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Core Canonical Pathways Involved in Developing Human Glioblastoma Multiforme (GBM)

Somiranjan Ghosh[1], Sisir Dutta[1], Gabriel Thorne[2],Ava Boston[2] Alexis Barfield[2], Narendra Banerjee[2],Rayshawn Walker[2] , Hirendra Nath Banerjee[2*]

[1]Molecular Genetics Laboratory, Department of Biology, Howard University, Washington, DC 20059, USA
[2]Department of Natural, Pharmacy and Health Sciences, Elizabeth City State University, University of North Carolina

ABSTRACT

Glioblastoma multiforme (GBM) is the most common and aggressive type of the primary brain tumors with pathologic hallmarks of necrosis and vascular proliferation. The diagnosis of GBM is currently mostly based on histological examination of brain tumor tissues, after radiological characterization and surgical biopsy. The ability to characterize tumors comprehensively at the molecular level raises the possibility that diagnosis can be made based on molecular profiling with or without histological examination, rather than solely on histological phenotype. The development of novel genomic and proteomic techniques will foster in the identification of such diagnostic and prognostic molecular markers. We analyzed the global differential gene expression of a GBM cell line HTB15 in comparison to normal human Astrocytes, and established a few canonical pathways that are important in determining the molecular mechanisms of cancer using global gene expression microarray, coupled with the Ingenuity Pathway Analysis (*IPA*®). Overall, we revealed a discrete gene expression profile in the experimental model that resembled progression of GBM cancer. The canonical pathway analysis showed the involvement of genes that differentially expressed in such a disease condition that included *Inositol pathway*, *Polo like kinases*, *nNOS signaling*, and *Tetrapyrrole biosynthesis*. Our findings established that the gene expression pattern of this dreaded brain cancer will probably help the cancer research community by finding out newer therapeutic strategies to combat this dreaded cancer type that leads to the identification of high-risk population in this category, with almost hundred percent mortality rate.

Keywords : Cancer, Glioblastoma Multiforme (GBM), Gene Expression, Pathway Analysis, Canonical Pathway

I. INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and most aggressive of the primary brain tumors with pathologic hallmarks of necrosis and vascular proliferation. The current World Health Organization classification of primary brain tumors lists GBM as a grade IV (malignant) astrocytoma [1]. Astrocytoma is one of the three distinct types of gliomas in the brain, although mixed cell types occur as well. GBMs are highly malignant, infiltrate the brain extensively and at times may become enormous before turning symptomatic. Among primary brain tumors, malignant astrocytomas are the most common in all age groups (however, among all brain tumors, metastases are the most common). GBMs are the most common primary brain tumors in adults, accounting for 12-15% of intracranial tumors and 50-60% of primary brain tumors.

Morbidity is a function of tumor location, progression, and pressure effects. The overall prognosis for GBM has changed little in the past two decades despite major improvements in neuroimaging, neurosurgery, radiation treatment techniques and supportive care. Few patients with GBM survive longer than three years and only a handful survive five years. Previously reported long-term survivors of GBM may be patients diagnosed with GBM who actually harbor low-grade glioma,

pleomorphic xantho astrocytoma, ganglioglioma, or other lesions. Occasional patients with a single necrotic, demyelinating plaque of multiple sclerosis also may be misdiagnosed with GBM, especially if only CT scans are obtained(2-4).

The diagnosis of GBM is currently based on histological examination of brain tumor tissues after radiological characterization and surgical biopsy. These approaches are successful in classifying and grading tumors in most cases, but in many situations these techniques do not allow accurate prediction of prognoses and therapeutic responses. The situation may be further complicated by the small size of some diagnostic biopsy samples. There is, therefore, a critical need to improve the diagnosis of these brain tumors to both improve current therapeutic management strategies and form a basis for the evaluation of novel approaches.

The ability to characterize tumors comprehensively at the molecular level raises the possibility that diagnosis could be based on molecular profiling with or without histological examination, rather than solely on histological phenotype. The development of novel genomic and proteomic techniques will help in identification of such diagnostic and prognostic molecular markers.

Molecular diagnostics is a rapidly advancing field in which insights into disease mechanisms are being elucidated by use of new gene-based biomarkers. Until recently, diagnostic and prognostic assessment of diseased tissues and tumors relied heavily on indirect indicators that permitted only geneal classifications into broad histologic or morphologic subtypes and did not take account the alterations in individual gene expression. Global expression analysis using microarrays now allows for simultaneous interrogation of the expression of thousands of genes in a high-throughput fashion and offers unprecedented opportunities to obtain molecular signatures of the state of activity of diseased cells and patient samples. Microarray analysis may provide invaluable information on disease pathology, progression, resistance to treatment and response to cellular microenvironments and ultimately may lead to improved early diagnosis and innovative therapeutic approaches for cancer(5).
In this study, we analyzed the differential gene expression of a GBM cell line HTB15 in comparison to normal human Astrocytes and searched for several canonical pathways important in determining the molecular mechanisms of cancer using the Ingenuity Pathway Software(***IPA®***).

II. METHODS AND MATERIAL

Cell Culture

Human normal astrocytes cells were a kind gift from Dr. K. Pahan of University of Nebraska Dental School (NE, USA), HTB15 human Astrocytoma cells were purchased from American Type Culture Collection (VA, USA). Astrocyte cells were cultured in DMEM-F12 medium supplemented with 10% calf serum and antibiotics penicillin-streptomycin (20µl/L of medium), 37°C in a carbon dioxide incubator. Astrocytoma cells were cultured in Leivowitz-15 medium (L-15 medium) supplemented with 10% calf serum and antibiotics penicillin-streptomycin under conditions similar to normal Astrocyte cultures.

Microarray analysis and gene expression profiling
RNA sample preparation

Total cellular RNA was isolated from HTB15 and normal human Astrocyte cells using Trizol (Invitrogen, CA, USA). The RNA quantity was analyzed using the Nano Drop ND1000 (SOP N° TAL009) and RNA quality checked using a Bio-analyzer 2100 (Agilent Technologies, CA, USA).

Sample amplification was performed with 200 ng of total RNA using Agilent Technologies Quick Amp Labeling Kit One Color to generate complementary RNA (cRNA) for oligo microarrays. cRNA microarray analysis was processed using a Whole Human Genome Oligonucleotide Microarray (G4112A, 41,000 genes; Agilent Technologies, CA, USA) according to the manufacturer's instructions.

Microarray Hybridization

To prepare samples for microarray analysis, slides were hybridized in buffer containing fluorescence labeled cRNA at 60°C, 17 h using HS Pro hybridization station. Slides were washed once with 63× SSPE buffer containing 0.005% N-lauryl sarcosine, 1 min at room temperature followed by a 1 min wash using pre heated (37°C) 0.06×SSPE buffer containing 0.005% N-lauryl

sarcosine. The final slide wash was performed for 30 sec using acetonitrile.

Image and Data Extraction

Fluorescence signals from hybridized microarrays were detected using an Agilent and DNA microarray scanner with a resolution of 5l M and using Agilent Feature Extraction Software (FES). FES determines feature intensities and normalized ratios by linear LOWESS with background subtraction, rejects outliers and calculates statistical confidences (P-values). Hybridization signals with P value less than 0.001 were considered significant. Only genes differentially expressed in the three repeat experiments were considered as relevant genes.

Ingenuity Pathway Analysis (IPA) towards the identification of cellular processes and pathways

Data sets containing gene identifiers and corresponding expression values (fold change) were uploaded into Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com). Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. We utilized the information in the Ingenuity Knowledge Base (Genes Only) as a reference set that consider both direct and indirect relationships. We included the molecules and/or the relationships only. To enrich our pathway analysis, we incorporated additional 661 gene transcripts from IPA knowledge base to our results. We used the data sources from ingenuity expert findings and use the "Core Analysis" function to interpret the data in the context of biological processes, pathways and networks. Differentially expressed gene identifiers were defined as value parameters for analysis and identified the relationship between gene expression alterations and related changes in biofunctions under the subcategories of Molecular and Cellular Functions, Physiological System Development and Function, and Disease and Disorders. Genes differentially expressed with $p<0.05$ were overlaid onto global molecular networks developed from information contained in the knowledge base. Networks were then algorithmically generated based on their connectivity. Networks were "named" on the most prevalent functional group(s) present. Canonical Pathway (CP) analysis identified function of specific genes significantly present within the networks.

III. RESULTS AND DISCUSSION

A. Results

Microarray data indicated several genes upregulated and downregulated in GBM cells relative to human normal Astrocyte cells. In our experimental studies, beside all gene transcript a handful number of genes 5049 in total that has been differentially altered, that ranges between -186 (Down-regulated) to + 215 (up-regulated), with a cut off p value <0.05. The top upstream regulator (gene) identified are: ***AR***, ***TGFß1***, ***TP53***, ***SLC2A1***. The top five molecular and cellular function that involve in the disease category are: *Cell Cycle* (p-value range 1.97E-02 – 2.17E-05 [11molecules involved]), *Cellular Movement* (2.26E-03 – 1.66E-03 [31 molecules involved];Cell-to-Cell Signaling and Interaction (2.06E-02 – 2.09E-03 [12 molecules involved]; *Cell Death and Survival* (1.65E-02 – 4.05E-03 [47 molecules involved], and Cellular Development (1.11E-02 – 1.11E-02 [6 molecules]. The IPA analysis showed involvement of the following pathways with genes differentially expressed in all of these pathways: a) *Inositol pathway*, b) *Polo like kinases*, c) *nNOS signaling*, and d) *Tetrapyrrole biosynthesis* with Cancer being at the top of the disease and disorder categories.

B. Discussion

Ingenuity® Pathway Analysis (IPA®) is a powerful analysis and search tool that uncovers the significance of 'omics data and identifies new targets or candidate biomarkers within the context of biological systems. IPA has broadly been adopted by the life science research community and is cited in thousands of articles for the analysis, integration, and interpretation of data derived from 'omics experiments, such as RNA-seq, small RNA-seq, microarrays including miRNA and SNP, metabolomics, proteomics, and small scale experiments. In this study, we analyzed the micro array data obtained from GBM and normal human Astrocytes by the IPA software, the results showed the involvement of several important cellular pathways with differentially expressed genes(6).

Myo-inositol (MI), a molecule that is located within astrocytes, is presumed to act as an osmolyte, and its concentration is altered in many brain disorders (ref). MI is also involved in the activation of protein C kinase. Protein C kinase leads to production of proteolytic enzymes, which are found more often in malignant and aggressive primary cerebral tumors. Thus, the levels of MI, as seen by proton magnetic resonance spectroscopy

(HMRS), may be helpful for predicting the histologic grade of brain tumors, the inositol pathway involved is shown in **Figure 1.**

A microarray profiling of 467 human GBMs discovered that polo-like kinase 1 (*PLK1*) was highly expressed in these tumors and that it clustered with the proliferative subtype. Patients with *PLK1*-high tumors were more likely to die from their disease suggesting that current therapies are inactive against such tumors given their association with treatment failure. In brain tumor initiating cells (BTICs) isolated from patients expressed 110-470 times more PLK1 than normal human astrocytes. Moreover, BTICs rely on PLK1 for survival because the PLK1 inhibitor BI2536 inhibited their growth in tumorsphere cultures. PLK1 inhibition suppressed growth, caused G(2) /M arrest, induced apoptosis, and reduced the expression of SOX2, a marker of neural stem cells, in SF188 GBM cells. Likewise, in U251 GBM cells, PLK1 inhibition suppressed cell growth, downregulated *SOX2*, and induced cell death. Furthermore, BI2536 delayed tumor growth of U251 cells in an orthotopic brain tumor model, demonstrating that the drug is active against GBM. In conclusion, PLK1 level is elevated in GBM and its inhibition restricts the growth of brain cancer cells(7-10). The PLK pathway is shown in **Figure 2**.

The role of nitric oxide (NO) as a mediator of cancer phenotype has led researchers to investigate strategies for manipulating *in vivo* production and exogenous delivery of this molecule for therapeutic gain. Unfortunately, NO· serves multiple functions in cancer physiology. In some instances, NO or nitric oxide synthase (NOS) levels correlate with tumor suppression and in other cases they are related to tumor progression and metastasis. Understanding this dichotomy has been a great challenge for researchers working in the field of NO· and cancer therapy. Due to the unique chemical and biochemical properties of NO·, it's interactions with cellular targets and the subsequent downstream signaling events can be vastly different based upon tumor heterogeneity and microenvironment. Simple explanations for the vast range of NO-correlated behaviors will continue to produce conflicting information about the relevance of NO· and cancer. Information on the relationship between neuronal NOS (nNOS) and cancer is scarce. One study examined 29 patients with grades II – IV astrocytoma and performed IHC staining for nNOS on surgically removed tumors. They found an increase in both distribution and intensity of staining with increasing grade of the disease (11). No functional information was pursued in these studies. An earlier study found an increase in IHC staining of nNOS in grade III and IV gliomas compared to grades I and II. When they attempted to perform NOS activity assays, however, they could not detect increased NOS activity despite the IHC results(12). It is possible that nNOS expression correlates with increased metastasis in some cases, but overall the data on nNOS and cancer are thin and inconclusive. The pathway deciphered in this study involving is shown in **Figure 3**.

Tetrapyrrole biosynthesis and increased accumulation is a characteristic of malignant cells(13-16).Therapeutic strategies have developed in targeting cancer cells by photodynamic therapy and killing these malignant cells by blocking tetrapyrrole biosynthesis and irreversible damage to the mitochondria where these reactions mostly occurs(ref).IPA analysis showed pathway of increased biosynthesis of tetrapyrrole compounds in GBM cells in contrast to normal Astrocytes (**Figure 4**).

Thus our findings by IPA analysis of the gene expression pattern of this dreaded brain cancer will probably help the cancer research community to explore newer therapeutic strategies to combat this cancer with almost hundred percent mortality rates.

IV. ACKNOWLEDGEMENT

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FIGURES:
Figure 1

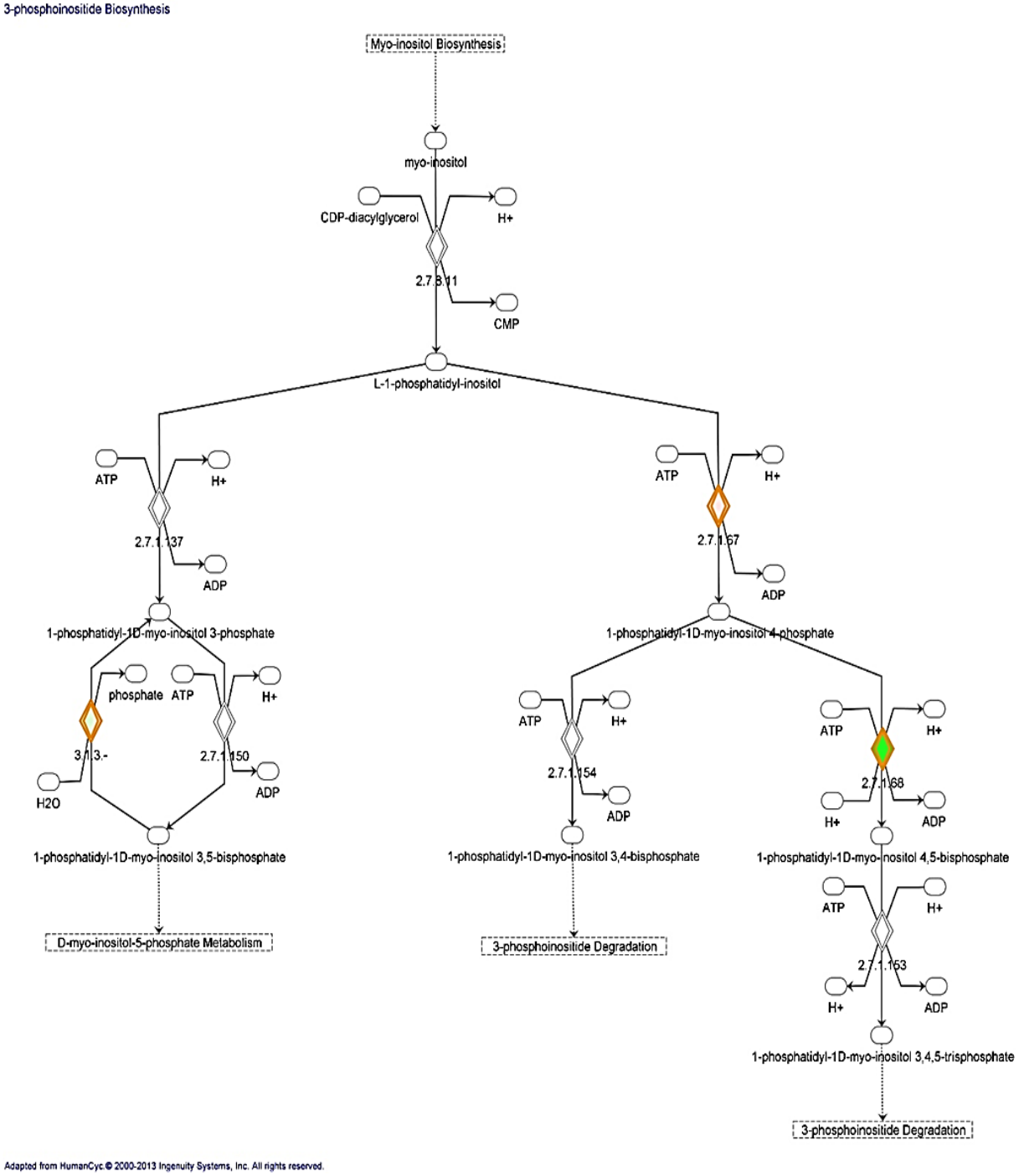

Figure 2

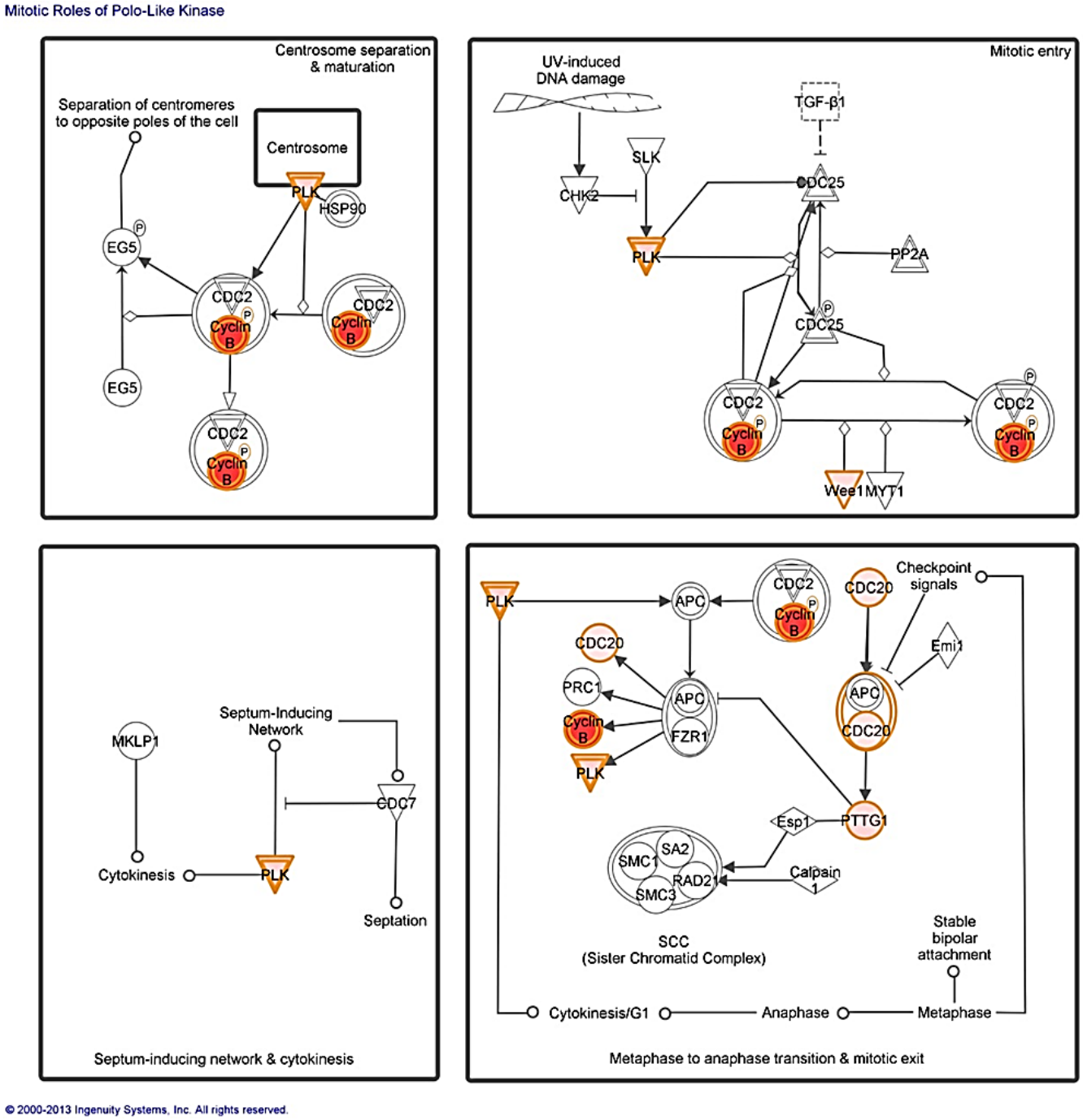
Mitotic Roles of Polo-Like Kinase
Centrosome separation & maturation
Separation of centromeres to opposite poles of the cell
Centrosome
PLK
HSP90
EG5
CDC2
Cyclin B
Mitotic entry
UV-induced DNA damage
TGF-β1
SLK
CHK2
CDC25
PLK
PP2A
Wee1
MYT1
Septum-Inducing Network
MKLP1
CDC7
Cytokinesis
Septation
Septum-inducing network & cytokinesis
Checkpoint signals
CDC20
Emi1
PRC1
APC
FZR1
Esp1
PTTG1
SMC1
SA2
RAD21
SMC3
Calpain 1
SCC (Sister Chromatid Complex)
Stable bipolar attachment
Cytokinesis/G1
Anaphase
Metaphase
Metaphase to anaphase transition & mitotic exit
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Figure 3

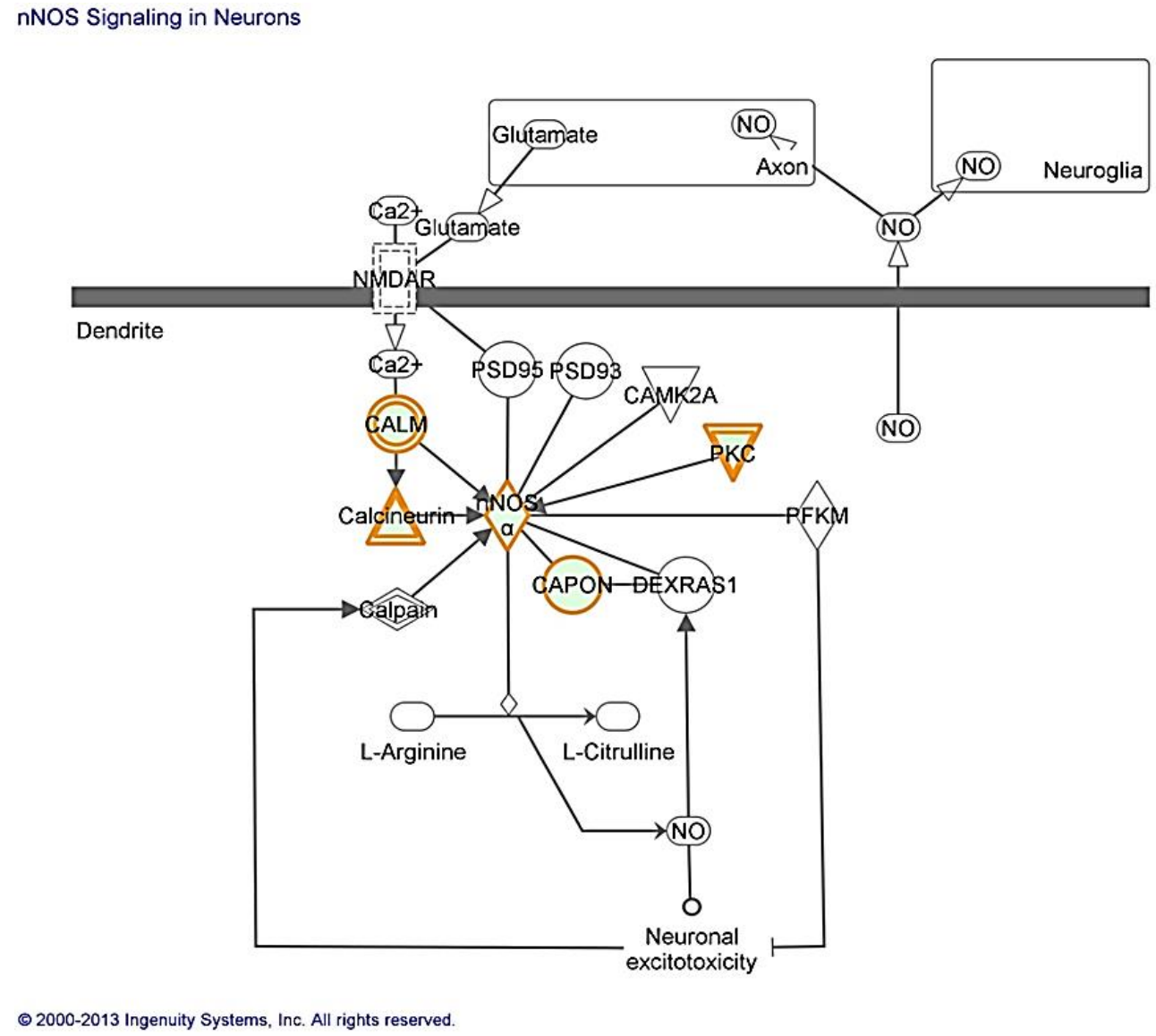

Figure 4