrogen until use. HOSE cells were obtained from normal ovaries of five patients at Indiana University School of Medicine (Department of Obstetrics and Gynecology) by scraping the ovarian surface epithelium and short term culture. Cell culture conditions and procedures were described previously [24]. HOSE cells were placed in short-term culture and expanded (two to four passages). The purity of the HOSE cells was confirmed by keratin and vimentin immunostaining as described [24]. Normal fallopian tube luminal epithelial cells were provided by Dr. Theresa Woodroff (Northwestern University, Chicago). All protocols and procedures for human subjects were approved by Indiana University School of Medicine.

Cell lines and culture conditions. OC cell lines OVCAR3, A2780 and SKOV3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). OVCAR5 cell line was obtained from the Developmental Therapeutics Program at the National Cancer Institute. All cell lines were authenticated by short tandem repeat (STR) analysis and tested for mycoplasma contamination in 2017 (IDEXX BioAnalytics, Columbia, MO) and mycoplasma testing was thereafter performed twice yearly. Cell culture conditions,

development of cisplatin resistant cells and extraction of DNA and RNA are described in Supplemental Methods (*SM*).

Methylome analysis. DNA extracted from patient samples and HOSE cells was bisulfite converted and DNA methylation was assayed by using the Infinium Human Methylation 450 BeadChip (HM450; Illumina) at the University of Chicago Genomics Facility. Data preprocessing and analyses were conducted in the statistical programming environment R v3.1.2 with the package RnBeads v0.99, as previously described [8, 15, 25, 26]. Normalization and background correction were applied to methylation data with manufacturer recommended algorithms implemented in *methylumi* package [27, 28]. Methylation levels were averaged for the replicates for each biopsy or HOSE after normalization and the difference in methylation β-value between two groups or the mean of the pairwise difference for paired samples was calculated. To correct *p*-values for multiple hypothesis testing, false discovery rates (FDR) were calculated by using an improved Benjamini-Hochberg procedure [29], and the methylation changes in CpG sites/regions with FDR < 0.05 were considered statistically significant. Ingenuity Pathway Analysis (IPA) was used to identify functional interactions between differentially methylated genes. Average methylation signals on the CpG sites within each CpG island, gene body, and/or promoter region were hierarchically clustered with Pearson dissimilarity and average linkage as clustering parameters. Illumina HumanMethylation450 BeadChip results are available for download at Gene Expression Omnibus (GEO) data repository at the National Center for Biotechnology Information (NCBI) under the accession number GSE102119.

Real-time RT-PCR. Expression (*mRNA*) levels of selected genes were measured in primary HGSOC tumors (n=20), normal fallopian tube epithelium (FTE; n=1), and cell lines by real-time RT-PCR (details are described in *SM*).

Immunohistochemistry (IHC). Expression of DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) was measured by IHC in archival paraffin-embedded HGSOC tumors obtained at time of diagnosis (before any therapy) from 32 patients included in Sample/Patient Group 2 (Recurrent). For IHC, tissue sections were treated with xylene to remove paraffin and then hydrated by immersion in decreasing concentrations of ethanol. Antigen retrieval was performed in 10 mM citrate buffer, pH 6.0, at 95°C for 30 minutes followed by removal of peroxidase activity using H_2O_2 for 15 minutes, blocking with 3% normal goat serum for 30 minutes at room temperature, and incubation with primary antibody at 4°C overnight. DNMT1 antibody (ab19905; Abcam,Cambridge, MA) was used at 10 µg/mL. Antibodies for DNMT3A (NB120-13888, clone 64B1446) and DNMT3B (NB100-266; Novus Biologicals, Littleton, CO) were used at 5 µg/mL and 1/100 dilution, respectively. Tissue sections were incubated with secondary antibody using the avidin-biotin peroxidase technique with DAKO Detection Kit (DAKO North America, Carpinteria, CA). Staining was developed using Liquid DAB+ Substrate Chromogen System (DAKO North America, Carpinteria, CA). Staining intensity (0-3+ scale), and percentage of stained cells were quantified by a board-certified pathologist. IHC scores were calculated as the product of staining intensity by the percentage of stained cells.

Statistical Analysis. Pearson correlation coefficients of DNMT expression levels and IHC scores were calculated using GraphPad Prism (San Diego, CA). Real-time RT-PCR data were analyzed using Student t-test on Microsoft Excel for Windows. Values of $P<0.05$ were considered significant. Associations between clinical outcomes (progression free survival, PFS; overall survival, OS) with protein (IHC scores) and mRNA expression levels of DNMT1, DNMT3A and DNMT3B, were examined using proportional hazards regression in SAS V9.4 (SAS Institute, Cary NC). IHC scores and mRNA measurements were treated as categorical values with optimal cut point defined by the maximal chi-square method. All models accounted for early (Phase I or MTD groups) vs late (treatment of choice group that crossed over to G + C after progression) exposure in NCT01696032 [16, 19]. For PFS, separate models were fitted that either included or did not include the subjects that crossed over to G + C after progression. Hazard ratios > 1 mean that if the marker is above the defined cut point then the hazard of having the event are higher. Kaplan-Meier curves were generated for key results. Because of the small sample sizes, the size of the hazard ratio, rather than the p-value, was to guide interpretation of results. Hazard ratios > 2 (or corresponding less than .5) are reported.

Results

Study population and workflow of data analysis. A total of 73 ovarian tumor samples were analyzed, consisting of 20 primary untreated HGSOC tumors, 42 recurrent platinum-resistant HGSOC tumors, and 11 guadecitabine-treated platinum resistant HGSOC tumors (Figure 1a, data analysis workflow). Tumor biopsies from patients with platinum-resistant HGSOC enrolled on a clinical trial testing the combination of guadecitabine and

carboplatin (NCT01696032) were collected on cycle 1 day 1 (C1D1; pre-guadecitabine), and guadecitabine-treated tumor biopsies were collected after two full cycles of daily X 5 doses of guadecitabine on day 8 (C2D8) (Supplementary Figure S1). Samples obtained from this clinical trial [8, 19] were subjected to the analysis work flow (Figure 1a).

Differential DNA methylation between tumors and HOSE. To begin investigating changes in DNA methylation in HGSOC tumors, we profiled methylated genes in HOSE, primary, recurrent, and guadecitabine-treated OC tumors using Infinium HumanMethylation 450 BeadChip arrays. Both fallopian tube and ovarian surface epithelia have been identified as cells of origin of HGSOC [30-33] and we used HOSE in the present study to define a normal baseline. Differential methylation between each tumor group (primary, recurrent, and HMA-treated) and HOSE was determined for all CpG sites, sites located within genes (promoter and body), promoter region, and CpG islands and shown as volcano plots in Figure 1b. Methylation changes between groups were defined on the basis of CpG sites with a $|\Delta\beta| > 0.1$ and FDR<0.05. Applying these criteria to differential methylation at promoter region (tumor vs. normal), we identified 2647, 2642 and 2234 differentially methylated genes between primary tumors vs. HOSE, recurrent tumor vs. HOSE, and guadecitabine-treated tumor vs. HOSE, respectively (Table 1, Supplementary Tables S1-S3). Pathways enriched/altered by differentially methylated genes in these three comparisons were determined by using IPA. The most significantly enriched pathways common to all comparisons were FXR/RXR activation and LXR/RXR activation (Supplementary Figures S2a, S3a, S4a). Cancer-related and tumorigenesis networks were enriched in primary OC when compared to HOSE

(Supplementary Figure S2b; red or green represents hypermethylated/inhibited or hypomethylated/activated molecules). DMGs in recurrent, platinum resistant OC tumors compared to HOSE were enriched for networks related to stem cell pluripotency and cytokine metabolism (Supplementary Figure S3b). Drug metabolism and transport-related networks were significantly downregulated among DMGs from tumors collected from patients post-HMA treatment compared to HOSE (Supplementary Figure S4b). We observed similar trends in sites unrelated to an island, i.e., differentially methylated sites generally occurring within the gene body (Fig. 1b). The entire list of networks enriched by DMGs in the OC groups compared to HOSE can be found in Supplementary Table S4.

Methylomic changes during acquired platinum resistance. To examine methylomic changes acquired during development of platinum resistance, we compared DMGs from primary vs. platinum resistant tumors to HOSE cells. We considered methylation changes specific to development of cisplatin resistance as those observed in recurrent tumors but not in primary tumors. The analysis identified 452 hypermethylated genes specifically occurring in recurrent platinum resistant cancer (Figure 2a, blue ellipse, gene list in Supplementary Table S5). Biological pathways associated with those 452 genes, determined by IPA, included gene ontology (GO) terms related to *migration and metastasis of OC cells*, *invasion of carcinoma cell lines*, *epithelial mesenchymal transition (EMT), differentiation of mesenchymal stem cells*, among others (Figure 2b). Furthermore, multiple pathways were represented by the 452 genes, including *cancer metastasis signaling*, *DNA damage-induced 14-3-3σ signaling*, and *regulation of EMT* (Figure 2c).

Hypomethylating agent-induced methylomic changes. When we intersected hypomethylated genes for all three comparisons (primary HGSOC vs. HOSE, platinum-resistant HGSOC vs. HOSE, and guadecitabine-treated HGSOC vs.HOSE), we observed that guadecitabine induced demethylation of 189 genes (Figure 3a, blue ellipse, gene list in Supplementary Table S6), which were hypermethylated in recurrent, platinum resistant ovarian tumors compared to HOSE. Networks included *EMT inhibition* and *reduced cell survival of cancer cells* (Figure 3b). Pathway analysis showed that guadecitabine altered methylation of genes associated with *cell cycle checkpoint regulation*, *vitamin-C transport* and *apoptosis signaling pathways* (Figure 3c). These data offer support that cellular processes impacted by HMA treatment contribute to platinum re-sensitatization.

Functional validation. To validate our findings, expression levels of specific genes in response to treatment with an HMA were validated in preclinical models of platinum-resistance developed by us (Supplementary Figure 5). Gene selection was based on the bioinformatics analyses performed in this study and on previously recognized positive correlations between methylated transcripts and OC prognosis/outcome in the existing literature [34]. For example, FXYD domain containing ion transport regulator 6 (*FXYD6),* pyruvate dehydrogenase complex component X (*PDHX*), and ubiquitination factor E4A (*UBE4A*) were previously identified by us to be silenced via methylation during acquired platinum resistance [31]. In addition, interferon regulatory factor 9 (*IRF9*), ecotropic viral integration site 2A (*EVI2A*) and C-C motif chemokine ligand 19 (*CCL19*) had been found to be demethylated and re-expressed in ovarian tumor biopsies from HMA-treated patients [8]. Platinum-sensitive/parental (wild-type) and -resistant EOC cell lines

OVCAR5, SKOV3 and A2780 were utilized (untreated or treated with vehicle (control) vs. guadecitabine). It is worth noting that recent genomic studies indicated that these cell lines may not have originated from HGSOC [32, 33]. Basal expression level of *FXYD56* was significantly lower in resistant OVCAR5 and resistant A2780 cells, while *UBE4A* was downregulated in resistant OVCAR5 cells relative to parental cells (Figure 4a-c). Although guadecitabine treatment upregulated *FXYD6, PDHX, UBE4A, IRF9*, *EVI2A* and CCL19 in the majority of EOC cell lines, the platinum-resistant cell lines were overall more responsive to the HMA (Figure 4d-f). Increased expression after guadecitabine treatment was observed for 5 of 6 genes in OVCAR3 cells (Figure 4g).

Expression of DNMTs in primary OC tumors. The DNMTs (1, 3A and 3B) are the enzymes responsible for maintenance and de novo DNA methylation, respectively. Given our observations related to DNA methylation changes associated with HGSOC progression obtained through methylomic analyses, we sought to determine the expression levels of DNMTs and potential associations between expression and clinical outcomes. DNMT1, DNMT3A, and DNMT3B *mRNA* expression levels were examined in primary HGSOC tumors relative to FTE (Sample/Patient Group 1). DNMT1 and DNMT3B expression levels were similar, and expression of both was higher than DNMT3A (Figure 5a). A positive correlation between DNMT1and DNMT3B was observed (Figure 5b), whereas no correlation was seen between DNMT3A and DNMT1 or DNMT3B (Figure 5c). A positive correlation between DNMT3B and DNMT1 mRNA levels was also noted in a subgroup of recurrent HGSOC tumors of Sample/Patient Group 2 (Supplementary Figure S6a-b), further indicating that an association between *de novo* (DNMT3B) and

maintenance (DNMT1) methylation [35] persisted through development of platinum resistance.

IHC was used to measure DNMT protein expression and localization in a subset of 32 HGSOC specimens obtained at time of diagnosis from patients included in Sample/Patient Group 2 (Figure 6a; representative immunostaining). IHC scores for DNMT1 were greater ($P<0.05$) than DNMT3A or 3B (Figure 6b). A positive correlation ($P=0.027$) was observed between DNMT3A and DNMT3B IHC scores (Figure 6c). Although DNMT1 and DNMT3B mRNA levels were correlated (Figure 5b), the correlation between IHC scores of these DNMTs did not reach statistical significance (P=0.07, Figure 6c), perhaps due to the limited numer of specimens.

Finally, it was of interest to examine whether DNMT protein levels in the primary ovarian cancer tumors associated with clinical outcomes (PFS, OS). Intriguingly, hazard ratio values indicated that low IHC scores for DNMT1 or DNMT3B were associated with both longer PFS (Figure 6d-e) and OS (Figure 6f-g), inferring perhaps that lower levels of methylation in primary tumors may be associated with improved outcomes.

Discussion

It has been hypothesized that aberrant DNA methylation plays an important role in HGSOC, including in processes related to tumor initiation and progression to platinum-

resistance, a common occurrence in the disease and a major cause of OC deaths. To address this important premise, we interrogated DNA methylomes from tumors collected before chemotherapy (treatment naïve), chemoresistant tumors (acquired, carboplatin) and chemo-resensitized tumors (by an HMA). By comparing epigenomic signatures from these patient cohorts to normal (HOSE) methylomes and integrating methylomic changes with transcriptomic data from these tumor cohorts, previously analyzed [34], key genes and pathways contributing to OC tumorigenesis were identified and subsequently validated in OC cell lines. Several compelling findings of this study are highlighted.

Firstly, we observe that a remarkable number of gene promoters are silenced by DNA methylation in all three OC stages (primary, recurrent or HMA-treated vs. normal ovarian epithelium; Fig. 1b), supporting the hypothesis that epigenetic reprogramming plays an important role in tumorigenesis. Network analysis highlights that both general processes of tumorigenesis as well as specific processes such as cell migration, cell invasion and EMT are altered by DNA methylation and potentially contribute to platinum resistance. Pathway analysis also showed that EMT, cancer metastasis signaling and DNA damage-induced 14-3-3σ signaling are altered during platinum resistance, in agreement with our previous work demonstrating that 14-3-3σ contributes to platinum sensitivity in OC [8]. These findings are congruent with other studies linking epigenetic abnormalities including DNA methylation with chronic DNA damage, tumor initiation and progression to platinum-resistance [36-40]. We recognize as a limitation the use of HOSE, as opposed to FTE, as the comparator in our study, as studies suggest that most epithelial OC originate in the fallopian tube[32, 33]. However, very recent studies also support the ovarian surface

epithelium as the origin of OC[30, 31]. A recent methylomic analysis noted a higher resemblance between methylomic profiles of HGSOC and FTE than between HGSOC and HOSE[33]. Nevertheless, our results contribute to the body of evidence supporting that targeting epigenetic abnormalities may represent a possible treatment strategy for OC.

HMAs have been approved by the U.S. Food and Drug Administration for treating hematological malignancies (myelodysplastic syndrome and acute myeloid leukemia) [41, 42]. By reversing cancer-specific CpG island methylation, HMAs cause broad changes in gene expression [43]. Previous bench-to-clinic therapeutic interventions by us and others targeting aberrant DNA methylation in OC support that platinum re-sensitization can be an effective strategy [7-9, 14-17, 19, 44, 45]. In support of those reports, we show here that an HMA induces significant hypomethylation in tumors compared to normal HOSE. The 189 hypomethylated genes in Fig. 3a (blue ellipse) are common to both primary tumors and tumors that had been treated by guadecitabine, suggesting that these genes were originally hypermethylated in the recurrent, platinum-resistant OC tumors. Pathway and network analyses demonstrate inhibition of EMT and reduced cell survival of cancer cells as key features of those 189 genes, along the compelling observation on the vitamin-C transport pathway (Fig. 3c). A role for vitamin C in regulating the cancer epigenome via TET and Jumonji enzymes has recently been reported [46-49]. Vitamin C increases viral mimicry induced by 5-aza-2'-deoxycytidine [50], and the findings here offer additional support for vitamin C in broadly reprogramming epigenomic medicines such as HMAs. In this regard, further assessment of both methylomic and transcriptomic

data from our recent study [19] shows upregulation of TET3 and the viral defense gene interferon regulatory factor 9 (IRF9) after guadecitabine treatment ([8]; data not shown), supportive of the novel concept that epigenetic changes induced by an HMA-vitamin C combination have the potential to improve patient outcomes [51].

To validate the bioinformatic findings, we selected multiple genes based on functional relevance and utilized epithelial OC cell lines, including platinum sensitive and acquired platinum resistant cells (Figure 4). We demonstrate that several chemoresistance-associated genes, which were also reactivated in patient tumors by HMA, are upregulated in OC model systems and/or also inducible by guadecitabine *in vitro*. *IRF9* is a viral defense gene, and *CCL19* has been show to regulate EMT via ERK signaling pathway in OC patients [52]. *EVI2A* can interact with *DOK2*, a TSG and epigenetically regulated transcript in cancers [53]. *PDHX* is a structural component of the PDH complex that plays a critical role in cell metabolism, and suppression of *PDHX* leads to cancer cell proliferation [54]. These *in vitro* findings corroborate the tumor bioinformatics analysis and provide additional support for a role of methylation-mediated gene silencing in platinum resistant OC

Finally, we investigated DNMT expression in the patient samples. In primary OC tumors, DNMT1 and DNMT3B mRNA levels were found to be positively correlated. In this regard, studies evaluating DNMT1 mRNA as a prognostic indicator in other cancer types [55, 56] support an important role for this enzyme in patient survival. We suggest that combined expression of DNMT1 and DNMT3B could serve as a predictor of response to epigenetic

therapy in OC. In recurrent OC tumors, at the protein level, DNMT1 was higher than DNMT3A or DNMT3B, perhaps indicating a central role for DNA methylation maintenance over *de novo* DNA methylation in OC tumors with acquired platinum resistance. Further, based on an integrated data analysis with PFS or OS, low DNMT1 or DNMT3B IHC scores correlated with better prognosis/clinical outcome, warranting further exploration of these DNMTs as potential predictors of response of OC patients to HMA therapy. Validation of these findings in larger cohorts is necessary.

Overall, this study provides important insight into the DNA methylation landscape of HGSOC tumorigenesis, platinum resistance and epigenetic resensitization. By analyzing methylomic changes in human ovarian tumors relative to clinical outcomes, we infer that key tumorigenicity-associated pathways can be altered through methylation gains and losses supporting future interventions targeting epigenetic events in this difficult to treat malignancy.

Acknowledgements

We thank the Genomics Facility of The University of Chicago for help with Illumina HumanMethylation450 BeadChip. We thank Dr. Theresa Woodroff (Northwestern University) for providing the normal fallopian tube luminal epithelial cells. This work was funded in part by the National Cancer Institute Award CA182832-01 and a V Foundation Translational Grant (to DM and KPN).

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Abbreviations

ARHI: DIRAS family GTPase 3; ARLTS1: ADP ribosylation factor like GTPase 11; BRCA1: BRCA1 DNA repair associated; C: Carboplatin; CCL19: C-C motif chemokine ligand 19; CGI: CpG island; DAPK: Death associated protein kinase; DLEC1: DLEC1 cilia and flagella associated protein; DNMT: DNA methyltransferase; DOK2: Docking protein2; EMT: Epithelial-mesenchymal transition; EVI2A: Ecotropic viral integration site 2A; FDR: False discovery rate; FXYD6: FXYD domain containing ion transport regulator 6; G: Guadecitabine; GEO: Gene expression omnibus; HGSOC: High grade serous ovarian cancer; HMA: Hypomethylating agent; HOSE: Human ovarian surface epithelial cells; HOXA10: Homeobox A10; HOXA11: Homeobox A11; IACUC: Institutional Animal Care and Use Committee; IHC: Immunohistochemistry; IPA: Ingenuity pathway analysis; IRF9: Interferon regulatory factor 9; MLH1: mutL homolog 1; MTD: Maximum tolerated dose; NSG mouse: NOD scid gamma mouse; OC: Ovarian cancer; OPCML: Opioid binding protein/cell adhesion molecule like; OS: Overall survival; PDHX: Pyruvate dehydrogenase complex component X; PFS: Progression free survival; RASSF1A: Ras association domain family member 1; RT-PCR: Reverse transcription polymerase chain reaction; STR: Short tandem repeat; TCEAL7: Transcription elongation factor A like 7; TSG: Tumor suppressor gene; UBE4A: ubiquitination factor E4A

Authors' contributions

HC and FF: acquisition, analysis and interpretation of the data, writing original draft. GJ: data curation, software, formal analysis. SMP: data curation, software, formal analysis. CZ: data curation, software, formal analysis. HNK: investigation, resources. YL: data curation, software, formal analysis. KNP and DM: conceived the idea, supervised research personnel and interpreted data. All authors read, critically reviewed and approved the final manuscript.

Funding

This work was supported by the National Cancer Institute Award CA182832-01 and the V- Foundation (to D.M. and K.P.N.).

Available of data and materials

Illumina HumanMethylation450 BeadChip results are available for download at Gene Expression Omnibus (GEO) data repository at the National Center for Biotechnology Information (NCBI) under the accession number GSE102119.

Ethics approval and consent to participate

All protocols and procedures for human subjects were approved by Indiana University School of Medicine.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. Differential methylation between each OC group and HOSE. (a) workflow of data analysis. (b) Volcano plots depicting differentially methylated CpG sites in different genomic regions (All CpG sites, Gene Body, Promoter, CpG island) of three OC groups compared to HOSE. Differentially methylated CpG sites are depicted as blue dots. The number of sites at different regions are indicated in each volcano plot. HOSE is human ovarian surface epithelium, which served as the normal control.

Figure 2. Methylomic changes during acquired platinum resistance. (a) Venn diagram shows unique and common hypermethylated genes in all three ovarian tumor groups, in the blue ellipse, 452 genes identified hypermethylated during acquired platinum resistance (hypermethylated in recurrent but not in primary OC). (b) Gene ontology analysis of the 452 genes hypermethylated in recurrent OC, different shapes on the out layer are genes from 452 gene list, whereas the ones in the middle are different gene ontology terms enriched by those genes from the outer layer. (c) Pathways enriched by 452 hypermethylated genes during acquired platinum resistance.

Figure 3. Hypomethylated genes induced by the HMA guadecitabine. (a) Venn diagram shows unique and common hypomethylated genes in all three ovarian tumor groups, in the blue ellipse, 189 genes identified as hypomethylated and induced by guadecitabine (genes hypomethylated in guadecitabine-treated but not in recurrent OC). Networks of

epithelial-mesenchymal transition (EMT) inhibition and reduced cell survival of cancer cells (b) and pathways (c) enriched by the189 guadecitabine-induced hypomethylated genes.

Figure 4. Effects of guadecitabine on expression of selected genes. (a-c) Basal mRNA expression levels of *FXYD6*, *PDHX* and *UEB4A* measured by real-time RT-PCR in platinum resistant (R) OVCAR5_R, A2780_R and SKOV3_R; (d-f) expression levels of *IRF9*, *EVI2A* and *CCL19* on parental and cisplatin resistant (R) A2780, SKOV3, and OVCAR5 treated with guadecitabine (100 nM) for 72 hours, and (g) OVCAR3 cells treated with the guadecitabine (100 nM) for 72 hours. Bars represent mean ± SD, n=3 (* $P< 0.05$, ** $P<0.01$).

Figure 5. Expression levels of DNMT1, DNMT3A, and DNMT3B mRNAs in OC tumors. (a) DNMT1 and DNMT3B mRNA levels are positively correlated in HGSOC tumors. Box plots show medians and 25-75% quartiles of DNMT mRNAs levels measured by real-time RT-PCR in HGSOC primary tumors (n=20, Sample/Patient Group 1) relative to fallopian tube epithelium. (b-d) Scatter plots and correlation coefficients between mRNA levels of DNMT1 and DNMT3B (b), DNMT1 and DNMT3A (c), or DNMT3A and DNMT3B (d) in HGSOC primary tumors (n=20, Sample/Patient Group 1).

Figure 6. DNMT1 and DNMT3B are associated with progression-free (PFS) and overall survival (OS) of ovarian cancer patients. (a) Examples of DNMT1, DNMT3A and DNMT3B

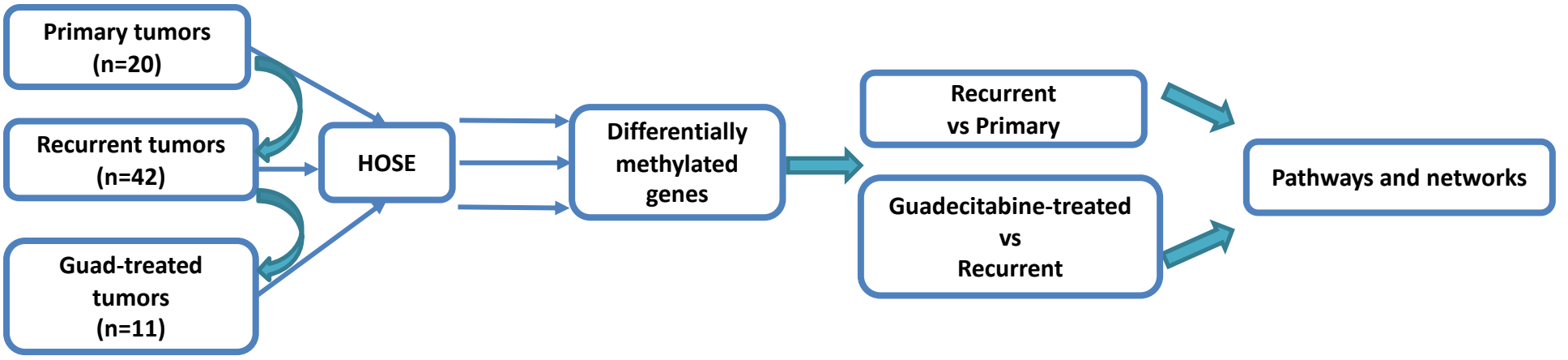
IHC immunostaining in sections from the same recurrent HGSOC tumor (original magnification: 200X). (b) Box plots show medians and 25-75 quartiles of DNMT1, DNMT3A, and DNMT3B IHC scores in recurrent HGSOC tumors (n=32, Sample/Patient Group 2) before patients received guadecitabine treatment (*P<0.05). (c) Scatter plots and correlation coefficients of IHC scores between DNMT1 and DNMT3A, DNMT1 and DNMT3B, or DNMT3A and DNMT3B in recurrent HGSOC tumors (n=32, Sample/Patient Group 2). (d-g) Kaplan-Meier plots of progression-free survival (PFS) (d-e) and overall survival (f-g) by IHC score level (low or high) of DNMT1, or DNMT3B in recurrent HGSOC tumors from patients included in C above, and subsequently treated with a combination of carboplatin and guadecitabine. HR, hazard ratio.

Table 1. Differentially methylated genes between different ovarian cancer groups and HOSE (FDR<0.05, |Δβ|>0.1).

	Numbers of Genes	
	Hypermethylated	Hypomethylated
Primary OC vs. HOSE	1810	837
Recurrent OC vs. HOSE	1541	1101
Guad-treated OC vs. HOSE	1364	870

Figure 1

a.


Primary tumors (n=20)
Recurrent tumors (n=42)
Guad-treated tumors (n=11)
HOSE
Differentially methylated genes
Recurrent vs Primary
Guadecitabine-treated vs Recurrent
Pathways and networks

b.

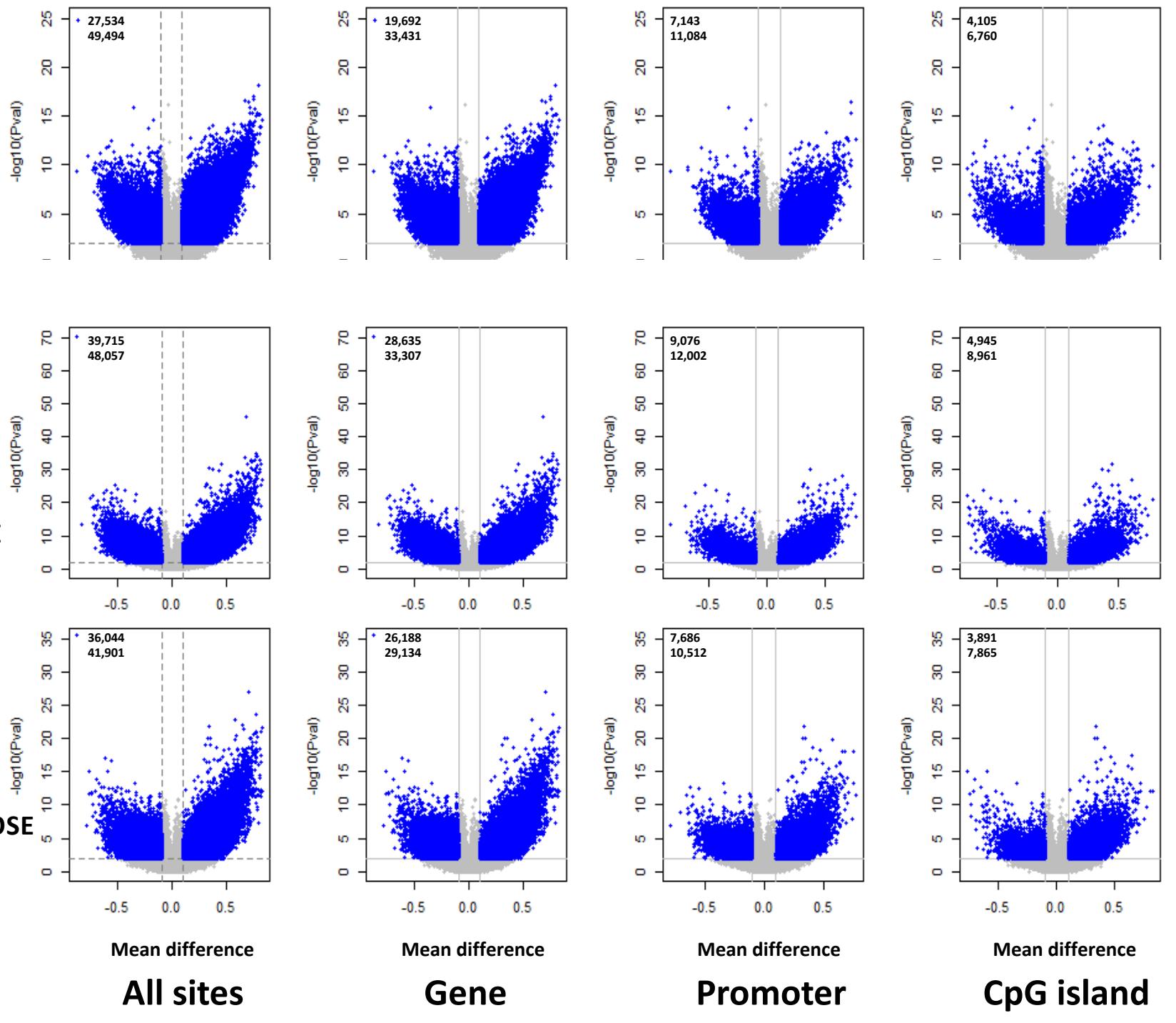
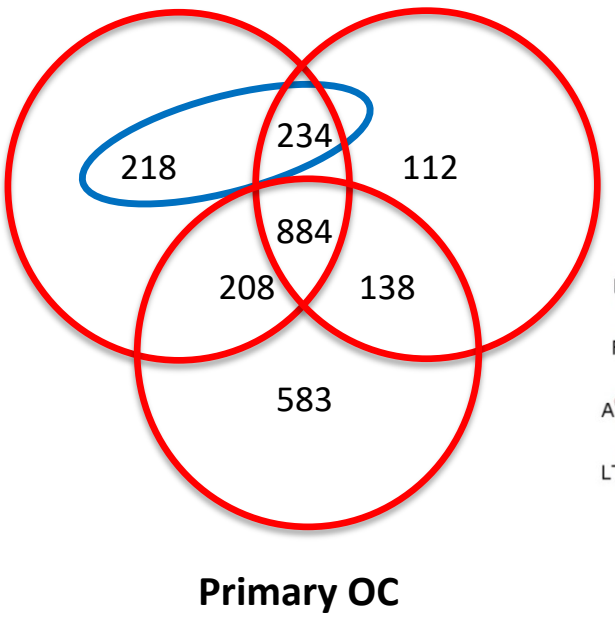

Primary vs. HOSE
Recurrent vs. HOSE
Guad-treated vs. HOSE
-log10(Pval)
Mean difference
27,534
49,494
19,692
33,431
7,143
11,084
4,105
6,760
39,715
48,057
28,635
33,307
9,076
12,002
4,945
8,961
36,044
41,901
26,188
29,134
7,686
10,512
3,891
7,865
All sites
Gene
Promoter
CpG island

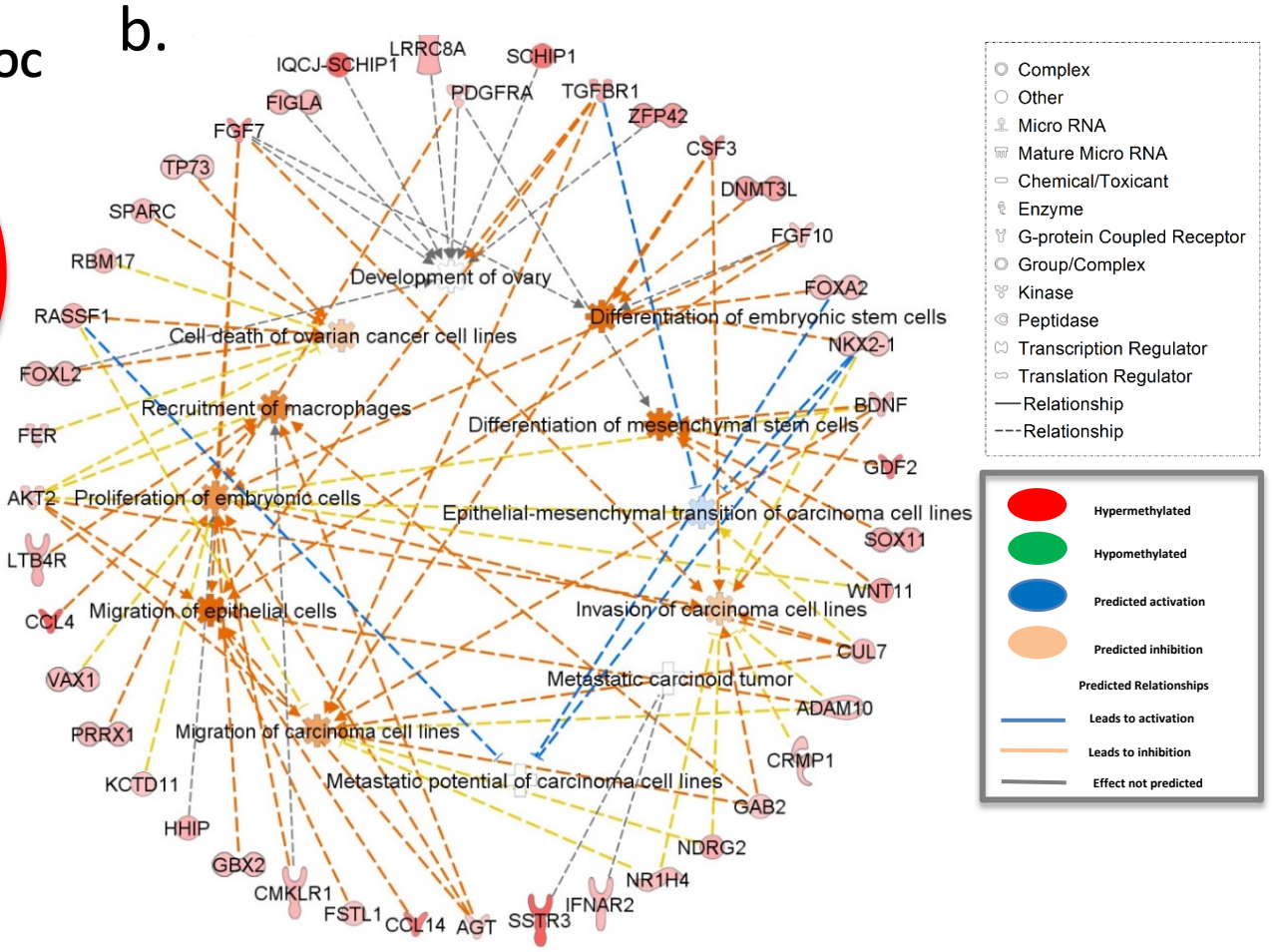
Figure 2

a.



Hypermethylated genes

b.



c.

Pathways enriched by 452 hypermethylated genes of acquired platinum resistance

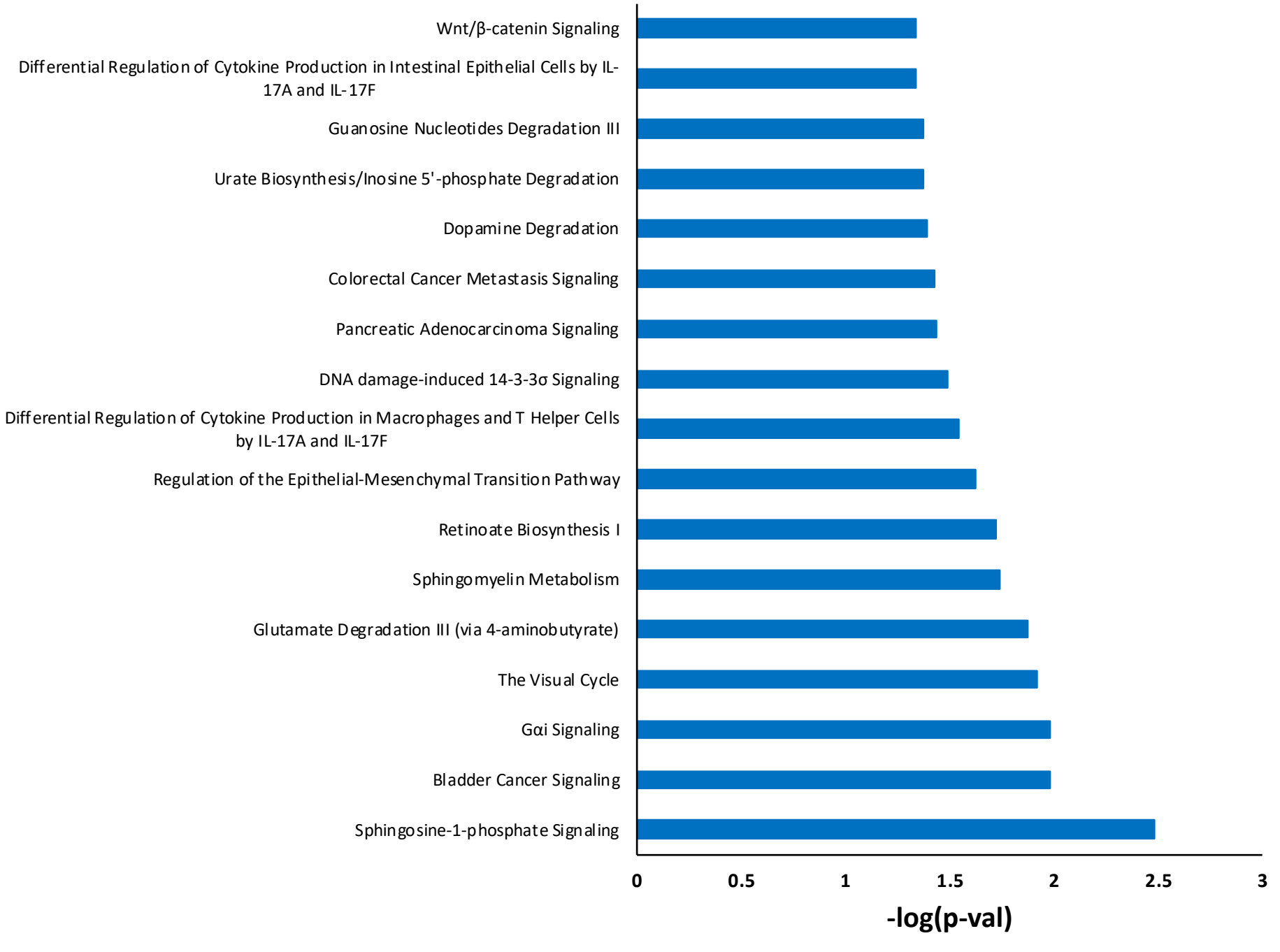
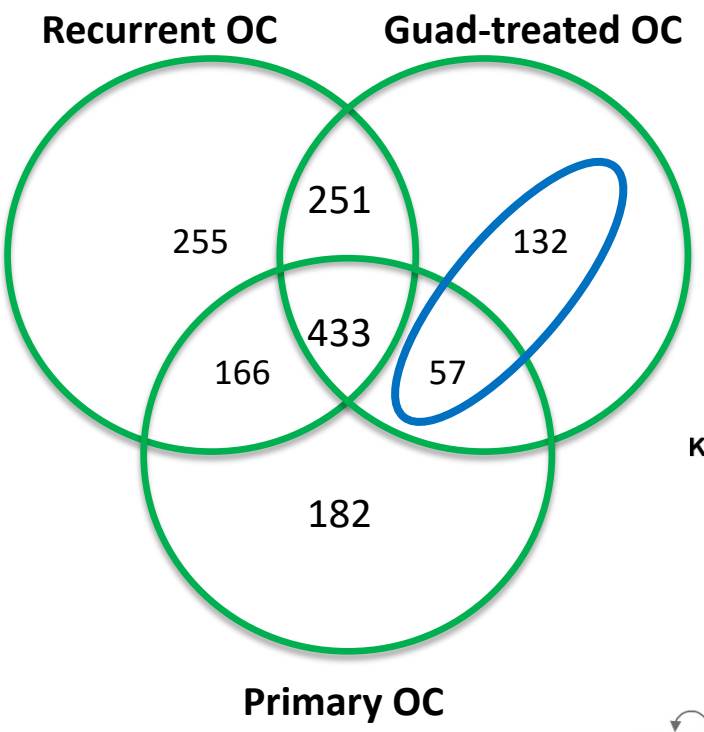


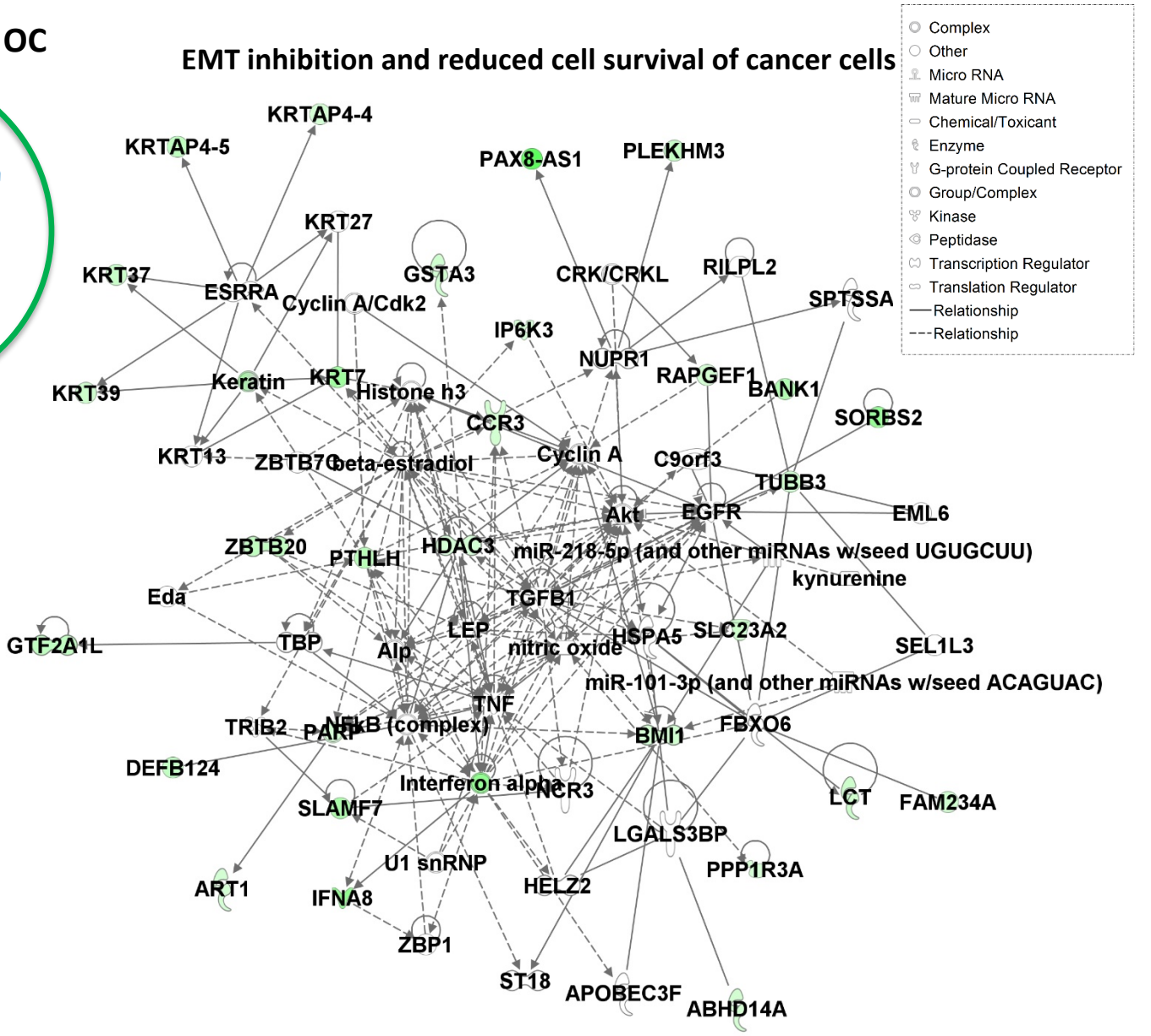
Figure 3

a.



Hypomethylated genes

b.



C.

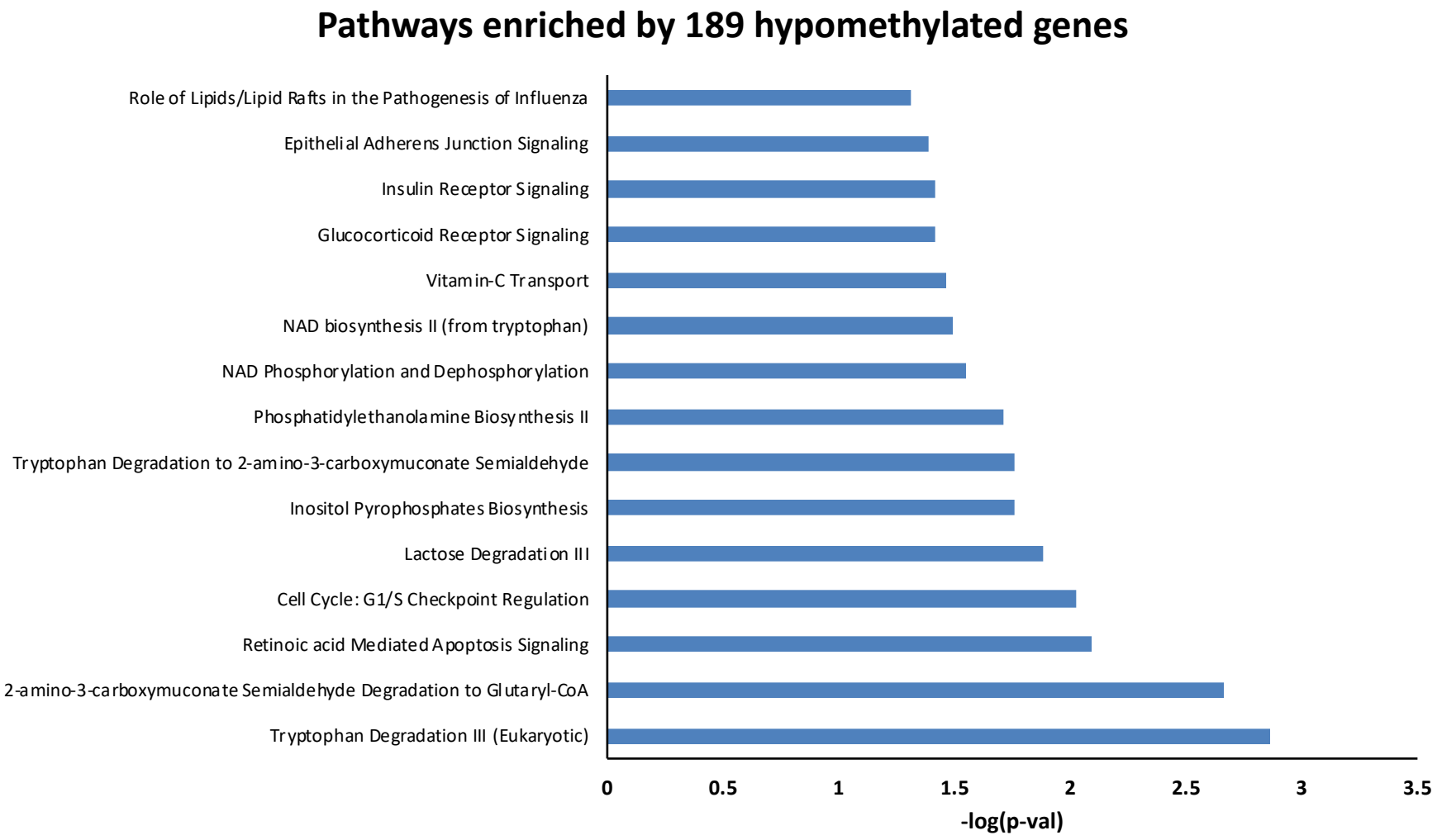
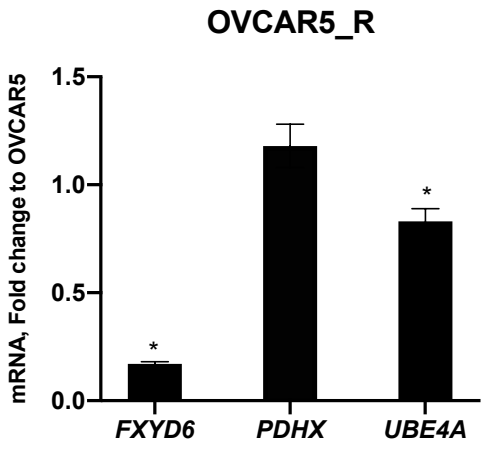


Figure 4

a.



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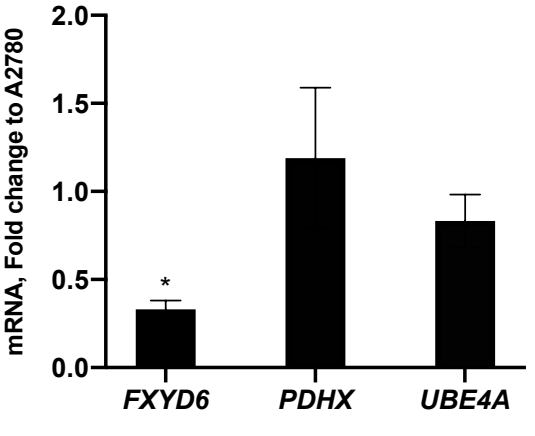
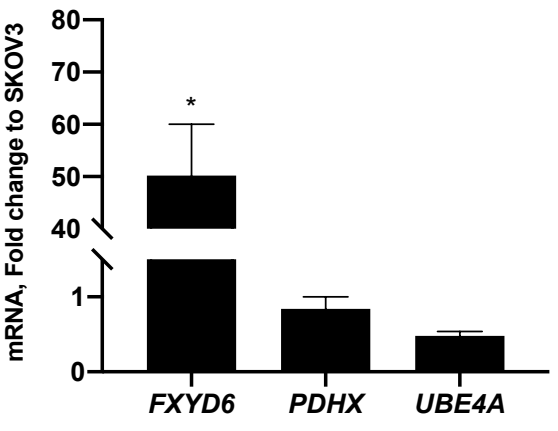


C.



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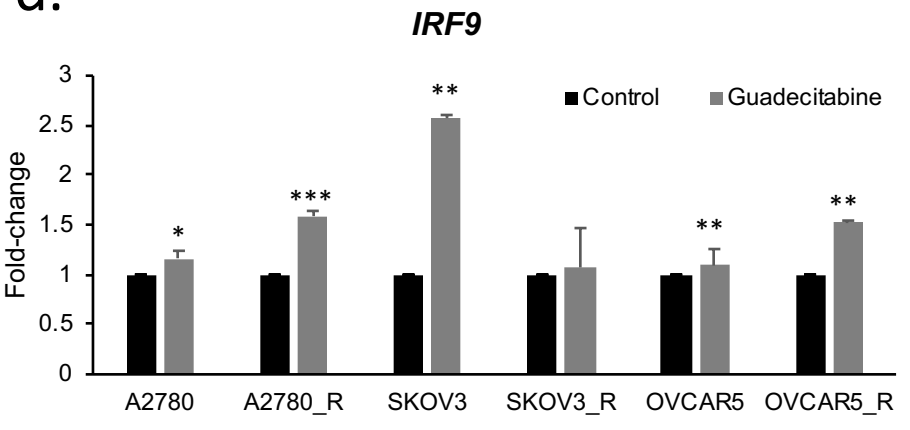

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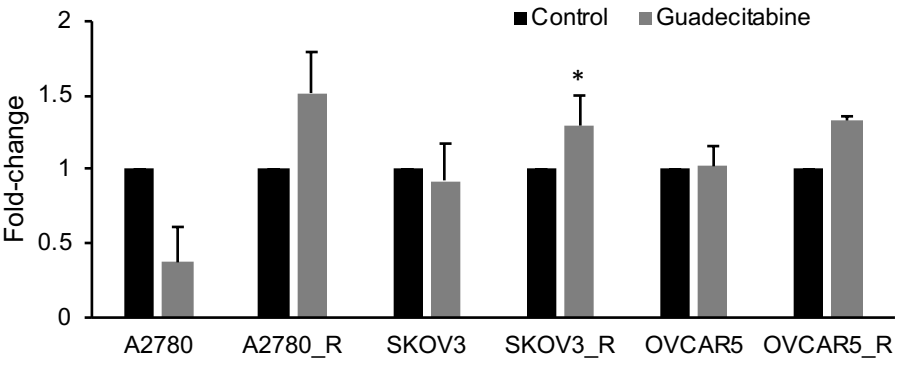

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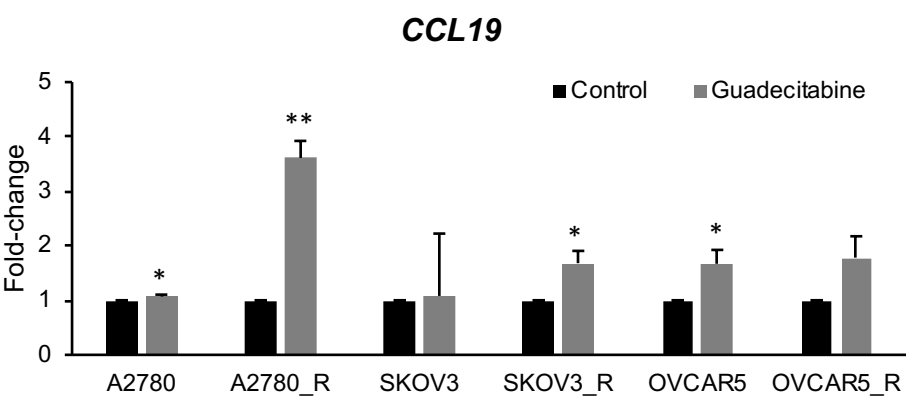


gg.

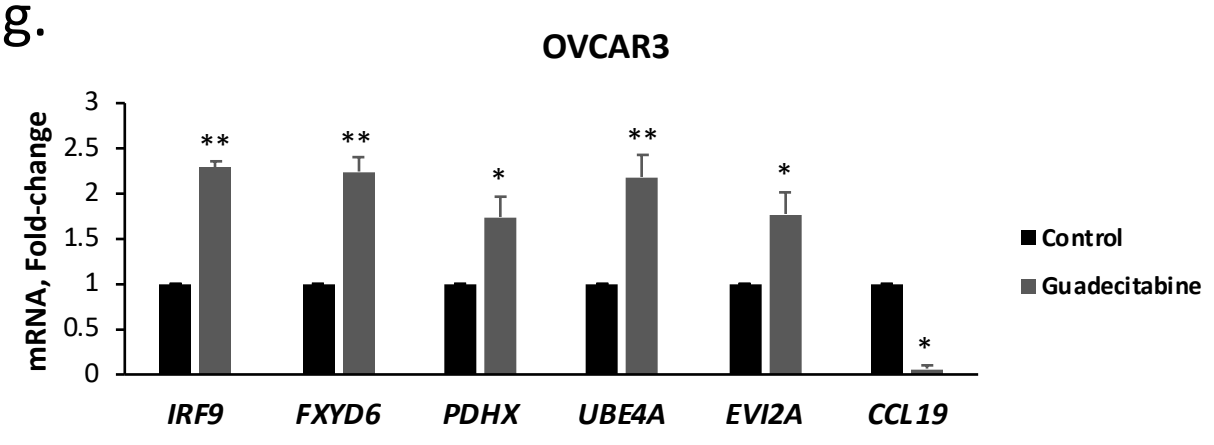


Figure 5

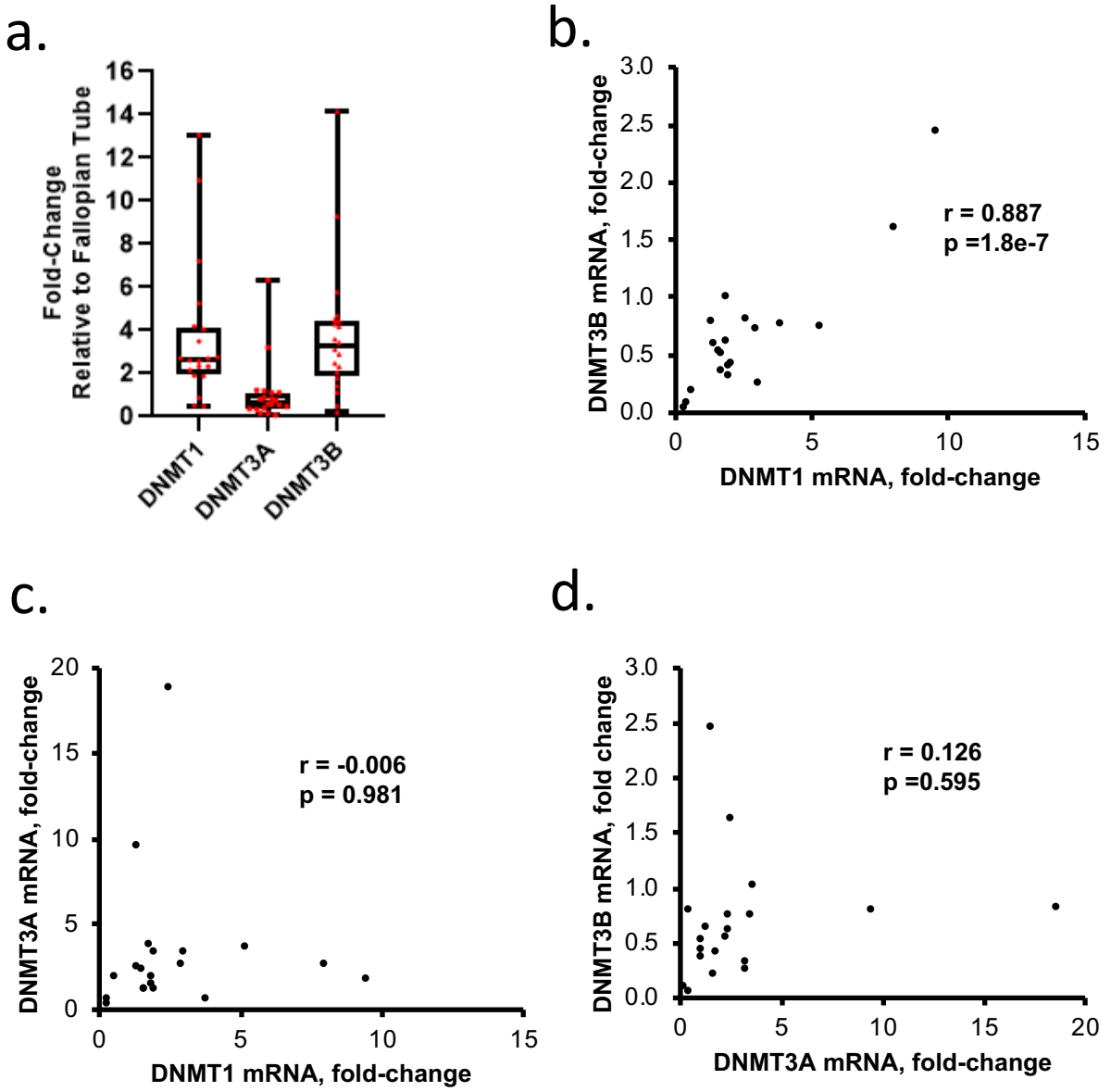
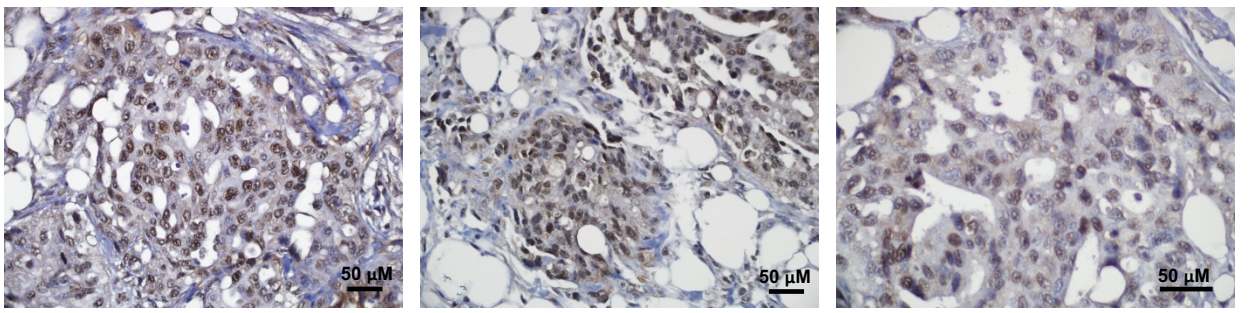

a.
Fold-Change Relative to Fallopian Tube
16
14
12
10
8
6
4
2
0
DNMT1
DNMT3A
DNMT3B
b.
DNMT3B mRNA, fold-change
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
DNMT1 mRNA, fold-change
r = 0.887
p =1.8e-7
c.
DNMT3A mRNA, fold-change
20
15
10
5
0
0
5
10
15
DNMT1 mRNA, fold-change
r = -0.006
p = 0.981
d.
DNMT3B mRNA, fold change
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
DNMT3A mRNA, fold-change
r = 0.126
p =0.595

Figure 6

a.



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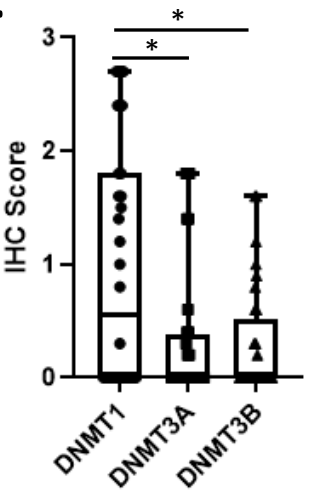

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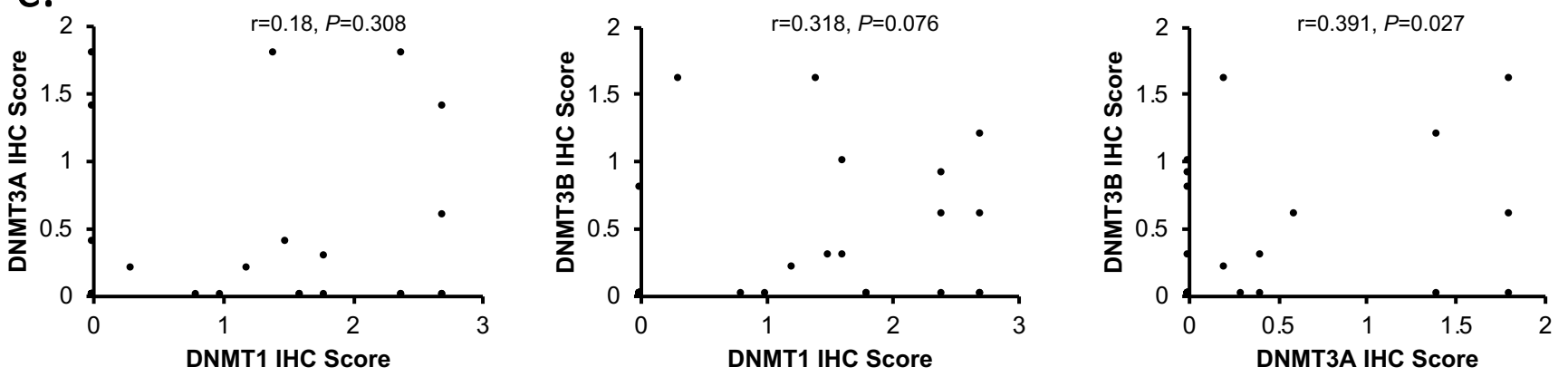

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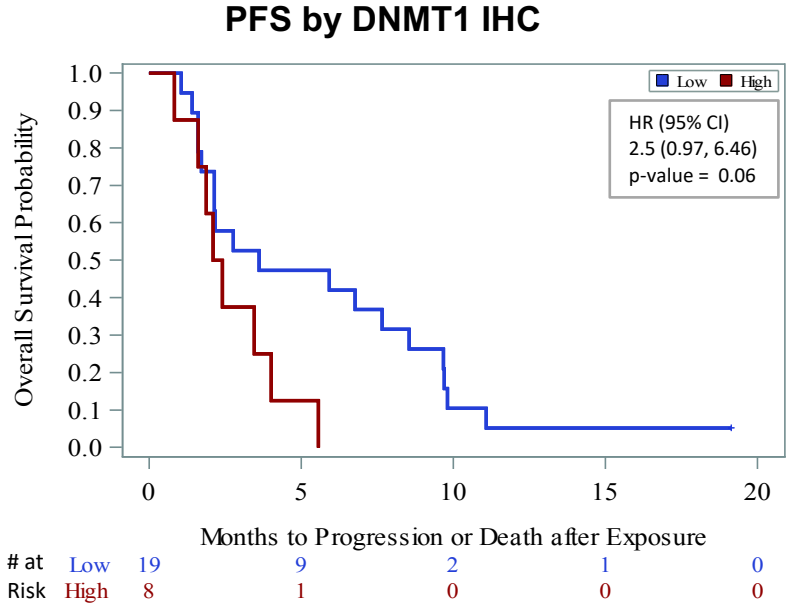

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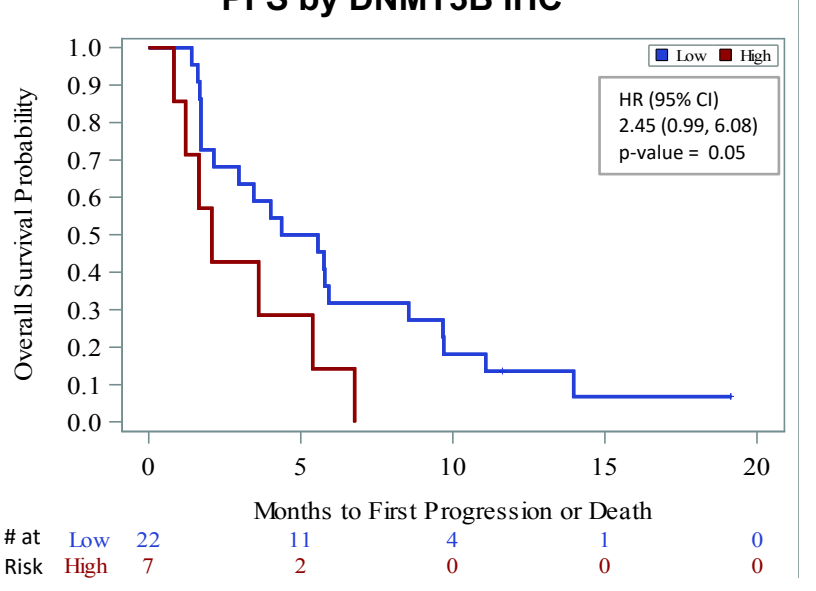

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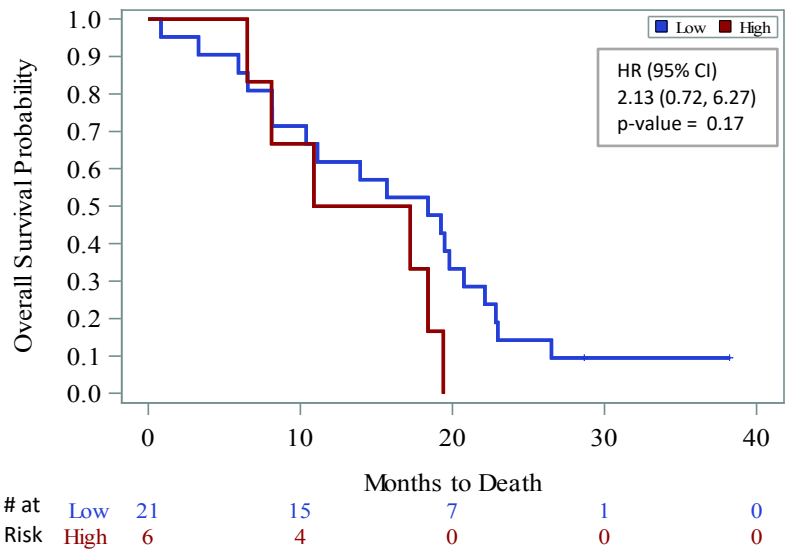

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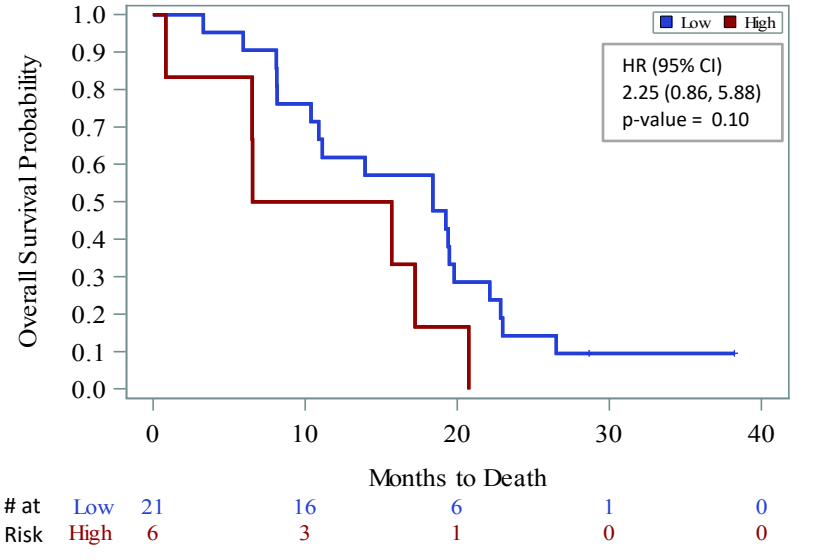
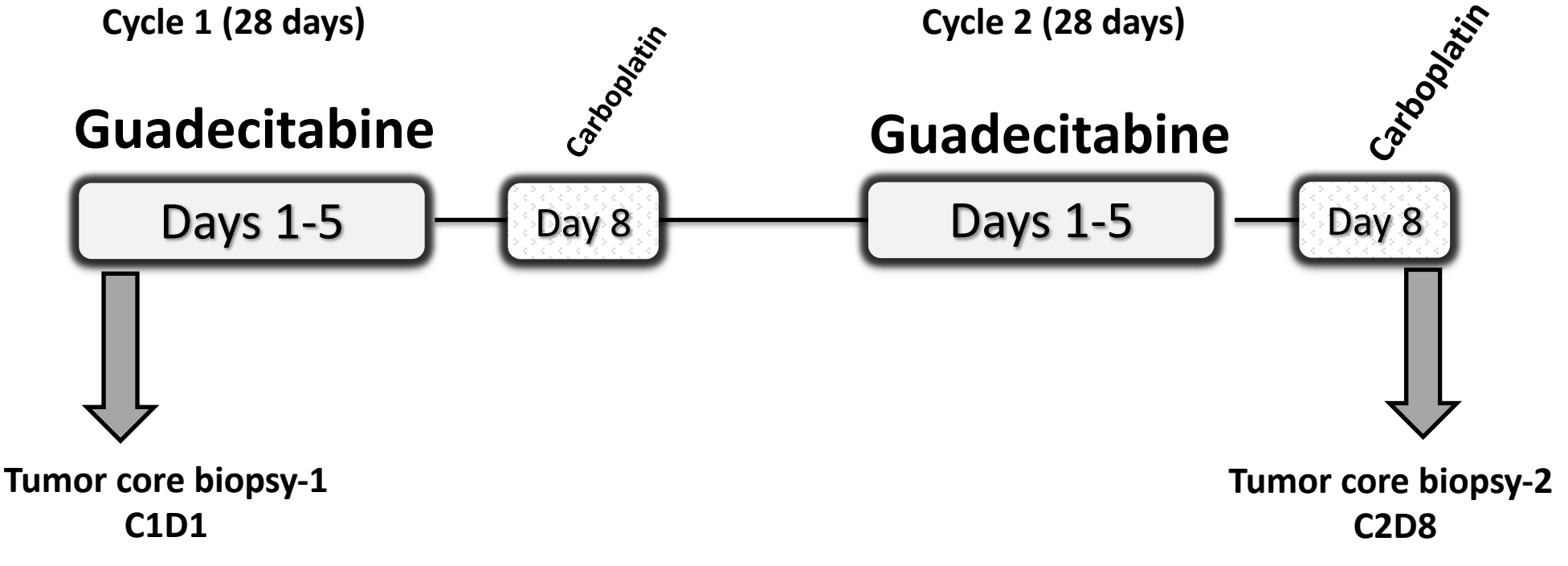


Supplemental Figure S1

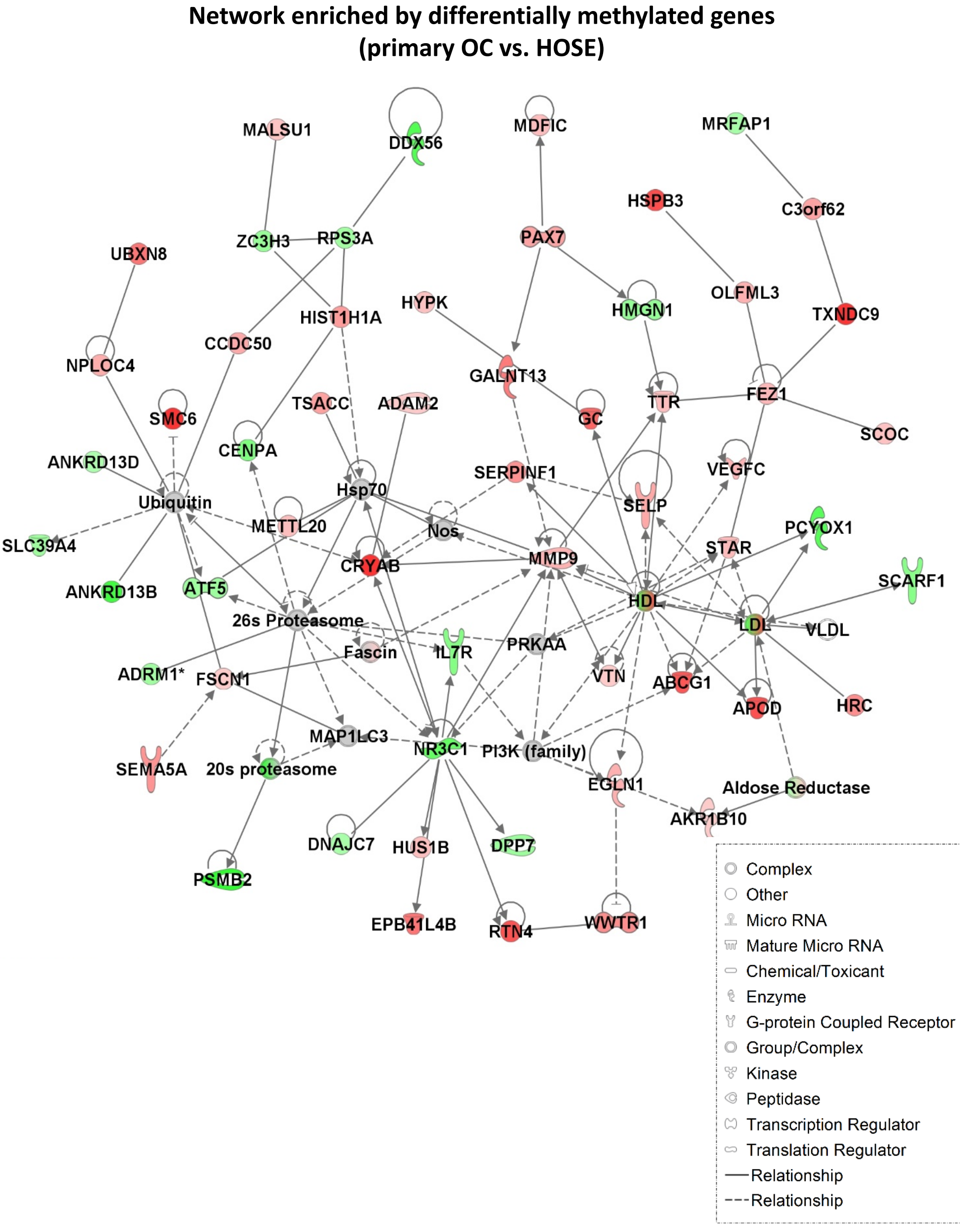


Supplemental Figure S2

a.

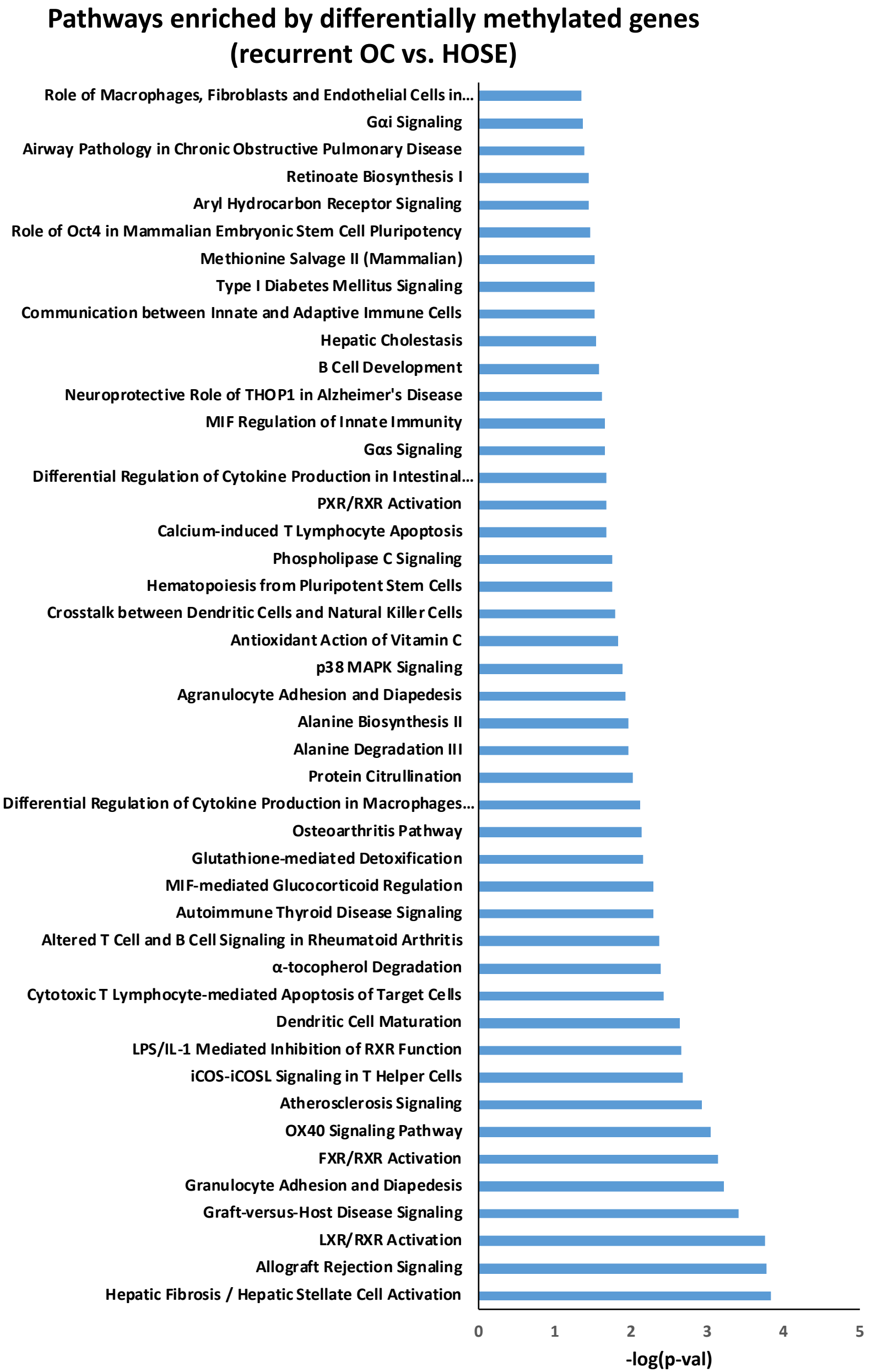
Supplemental Figure S2

b.



Supplemental Figure S3

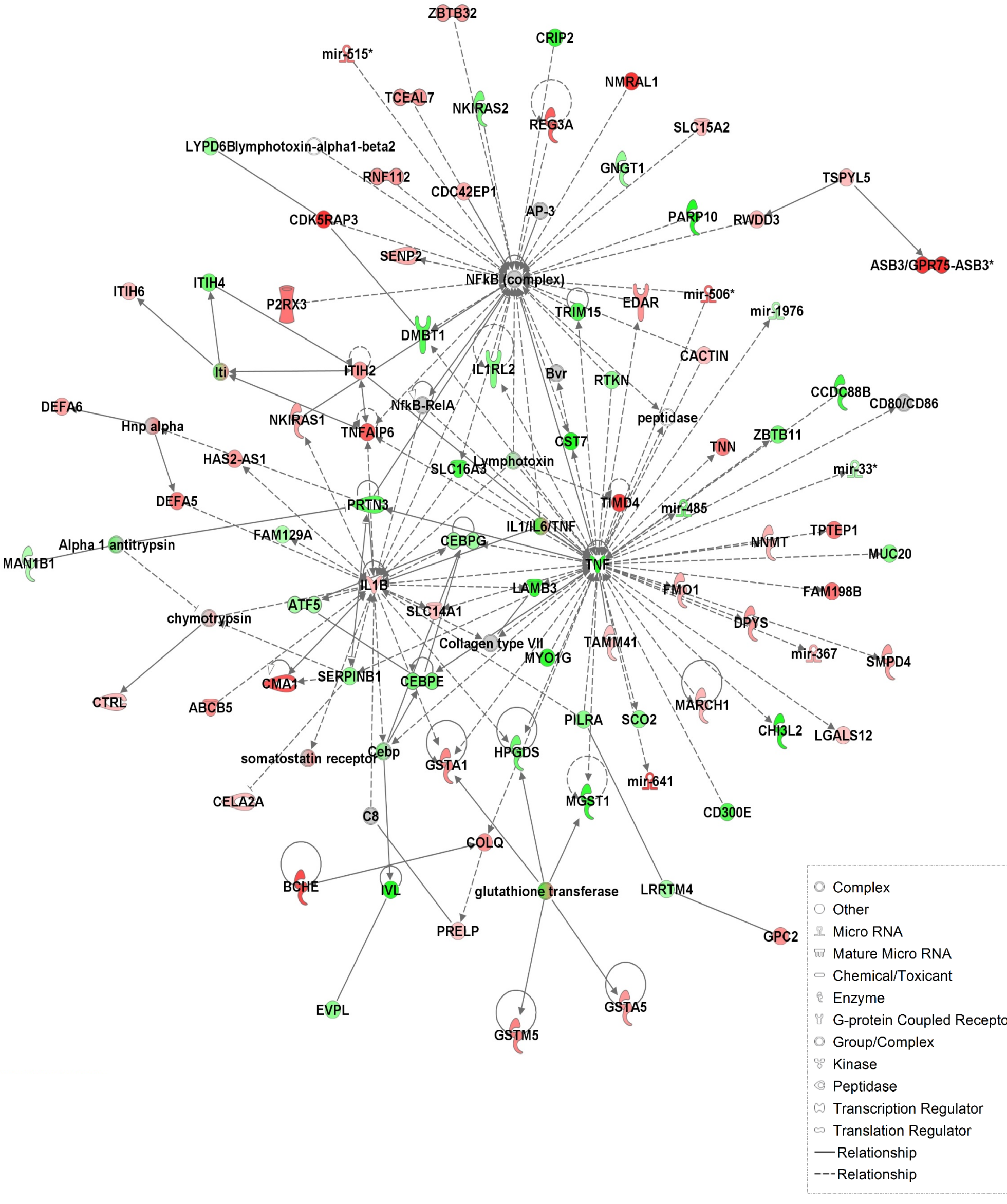
a.



Supplemental Figure S3

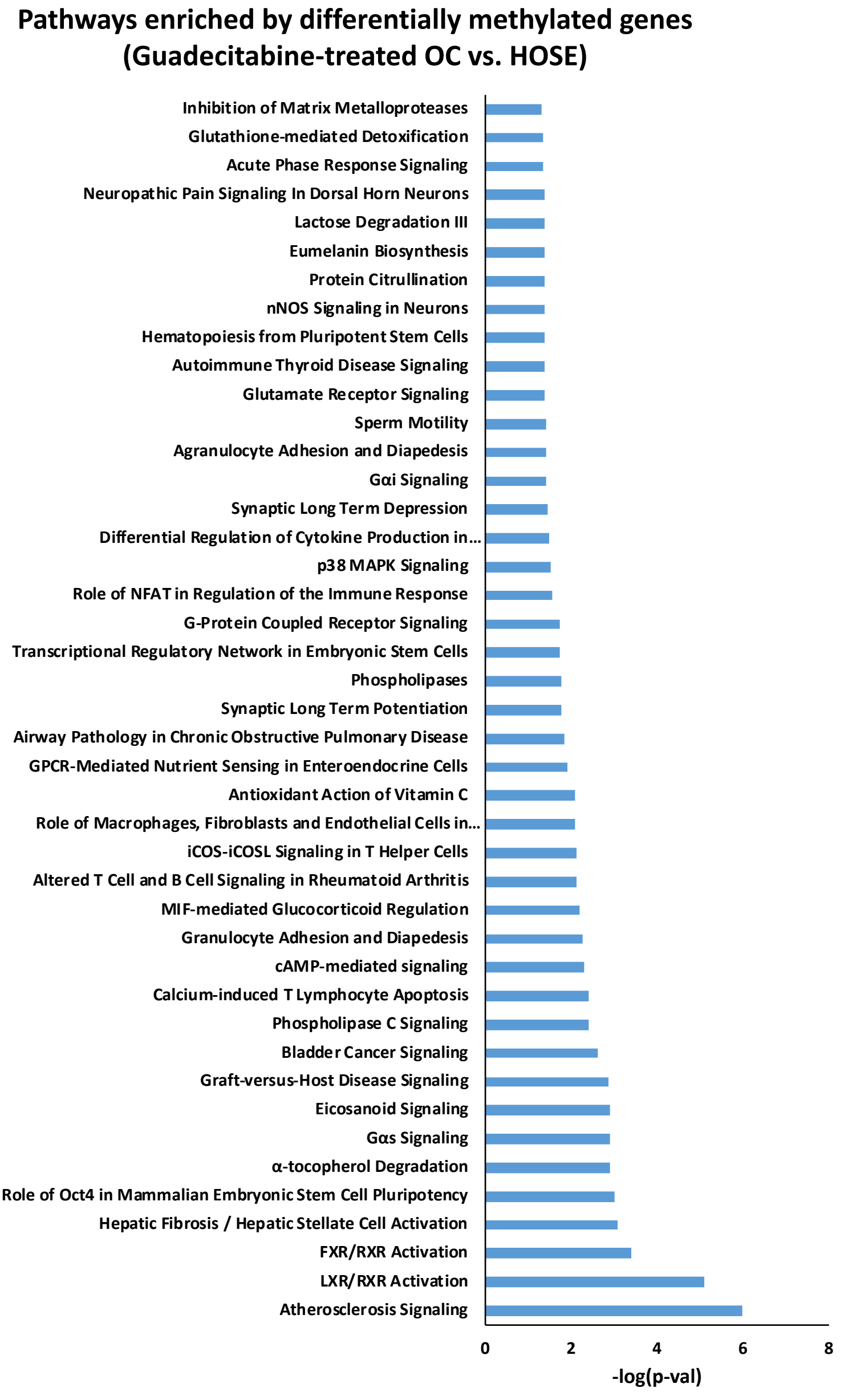
b.

Network enriched by differentially methylated genes (recurrent OC vs. HOSE)



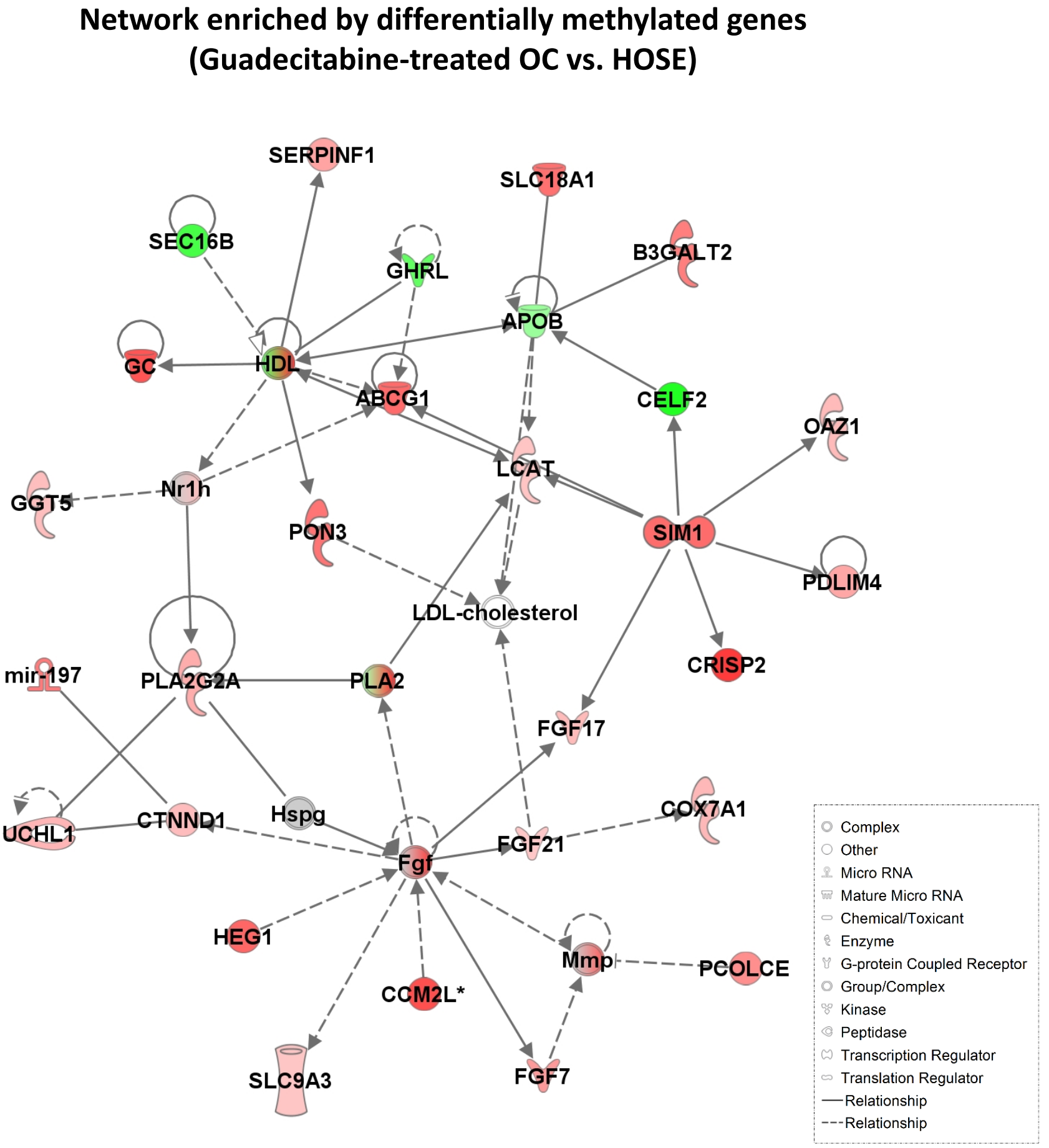
Supplemental Figure S4

a.

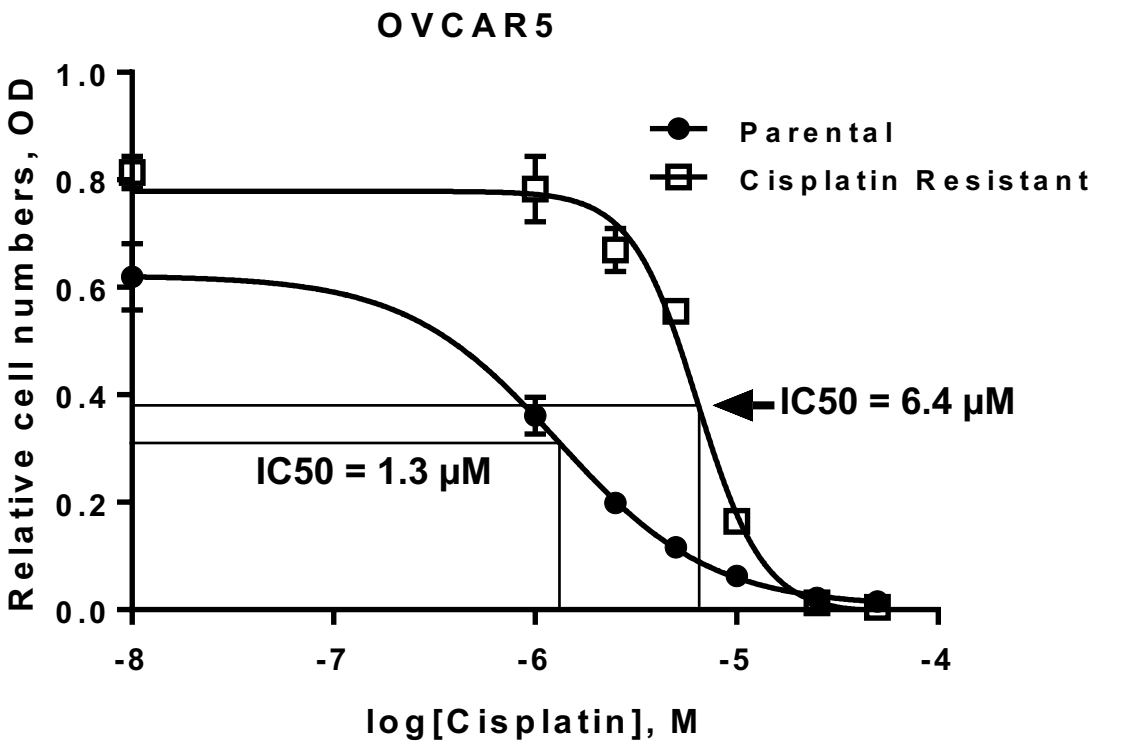


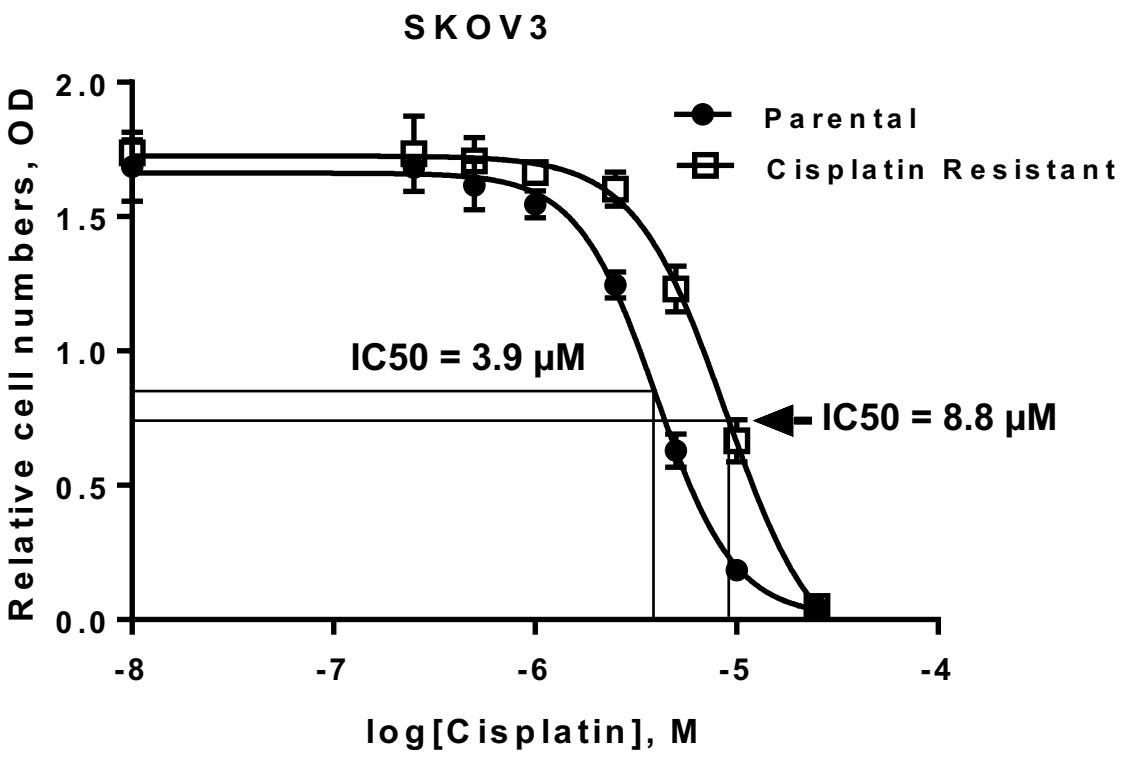
Supplemental Figure S4

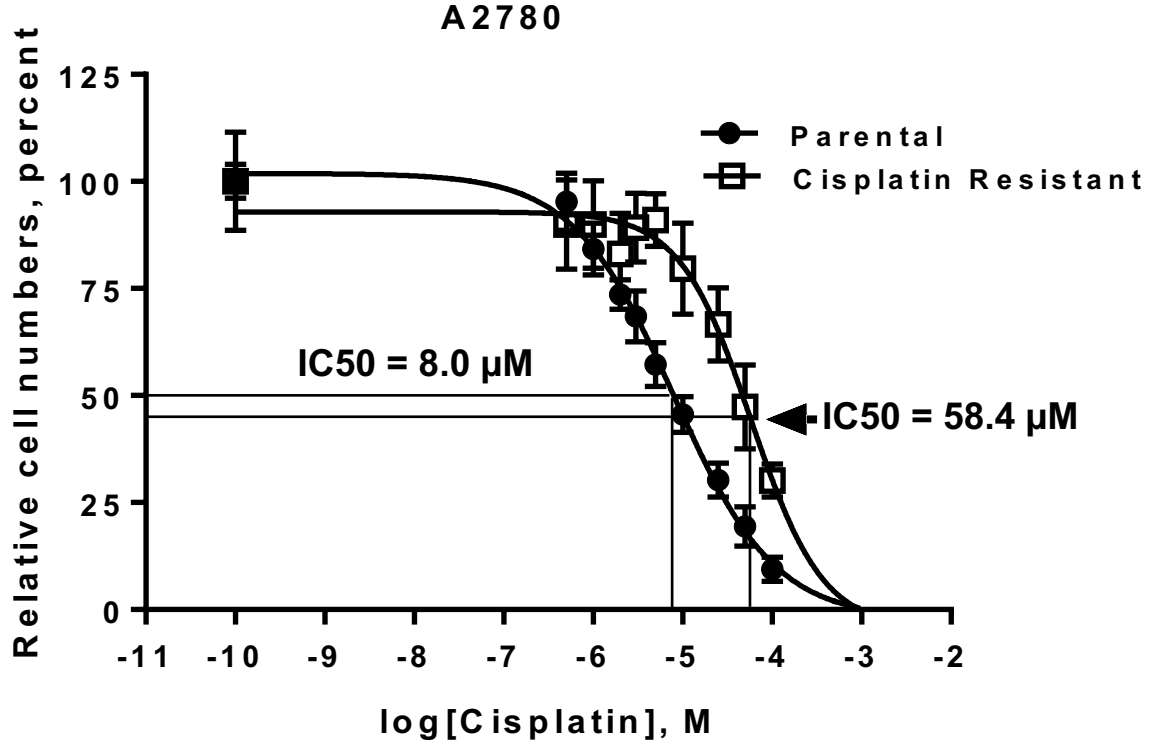
b.



Supplemental Figure S5

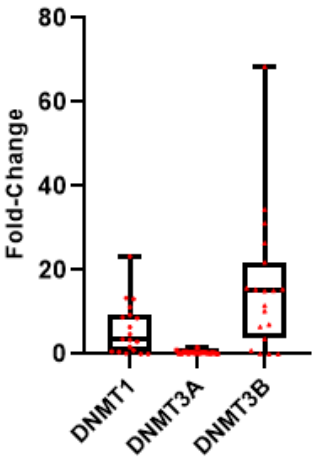




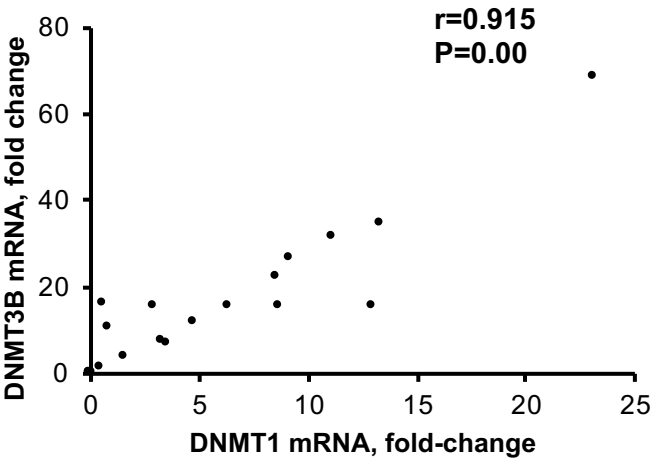


Supplemental Figure S6

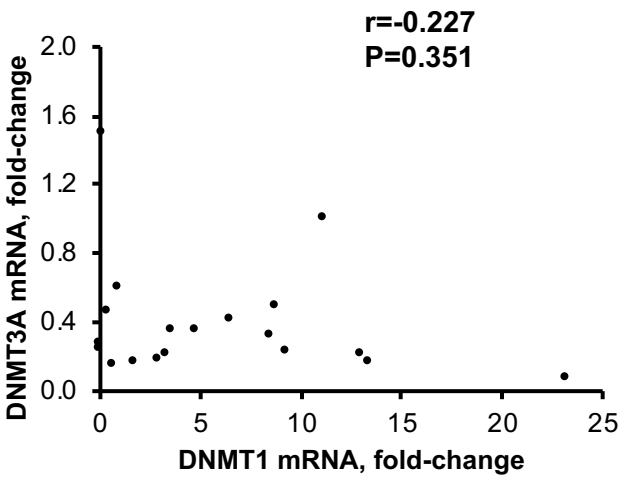
a.



b.



c.



d.