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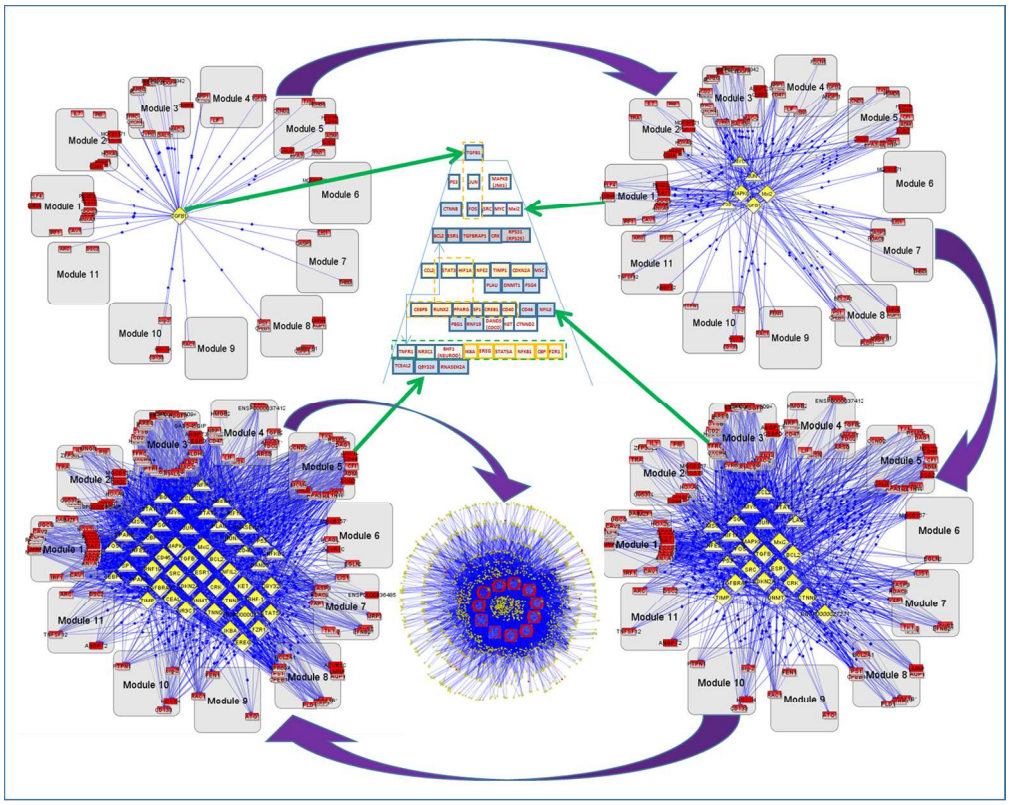

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Hierarchical Control of Coherent-Gene Clusters Defines the Molecular Mechanisms of Glioblastoma

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Running title: Hierarchical mechanisms of gene regulation in glioblastoma

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Abstract

Glioblastoma is a highly aggressive and rapidly lethal tumor characterized by resistance to therapy. Though data on multiple genes, proteins, and pathways are available, the key challenge is deciphering this information and identifying central molecular targets. Therapeutically targeting individual molecules is often unsuccessful due to the presence of compensatory and redundant pathways, and crosstalk. A systems biology approach that involves a hierarchical gene-group networks analysis can delineate the coherent function of different disease mediators. Here, we report an integrative networks-based analysis to identify a system of coherent-gene modules in primary and secondary glioblastoma. Our study revealed a hierarchical transcriptional control of genes in these modules. We elucidated such modules responsible for conversion of the glioma-associated microglia/macrophages into glioma-supportive, immunosuppressive cells. Further, we identified clusters comprising mediators of angiogenesis, proliferation, and cell death for both primary and secondary glioblastomas. Data obtained for these clusters point to a possible role of transcription regulators that function as the gene-modules mediators in glioblastoma pathogenesis. We elucidated a set of possible transcription regulators that can be targeted to affect the selected gene clusters at specific levels for glioblastoma. Our innovative approach to construct informative disease models may hold the key to successful management of complex diseases including glioblastoma and other cancers.

Keywords: Networks, Systems Biology, Modeling, Glioblastoma, Cancer

Acronyms and Abbreviations

ANGPT2—angiopoietin 2; ANXA1—annexin A1; ARNTL—aryl hydrocarbon receptor nuclear translocator-like; B2M—beta-2 microglobulin; BMP—bone morphogenic protein; C-L—

centrality-lethality; CD8A— cluster of differentiation 8a; CD31— cluster of differentiation 31; CDH2—neural cadherin (N-cadherin); CDKN2A—cyclin-dependent kinase inhibitor 2A; CGM—coherent-gene module; CHI3L1—chitinase-3-like protein 1; CTSB—cathepsin B; CSCR4—C_X_C chemokine receptor type 4; CYR61—cysteine-rich, angiogenic inducer, 61; DB—database; EMT— epithelial-mesenchymal-transformation; FBLN5—fibulin 5; GAM—glioma-associated microglia/macrophages; GBM—glioblastoma multiform; GBM1—primary glioblastoma; GBM2—secondary glioblastoma; GEO—Gene Expression Omnibus; HBEGFR—heparin-binding EGF-like growth factor receptor; IPA—Interactive Pathway Analysis; JMJD6—jumonji domain containing 6; LAMA2—laminin, alpha 2; LGALS1—lectin, galactoside-binding, soluble, 1; MeV—MultiExperiment Viewer (Bioconductor); MF—macrophage, NRP1—neuropilin 1; $Norm_{AVE}$—average value for the Normal sample group; PDGFRB—platelet-derived growth factor receptor, beta polypeptide; PLAU—urokinase-type plasminogen activator; PSG4—pregnancy specific beta-1-glycoprotein 4; TDEC—tumor-derived endothelial cell; TCR— T cell receptor; TF—transcription factor; TGFB (TGFβ)—transforming growth factor, beta; TNF—tumor-necrosis-factor; TR—transcription regulator; VEGF—vascular endothelial growth factor.

Introduction

Glioblastoma (GBM) is a one of the most malignant cancers that is rapidly lethal and treatment resistant. Similarly to other cancers, GBM has complex interactions on genetic and epigenetic pathways, signaling networks, and cellular levels, to name a few. Numerous gene-expression analyses were conducted on GBM tumor tissues in the past decade. Extracting the knowledge from these data as well as associated integrative knowledge is an important issue in GBM studies and in bioinformatics in general.

The prominent genetic alterations associated with GBM are elucidated in numerous publications.[1–6] These abnormalities include significantly increased angiogenesis, high rate of tumor proliferation, anti-apoptosis gene activation, and invasive immunosuppressing microglia. GBM is a highly vascular tumor associated with a high degree of microvascular proliferation. It is critical to understand molecular pathways regulating these events, especially, angiogenesis, because it represents a highly attractive therapeutic target. In addition, GBM is characterized by high, unregulated cellular proliferation, owing primarily to alterations in the cell cycle regulatory genes. The process of tumorigenesis is often associated with the cells evading apoptosis that determines susceptibility of cells to various chemotherapeutic agents and to radiation. Finally, one of the distinguishing features of GBM that makes it intractable is its high degree of infiltration into surrounding normal brain tissue.[7]

Considering the complex interplay of mediators involved in GBM pathophysiology, it is clear that examining individual signaling molecules or even pathways may not give

information accurately representing the underlying molecular abnormalities. We believe that a comprehensive bioinformatics analyses using an integrative systems-biology approach may provide a means to define or delineate the role of these and other signaling mediators in the pathophysiology and in turn prognosis of GBM. The systems biology approach allows to analyze in conjunction gene expression, signaling and metabolic pathways, gene-regulation and protein-interaction networks, and gene-ontology data, to visualize and query these networks and pathways, and to predict new gene targets for treatment of disease and identification of pathways that can be affected by drugs. Here, using this approach, we present a network analysis that defines coherent-gene modules (CGMs) and highlights the hierarchical control of them. Our analysis identifies "highly-connected" signaling entities for GBM. Furthermore, we define gene modules that comprise specific mediators involved in the pathophysiology of GBM. These data will not only improve our understanding of basic disease pathology but will also identify effective therapeutic targets for GBM.

Results

Microarray data analysis

We used microarray data obtained from the Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array (Santa Clara, Calif., USA) deposited by Michal Grzmil and colleagues in the Gene Expression Omnibus (GEO) database (Entry GEO15824, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15824). The microarray contains 54675 probes and several sample groups from which we used three groups: 12 samples

from the primary glioblastoma (GBM1), 3 samples from the secondary (GBM2), and 2 samples from normal brains (Norm). For details see *Methods and Materials* section.

After Kruskal-Wallis nonparametric test of the ANOVA[8] with $p = 0.025$ for the 2229 significant genes obtained (4%), we subjected this data to expression threshold filtering. Only differentially expressed genes that overcome the 1.5-fold threshold of GBM gene expression compared to normal were taken in account, separately for primary (GBM1) and secondary (GBM2) glioblastomas. 442 genes for GBM1 and 586 genes for GBM2 were the subjects for further analysis. For these genes the average values for each probe for normal group ($Norm_{AVE}$) were calculated and their expressions were normalized to GBM1/$Norm_{AVE}$ and GBM2/$Norm_{AVE}$ rates (creating two sets of genes: GBM1—for primary, and GBM2—for secondary glioblastomas), which underwent further analysis. Apart of genes belong to both GBM1 and GBM2 sets but their expressions significantly differ.

Hierarchical networks of TFs and TRs regulating glioblastoma genes

As described above, using BiologicalNetworks,[9,10] we conducted analysis of calculated values for 12 sets of GBM1 and 3 sets of GBM2, using the method described earlier.[11] On the basis of analysis of microarray data and the existing Integrome database (IntegromeDB) of connectivity of transcription regulators (TRs) and transcription factors (TFs), the program created networks of "coherent-gene modules" (CGMs), TFs, and TRs[11] for both GBM1 and GBM2. One of the most important parameters in analysis of TFs and TRs is the connectivity.

Primary GBM (GBM1)

The network for GBM1 contains 11 CGMs (generated by BiologicalNetworks program), each including different genes from the aforementioned 442 genes set. All these genes are differentially expressed with fold ratio to normal greater than 1.5 (Fig. 1).

[Fig. 1]

The complete set includes 442 genes, 155 TFs, and 1605 TRs (Fig. 1E). Because of the number of connections involved, we cannot use image analysis and instead studied this computationally. Fig. 1A–D, shows the TF–TR–CGM networks "sliced" on consecutive levels of network connectivity. The level of connectivity of a particular gene in the network for a long time has been considered as a parameter related to its importance. There exists a so-called centrality-lethality (C-L) rule, which states that highly-connected genes in a network likely are more important for cell viability.[12,13] It also was shown that connectivity parameter for the particular genes can be important not only for general cell function but for specified functions of the gene groups, like environmental response, cell cycle, DNA damage, etc.[14] From our study on GBM1, we demonstrate that a TR with the greatest connectivity in this network is transforming growth factor-beta (TGFB—TGFβ) that appeared on the first layer (see Figs. 1A and 2). TGFB is increasingly recognized as an important player in GBM[15,16] and our group is conducting clinical trials of TGFB inhibitors that show activity against GBM. Other slices of the connectivity pyramid were made on different levels: level 3 (Fig. 1B), level 9 (Fig. 1C), and level 12 (Fig.1D). The central pyramid (Figs. 1E and 2) gives a hierarchical presentation of entire network of the TFs and TRs based on connectivity level that actually regulate all the CGMs. One can see that

different levels have significant functional separation by the connectivity. The close-up of the top levels is presented on Fig. S1A.

[Fig. 2]

To illustrate the gene functional distribution inside the modules, the heat maps were created for every important CGM (Figs. S2 and S3). The color represents *p*-value and the size—quantity of genes with the mentioned function. In Fig. S2 one can see that in GBM1 genes from the CGM1 participate in proliferation, differentiation, and migration of cells with *p*-values 1.09×10^{-8}, 3.18×10^{-5}, and 4.85×10^{-5}, accordingly. Genes from CGM2 are responsible for abnormal morphogenesis and inflammation. Genes of CGM3 participate in development of blood vessels ($p = 1.03\times10^{-8}$), vasculogenesis ($p = 1.86\times10^{-8}$), angiogenesis ($p = 3.43\times10^{-5}$), cell differentiation, proliferation, migration, and movement ($p = 2.16\times10^{-7}$, 4.97×10^{-7}, 3.02×10^{-7}, and 3.74×10^{-7}), and especially, in proliferation of tumor cells ($p = 3.85\times10^{-5}$), metastasis ($p = 1.97\times10^{-5}$), and growth of tumor ($p = 3.18\times10^{-5}$). CGM5 participates of cell proliferation.

Level 1 of TFs and TRs comprisesTGFB.

Level 2 of TFs and TRs includes p53 involved in the MAPK8 regulation pathway; MAPK8 phosphorylates p53 and also activates the TFs and TRs in a lower level. These genes are related to cancer, cellular growth, and proliferation.

Level 3 includes FOS that also in part is activated by TGFB. FOS interacts with RAS–RAF–MEK, JNK, and MYC–MAX groups. These genes are related to cancer and connective tissue development.

Level 4. TGFBRAP1 is involved in TGFB regulation.[17] Also the level includes TFs and TRs related to AKT–mTOR–PI3K signaling pathway.[18] v-Crk activates the PI3K/AKT

pathway;[19] BCL2 is activated by AKT/mTOR pathway.[20] These genes are related to development.

Level 5. Note that level 5 contains a majority of genes related to microglia activation. It has been shown[21] that microglia became immunosuppressive. Glioma-associated macrophages (MFs) and microglia cells promote cancer by increasing pro-angiogenic factors and enhancing invasion.[21–23] Expression of one of the microglia-activating genes **HIF-1** is associated with **CCL2** chemokine expression.[24] CCL2 interacts with MSP-1 and modulated the microglia activation and proliferation.[25] STAT activates creation of the immunosuppressive microglia that supports the cancer cells proliferation.[26] Gabrusiewicz and Heimberger[26] commented that "Mounting evidence indicates that the glioma microenvironment converts the glioma-associated microglia/macrophages (GAMs) into glioma-supportive, immunosuppressive cells." This way the level 5 TFs and TRs can play a significant role in GBM development and needs appropriate therapeutic intervention.

Level 6 of the TRs pyramid of GMB1 includes a majority of epithelial-mesenchymal-transformation (EMT)-related genes. First, we note that the top gene TGFB is known to induce EMT and the level 6 and some other genes on different levels actually provide the signaling for this transformation. For example, PPARG is directly responsible for TGFB-induced EMT and consequent tumor invasion.[27] SP1 gene in the level 6 cooperating with ZEB2 upregulates integrin alpha5 expression participating in EMT in cancer cells.[28]

Level 7 of the pyramid is mostly related to cell death. Part of the TFs and TRs activate cell death pathways: TNFR1, NR3C1, BNF1 (NeuroD)—and others (IKBA, EPEG, STAT5A, NFKB1, FZR1) inhibit this process.

Most important coherent-gene modules responsible for carcinogenesis

In the previous section, we described and analyzed the "pyramid" hierarchy of the TR and TF networks (that control the CGMs) based on their connectivity. Here we analyze the most populated CGMs using Ingenuity Interactive Pathway Analysis (IPA) software (Ingenuity Systems, Inc., Redwood City, Calif.) and describe an influence of their interactions with the TRs and TFs in terms of their functions and their effects on tumorigenesis. The BiologicalNetworks software generated 11 CGMs based on 442 differentially expressed genes for GBM1 and 13 CGMs based on 586 genes for GBM2. We considered only statistically significant CGMs and described them in the ascending *p*-value order.

Analyzing the most populated CGMs using IPA, we obtained several gene networks. For further analysis we selected only statistically significant and functionally meaningful networks.

For GBM1 we selected CGMs 1 and 3 that are statistically significant. Note that most populated CGM3 has the lowest *p*-values (highest 1/*p* values) for the functions of development of blood vessels (Fig. 3C). As noted by El Hallani and co-authors, GBM is one of the most angiogenic human tumors.[29] The other main feature noted by these authors is the highest rate of endothelial proliferation. These factors are also at the top of the functions of the CGM3 (Fig. 3C). In the GBM1, the CGM3 has 11 connections with a higher-level controlling TR—TGFB1—the maximum number of connections in comparison with other CGMs.

[Fig. 3]

Angiogenic function of networks 1 *and* 3 *of the CGM3*

Highlighted red on Fig. 3A and B, are genes that participate in angiogenesis. The *p*-value of angiogenic function of this module estimated on the basis of activated genes is 1.03×10^{-8}. One can see eight such genes in the *network 1* (CGM3) and six in the *network 3* (CGM3).

Among the genes of the *network 1* are well known mediators of angiogenesis, including ANGPT2 (angiopoietin 2), CDH2 (N-cadherin), CTSB (cathepsin B), CYR61 (cystein-rich, angiogenic inducer 61), FBLN5 (fibulin 5), LAMA2 (laminin-alpha 2), PDGFRB (platelet-derived growth factor receptor, beta polypeptide). These genes are regulated by TGFB1 and PDGF. MEK–ERK1/2 pathway and Ap1 are also included in the genes group that control the network.

Among the genes of the *network 3* (CGM3) are ARNTL (aryl hydrocarbon receptor nuclear translokator-like), CXCR4 (C_X_C chemokine receptor type 4), HBEGFR (heparin-binding EGF-like growth factor receptor), and LGALS1 (lectin, galactoside-binding 1). These genes are controlled by HBEGFR and VEGF, which in their turns are activated by TGFB1. AKT protein is involved in expression of CXCR mRNA.[30]

Proliferation function of networks 1 *and* 2 *of the CGM1*

Proliferation is a top function of the CGM1 of GBM1. Genes related to proliferation of cells: **ADAM12**, *ADAMTS9*, **ANXA1**, *CAV1*, **CAV2**, *CFB*, **CHI3L1**, **DAB2, ELF4**, GALNS, **GCH1**, *HOXB3, HOXB7*, IFI30*, IRF1*, LEPREL1, **LMNA**, MYOF, *MZF1*, **NAMPT**, *NELFE*, **PLA2G5**, **POSTN**, *SAT1, SOCS3*, **SOD2**, **UGCG**, *VAMP8*, and *VMP1* feature in this module. The *p*-value of this function for CGM1 is 1.9×10^{-8}, which give a

clear functional assignment for this module GBM1. There are two main gene networks assigned to this module by IPA program. Gene symbols represented by bold typeface are from the *network 1* (Fig. 4A), which is related to cell death, survival, and cellular movement. Italicized gene symbols are from the *network 2* (Fig. 4B), which is related to connective tissue development and function and organ development. The proliferation activity of CGM1 gene *network 1* (Fig. 4A) is controlled by TGFB and PDGF in cooperation with MAPK, ERK1, and AP1 (heterodimer containing most often JUN kinase and FOS). Colored brown genes and gene connections are related to the proliferation process governed by this network (Fig. 4). AP1 is known to activate proliferation of cells; ETS gene in CGM1 is a well-known cancer-related TF that is responsible for proliferation and cell migration, along with ETS-related TF ELF4 (overexpressed in CGM1 of the GBM1). ETS activates the chitinase-3-like protein 1 (CHI3L1, also known as YKL-40) responsible for cancer progression and specifically for glioma cell migration.[31] Note that PDGF protein that is included in the CGM1 *network 1* controls specific mesenchyme proliferation and differentiation.

[Fig. 4]

Network 2 of CGM1 also contains a number of proliferation-related genes (These genes and their connections are colored brown on Fig. 4B). The top regulation of proliferation is conducted by TFGB1 and VEGF in cooperation with ERK, MAPK, AKT, and PI3K. Note that Ck2 kinase also activates the proliferation-related genes. It increases phosphorylation of IRF (interferon-regulatory factor 1),[32] SAT1,[33] and HOXB7 (and most probably HPXB3 and HOX8) genes.[32]

Secondary GBM (GBM2)

The global network for GBM2 contains 13 CGMs, each including different genes from the aforementioned 586 genes set (Fig. 5). All these genes are differentially expressed with fold ratio to normal greater than 1.5. The full network is presented in Fig. 5E. It includes 586 genes, 147 TFs, and 1694 TRs. Fig. 5A–D, shows the TF–TR–CGM networks "sliced" on consecutive levels of network connectivity. The close-up of the top levels is presented on Fig. S1B.

[Fig. 5]

The heat map of the most representative CGMs for GBM2 (Fig. S3) shows that they play an important role in cell death. 146 genes of CGM1 are responsible for cell death ($p = 2.93\times10^{-8}$), necrosis ($p = 4.58\times10^{-7}$), and apoptosis ($p = 9.01\times10^{-7}$). 128 genes of CGM2 also participate in necrosis ($p = 7.60\times10^{-7}$), apoptosis ($p = 5.17\times10^{-6}$), and cell death ($p = 2.23\times10^{-5}$). Large amount of genes of CGM2 also trigger cell proliferation ($p = 1.60\times10^{-6}$).

The GBM2 controlling TF/TR pyramid (Fig. 5F) and CGMs are shown in Fig. 6. They were analyzed similarly to the GBM1 analysis reported above.

[Fig. 6]

Level 1 of TFs and TRs comprises JUN-kinase (JNK, MAPK8). Its interaction with FOS creates the most connected TF of GBM2.

Level 2 includes p53 involved in the JNK (MAPK8) regulation pathway: MAPK8 phosphorylates p53 and also activates the TFs and TRs in a lower level. FOS is in part activated by TGFB. FOS interacts with RAS–RAF–MEK, JNK, and MYC–MAX groups. These genes are related to cancer, cellular growth and proliferation. Note that Mxi2 sustains ERK1/2 phosphorylation in the nucleus by preventing ERK1/2 binding to phosphatases.[34]

Level 3 includes FOS that also in part is activated by TGFB. FOS interacts with RAS–RAF–MEK, JNK, and MYC–MAX groups. These genes are related to cancer and connective tissue development.

Level 4 includes TFs and TRs related to AKT–mTOR–PI3K signaling pathway.[18] v-Crk activates the PI3K/AKT pathway.[19] Note also DNMT1 methyltransferase gene that maintains DNA methylation status in tumor-suppressing genes. DNMT1 silencing significantly increases levels of apoptosis in cancer cells.[35] NFE1 is involved in a set of cancer-related processes including regulation of the antiapoptotic BCL2.[36] PI3K–AKT pathway genes affect NFE2.[37] NFE2 also participate in the oncogenesis affecting KRAS, BRAF, and MYC genes activity.[38]

Level 5. It is also significantly related to AKT–mTOR–PIR3 pathway. AKT activates CREB1 that regulates transcription of BCL2.[20,39] CREB is a regulatory target for the protein kinase Akt/PKB;[39] ESR1 activates PI3K.[40,41] CDKN2A (cyclin-dependent kinase inhibitor 2A) is a tumor suppressor through binding of CDK4/6 and preventing their proper contact with these kinases. TGFBRAP1 (TRAP1) is involved in TGFB regulation.[17]

Level 6. NFIL3, overexpressed in various cancers, supports tumor cell survival through repression of TRAIL.[42] SP1 regulates known cancer-related genes: androgen receptor, TGFβ, c-Met, and others.[43] Interesting to note a presence of PSG4 (pregnancy specific beta-1-glycoprotein 4) that usually plays a role of immunomodulator to protect the growing fetus. We hypothesize that such function can be mimicked to protect the growing tumor. PLAU (urokinase-type plasminogen activator) is involved in cancer cells proliferation. Tumor promoter HIF1A, MYC, p300, NFKB1 are the well-known targets of anticancer

medicine. FRZ1 mediates multidrug resistance in cancer through regulating of WNT pathway.[44]

Level 7. Interesting to note COCO (DAND5) gene that is known as BMP (bone morphogenic protein—tumor suppressor) antagonist that reactivates tumor cells leading to metastasis in lungs.[45] It is possible that COCO plays a more important role in development of secondary GBM that need to be explored. It has been shown that PPARG plays a significant role in blocking the invasiveness of glioma cells and that its agonists like pioglitazone (Actos), troglitazone (Rezulin), rosiglitazone (Avandia), and the experimental PPARγ (agonist ciglitazone[46]) can be useful for increasing its antitumor activity.

Above, we have presented some examples of important TFs and TRs from different layers of the pyramid. One can see that on all layers can be found to be known cancer-related proteins. Our finding is that we outlined that these genes can play a concerted roles in secondary GBM and the treatment of GBM would need to take an approach that targets most of them in order to effect outcomes.

Cell-death-related functions of CGM1 of GBM2

In spite of aforementioned lack of samples, the CGM1 of GBM2 shows very significant functional assignment. As assigned by IPA, the top function for both CGM1 and CGM2 is "cell death," with *p*-value 2.9×10^{-8} for CGM1 and *p*-value 2.35×10^{-7} for CGM2. There are a total of 37 genes in CGM1 and 29 genes in CGM2 that are assigned to cell death function. IPA generated four networks for each module. CGM1 *networks 4* and *2* and CGM2 *networks 4* and *1* are statistically significant and were selected for further analysis. CGM1 networks include 37 genes related to cell death. These genes are: **ANG**,

ARHGAP18, *CASP4, CASP7*, *CD44*, **CEBPD, CEBPG**, CHI3L1, CTSS, CXCR4, CYR61, *CYR64*, DAB2, DRAM1, ELF4, FCGR2B, FSTL1, GZMA, HBEGFR, *KLF6*, LCP2, LGALS1, LGALS3, *LMNA*, LYZ, **MICA**, MSR1, *NRP1*, *RAB27A*, **SAT1**, SLC47A1, SOCS3, SOD2, TFEC, TNFSF12, TNFSF8, **VIM**, and YAP1. Genes with Italicized symbols belong to *network 4* (Fig. 7A), with underlined—to *network 2* (Fig. 7B), and with bolded symbols—to *network 3* (Fig. 8).

[Fig. 7]

In the *network 4 of CGM1* (Fig. 7A) cell-death-related genes (colored green) including caspases are regulated by TGFB1 and PDGF_BB. The latter decreases expression of CASP4.[47] PDGF_BB also increases activity of proto-oncogene BCD (KLF6).[48] MEK gene decreases apoptotic activity of caspase3/7.[49] NRP1 (neuropilin 1) expression is increased by HBEGFR.[50] Overall MEK, MAP2K1/2, and AKT participate significantly on the cell-death signaling.

[Fig. 8]

Network 2 of CGM1 (Fig. 7B) is significantly related to the cell death and immune responses. BCR–ABL/GRB2 complex recruits SOS, and BCR–ABL/GRB2/SOS complex activates RAS, MEK, and MAPK pathways.[51] Tumor-necrosis-factor (TNF)-related pathway sends a cell-death signal that is overridden by ERK1/2 activation amplified by the same TNF genes.[52] Activation of the RhoA/ROCK signaling pathway has been shown to contribute to dissociation-induced apoptosis of embryonic and neural stem cells.[53] Expression of ROCK is increased by ERK1/2.[54] At the same time, as a negative feedback loop, RHO kinase (ROCK) decreases the activity of ERK1/2.[55] Studies have shown a

significant role for the BCR complex that increases expression of MHC (major histocompatibility complex) class II gene[56] and activates ERK1/2 protein.[57] Moreover, FC-gamma receptor can be a target for anticancer therapy.[58]

CGM2 of the GBM2 contains 29 genes related to cell death: **ANTXR2**, *ANXA1*, *B2M*, **BCL2A1**, *CALR*, **CASP4**, *CD8A, CDCP1*, **CEBPD**, **CHI3L1, DAB2**, DPYD, EMP3, GLIPR1, GLRX, **IL33**, *MSN*, **NAMPT**, **PECAM1**, *PLAGL1, PRKAA1, RAB32*, RASSF3, **SOD2**, **SWAP70**, **TGFB2**, **THBD, TYMP, VAV3**. The bolded symbols of genes are in the *network 1* and Italicized—in the *network 4*. The cell-death-related genes are colored green on both networks (Fig. 8A and B).

Network 1 of CGM2 of GBM2 (Fig. 8B) contains a number of cell-death-related genes (highlighted in green). It is controlled by the TGFB1 and TGFB2 in contact with PDGF. Complex Ap1 is also involved in this network regulation. CASP4 is a pro-apoptotic protein. CHI3L1 (chitinase 3-like 1, cartilage glycoprotein 39) promotes cancer cell proliferation, macrophage recruitment, and angiogenesis. PECAM1 known as cluster of differentiation 31 (CD31) is also involved in angiogenesis.

Inflammation and Immune response functions of CGM1 of GBM2

Network 2 of CGM1 of the GBM2 is significantly related to inflammation and immune response. This can be explained by the presumption that the growing GBM2 activates the immune system that tries to fight the growing tumor.

Network 4 of CGM2 of the GBM2 also contains immune response genes: MHC Class 1, activating it beta-2-microglobulin (B2M), immunoglobulins, T-cells receptor recognizing antigens (TCR), and CD8A (CD8 antigen is a cell surface glycoprotein found on most

cytotoxic T lymphocytes that mediates efficient cell-cell interactions within the immune system). CD3 (together with other members of this family) couples the antigen response to intracellular pathways. ANXA1 (annexin A1) is also important for the immune response and inhibits the NFKB pathway that can lead to proliferation in cancer. It is interesting to note that interference of active CD3 by antibody increases activation of AKT.[59] This indicates that activated CD3 can inhibit the AKT pathway, while MAPK is activated by TCR complex.[60]

Correlation of top level network genes to survival and GBM subtypes

We looked at the top level genes in the networks identified above and tested whether they correlated with survival or subgroups in the Cancer Genome Atlas (TCGA) dataset (http://cancergenome.nih.gov). Using SurExpress platform, we found that MAPK8, SRC, CCL2, and MAPK14 expression levels are related to patients' survival for general GBM. The volcano plots in the insets show that for MAPK8, SRC, and MAPK14 higher expression level of these genes in the tumor correlates with the increase of survival, and for CCL correlates with the decrease of survival (Fig S4). These results contradicting the initial "naïve" hypothesis of direct correlation of oncogenes expression with patients' survival are discussed in the Discussion section.

Using cBioPortal portal (http://www.cbioportal.org), we confirmed that MAPK8, SRC, CCL2 expression levels were associated with overall survival and disease-free status of GBM patients (Fig S5). Several top level network genes also are correlated with survival in different molecular subtypes in TCGA dataset (Fig S6). For TGFB1, TP53, CTNNB1, SRC, and MYC the expression of these genes corresponds to the increase of survival only

for the neural subtype of GBM. For other subtypes we see the various correlations that are interesting and may lead to more comprehensive understanding of GBM molecular mechanism (Fig. S6).

Strategies for drug administration

Based on our analysis of the genes controlling connections, one can make a decision according to the level of hierarchy of the genes involved in GBM. Table 1 shows the possible drugs to target the top five levels of the GBM1 pyramid (Fig. 2). Note that we propose drugs only for the selected genes of each level. The current state of drug development does not provide reliable drugs solution for all the genes elucidated in the pyramid but it gives a possible motivation for drug design based on the identification of specific genes in the top levels.

Discussion

GBM is an aggressive cancer characterized by a high degree of treatment resistance and dismal prognosis. The lack of effective therapeutic options makes GBM a major unmet clinical need. In this study, we identified the GBM molecular networks using the unique multidimensional networks of TFs and TRs and coherent-gene modules including co-regulated genes. Such analysis made it possible for us to find unexpected points of intervention and combinations of drugs that serve as effective GBM therapeutics.

Using an integrative systemic approach, we have elucidated and examined hierarchical networks pyramids including TRs and TFs that serve as driving forces for activation of specific CGMs. The analysis of genes at different layers of these pyramids for primary and secondary GBMs have identified a vast majority of TFs and TRs related to cancers. Interestingly, some of the mediators that our analysis uncovered, such as PSG4 or COCO have been for the first time shown to be associated with GBM. At the same time, data suggests their possible role in the pathogenesis of primary and/or secondary GBMs.

Our analysis based on the hierarchical pyramid revealed TGFB to have the highest connectivity in all molecules identified in the coherent gene modules for primary GBM. TGFB has been shown in unrelated studies to be important in the pathogenesis of cancers[61] and specifically GBM[15,16] but it has never been demonstrated to be one of the key genes responsible for GBM. Inhibitors of TGFB are being developed and tested in various preclinical and clinical studies. One such inhibitor, LY2157299, is a TGFβ receptor type I (R1) inhibitor is currently being tested in a phase I/II clinical study for GBM, in combination with radiation and chemotherapy (ClinicalTrials.gov identifiers

NCT01582269 and NCT01220271). This drug has potent antitumor activity in animal models of cancer. These observations suggest that the 3D network analysis we present here can identify therapeutically relevant molecules that can form effective clinical targets.

Following identification of specific CGMs, we used detailed IPA-driven network analyses to pinpoint specific genes. We identified clusters comprising different genes linked to angiogenesis for both primary and secondary GBM. A cancer characterized by high vascularity, GBM shows overexpression of VEGF, an important regulator of tumor-associated angiogenesis. Anti-angiogenesis therapies targeting VEGF are being used in GBM with variable success. Monoclonal antibodies to VEGF,[62] small molecule inhibitors of VEGFR,[63] and decoy receptors inhibiting VEGF activity[64] represent major therapeutic approaches currently being tested to target the VEGF/VEGFR pathway. Multiple phase I/II studies using the VEGF inhibitor bevacizumab (Avastin) for recurrent GBM show modest improvement in progression-free survival.[65]

In addition, in keeping with the highly proliferative nature of GBM, one of the high scoring networks that IPA analysis identified for primary GBM was related to proliferation (CGM1). Two mutations commonly seen in most cancers including GBM are those of the retinoblastoma (RB) and p53 pathways.[66,67] In addition to these two tumor suppressor pathway abnormalities, GBM also features increased expression and/or activity of mitogens and associated membrane receptors with their downstream signaling pathways, such as MAPK, PI3K/PTEN, and different RTKs.[1] Not surprisingly, in our analyses, one proliferative gene network in primary GBM (*network 1* of CGM1) was controlled by TGFB and PDGF, in cooperation with the MAPK pathway. Similarly, *network 2* of CGM1 in primary GBM comprised of proliferation-related genes that were regulated by TGFB1 and

VEGF in cooperation with the MAPK pathway, AKT and PI3K. These data from our network analyses in primary GBM point to the importance of the TGFB and VEGF molecules in regulating proliferation, an important phenotypic feature of GBM.

On the other hand, network analyses on secondary GBM revealed a network that was linked to cell death (*network 4* of CGM1) and comprised of 37 genes related to cell death or apoptosis. There are several molecular mediators of apoptosis, prominent among which is the Bcl2 family of proteins comprising both pro- and anti-apoptotic members. It has been shown that the treatment resistance seen in recurrent GBM is primarily related to an anti-apoptotic shift in the dynamics of the Blc2 family members.[68] However, counter-intuitively, increased expression of anti-apoptotic Bcl2 members has also been shown to improve prognosis in GBM.[69] Interpretation of data on apoptotic mediators may not straightforward and may be influenced by different signaling mediators. In our study, TGFB1 and PDGFB regulated many of these cell-death-related genes, including caspases and other proto-oncogenes. The net effect was a reduced apoptosis in cells leading to uncontrolled growth. Secondary GBM was also characterized by the presence of a set of genes that was significantly related to immune response, in addition to cell death (*network 2* of CGM1). Together, our network analyses data are in line with the high inflammation, immune response, and anti-apoptotic effects observed in GBM.

Notably, when we analyzed survival data in GBM patients with specific genetic aberrations (TCGA data analyses, Fig. S4, S5, and S6), we observed that low expression of MAPK8, MAPK14, and SRC correlated with high risk and shorter overall survival in patients. On the other hand, low expression of CCL2 correlated with higher risk and shorter overall survival. The apparent discrepancy wherein high-risk patients (shorter overall

survival) demonstrated low MAPK8 and MAPK14 expression can be explained by the fact that highly proliferative cells have higher expression of these kinases. However, these cells, though more aggressive, are preferentially targeted by anti-proliferative agents resulting in elimination of cell populations with high kinase expression. Furthermore, these kinases are also targets of many molecularly targeted agents in clinical practice. As a consequence, though high MAPK8 and MAPK14 expression confers an aggressive phenotype (supported by our data here), these cells are better targeted by current therapies and hence may demonstrate an apparent contradiction in the form of longer survival in patients with higher expression.

Conclusions

Cancer, particularly GBM, is a complex disease that involves interplay of various genes and proteins. Understanding this complexity will help decipher the exact molecular mechanisms responsible for GBM and will facilitate optimal therapeutic targeting. However, this complexity is difficult to comprehend and unlikely to be understood solely by focusing on individual molecules or even single pathways. Our approach of identifying major multilevel hierarchical networks and coherent-gene modules within these networks would improve our understanding for clinical translation. This analysis is ready to be introduced in concrete medical practice.

A global view of the network of signaling molecules presents a viable mode of studying cancer. We believe that this approach will lead to construction of informative disease models that facilitate development of novel strategies for clinical management, including optimal individualized therapies for different patients.

Methods and Materials

The strategy

Our strategy is based on application of the contemporary systems-biology programs to the results of microarray data analyses and consisted of the following steps: (A) initial processing of microarray data (which continues by the three branches); (B) functional and hierarchical network analysis and network visualization; (C) elucidation of genes associated with brain cancers, namely, primary and secondary glioblastomas and analysis of pathways and networks containing these genes; and (D) development strategies of drugs administration.

Step A included (1) calculation of mean from replicates, (2) selecting set of genes by significance; and (3) calculating ratios *cancer expression* value to *normal average expression* value. In Step B we selected the top of differentially expressed genes and analyzed them by BiologicalNetworks program[9–11] creating integrative multilevel hierarchical pyramid of coherent-gene modules, TFs and TRs. In Step C we elucidated and analyzed the signaling networks and pathways generated from genes extracted from multilevel hierarchical pyramid by Ingenuity Pathway Analysis software (IPA®, Ingenuity Systems Inc., Redwood City, Calif.). Outcomes of this analysis allowed to elucidate specific gene–TF interactions for each glioblastoma and to catch sight of differences between them. Analysis of obtained pathways with IPA led to listing biological processes characteristic of each of them. Step D showed further directions for developing strategies of drugs administration based on analysis of hierarchical networks.

Sample preparation, microarray and data analysis

We used microarray data published by Michal Grzmil and colleagues.[70] As mentioned in[70] primary glioblastoma tissues were obtained from the operating room in line with the guidelines of the Ethical Committee of the University Hospitals of Basel and Dusseldorf and were processed as described by Maier and co-authors.[71] The microarray was created by RNA hybridization as described in,[72] the Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array (Santa Clara, Calif., USA), and its data was deposited in the Gene Expression Omnibus (GEO) database (Entry GEO15824, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15824). The microarray contains 54675 probes and three sample groups: 12 samples from the primary glioblastoma (GBM1), 3 samples from the secondary (GBM2), and 2 samples from normal brains (Norm) (other samples present in microarray were omitted due beyond the study scope).

Because the data distribution is non-normal, the data were subjected to a one-factor Kruskal-Wallis nonparametric test[8] with MultiExperiment Viewer (MeV), v. 4.8.1.[73] Like most nonparametric tests, it is performed on ranked data. The test statistic for a Kruskal-Wallis test is given by:

$$H = ((12 / (N \times (N + 1))) \times (\Sigma(T_i^2 / n_i))) - 3 \times (N + 1)$$

where N is the total amount of participants so $N = 17$ and n_i is the amount of samples in each group so $n_1 = 12$, $n_2 = 3$, and $n_3 = 2$; T_i is an average rank for the all samples. Significance was based on an Input Alpha: $p < 0.025$.

Gene–TF networks elucidation

To find genes that are regulated with the same transcription factors (TFs) and novel potential regulators of significant genes and TFs in glioblastoma, we used the *BiologicalNetworks* program,[9–11] an environment for integrative systems biology research. Genes exhibiting correlated expression patterns may be co-regulated by common TFs or may be functionally related, forming a functional module or a molecular complex.[74] Finding of the transcription factor binding sites (TFBSs) faces a number of problems.[75] For example, because of the ambiguity of binding TFs, the number of false-positives and false-negatives can be unexpectedly high.[75] Evolutionary conserved motifs or regions in the promoters of the homologous genes may function as TFBSs. In an attempt to identify those genes that are considered significant for glioblastoma, a systematic, large-scale search for TFs, their respective binding sites (i.e., TFBSs), and for a phylogenetically conserved promoter structures was conducted.[11] Starting with a set of gene pairs found to be differentially expressed in glioblastoma and normal tissues, *BiologicalNetworks* identified orthologous promoter regions from three species (*H. sapiens*, *Mus musculus*, and *Rattus norvegicus*), using a systematic approach. TFBSs common to promoter regions across species were first identified for individual genes. Using a modified phylogenetic footprinting method, a search was conducted for TFBSs that are enriched in the region of 6000 bases upstream of each transcription start site to 500 bases downstream of each transcription start of every gene in the gene pairs found and are conserved in the human, mouse, and rat genomes.

Binding sites and correspondent TFs were filtered for $p < 10^{-3}$ and examined for consistency.[11]

Gene–TF networks and pathway analysis

For network and pathway analysis the Ingenuity Interactive Pathway Analysis (IPA) package (Ingenuity Systems, Inc., Redwood City, Calif., USA) was used.

Statistical analysis

Because the microarray data has non-normal distribution and often a small sample size (in our case 2, 3, and 12), we used the Kruskal-Wallis nonparametric test[8]—a one-factor analysis for multiple samples with non-normal distribution. In *BiologicaNetworks*, for Gene Ontology term overrepresentation analysis the Fisher's exact test was used.[76] To build correlation networks the Pearson's correlations were calculated.[76]

Cancer Genome Atlas (TCGA) analysis

We investigated whether the expression levels of genes identified above in network analysis correlated with GBM patient's clinical outcomes by SurvExpress [77]. Biomarker and Cox Survival Analysis censored by survival months, and patient's survival data stratified by death. Genes in the GBM pyramid level 1, 2, 3 were analyzed in total 538 samples of TCGA.

Kaplan-Meier survival curve comparison and Cox proportional-hazards model were used to investigate the gene expression level and patients outcome according to their molecular subtypes [78] The Glioblastoma Bio Discovery Portal (GBM-BioDP) was queried and visualized for association of gene expression levels with clinical outcomes. The mRNA levels were defined in 3-Platform Aggregates and participants were patients in the Verhaak Extended database from TCGA (http://robtcga.nci.nih.gov).

We also investigated Kaplan-Meier Overall Survival and Disease-Free Survival data comparing gene expression levels with patient survival and disease-free survival by cBioPortal (http://www.cbioportal.org). The cBioPortal is developed and maintained by

the Computational Biology (cBio) Center at Memorial Sloan-Kettering Cancer Center. We analyzed and grouped patient data according to their gene expression levels as: up-regulated, normal and down-regulated (mRNA/miRNA expression Z-scores>1, >-1 and <1, <-1 respectively).

Table 1. Possible drugs for intervention at top levels of the GBM1 and GBM2 TFs and TRs hierarchy.

Pyramid level	Target gene	Drug	Approval status	Description	References
1	**TGFB**	**LY2157299**	Trials II	LY2157299 is an inhibitor of TGFB with IC_{50} = 56 nM.	79
2	**MAPK**	**trametinib and selumetinib**	tramatinib FDA approved	**MAPK/Ras/Raf/Mek/Erk** pathway is one of the targets. MEK inhibitors trametenib and selumetinib.	80
3	**CTNNB**	**celecoxib**	FDA approved	Celecoxib—The selective **COX2** inhibitor celecoxib has been FDA approved in patients with FAP as it has been shown to reduce polyp formation as well It has also been shown to reduce nuclear β-catenin (CTNNB1) in colorectal cancer cell lines similarly to other NSAIDs.	81
3	**SRC**	**dasatinib (Sprycel), saracatinib, bositinib**	dasatinib FDA approved	Constitutively active v-Src [**SRC**] protein increases activation of Estrogen receptor alpha [**ESR1**] protein that is mediated by Mekk **[MAP3K1]** protein. ESR1 also can be a target for drug intervention. Dasatinib is an oral multi-**BCR/Abl** and **Src** family tyrosine kinase inhibitor approved for first line use in patients with chronic myelogenous leukemia (CML).	82 83
3	**Mxi2**	**trametinib and selumetinib**	tramatinib FDA approved	**Mxi2** sustains **ERK1/2** phosphorylation in the nucleus by preventing **ERK1/2** binding to phosphatases. **MAPK/Ras/Raf/Mek/Erk** pathway is one of the targets. MEK inhibitors trametenib and selumetinib	84
4	**BCL2**	**obatoclax**	Trials I and II	BCL2 inhibitor with K_i = 220 nM	85,86
4	**CRK**	**tozasertib** (VX-680)	Trials I and II	Tozasertib—Aurora kinase inhibitor that reduces phosphorylation of CRK.	87
5	**CDKN2A**	**everolimus defrolimus, temsirolimus, tacrolimus, ridaforolimus**	everolimus FDA approved	Akt increases activation of CDKN2A through PI3K/AKT/mTOR pathway: everolimus (Zortress, Certican,Afinitor), defrolimus, temsirolimus, tacrolimus, ridaforolimus.	88
5	**STAT3**	**celecoxib**	FDA approved	**Celecoxib** (a COX-2 inhibitor) with a novel function to target STAT3 [Predictive STAT3 binding dissociation constant K_d = 8.6 μM].	89
3	**ERK2**	**trametinib and selumetinib**	tramatinib FDA approved	**MAPK/Ras/Raf/Mek/Erk** pathway is one of the targets. MEK inhibitors trametenib and selumetinib.	80
4	**ESR1**	**tamoxifen**		**Tamoxifen**—ESR antagonist (selective	90

				estrogen receptor modulators (SERM).	

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References

1. F. B. Furnari, T. Fenton, R. M. Bachoo, A. Mukasa, J. M. Stommel, A. Stegh, W. C. Hahn, K. L. Ligon, D. N. Louis, C. Brennan, L. Chin, R. A. DePinho and W. K. Cavenee, Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev.,* 2007, **21**, 2683–2710.
2. S. Kesari, Understanding glioblastoma tumor biology: the potential to improve current diagnosis and treatments. *Semin. Oncol.* 2011, **38 Suppl 4**, S2–S10.
3. Y. P. Ramirez, J. L. Weatherbee, R. T. Wheelhouse and A. H. Ross, Glioblastoma multiforme therapy and mechanisms of resistance. *Pharmaceuticals* (Basel), 2013 **6**, 1475–1506.
4. P. Wesseling, D. J. Ruiter and P. C. Burger, Angiogenesis in brain tumors; pathobiological and clinical aspects. *J. Neurooncol*., 1997, **32**, 253–265.
5. P. Y. Wen and S. Kesari, Malignant Gliomas in Adults. *N. Engl. J. Med.*, 2008, **359**, 492–507.

6. D. J. Brat and E. J. Van Meir, Vaso-occlusive and pro-thrombotic mechanisms associated with tumor hypoxia, necrosis and accelerated growth in glioblastoma. *Lab. Invest.,* 2004, **84**, 397–405.
7. F. Lefranc, J. Brotchi and R. Kiss, Possible future issues in the treatment of glioblastomas: special emphasis on cell migration and the resistance of migrating glioblastoma cells to apoptosis. *J. Clin. Oncol.*, 2005, **23**, 2411–2422 (2005).
8. W. H. Kruskal and W. A. Wallis, Use of ranks in one-criterion variance analysis. *J. Am. Stat. Assoc.*, 1952, **47**, 583–621.
9. S. Kozhenkov, Y. Dubinina, M. Sedova, A. Gupta, J. Ponomarenko and M. Baitaluk, BiologicalNetworks 2.0—an integrative view of genome biology data. *BMC Bioinformatics*, 2010, **11**, 610.
10. M. Baitaluk, S. Kozhenkov, Y. Dubinina and J. Ponomarenko, IntegromeDB: an integrated system and biological search engine. *BMC Genomics*, 2012, **13**, 35.
11. I. F. Tsigelny, V. L. Kouznetsova, M. Baitaluk and J.-P. Changeux, A hierarchical coherent-gene-group model for brain development. *Genes Brain Behav.*, 2013, **12**, 147–165.
12. H. Jeong, S. P. Mason, A. L. Barabasi and Z. N. Oltvai, Lethality and centrality in protein networks. *Nature*, 2001, **411**, 41–42.
13. J. Kim, I. Kim, S. K. Han, J. U. Bowie and S. Kim, Network rewiring is an important mechanism of gene essentiality change. *Sci. Rep.,* 2012, **2**, 900.
14. M. R. J. Carlson, B. Zhang, Z. Fang, P. S. Mischel, S. Horvath and S. F. Nelson, Gene connectivity, function, and sequence conservation: predictions from modular yeast co-expression networks. *BMC Genomics*, 2006, **7**, 40.

15. W. Wick, U. Naumann and M. Weller, Transforming growth factor-beta: a molecular target for the future therapy of glioblastoma. *Curr. Pharm. Des.,* 2006, **12**, 341–349.

16. A. Bruna, R. S. Darken, F. Rojo, A. Ocaña, S. Peñuelas, A. Arias, R. Paris, A. Tortosa, J. Mora, J. Baselga and J. Seoane, High TGFbeta-Smad activity confers poor prognosis in glioma patients and promotes cell proliferation depending on the methylation of the PDGF-B gene. *Cancer Cell,* 2007, **11**, 147–160.

17. J. U. Wurthner, D. B. Frank, A. Felici, H. M. Green, Z. Cao, M. D. Schneider, J. G. McNally, R. J. Lechleider and A. B. Roberts, Transforming growth factor-beta receptor-associated protein 1 is a Smad4 chaperone. *J. Biol. Chem.*, 2001, **276**, 19495–19502.

18. X. He, *Identification of the novel Akt2 interacting proteins RPS25 and eIF3c: characterization of interaction and physiological consequences.* Doctor Degree Dissertation, Department of Biology, Eberhard Karls University of Tübingen, Germany, 2007.

19. T. Akagi, K. Murata, T. Shishido and H. Hanafusa, v-Crk activates the phosphoinositide 3-kinase/AKT pathway by utilizing focal adhesion kinase and H-Ras. *Mol. Cell. Biol.*, 2002, **22**, 7015–7023.

20. S. Pugazhenthi, A. Nesterova, C. Sable, K. A. Heidenreich, L. M. Boxer, L. E. Heasley and J. E. Reusch, Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J Biol Chem.*, 2000, **275**, 10761–10766 (2000).

21. A. Wu, J. Wei, L. Y. Kong, Y. Wang, W. Priebe, W. Qiao, R. Sawaya and A. B. Heimberger, Glioma cancer stem cells induce immunosuppressive macrophages/microglia. *Neuro Oncol.*, 2010, **12**, 1113–1115.

22. J. W. Pollard, Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer*, 2004, **4**, 71–78.

23. M. Sliwa, D. Markovic, K. Gabrusiewicz, M. Synowitz, R. Glass, M. Zawadzka, A. Wesolowska, H. Kettenmann and B. Kaminska, The invasion promoting effect of microglia on glioblastoma cells is inhibited by cyclosporin A. *Brain*, 2007, **130**, 476–489.

24. G. J. Baay-Guzman, I. G. Bebenek, M. Zeidler, R. Hernandez-Pando, M. I. Vega, E. A. Garcia-Zepeda, G. Antonio-Andres, B. Bonavida, M. Riedl, E. Kleerup, D. P. Tashkin, O. Hankinson and S. Huerta-Yepez, HIF-1 expression is associated with CCL2 chemokine expression in airway inflammatory cells: implications in allergic airway inflammation. *Respir. Res.*, 2012, **13**, 60.

25. A. E. Hinojosa, B. Garcia-Bueno, J. C. Leza and J. L. M. Madrigal, CCL2/MCP-1 modulation of microglial activation and proliferation. *J. Neuroinflammation,* 2011, **8**, 77.

26. J. Wei, K. Gabrusiewicz and A. Heimberger, The controversial role of microglia in malignant gliomas. *Clin. Dev. Immunol.,* 2013, **2013**, 285246.

27. L. C. Lin, S. L. Hsu, C. L. Wu, W. C. Liu, H. T. Chiu and C. M. Hsueh, Peroxisome proliferator-activated receptor Gamma responsible for TGFβ-induced epithelial mesenchymal transition (EMT) and tumor invasion of NSCLC cells (H460). *Eur. J. Cancer,* 2009, Suppl. **7**, 124.

28. E. H. Nam, Y. Lee, Y. K. Park, J. W. Lee and S. Kim, ZEB2 upregulates integrin α5 expression through cooperation with Sp1 to induce invasion during epithelial-mesenchymal transition of human cancer cells. *Carcinogenesis,* 2012, **33**, 563–571.

29. S. El Hallani, B. Boisselier, F. Peglion, A. Rousseau, C. Colin, A. Idbaih, Y. Marie, K. Mokhtari, J.L. Thomas, A. Eichmann, J. Y. Delattre, A. J. Maniotis and M. Sanson, A new alternative mechanism in glioblastoma vascularization: tubular vasculogenic mimicry. *Brain,* 2010,**133**, 973–982.

30. R. J. Phillips, J. Mestas, M. Gharaee-Kermani, M. D. Burdick, A. Sica, J. A. Belperio, M. P. Keane and R. M. Strieter, Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha. *J. Biol. Chem.,* 2005, **280**, 22473–22481.

31. S. K. Singh, R. Bhardwaj, K. M. Wilczynska, C. I. Dumur and T. Kordula, A complex of nuclear factor I-X3 and STAT3 regulates astrocyte and glioma migration through the secreted glycoprotein YKL-40. *J. Biol. Chem.,* 2011, **286**, 39893–39903.

32. F. Meggio and L.A. Pinna, One-thousand-and-one substrates of protein kinase CK2? *FASEB J.,* 2003, **17**, 349–368.

33. L. Bordin, C. Vargiu, S. Colombatto, G. Clari, G. Testore, A. Toninello and M. A. Grillo, Casein kinase 2 phosphorylates recombinant human spermidine/spermine N1-acetyltransferase on both serine and threonine residues. *Biochem. Biophys. Res. Commun.,* 1996, **229**, 845–851.

34. B. Casar, J. Rodríguez, G. Gibor, R. Seger and P. Crespo, Mxi2 sustains ERK1/2 phosphorylation in the nucleus by preventing ERK1/2 binding to phosphatases. *Biochem. J.,* 2012, **441**, 571–578.

35. Y. Zhang, F. Q. Chen, Y. H. Sun, S. Y. Zhou, T. Y. Li and R. Chen, Effects of DNMT1 silencing on malignant phenotype and methylated gene expression in cervical cancer cells. *J. Exp. Clin. Cancer. Res.,* 2011, **30**, 98.

36. S. K. Niture and A. K. Jaiswal, Nrf2 protein up-regulates antiapoptotic protein Bcl-2 and prevents cellular apoptosis. *J. Biol. Chem.,* 2012, **287**, 9873–9886.

37. P. Shelton and A. K. Jaiswal, The transcription factor NF-E2-related Factor 2 (Nrf2): a protooncogene? *FASEB J.*. 2013, **27**, 414–423.

38. R. M. Perera and N. Bardeesy, Cancer: when antioxidants are bad. *Nature*,2011, **475**, 43–44.

39. K. Du and M. Montminy, CREB is a regulatory target for the protein kinase Akt/PKB. *J. Biol. Chem.,* 1998, **273**, 32377–32379.

40. T. Simoncini, A. Hafezi-Moghadam, D. P. Brazil, K. Ley, W. W. Chin and J. K. Liao, Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature,* 2000, **407**, 538–541.

41. P. Mannella and R. D. Brinton, Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action. *J. Neurosci.,* 2006, **26**, 9439–9447.

42. M. Keniry, M. M. Pires, S. Mense, C. Lefebvre, B. Gan, K. Justiano, Y. K. Lau, B. Hopkins, C. Hodakoski, S. Koujak, J. Toole, F. Fenton, A. Calahan, A. Califano, R. A. DePinho, M. Maurer and R. Parsons, Survival factor NFIL3 restricts FOXO-induced gene expression in cancer. *Genes Dev.,* 2013, **27**, 916–927.

43. U. T. Sankpal, S. Goodison, M. Abdelrahim and R. Basha, Targeting Sp1 transcription factors in prostate cancer therapy. *Med Chem.,* 2011, **7**, 518–525.

44. H. Zhang H, X. Zhang X, X. Wu, W. Li, P. Su, H. Cheng, L. Xiang, P. Gao and G. Zhou. Interference of Frizzled 1 (FZD1) reverses multidrug resistance in breast cancer cells through the Wnt/β-catenin pathway. *Cancer Lett.,* 2012, **323**, 106–113.

45. H. Gao H, G. Chakraborty, A. P. Lee-Lim, Q. Mo, M. Decker, A. Vonica, R. Shen, E. Brogi, A. H. Brivanlou and F. G. Giancotti. The BMP inhibitor Coco reactivates breast cancer cells at lung metastatic sites. *Cell,* 2012, **150**, 764–779.

46. J. E. Cobb, S. G. Blanchard, E. G. Boswell, K. K. Brown, P. S. Charifson, J. P. Cooper, J. L. Collins, M. Dezube, B. R. Henke, E. A. Hull-Ryde, D. H. Lake, J. M. Lenhard, W. Oliver Jr, J. Oplinger, M. Pentti, D. J. Parks, K. D. Plunket and W. Q. Tong, N-(2-Benzoylphenyl)-L-tyrosine PPARgamma agonists. 3. Structure-activity relationship and optimization of the N-aryl substituent. *J. Med. Chem.*, 1998, **41**, 5055–5069.

47. N. Kaplan-Albuquerque, Y. E. Bogaert, V. Van Putten, M. C. Weiser-Evans and R. A. Nemenoff, Patterns of gene expression differentially regulated by platelet-derived growth factor and hypertrophic stimuli in vascular smooth muscle cells: markers for phenotypic modulation and response to injury. *J. Biol. Chem.,* 2005, **280**, 19966–19976..

48. J. W. Tullai, M. E. Schaffer, S. Mullenbrock, S. Kasif and G. M. Cooper, Identification of transcription factor binding sites upstream of human genes regulated by the phosphatidylinositol 3-kinase and MEK/ERK signaling pathways. *J. Biol. Chem.,* 2004, **279**, 20167–20177.

49. R. R. Mattingly, J. M. Kraniak, J. T. Dilworth, P. Mathieu, B. Bealmear, J. E. Nowak, J. A. Benjamins, M. A. Tainsky and J. J. Reiners, The mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor PD184352 (CI-1040) selectively induces apoptosis in malignant schwannoma cell lines. *J. Pharmacol. Exp. Ther.,* 2006, **316**, 456–465.

50. P. Kurschat, D. Bielenberg, M. Rossignol-Tallandier, A. Stahl and M. Klagsbrun, Neuron restrictive silencer factor NRSF/REST is a transcriptional repressor of neuropilin-1 and diminishes the ability of semaphorin 3A to inhibit keratinocyte migration. *J. Biol. Chem.,* 2006, **281**, 2721–2729.

51. D. Cilloni and G. Saglio, Molecular pathways: BCR-ABL. *Clin. Cancer Res.,* 2012, **18**, 930–937.

52. S. E. Tran, T. H. Holmstrom, M. Ahonen, V. M. Kahari and J. E. Eriksson, MAPK/ERK overrides the apoptotic signaling from Fas, TNF, and TRAIL receptors. *J. Biol. Chem.,* 2001, **276**, 16484–16490.

53. L. Zhang, J. M. Valdez, B. Zhang, L. Wei, J. Chang and L. Xin, ROCK inhibitor Y-27632 suppresses dissociation-induced apoptosis of murine prostate stem/progenitor cells and increases their cloning efficiency. *PLoS One,* 2011, **6**, e18271.

54. E. Sahai, M. F. Olson and C. J. Marshall, Cross-talk between Ras and Rho signalling pathways in transformation favours proliferation and increased motility. *EMBO J.,* 2001, **20**, 755–766.

55. S. Zhou, A. Schmelz, T. Seufferlein, Y. Li, J. Zhao and M. G. Bachem, Molecular mechanisms of low intensity pulsed ultrasound in human skin fibroblasts. *J. Biol. Chem.,* 2004, **279**, 54463–54469.

56. R. J. Benschop, D. Melamed, D. Nemazee and J. C. Cambier, Distinct signal thresholds for the unique antigen receptor-linked gene expression programs in mature and immature B cells. *J. Exp. Med.,* 1999, **190**, 749–756.

57. A. Jacob, D. Cooney, M. Pradhan and K. M. Coggeshall, Convergence of signaling pathways on the activation of ERK in B cells. *J. Biol. Chem.,* 2002, **277**, 23420–23426.

58. P. M. Hogarth and G. A. Pietersz, Fc receptor-targeted therapies for the treatment of inflammation, cancer and beyond. *Nat. Rev. Drug Disc.,* 2012, **11**, 311–331.

59. K. W. Kim, M. L. Cho, M. K. Park, C. H. Yoon, S. H. Park, S. H. Lee and H. Y. Kim, Increased interleukin-17 production via a phosphoinositide 3-kinase/Akt and nuclear factor kappaB-dependent pathway in patients with rheumatoid arthritis. *Arthritis Res. Ther.,* 2005, **7**, R139–R148.

60. A. S. Naura, H. Kim, J. Ju, P. C. Rodriguez, J. Jordan, A. D. Catling, B. M. Rezk, Z. Y. Abd Elmageed, K. Pyakurel, A. F. Tarhuni, M. Q. Abughazleh, Y. Errami, M. Zerfaoui, A. C. Ochoa and A. H. Boulares, Minocycline blocks asthma-associated inflammation in part by interfering with the T cell receptor-nuclear factor κB-GATA-3-IL-4 axis without a prominent effect on poly(ADP-ribose) polymerase. *J. Biol. Chem.,* 2013, **288**, 1458–468.

61. R. Derynck, R. J. Akhurst and A. Balmain, TGF-beta signaling in tumor suppression and cancer progression. *Nat. Genet.,* 2001, **29**, 117–129.

62. F. Winkler, S. V. Kozin, R. T. Tong, S. S. Chae, M. F. Booth, I. Garkavtsev, L. Xu, D. J. Hicklin, D. Fukumura, E. di Tomaso, L. L. Munn and R. K. Jain, Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of

oxygenation, angiopoietin-1, and matrix metalloproteinases. *Cancer Cell,* 2004, **6**, 553–563.

63. T. T. Batchelor, A. G. Sorensen, E. di Tomaso, W. T. Zhang, D. G. Duda, K. S. Cohen, K. R. Kozak, D. P. Cahill, P. J. Chen, M. Zhu, M. Ancukiewicz, M. M. Mrugala, S. Plotkin, J. Drappatz, D. N. Louis, P. Ivy, D. T. Scadden, T. Benner, J. S. Loeffler, P. Y. Wen and R. K. Jain, AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. *Cancer Cell,* 2007, **1**, 83–95.
64. J. Folkman, Angiogenesis: an organizing principle for drug discovery? *Nat. Rev. Drug. Discov.,* 2007, **6**, 273–286.
65. M. H. Cohen, Y. L. Shen, P. Keegan and R. Pazdur, FDA drug approval summary: bevacizumab (Avastin) as treatment of recurrent glioblastoma multiforme. *Oncologist,* 2009, **14**, 1131–1138.
66. E. E. Schmidt, K. Ichimura, G. Reifenberger and V. P. Collins, CDKN2 (p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. *Cancer Res.,* 1994, **54**, 6321–6324.
67. K. Ueki, Y. Ono, J. W. Henson, J. T. Efird, A. von Deimling and D. N. Louis, CDKN2/p16 or RB alterations occur in the majority of glioblastomas and are inversely correlated. *Cancer Res.,* 1996, **56**, 150–153.
68. H. Strik, M. Deininger, J. Streffer, E. Grote, J. Wickboldt, J. Dichgans, M. Weller and R. Meyermann, BCL-2 family protein expression in initial and recurrent glioblastomas: modulation by radiochemotherapy. *J. Neurol. Neurosurg. Psychiatry,* 1999, **67**, 763–768.

69. F. E. McDonald, J. W. Ironside, A. Gregor, B. Wyatt, M. Stewart, R. Rye, J. Adams and H. W. Potts, The prognostic influence of bcl-2 in malignant glioma. *Br. J. Cancer,* 2002, **86**, 1899–1904.

70. M. Grzmil, P. Morin Jr., M. M. Lino, A. Merlo, S. Frank, Y. Wang, G. Moncayo and B. Hernmings, MAP kinase-interacting kinase 1 regulates SMAD2-dependent TGF-β signaling pathway in human glioblastoma. *Cancer Res.,* 2011, **71**, 2392–2402.

71. D. Maier, D. Comparone, E. Taylor, Z. Zhang, O. Gratzl, E. G.Van Meir, R. J. Scott and A. Merlo, New deletion in low-grade oligodendroglioma at the glioblastoma suppressor locus on chromosome 10q25–26. *Oncogene,* 1997, **15**, 997–1000.

72. M. Grzmil, T. Rzymski, M. Milani, A. L. Harris, R. G. Capper, N. J. Saunders, A. Salhan, J. Ragoussis and C. J. Norbury, An oncogenic role of eIF3e/INT6 in human breast cancer. *Oncogene,* 2010, **29**, 4080–4089.

73. A. I. Saeed, V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa, T. Currier, M. Thiagarajan, A. Sturn, M. Snuffin, A. Rezantsev, D. Popov, A. Ryltsov, E. Kostukovich, I. Borisovsky, Z. Liu, A. Vinsavich, V. Trush and J. Quackenbush, TM4: a free, open-source system for microarray data management and analysis. *Biotechniques,* 2003, **34**, 374–378.

74. S. Fessele, H. Maier, C. Zischek, P. J. Nelson and T. Werner, Regulatory context is a crucial part of gene function. *Trends Genet.*, 2002, **18**, 60–63.

75. E. Barta, Comparative genomics-based orthologous promoter analysis using the DoOP database and the DoOPSearch web tool. *Methods Mol. Biol.*, 2007, **395**, 319–328.

76. M. Baitaluk, M. Sedova, A. Ray and A. Gupta, BiologicalNetworks: visualization and analysis tool for systems biology. *Nucleic Acid Res.*, 2006, **34**, W466–W471.

77. R. Aguirre-Gamboa, H. Gomez-Rueda, E. Martinez-Ledesma, A. Martinez-Torteya, R. Chacolla-Huaringa, A. Rodriguez-Barrientos, J. G. Tamez-Pena and V. Trevino, *PLoS One*, **8**, e74250.

78. R. G. Verhaak, K. A. Hoadley, E. Purdom, V. Wang, Y. Qi, M. D. Wilkerson, C. R. Miller, L. Ding, T. Golub, J. P. Mesirov, G. Alexe, M. Lawrence, M. O'Kelly, P. Tamayo, B. A. Weir, S. Gabriel, W. Winckler, S. Gupta, L. Jakkula, H. S. Feiler, J. G. Hodgson, C. D. James, J. N. Sarkaria, C. Brennan, A. Kahn, P. T. Spellman, R. K. Wilson, T. P. Speed, J. W. Gray, M. Meyerson, G. Getz, C. M. Perou and D. N. Hayes, *Cancer Cell*, **17**, 98-110.

79. R. J. Akhurs and A. Hata, Targeting the TGFβ signalling pathway in disease. *Nat. Rev. Drug Discov.,* 2012, **11**, 790–811.

80. J. R. Infante, K. P. Papadopoulos, J. C. Bendell, A. Patnaik, H. A. Burris 3rd, D. Rasco D, S. F. Jones, L. Smith, D. S. Cox, M. Durante, K. M. Bellew, J. J. Park, N. T. Le and A. W. Tolcher, A phase 1b study of trametinib, an oral Mitogen-activated protein kinase kinase (MEK) inhibitor, in combination with gemcitabine in advanced solid tumours. *Eur. J. Cancer,* 2013, **49**, 2077–2085.

81. J. J. Xia, L. B. Pei, J. P. Zhuang, Y. Ji, G. P. Xu, Z. P. Zhang, N. Li and J. L. Yan, Celecoxib inhibits β-catenin-dependent survival of the human osteosarcoma MG-63 cell line. *J. Int. Med. Res.,* 2010, **38**, 1294–1304.

82. W. Feng, P. Webb, P. Nguyen, X. Liu, J. Li, M. Karin and P. J. Kushner, Potentiation of estrogen receptor activation function 1 (AF-1) by Src/JNK through a serine 118-independent pathway. *Mol. Endocrinol.,* 2001, **15**, 32–45.

83. FDA. FDA approves additional medical indication for Sprycel. www.fda.gov. FDA. Retrieved 22 March 2014.

84. B. Casar, J. Rodríguez, G. Gibor, R. Seger and P. Crespo, Mxi2 sustains ERK1/2 phosphorylation in the nucleus by preventing ERK1/2 binding to phosphatases. *Biochem. J.,* 2012, **441**, 571–578.

85. M. Nguyen, R. C. Marcellus, A. Roulston, M. Watson, L. Serfass, S. R. Murthy Madiraju, D. Goulet, J. Viallet, L. Bélec, X. Billot, S. Acoca, E. Purisima, A. Wiegmans, L. Cluse, R. W. Johnstone, P. Beauparlant and G. C. Shore, Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proc. Natl. Acad. Sci. U. S. A.,* 2007, **104**, 19512–19517.

86. F. Basit, S. Cristofanon and S. Fulda, Obatoclax (GX15-070) triggers necroptosis by promoting the assembly of the necrosome on autophagosomal membranes. *Cell Death Differ.,* 2013, **20**, 1161–1673.

87. S. Okabe, T. Tauchi, Y. Tanaka, S. Kimura, T. Maekawa and K. Ohyashiki, Activity of histone deacetylase inhibitors and an Aurora kinase inhibitor in BCR-ABL-expressing leukemia cells: Combination of HDAC and Aurora inhibitors in BCR-ABL-expressing cells. *Cancer Cell Int.,* 2013, **13**, 32.

88. C. O'Neill, Y. Li and X. L. Jin, Survival signaling in the preimplantation embryo. *Theriogenology,* 2012, **77**, 773–784.

89. L. Lin, H. Li, M. Xu, Z. Zhao, V. Olson, Y. Xu, C. Li and J. Lin, Abstract 4772: Novel drug discovery approach targeting STAT3 for breast cancer therapy using MLSD and drug repositioning. *Cancer Res.,* 2012, **72**, Supp. 1, 4772.

90. V. C. Jordan, Tamoxifen (ICI46, 474) as a targeted therapy to treat and prevent breast cancer. *Br. J. Pharmacol.,* 2006, **147**, (Suppl 1), S269–S276.

Figures Legends

Fig. 1 Primary glioblastoma (GBM1) hierarchy. Gray rounded squares—coherent-gene modules, red rectangles—genes, (CGMs), yellow triangles—transcription factors (TFs), yellow diamonds—transcription regulators (TRs). (**A**) Level 1 hierarchical network. Several genes included in CGMs and the only top TR—TGFB (TGFβ), (**B**) Level 3 of hierarchy. (**C**) Level 9. (**D**) Level 12. These three panels (B–C) show gradual conversion from the top level (**A**) to the bottom (**E**). (**E**) Bottom level full network including 442 genes, 155 TFs, and 1605 TRs. (**F**) A hierarchical control pyramid of TFs and TRs that regulate all the CGMs.

Fig. 2 Hierarchical pyramid for primary glioblastoma. This figure shows a close-up of the control pyramid shown in Fig. 1F including TFs and TRs. Each level is responsible for control of specific functions.

Fig. 3 Angiogenesis-controlling CGM3's of GBM1. Networks of genes included in CGM3 used for analysis. Blue—TGFB1, red—genes responsible for angiogenesis. (**A***) Network 1*. TGFB1 is hub regulating a number of high-connected genes, TRs, and TFs: ERK1/2, CDH2, VIM, and PDGFRB. (**B**) *Network 3*. One can see that TGFB1 regulates two of the most important hubs—VEGF and HBEGFR—that have high connectivity. (**C**) Distribution of main gene functions for CGM3 of GBM1.

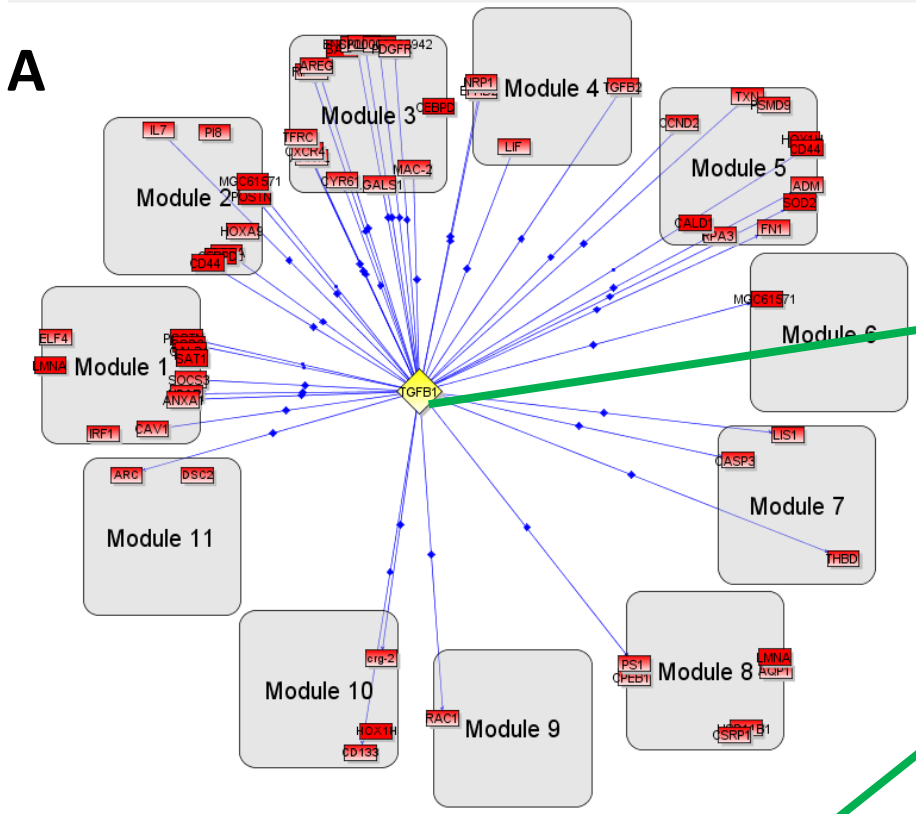
Fig. 4 Cell-proliferation-controlling CGM1's of GBM1. Networks of genes included in CGM1 taken for the analysis. Blue—TGFB1, orange—genes responsible for cell proliferation. (**A**) *Network 1*. TGFB1 is hub regulating a number of high-connected genes: PDGF_BB, DAB2, MAPK, and ERK1/2. (**B**) *Network 2*. One can see that TGFB1 regulates three of the most important hubs—VEGF, ERK, and p38 MAPK—that have high connectivity. (**C**) Distribution of main gene functions for CGM1 of GBM1.

Fig. 5 Secondary glioblastoma (GBM2) hierarchy. Gray rounded squares—coherent-gene modules, red rectangles—genes, (CGMs), yellow triangles—transcription factors (TFs), yellow diamonds—transcription regulators (TRs). (**A**) Level 1 hierarchical network. Several genes included in CGMs and the only top TR—JUN, (**B**) Level 3 of hierarchy. (**C**) Level 9. (**D**) Level 12. These three panels (**B–C**) show gradual conversion from the top level (**A**) to the bottom (**E**). (**E**) Bottom level full network including 586 genes, 147 TFs, and 1694 TRs. (**F**) A hierarchical control pyramid of TFs and TRs that regulate all the CGMs.

Fig. 6 Hierarchical pyramid of secondary glioblastoma. This figure shows a close-up of the control pyramid shown in Fig. 5F including TFs and TRs. Each level is responsible for control of specific functions.

Fig. 7 Cell-death-controlling CGM1's of GBM2. Networks of genes included in CGM1 taken for the analysis. Blue—TGFB1, green—genes, TFs, and TRs responsible for cell death and survival. (**A**) *Network 4*. Cell-death-related genes including caspases are regulated by TGFB1 and PDGF_BB. (**B**) *Network 2* is significantly related to the cell death and immune response. It includes tumor-necrosis factor (TNF) and its family, which regulate hubs such as ERK1/2, IgG, SOCS3, and MHC Class II complex. (**C**) Distribution of main gene functions for CGM1 of GBM2.

Fig. 8 Cell-death- and immune-response-controlling CGM2 of GBM2. Networks of genes included in CGM2 taken for the analysis. Blue—TGFB1 and TGFB2 TRs, green—genes, TFs, and TRs responsible for cell death and survival. (**A**) *Network 4*. Cell-death-related genes including caspases are regulated by TGFB1 and PDGF_BB. It also contains immune response genes: MHC Class 1 and activating it B2M, Immunoglobulins, TCR, and CD8A. (**B**) *Network's 1* cell-death function is controlled by the TGFB1 and TGFB2 in contact with PDGF. Complex Ap1 is involved in this network regulation. (**C**) Distribution of main gene functions for CGM2of GBM2.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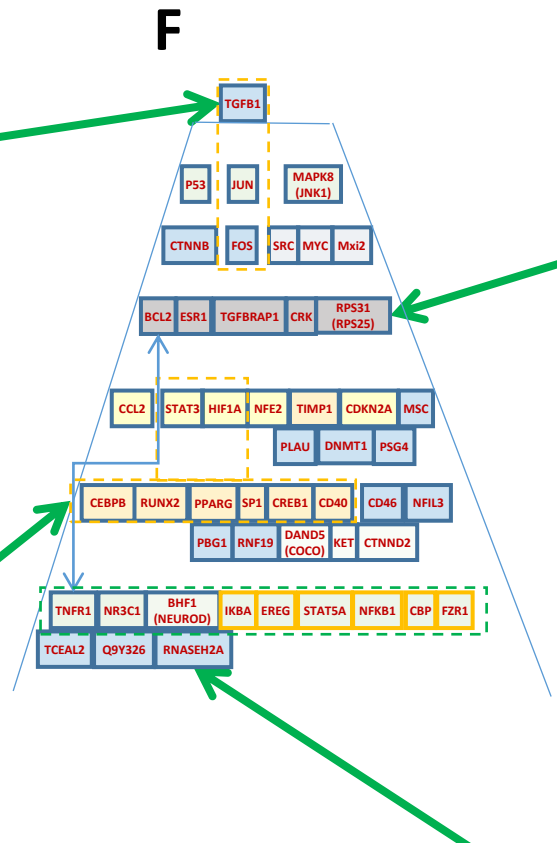
TGFB1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MAPK8 (JNK1)
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BCL2
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CRK
RPS31 (RPS25)
CCL2
STAT3
HIF1A
NFE2
TIMP1
CDKN2A
MSC
PLAU
DNMT1
PSG4
CEBPB
RUNX2
PPARG
SP1
CREB1
CD40
CD46
NFIL3
PBG1
RNF19
DAND5 (COCO)
KET
CTNND2
TNFR1
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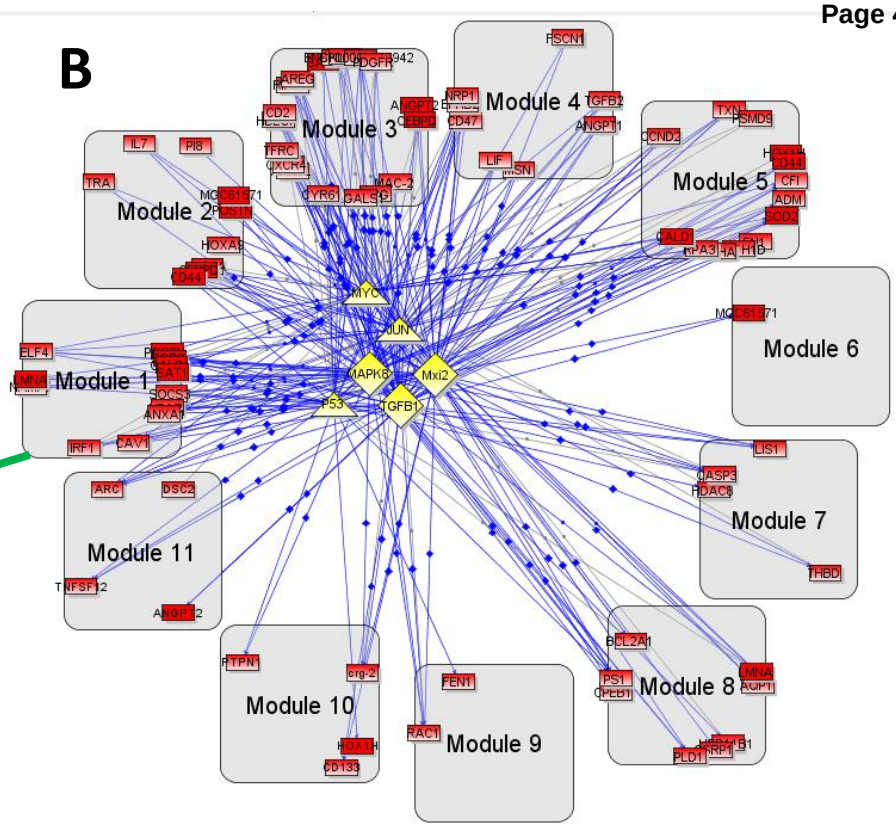
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MYC
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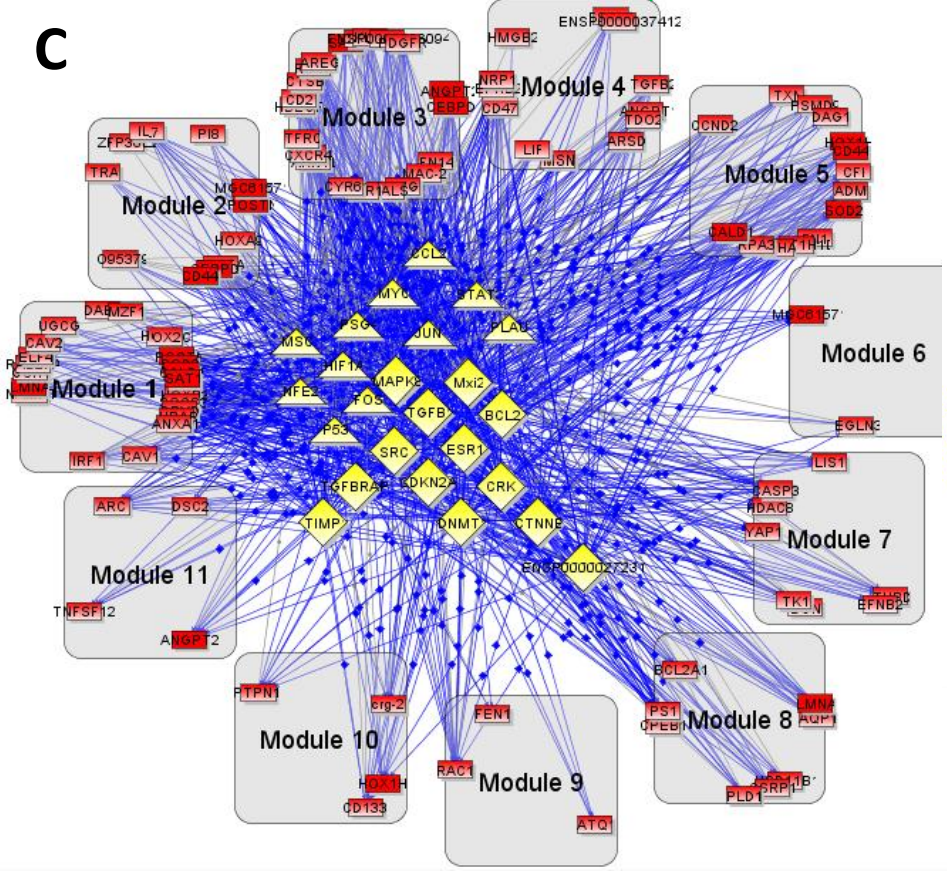
MAPK8
Mxi2
TGFB1
PS3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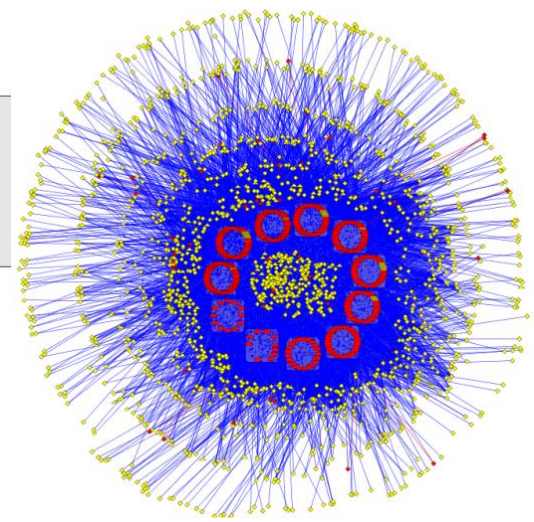
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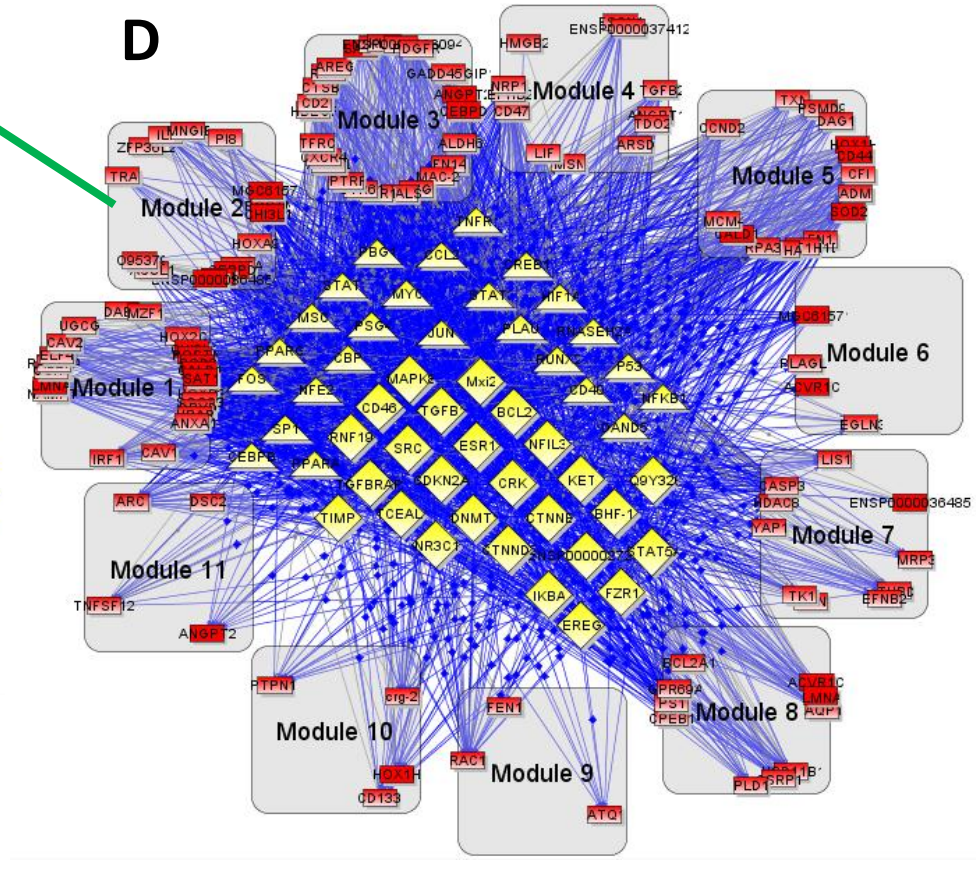
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Module 11

Figure 1

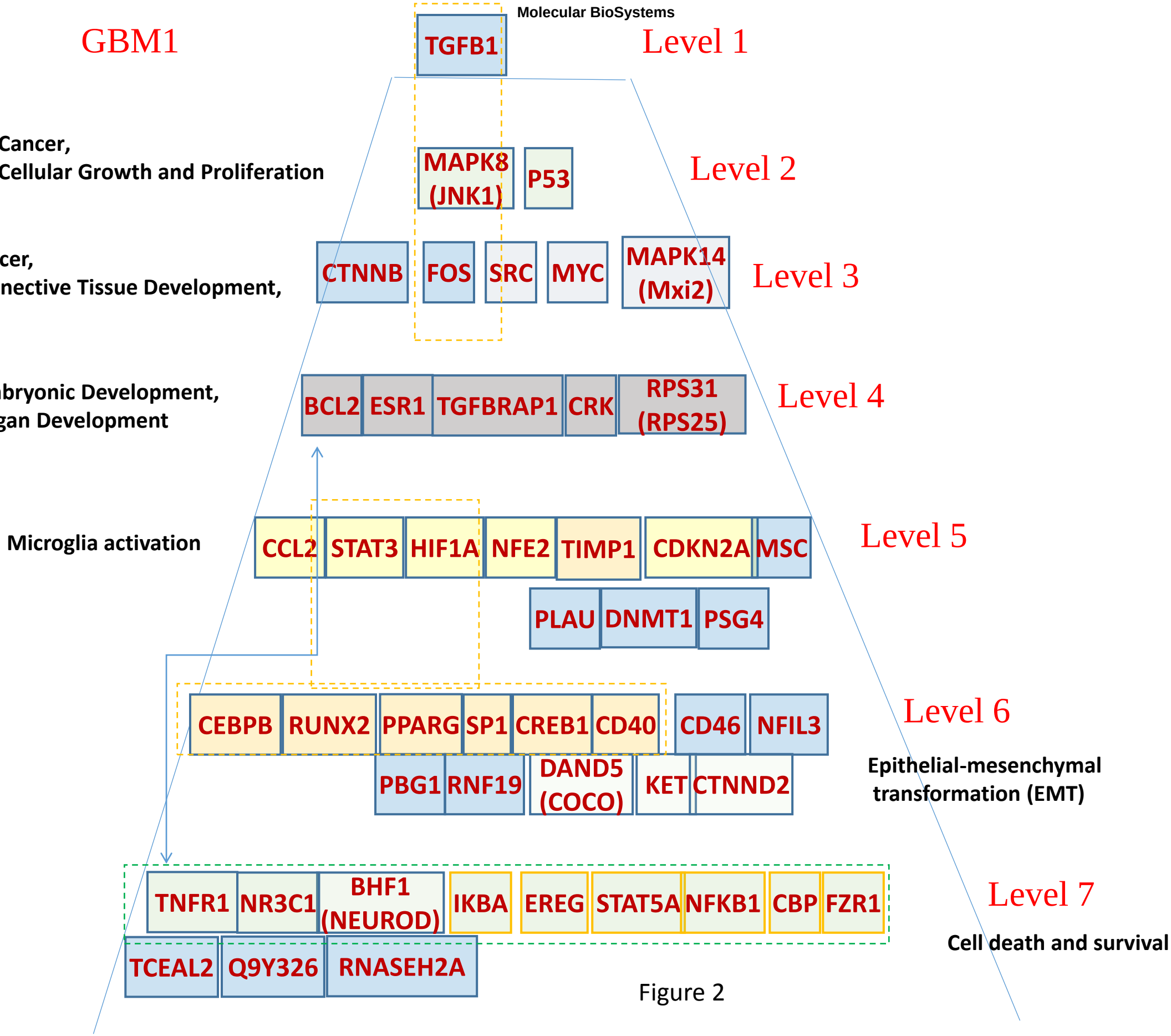
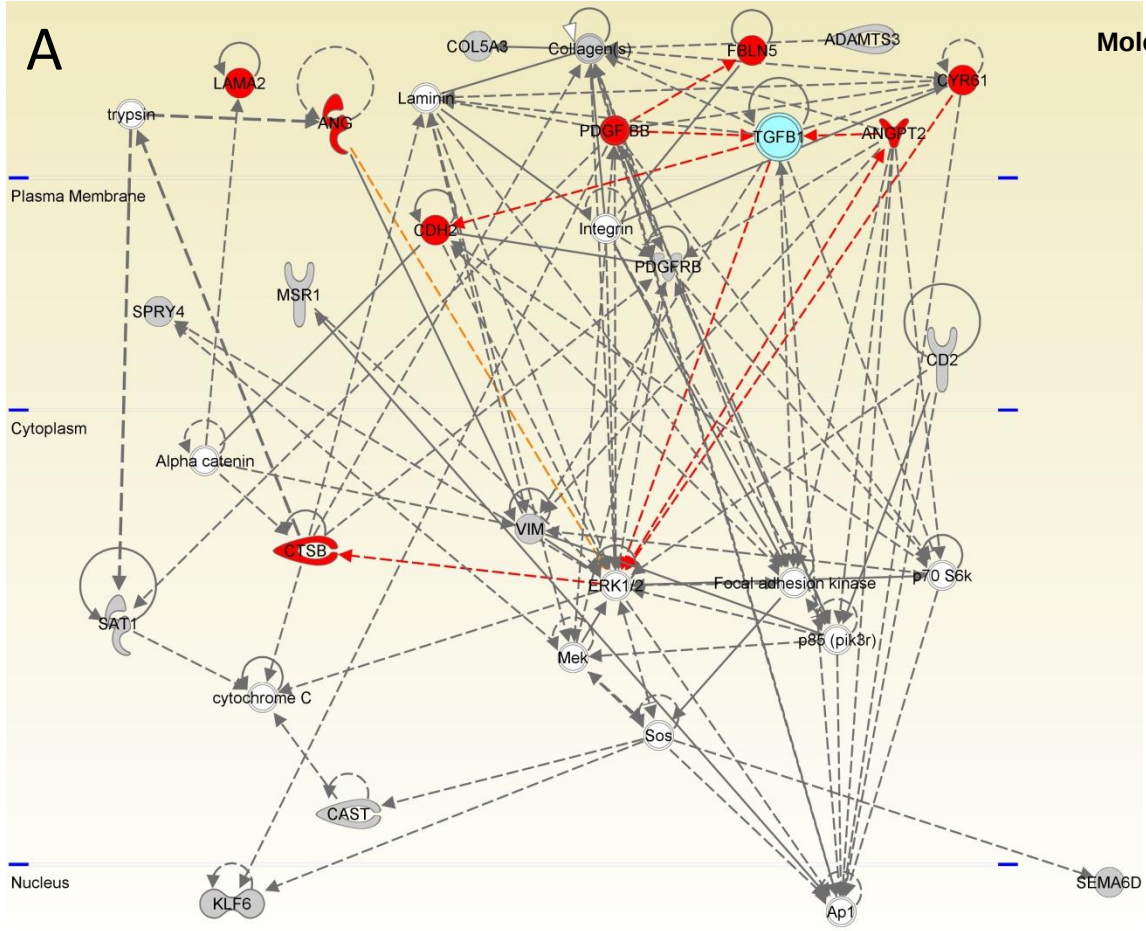
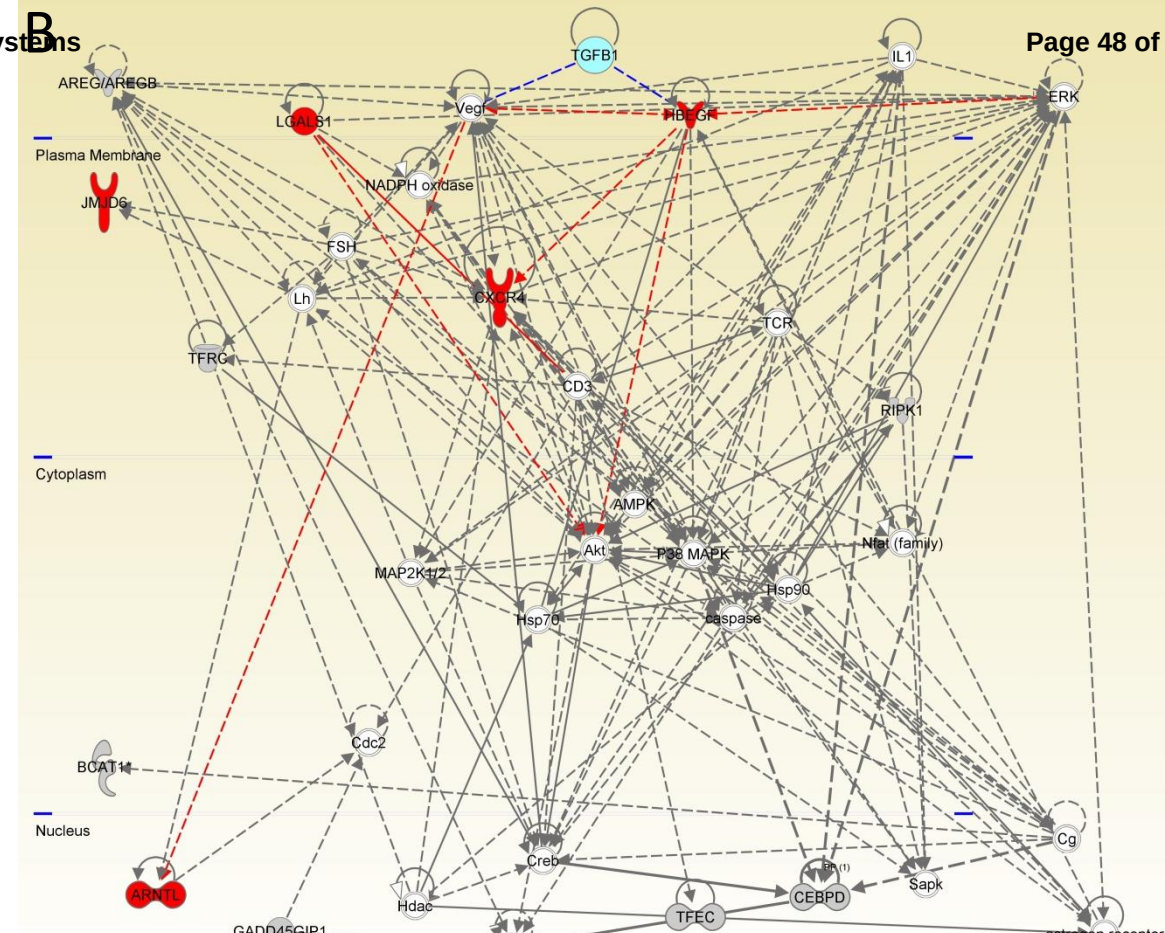

GBM1
TGFB1
Level 1
Cancer,
Cellular Growth and Proliferation
MAPK8 (JNK1)
P53
Level 2
Cancer,
Connective Tissue Development,
CTNNB
FOS
SRC
MYC
MAPK14 (Mxi2)
Level 3
Embryonic Development,
Organ Development
BCL2
ESR1
TGFBRAP1
CRK
RPS31 (RPS25)
Level 4
Microglia activation
CCL2
STAT3
HIF1A
NFE2
TIMP1
CDKN2A
MSC
PLAU
DNMT1
PSG4
Level 5
CEBPB
RUNX2
PPARG
SP1
CREB1
CD40
CD46
NFIL3
PBG1
RNF19
DAND5 (COCO)
KET
CTNND2
Level 6
Epithelial-mesenchymal transformation (EMT)
TNFR1
NR3C1
BHF1 (NEUROD)
IKBA
EREG
STAT5A
NFKB1
CBP
FZR1
TCEAL2
Q9Y326
RNASEH2A
Level 7
Cell death and survival

Figure 2



Network 1 of CGM3 primary GBM



Network 3 of CGM3 primary GBM

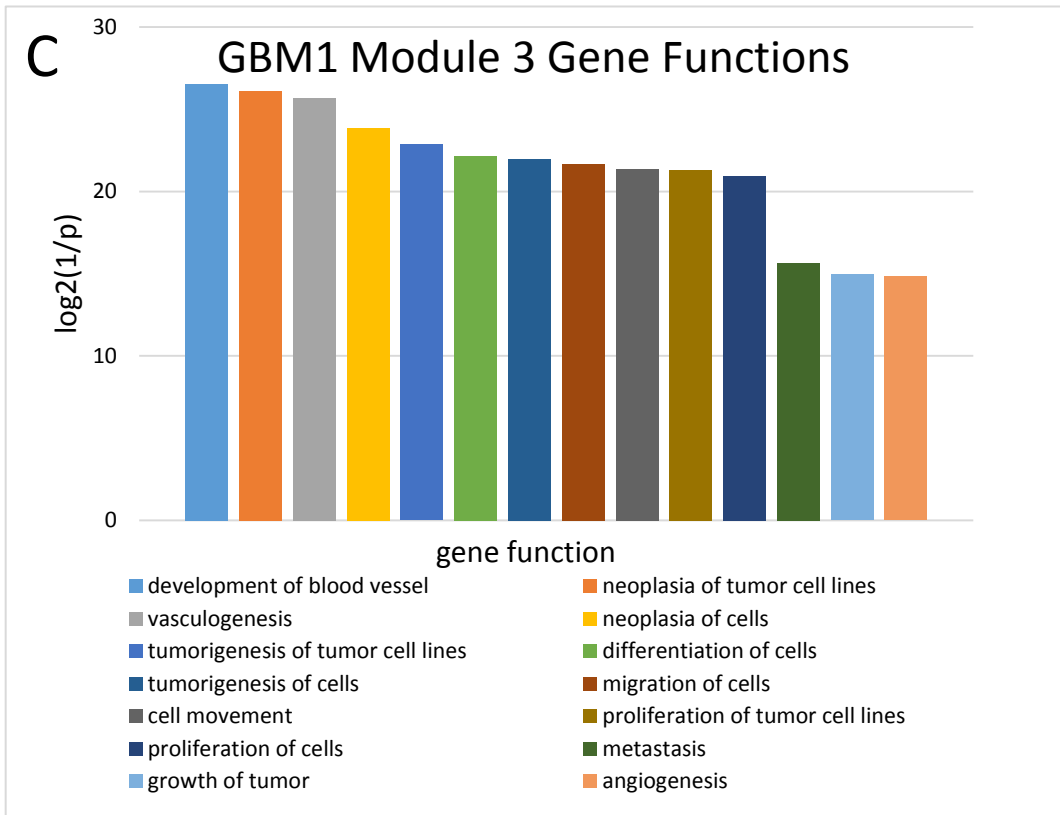
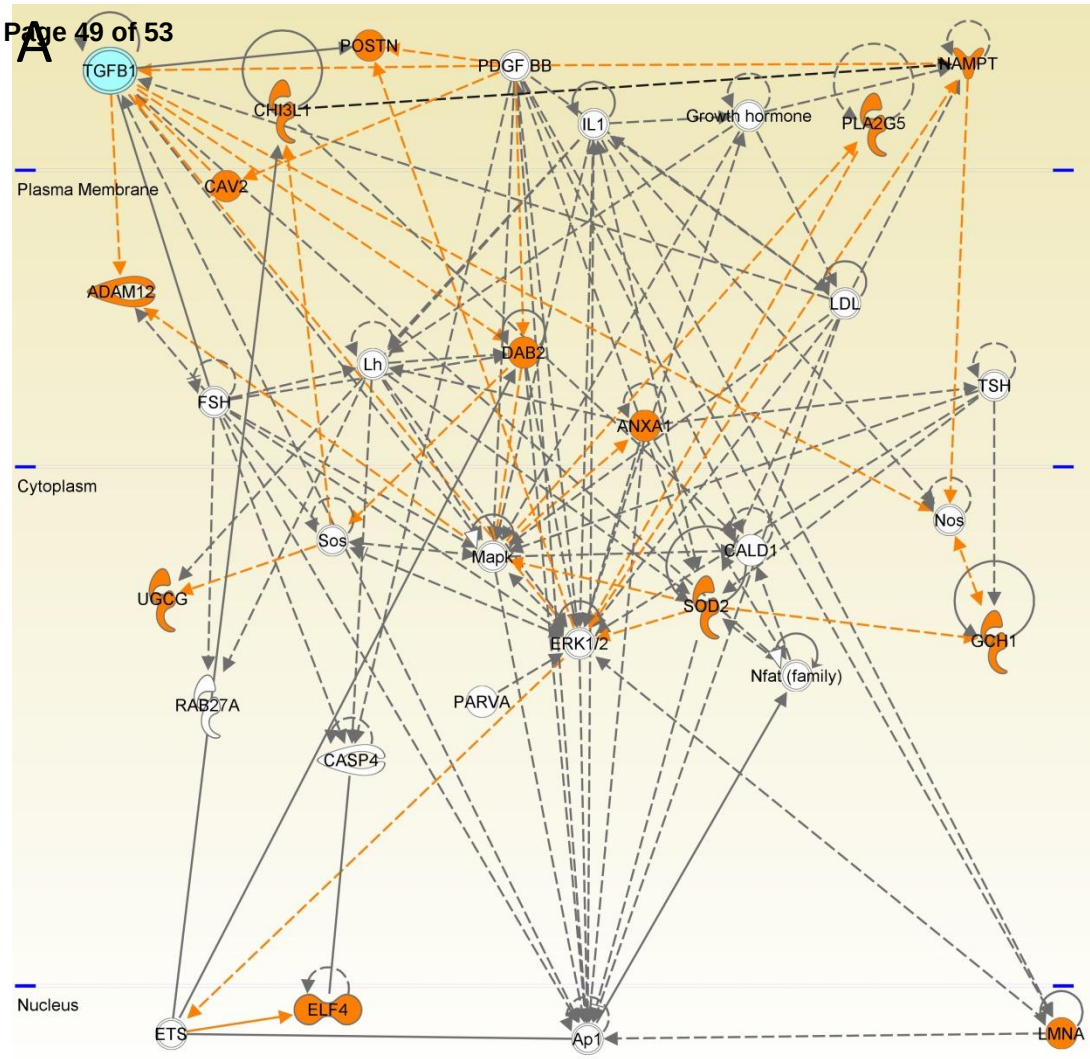
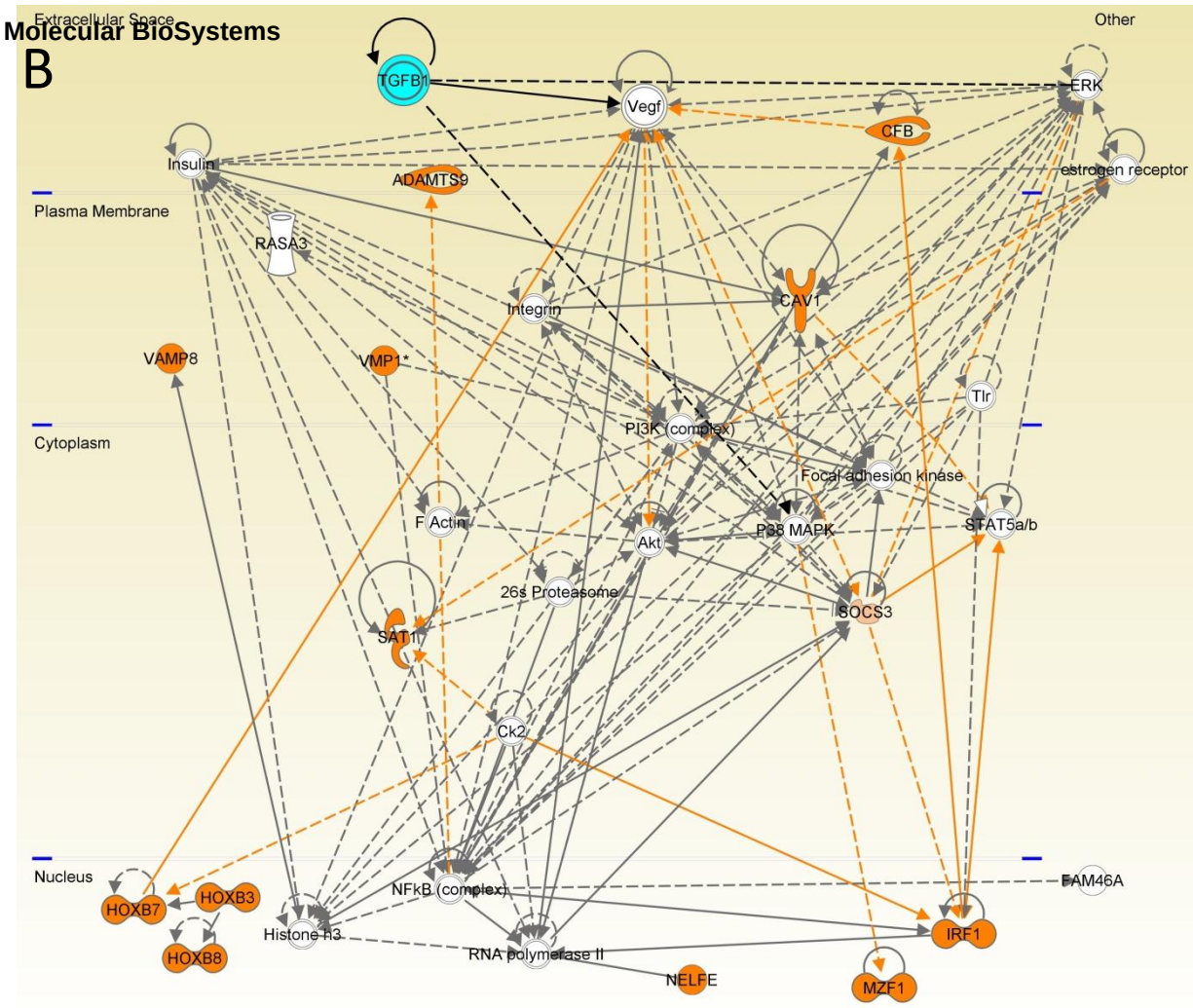


Figure 3



Network 1 of CGM1 primary GBM



Network 2 of CGM1 primary GBM

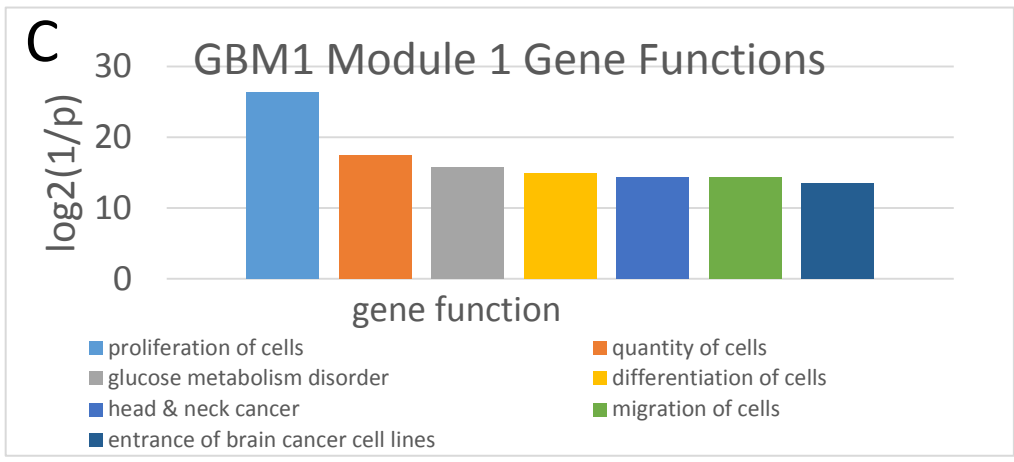
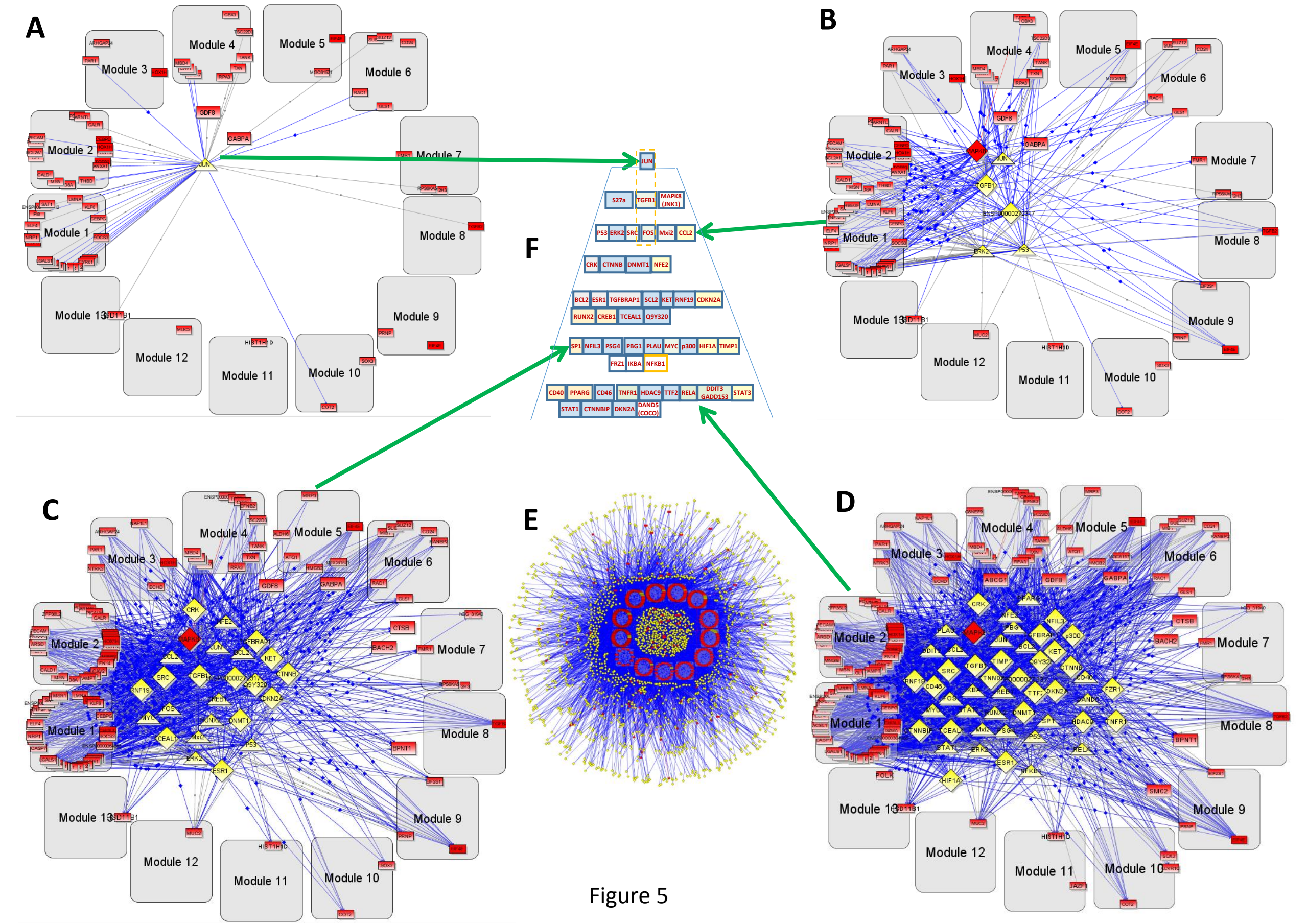


Figure 4


A
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Module 12
Module 13
JUN
GDF8
GABPA
F
JUN
S27a
TGFB1
MAPK8 (JNK1)
P53
ERK2
SRC
FOS
Mxi2
CCL2
CRK
CTNNB
DNMT1
NFE2
BCL2
ESR1
TGFBRAP1
SCL2
KET
RNF19
CDKN2A
RUNX2
CREB1
TCEAL1
Q9Y320
SP1
NFIL3
PSG4
PBG1
PLAU
MYC
p300
HIF1A
TIMP1
FRZ1
IKBA
NFKB1
CD40
PPARG
CD46
TNFR1
HDAC9
TTF2
RELA
DDIT3
GADD153
STAT3
STAT1
CTNNBIP
DKN2A
DAND5 (COCO)
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MAPK8
TGFB1
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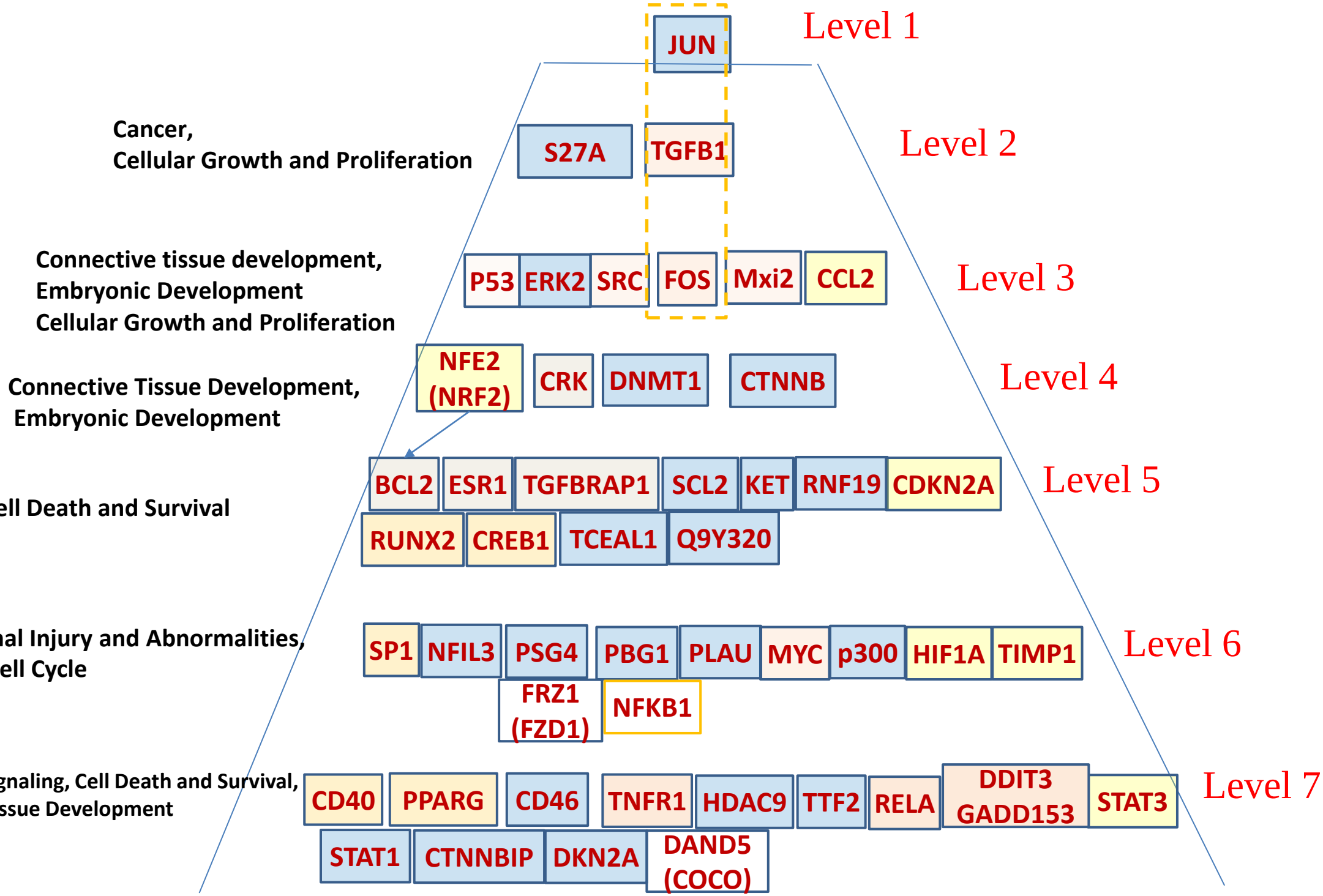
ERK2
P53
C
MAPK8
ESR1
D
E
Figure 5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JUN
Level 2
Cancer,
Cellular Growth and Proliferation
S27A
TGFB1
Level 3
Connective tissue development,
Embryonic Development
Cellular Growth and Proliferation
P53
ERK2
SRC
FOS
Mxi2
CCL2
Level 4
Connective Tissue Development,
Embryonic Development
NFE2 (NRF2)
CRK
DNMT1
CTNNB
Level 5
Cell Death and Survival
BCL2
ESR1
TGFBRAP1
SCL2
KET
RNF19
CDKN2A
RUNX2
CREB1
TCEAL1
Q9Y320
Level 6
Organismal Injury and Abnormalities,
Cancer, Cell Cycle
SP1
NFIL3
PSG4
PBG1
PLAU
MYC
p300
HIF1A
TIMP1
FRZ1 (FZD1)
NFKB1
Level 7
Cell-to-Cell Signaling, Cell Death and Survival,
Connective Tissue Development
CD40
PPARG
CD46
TNFR1
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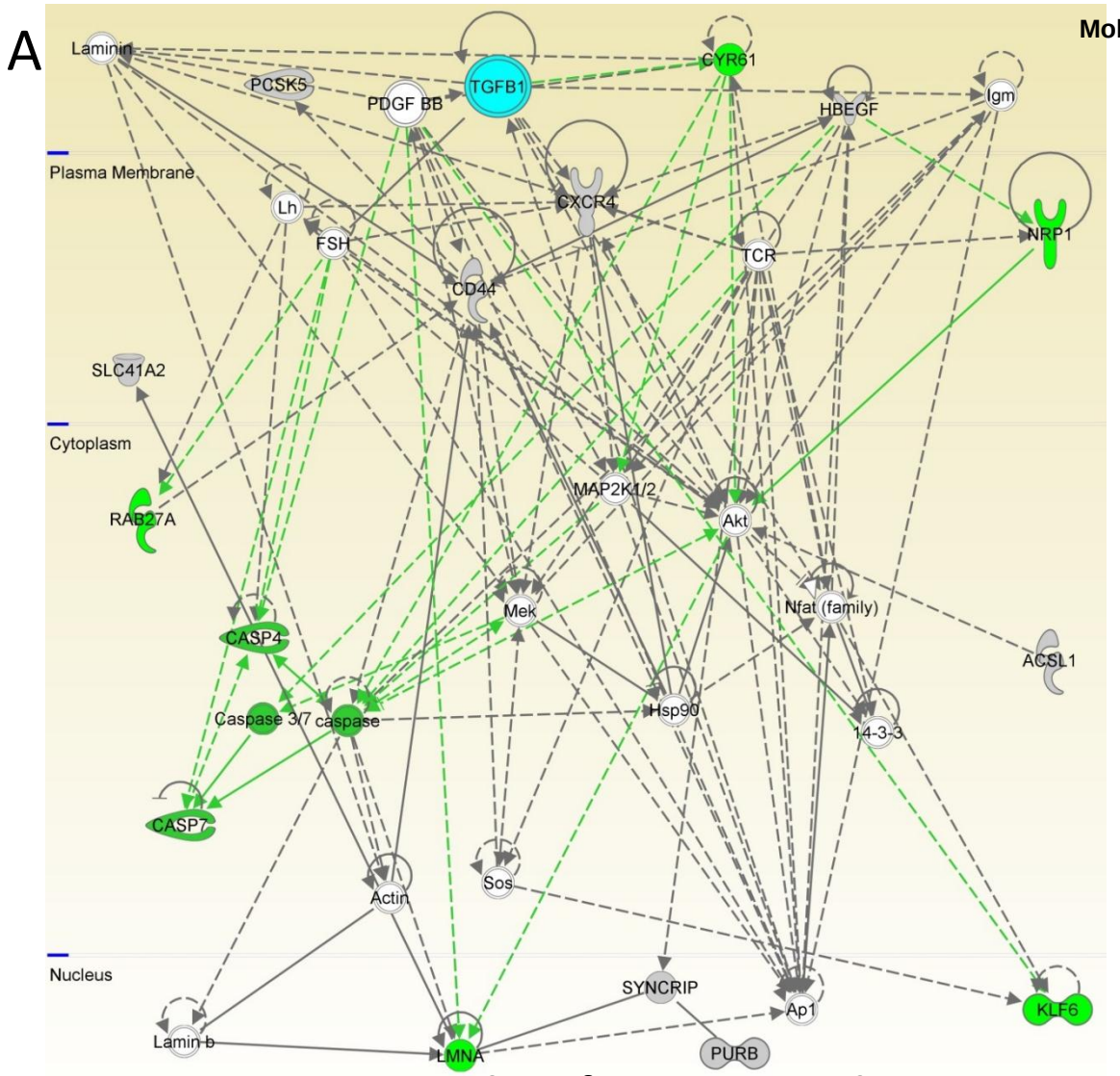
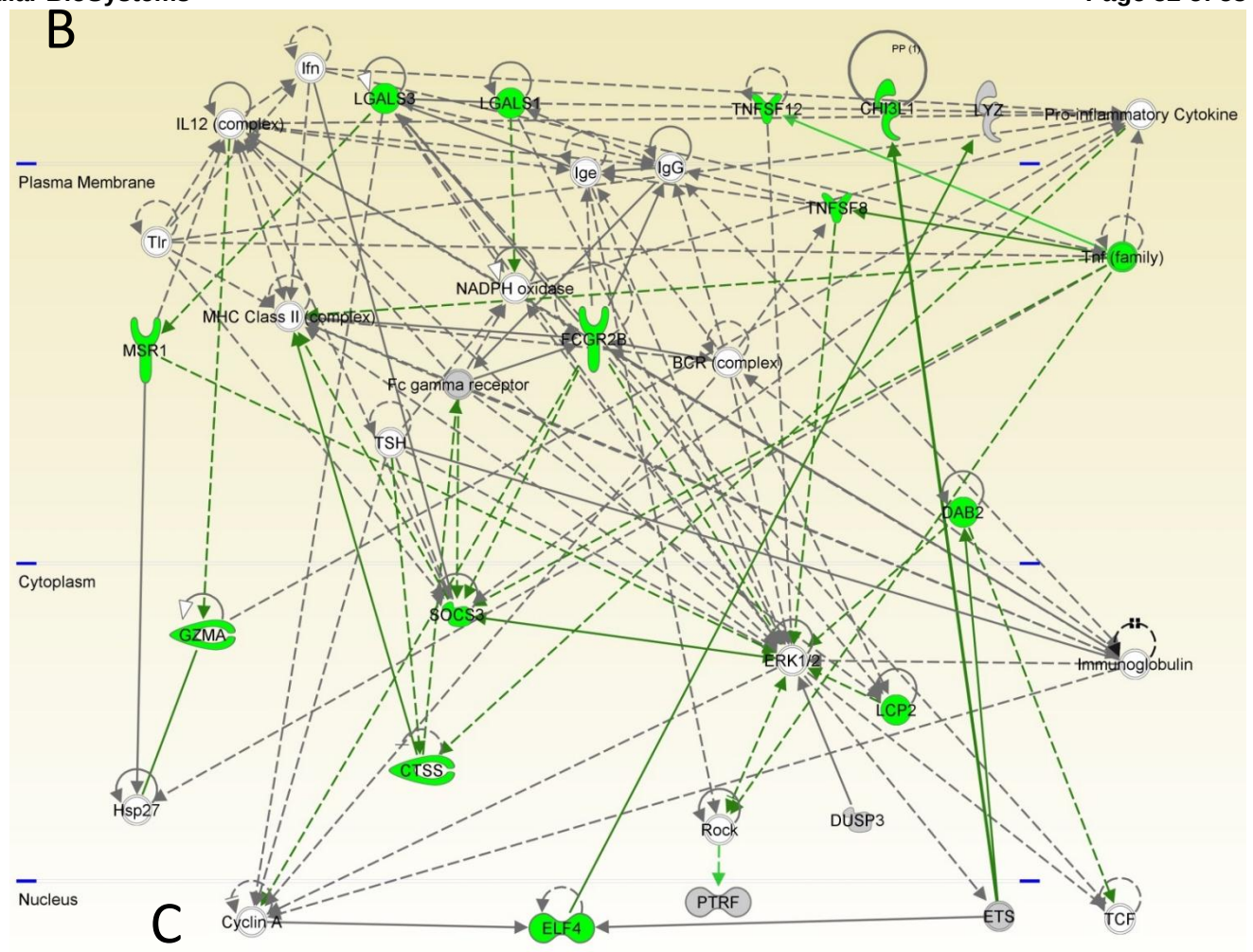
STAT1
CTNNBIP
DKN2A
DAND5 (COCO)

Figure 6



Network 4 of CGM1 secondary GBM



Network 2 of CGM1 secondary GBM

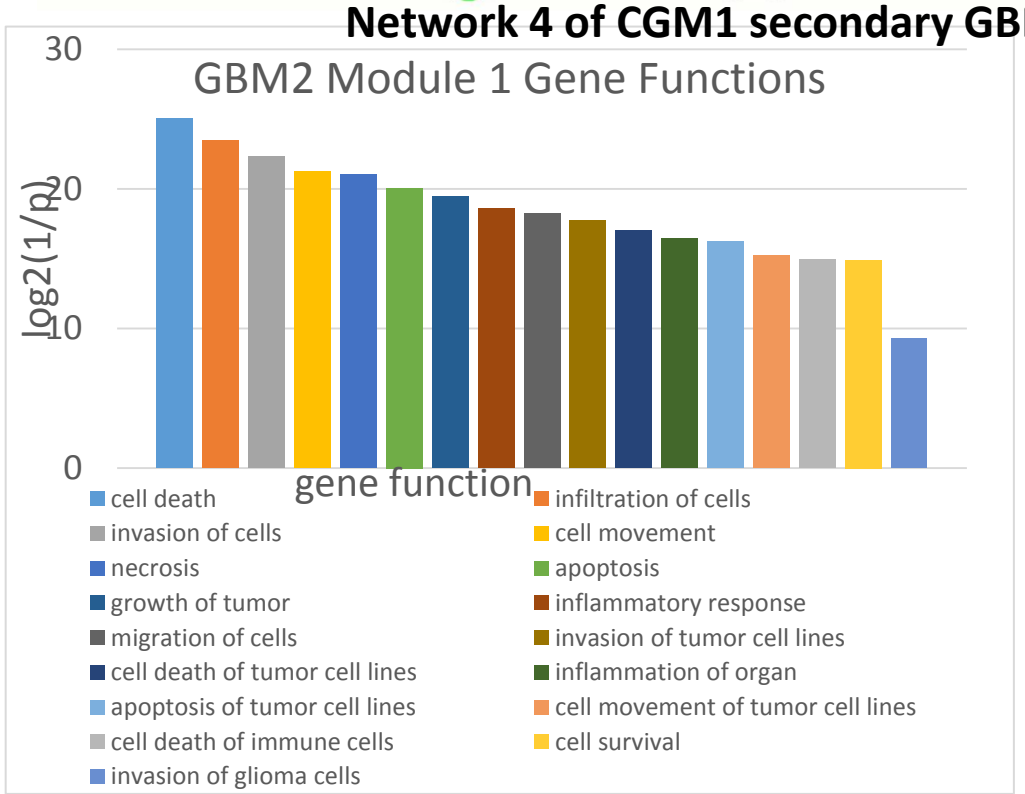
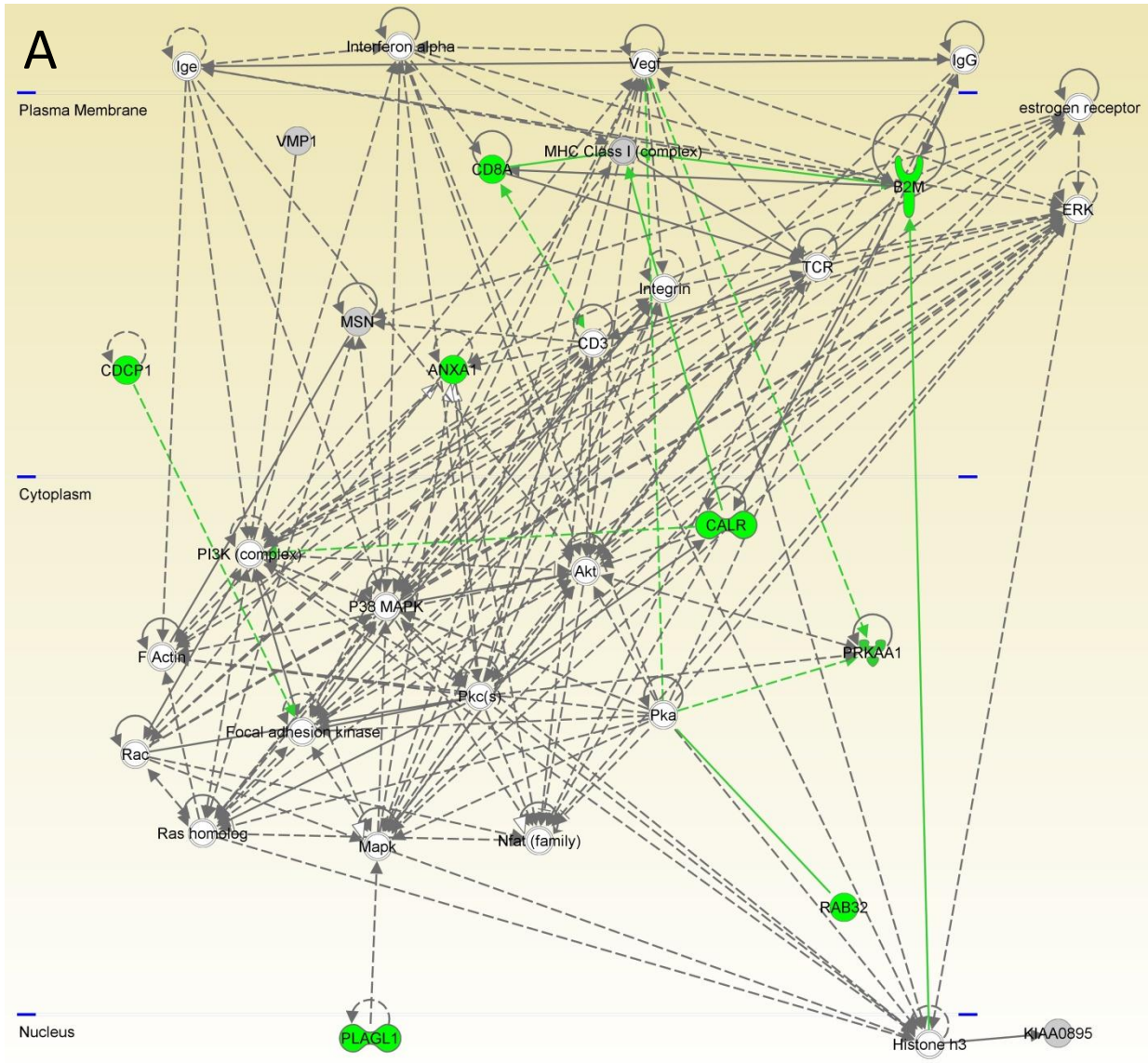
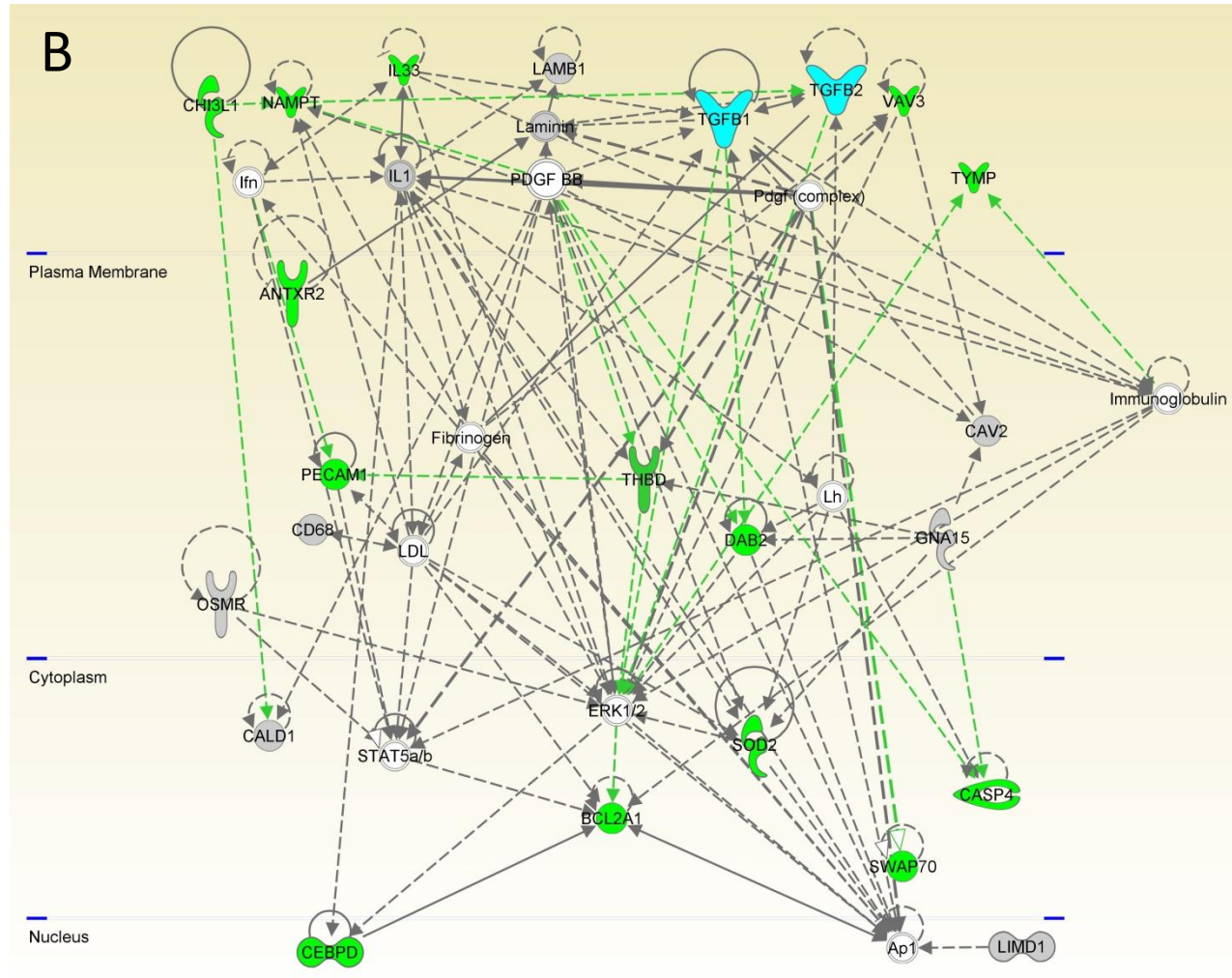


Figure 7



Network 4 of CGM2 secondary GBM



Network 1 of CGM2 secondary GBM

C

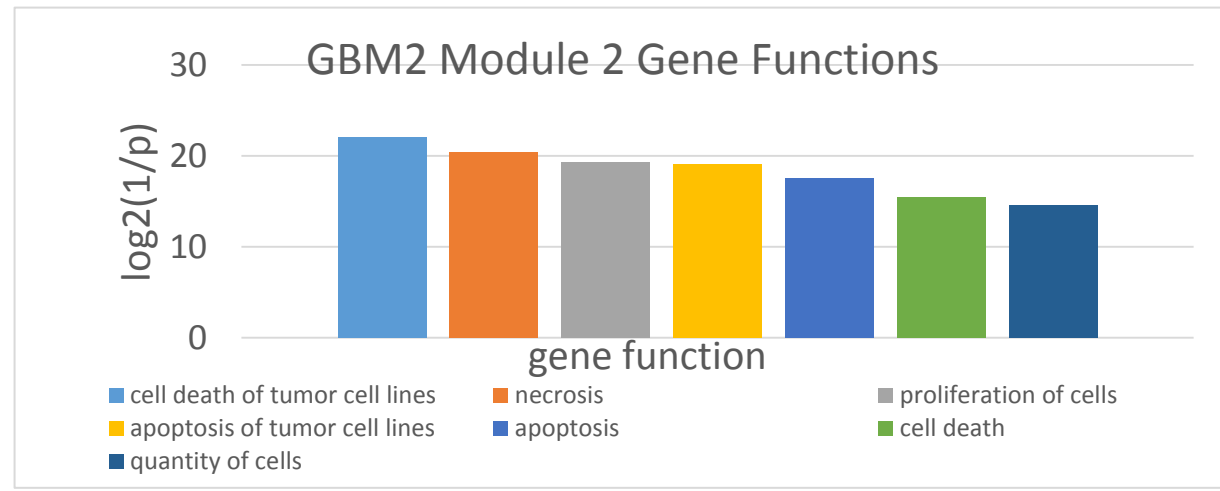


Figure 8