DAC-driven Integrative Network Regulation and Pathway Coordination in Breast Cancer

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Abstract. Epigenetic variation represents a mechanism of regulation for genes expressed in different cancer histotypes. We considered breast cancer, and investigated differential expression following treatment with the 5-Aza-2'-deoxycytidine or DAC, a demethylating agent. Several oncogenic signalling pathways altered upon DAC treatment were detected with significant enrichment, and a regulatory map integrating Transcription Factors and microRNAs was derived. The ultimate goal is deciphering the potential molecular mechanisms induced by DAC therapy in MCF7 cells.

Keywords: Breast Cancer; Demethylation; Pathways; Regulatory Networks.

1 Introduction

Recent advances in the field of epigenetics have provided new insights of global epigenetic modifications that promote cancer development and progression [1- 8]. Notably, epigenetic therapy, which is a consequence of the reversible nature of the epigenetic changes that alter gene expression in many tumor histotypes [9, 10], is the ultimate interest of our proposed research. Among several drugs with anti-tumorigenic effect regulating the epigenetic status of cells, the 5-Aza-2'-deoxycytidine (DAC, Dagogen) is considered here. DAC is a potent demethylating agent known for anti-leukemic effect in the mouse model [11- 14]. Also, DAC acts to correct epigenetic defect including reactivation of tumor suppressor genes (TSG) [15] silenced by epigenetic mechanisms in tumor tissues [2, 8]. Various phase II trials have demonstrated substantial DAC activity in patients with relapsed or untreated Acute Myeloid Leukemia (AML) [16, 17]. Combining DAC with a chemotherapeutic agent (Carboplatin)

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in patients with recurrent, platinum-resistant, Epithelial Ovarian Cancer (EOC), was shown to exert a potent demethylating effect justifying the need of further testing for clinical efficacy [18]. The DAC effect was evaluated in breast cancer (BC) cell lines by gene expression analysis: two DAC concentrations (5 μM and 10 μM) were used, which revealed a role for treatment in different cellular processes linked to *TNF-α*-dependent apoptosis [19]. In colon cancer (CC) cell lines, activation of a set of hypermetilated genes and transcriptional down-regulation were observed [20, 21].

In our work, the goal is to investigate the effect of DAC treatment in MCF-7 BC cell lines. We look into two main directions: pathway signatures and regulatory networks. We thus performed gene expression profiling to identify epigenetically modified genes, before studying functionally enriched pathways. We built both transcriptional and post-transcriptional regulatory networks with the goal of driving future developments towards novel anticancer target therapies. The structure of the paper is as follows: Methods are presented in Section 2, Results are reported in Section 3, and Discussion is proposed in Section 4.

2 Methods

2.1 Microarray Analysis.

The MCF-7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, at split ratio of 1:4 twice a week. After 24 hours of spit the culture medium was changed with media containing 2,5 μM 5-Aza-2-dC (DAC). The treated cells were collected after 48 hours and the mRNA was extracted for pelleted cells. Steps for cDNA microarray analysis were: 1. Total RNA samples were isolated from treated/untreated cells using TRIZOL reagent (Invitrogen); 2. Concentration of purified RNA samples were determined by A260 measurement and the quality was checked by Lab-on-a-chip analysis (total RNA nano biosizing assay, Agilent) with the Agilent 2100 Bioanalyzer.

RNAs isolated from different tumor tissues, and transcribed in cDNAs, were used to carry out the analysis. The cDNAs from treated BC were labeled with cy5 red fluorescent dye and untreated BC with cy3 green fluorescent dye. Hybridization was done on a microarray chip called MWG Human Cancer Array containing 50-mer oligo probes for 1920 genes (1853 human genes associated with cancer, 27 control genes and 40 replicated genes). Spots of fluorescence intensity were read by dual laser scanner (BioDiscovery) and the values were processed with Mavi Pro-2.6.0. (MWG Biotech), by computing background subtraction, normalization to a number of housekeeping genes, and comparison with untreated cancers. In order to select deregulated genes, we considered the cy5/cy3 normalized ratio (NR), calculated for each gene and by taking the ratio of the intensity in cy5 (Ic5) and the normalized intensities in cy3 (nIc3). Then, to reduce variability, all ratio values were transformed in log base 2. For inclusion of highly deregulated genes, we considered as up-regulated or down-regulated genes those with $log2\ (NR) > 2.0$ and $log2\ (NR) < -2.0$, respectively.

2.2 Functional Enrichment and Pathways Analysis

The analysis of the list of significantly deregulated genes is based on the F-Census database [22] (http://bioinfo.hrbmu.edu.cn/fcensus/Home.jsp), to extract information from highly inconsistent cancer gene data sources including CGC (Cancer Gene Census) [23], OMIM (Online Mendelian Inheritance in Man) [24], AGCOH(Atlas of Genetics and Cytogenetics in Oncology and Haematology) [25], CancerGenes [26], TSGDB (Tumor Suppressor Gene Database) [27], TGDBs (Tumor Gene Family Databases) [28]. Then, two gene lists have been considered: H-list [29- 32] and R-list [33- 34] identified by two types of high-throughput techniques, and post-transcriptional regulation predicted by some microRNA (miRNA) target prediction algorithms including *TargetScan* [35], *PicTar* [36], *DIANA-microT* [37] and *MirTarget2* [38].

For bioinformatics and functional annotation, the tools that were considered were *FatiGO,* available in *Babelomics* [39, 40] (http://babelomics.bioinfo.cipf.es), *DAVID Bioinformatics* [41, 42] (http://david.abcc.ncifcrf.gov/gene2gene.jsp), and also over-representation analysis (*ORA*) available in *ConsensusPathDB* [43] (http://cpdb.mol-gen.mpg.de/). The tool combination aimed at identifying the functional categories and biological pathways among the genes that were differentially expressed (DE) between treated and untreated BC. *FatiGO*, Functional annotation analysis and ORA analysis are tools for easy and interactive querying that perform a functional enrichment (FE) analysis by comparing two lists of genes (one corresponds to the list of deregulated genes, and the other to the list of human genome). The output links the genes to the corresponding annotations, found in the databases (like GO terms, KEGG, Biocarta, Reactome etc.). *Babelomics* and *David Bioinformatics* contain an old version of data sources, while *ConsensusPathDB* contains the latest version of several dbs also included in the previously listed tools.

2.3 Transcriptional and Post-transcriptional Regulatory Network Analysis

The detection of Transcription Factors (TFs) predicted to regulate the list of significantly deregulated genes upon DAC treatment in Breast cancer, was performed using the web resource *TFactS* [44] (http://www.tfacts.org/). *TFactS* db contains genes responsive to TFs, according to experimental evidence reported in literature. It reports two datasets: (i) A sign sensitive catalogue that indicates the type (up or down) of TF regulation exerted on its targets; (ii) A sign less catalogue that includes all regulatory interactions contained in sign sensitive and further interactions without the specific type of regulation. *TFactS* takes as a query the two lists of up/down regulated genes and compares them with sign-sensitive catalog of manually curated annotated target genes, then returning the lists of activated and inhibited TFs whose annotated target genes show a significant overlap with the query genes.

We merged the last versions of experimentally validated miRNA-target gene databases: *miRTarBase V.2.4* [45] (http://mirtarbase.mbc.nctu.edu.tw/), *miRecords V.3* [46] (http://mirecords.biolead.org/), and *miR2Disease* [47] (http://www.mir2disease.org/), to build a non-redundant dataset of miRNA-target genes regulatory interactions in human. We used this non-redundant dataset to predict the miRNA regulators among the list of significantly deregulated genes. We used Cytoscape [48]

(http://www.cytoscape.org/), to show the transcriptional and post-transcriptional regulatory network. For network analysis, we used a tool called *"AdvancedNetworkMerged"* merging networks by set operations (union, intersection and difference).

3 Results

3.1 Pathway signatures after DAC treatment

Overall we have identified 336 DE genes upon DAC treatment. In particular, after treatment of MCF-7 cell line, we performed gene expression profiling to identify the epigenetically modified genes. We found 222 up-regulated genes with a $log_2 (NR) > 2$ and integrated them with the *F-census* (Table 1S). Then we found 114 down regulated genes with a $log_2 (NR) < -2$ (Table 2S). The following examples from the list of top up-regulated genes (Table 1S) are emphasized: -**CTAG1B** *(cancer/testis antigen 1B)* is a member of cancer/testis (CT) antigens found expressed in normal testis and in many cancers [49, 50]. Thanks to the capacity of a subset of these antigens to activate a spontaneous cellular immune responses in cancer patients [51], this makes them ideal cancer antigen targets for tumor immunotherapy, especially for adult T-cell leukemia/lymphoma (ATLL) [52]; -**RAB30** is a member of Ras-associated binding proteins (Rabs), that are involved in regulating different steps during exocytosis [53]. In particular Rab30 is Golgi-specific Rabs, required for the structural integrity of the Golgi apparatus [54]; -**MAGEA1** is also one of CT antigens, in particular found expressed in 10% of tumour cells in conjunctival melanomas [55], and is a potential candidate in combination with immunotherapy.

We then found the following top down-regulated genes: -**CDKN2B**, a cyclin-dependent kinase inhibitor 2B (known also as p15, inhibits CDK4) and a key regulator of biological processes repressing cell cycle progression by inhibition of cdk4 and cdk6 [56]. The down-regulation may represent a side effect of treatment and the balance between DNA methylation and demethylation is a critical regulator of the methylation status of cyclin-dependent kinase inhibitor [57]. In fact it is known that the demethylating agents function as DNA methyltransferase inhibitors and can be incorporated into the genome during DNA replication, and bind DNA methyltransferases that have the catalytic domain. This may lead to global hypomethylation and re-expression of both tumor suppressor genes and misregulation of proto-oncogenes [58]; - **CDH6** (*cadherin 6*) is a membrane glycoprotein and a member of the cadherin superfamily type II involved in cell-cell adhesion, differentiation and morphogenesis. The aberrant expression of cadherin-6 correlates with a poor prognosis in patients with E-cadherin-absent Renal cell carcinomas (RCC) and could be a useful tool to estimate the malignancy potential of the tumor [59]; - **CDH7** (*cadherin 7*) is another member of the cadherin superfamily type II. It was shown that CDH7 play a role in tumor development of malignant melanoma cells by interacting with melanoma inhibitory activity protein (MIA) and migration melanoma cell [60].

Using different functional enrichment analysis tools, we found a list of the over-represented functional categories and pathways referring to up- and down-regulated gene. The results using *FatiGO* are reported in Table 3S and Table 4S. The results using functional enrichment analysis by *David Bioinformatics* are reported in Table 5S

and Table 6S. The enriched pathways resulted from *ORA* analysis available in *consensusPathDB* are reported in Table 7S and Table 8S. We then used the graphical tool available in *consensusPathDB* to select the most interesting pathways involved in up- and down-regulated genes (Figure 1 and 2). We next report the results obtained using ORA. Figure 1 shows the most interesting pathways regulated upon DAC treatment. A summary is reported in Table 1 together with the list of up-regulated genes in each specific pathway. Our result show that the top enriched pathways associated to up-regulated genes are the ATM signalling (Ataxia telangiectasia mutated) and *the MDM2* (Mouse double minute 2), an upstream components in ATM and DNA damage response mechanism.

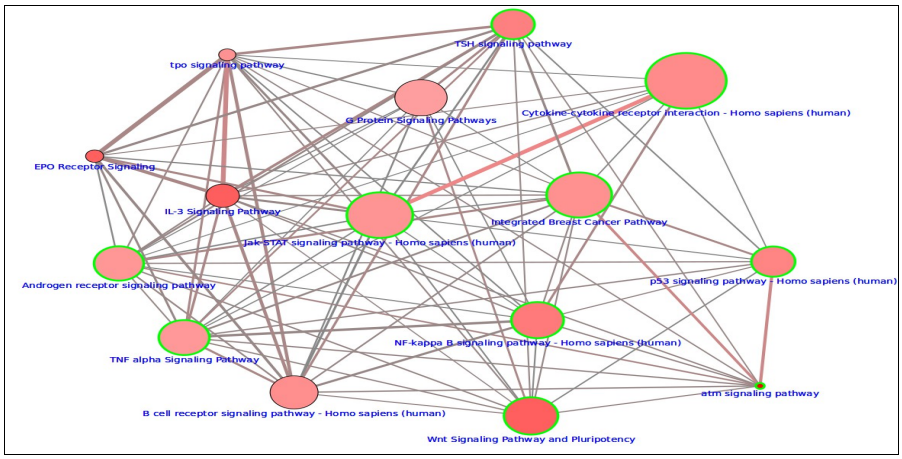

Figure 1: The over-represented pathways among the input list of 222 up-regulated genes upon DAC treatment. The node size indicates the size of the gene set. The *node color* corresponds to the p-value (deeper red means smaller P-value). The green node border is used to select the most interesting pathways. The *edge width* indicates the size of the overlap between two nodes, while the *edge color* represents the number of shared genes in the predefined datset.

Table 1: The enriched pathways associated to up-regulated genes.

Pathways ID	Genes	p-value	q-value
Atm signaling	ATM; MDM2; RBBP8; JUN; TP73	3.23E-06	1.23E-03
Wnt Signaling and Pluripotency	WNT2B; PRKCH; PPP2R1B; PRKCE; WNT1; JUN; FRAT1	3.68E-04	1.25E-02
EPO Receptor Signaling	STAT5B; PTPRU; RAF1; EPO	3.90E-04	1.25E-02
NF-kappa B signaling	PTGS2; ATM; CFLAR; TNFRSF11A; MALT1; IL8	1.48E-03	3.11E-02
TSH signaling	JUN; GNA12; RAF1; GNA13; PAX8	1.87E-03	3.36E-02
p53 signaling	ATM; MDM2; PPM1D; APAF1; TP73	2.28E-03	3.60E-02

Cytokine-cytokine receptor interaction	IFNW1; TGFB3; IL22; EPO; TNFRSF14; CSF2RA; TNFRSF8; TNFRSF11A; IL8; LIFR	3.31E-03	3.80E-02
Androgen Receptor	NR2C2; CEBPA; RUNX2; JUN; FHL2; AHR; MDM2	3.76E-03	4.07E-02
Integrated breast cancer	RAD54L; ATM; JUN; AHR; RAD50; MDM2; TSC1	4.36E-03	4.15E-02
Jak-STAT signaling	IFNW1; IL13RA2; CSF2RA; EPO; STAT5B; IL22; LIFR	5.21E-03	4.30E-02
TNF alpha Signaling	JUN; RIPK3; RAF1; APAF1; CFLAR	6.59E-03	4.90E-02

The mechanisms underlying the cytotoxicity of DAC treatment in gastric cancer AGScells was recently revealed, showing that ATM activation was associated with P53 phosphorylation and the cytotoxicity could be abolished using PI3K inhibitor [61]. In our case the ATM activation pathway is associated with the activation of p73 which is a member of p53 tumor suppressor family, important in regulation of cell cycle and apoptosis after DNA damage and found inactivated in many tumor histotypes [62, 63]. The methylation status of p73 was shown to be common in patients with MDS and associated with poor prognosis [63, 64]. The p73 play also a key role in p53 signalling pathways exerting a anticarcinogenic effect [65].

The reactivation of tumor suppressor represent one of the antitumor actions of demethylating agent therapy. The activation of the Wnt Signalling and nuclear factor kappa *B(NFKB)* pathways are important for the control of self-renewal in normal stem cell and also in the biology of breast cancer stem cells [66]. However the activation of *NFKB* pathway could leads to the production of cytokines that trigger Cytokine-cytokine receptor interaction, differentiation an proliferation signals, all of which contribute to chronic inflammation and malignant progression [67- 69]. The key components of thyroid stimulating hormone (TSH) signalling pathway are the up regulation of guanine nucleotide binding protein GNA12 and GNA13, also known as G12 subfamily, which mediate G protein signaling and have been implicated in various biological and pathological processes [70].

The Jun TF, known also as AP-1 or c-Jun, represents a key downstream effector of G12 signaling pathway [71]. PAX8 is a nuclear protein, involved in regulation of thyroid-specific genes and development of thyroid follicular cell development. The inhibition of Pax8 may be a very attractive targeted therapy for patients with endometrial cancer [72]. RAF1 is a protoncogene and functions downstream of the Ras family; it is activated in TSH and TNF alpha signaling pathway. The up regulation of RAF1 have been identified as predictive biomarker and new target for breast cancer therapy [73]. Figure 2 shows the most interesting pathways regulated upon DAC treatment and a summary appears in Table 2 to show the list of down-regulated genes in each specific pathway. It is important to notice that DAC treatment in MCF-7 cells induces down-regulation of different cytokines and interferons genes that represent key regulators of the Cytokine-cytokine receptor interaction, PI3K-AKT, Jak-STAT and IL-2 signaling pathways.

From Table 2, IL2RA and IL-2 are the common genes among all these pathways. The up-regulation of the interleukin-2 (IL-2) and its receptor alpha (IL2RA) are associated with the malignancy of the infiltrating human breast cancer [74]. It seems that their down-regulation could produce an anticancer effect. The deregulation of these cytokines and interferons genes may suggest a potential role as breast cancer biomarker or use as novel treatment in combination with DAC treatment.

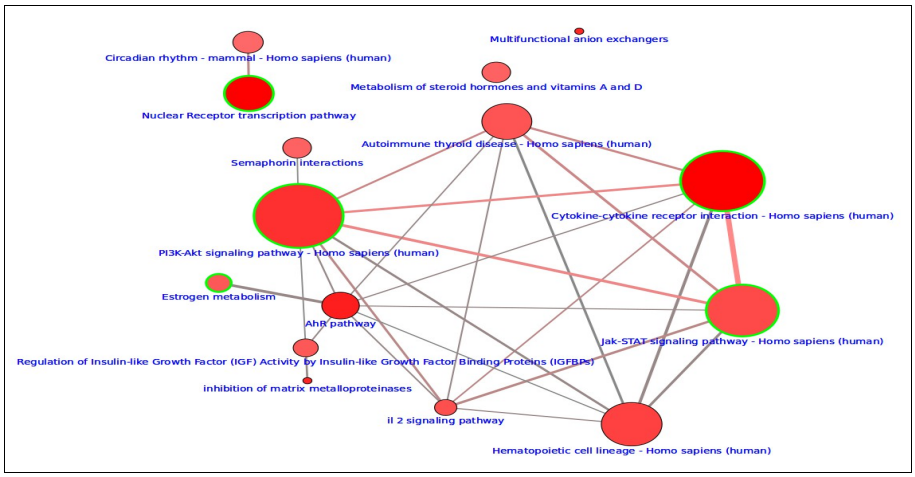

Figure 2: The over-represented pathways among the input list of 114 down regulated genes upon DAC treatment. The *node size* indicates the size of the gene set. The *node color* corresponds to the p-value (deeper red means smaller P-value). The green node border is used to select the most interesting pathways. The e*dge width* indicates the size of the overlap between two nodes, while the *edge color* the number of shared genes in the predefined dataset.

Table 2: The enriched pathways associated to down-regulated genes.

Pathways ID	Genes	p-value	q-value
Cytokine-cytokine receptor interaction	IL2RA; IFNA8; IFNA1; BMPR1B; TNFSF8; IL2; IL26; TNFRSF18.	3.54E-04	2.93E-02
Nuclear Receptor transcription	NR1D1; NR2E1; RXRA; RORB	3.55E-04	2.93E-02
PI3K-Akt signaling	IL2RA; IFNA8; IFNA1; THBS2; IL2; FGF12; TSC2; FGF20	1.61E-03	4.42E-02
Jak-STAT signaling	IL2RA; IFNA1; IFNA8; IL2; IL26.	3.65E-03	7.52E-02
IL-2 signaling	IL2RA; IL2	4.34E-03	7.66E-02

3.2 Transcriptional and post-transcriptional regulatory networks

We built with the cytoscape tool *"AdvancedNetworkMerged"* the union of transcriptional network resulted from *TFactS* analysis (Table 9S), and the miRNA regulatory network to show TFs and miRNAs experimentally validated as candidate regulators of the epigenetically modified genes in BC (Figure 3). Then, we selected only

TFs up-regulated upon DAC treatment and extracted the first neighbors of the selected TFs that resulted epigenetically modified (Figure 4). Therefore, the epigenetic profile resulting from the analysis is here described in an integrative network fashion.

The following box introduces the symbols of the next two figures.

We found that five TFs correspond to up-regulated genes. The top TF is CEBPA ($log_2 (NR) = 3.68$), called CCAAT/enhancer binding protein alpha, a key TF involved in the regulation of cellular processes, especially in Hematopoietic system [75]. It was found epigenetically modified and post-transcriptionally regulated by miR-124 after DAC and trichostatin treatment in AML [76]. The mechanism of regulation may be the same also in BC (Figure 4). Also miR-138 regulates CEBPA, found to inhibit adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells [77]. We didn't find any association to BC and epigenetic status. However, we found that CEBPA together with RUNX2 (runt-related TF 2) activates PTGS2 (prostaglandin-endoperoxide synthase 2), also known as cyclooxygenase-2(COX-2), which is an inducible enzyme responsible in the prostanoid biosynthesis and is involved in inflammation and mitogenesis [78]. PTGS2 is a target for development of anticancer therapy, and the use of anti-inflammatory PTGS2 inhibitors represents a promising strategy in the treatment of solid tumors [79]. Interestingly, the up-regulation of Jun TF is connected to the activation of TIMP1, which is a mettallopeptidase inhibitor whose overexpression contributes to antimetastatic effect in BC [80] and to the activation of NQO1 (NADPH quinone oxidoreductase 1), playing a cytoprotective role and found associated to increase cell-sensitivity to BC anticancer treatment [81]. Another important JUN target is the SERPINB5 (serpin peptidase inhibitor clade B, member 5) found also up-regulated upon DAC treatment and known as an important suppressor of the invasion and migration of cancer cells [82]. STAT5B (signal transducer and activator of transcription 5B) is a member of the STAT family of TFs, normally activated in response to cytokines and growth factors signals. It has been shown that the regulation of STAT1/STAT5 signalling pathway mediated by STAT5B is important in the regulation of essential functions in the mammary gland [83]. The TFact tool reported among the up-regulated target of STAT5B the DPAGT1 target, which is an enzyme that catalyzes glycoprotein biosynthesis and was found to be targeted also by wnt/β-Catenin signalling pathways, mediating a variety of critical developmental processes [84].

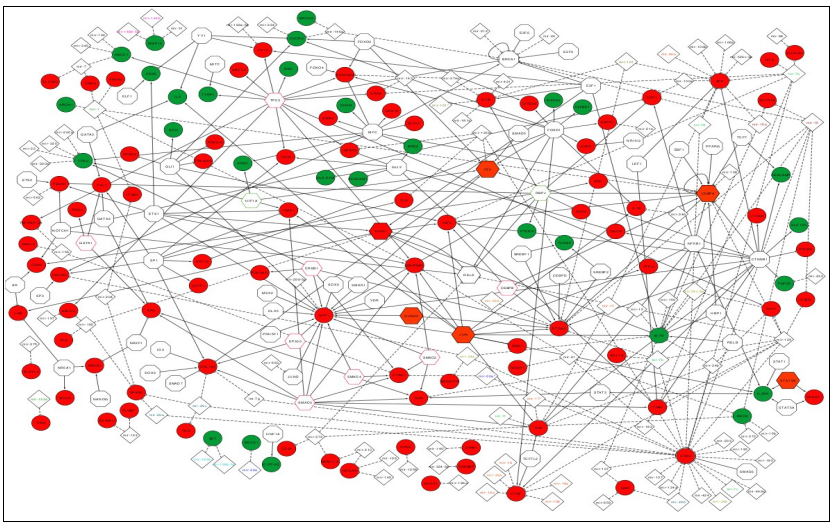

Figure 3: The global network resulted from the union of transcriptional and post-transcriptional networks. *Node shape* indicates the physical entity (ellipse for epigenetically modified genes, Hexagon for TFs and Diamond for miRNAs). *Node color* indicates the differentially expressed genes (red for up-regulated genes and green for down-regulated genes). *Node border color* indicate the significant TFs enriched (with p value<0.05) from *TFact*S analysis (red for activated TFs and green for inhibited TFs). The non-colored nodes correspond to TFs that regulate at least one target of the epigenetically modified genes.

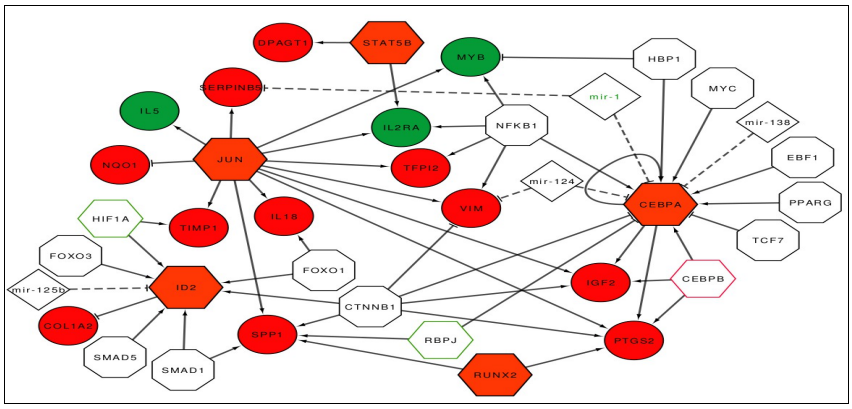

Figure 4: Selected first neighbors of TF epigenetically modified genes from Figure 3.

4 Discussion

The improved curation of database reporting pathway definitions and regulatory interactions allowed to to draw a representative picture of the major gene players and pathway landscape that are regulated in DAC treatment in BC. The dissection of the

key pathways and the knowledge of the interconnections between its components, are fundamental to conceive new therapeutic approaches.

Our results are in agreement with other studies that showed the mechanism underlying the anticancer effect of DAC treatment and its involvement in activation ATM-p53 signalling pathways in cancer cell lines [61] and oncogenic pathways in MCF-7 brest cancer cells [19]. The identification of a potential TF-miRNA regulatory network contributes to shed light on the potential molecular mechanism played by DAC treatment in MCF-7 BC. One of this molecular mechanism is the activation of an important TF, the CEBPA and its post-transcriptional regulator miR-124 by demethylating agent, suggested as predictors for effectiveness of epigenetic therapy in AML [76] and that could be further investigated also in breast cancer.

All these evidences might help the design of new therapeutic formulations, and to find the best therapeutic combinations between the use of demethylating agent as DAC and immonotherapy, e.g directly targeting the CT antigens the cancer antigens or other therapeutic tools that could improve the anticancer effect of the demethylating agent, obtaining therefore more efficient therapy for breast cancer. Moreover, we are confident that gene expression and pathway signatures could give aid to the discovery of relevant connections among molecular mechanisms and drugs so as to significantly contribute to the improvement of drug for cancer therapy.

As a follow up agenda for future research, we are planning to extend our analysis to other tumor histotypes that are already available, and also to use novel gene expression profiling (exploiting transcriptome landscapes from RNA-Seq) for refinement of pathway signatures.

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