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Supplementary Information

The Mitochondrial Uncoupling Agent DNP Triggers Adaptive Brain Cell Signaling Network Reprogramming Involving Suppression of mTOR and Insulin Signaling, and Upregulation of CREB

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Supplementary Figures S1-S4

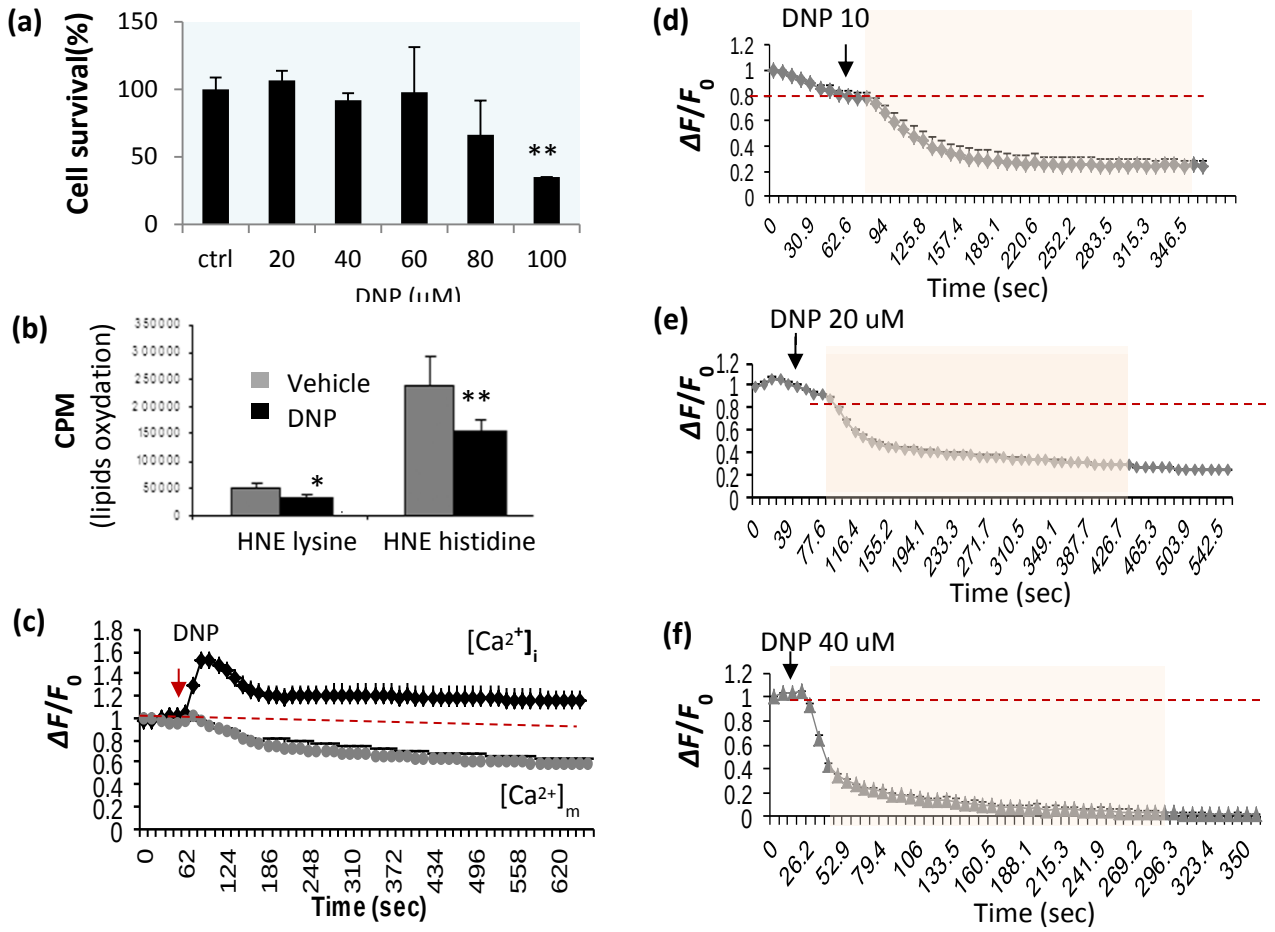


Figure S1 Bioenergetics actions of DNP on cultured rat cortical neurons. (**a**) Rat cortical neuronal cells survival to DNP at indicated concentrations of DNP or vehicle control (DMSO, ≤ 0.2%) for 24 hours. Values are the mean and SD of 4 cultures for each concentration. **p<0.01. (**b**) Cortical neurons were treated with DNP (20 μM) or vehicle (DMSO) for 24 h, and levels of lipid peroxidation products (lysine and histidine adducts of 4-hydroxynonenal; HNE) were measured by tandem mass spectrometry as described in Methods. Values were normalized to the phosphoenolamine level and are the mean and SD of 3 independent experiments. **p<0.01. (**c**) Time-laps imaging-based measurement of cytoplasmic free calcium ($[Ca^{2+}]_i$) with Fluo-4 and mitochondrial calcium ($[Ca^{2+}]_m$) withRhod-2 in cortical neurons prior to and after exposure to DNP (20 μM). Values are the mean and SD change from baseline ($\Delta F/F_0$) in 30-40 neurons from 3 cortical cultures. (**d-f**) Representative time-lapse confocal imaging on rat cortical neurons loaded with mitochondrial membrane potential ($\Delta\Psi_m$) indicator TMRE and acquired at 10 sec intervals. DNP induced depolarization of $\Delta\Psi_m$ on neurons at the indicated doses. The dashed line indicates the baseline level (red). Data presented as an average cell intensity change from the baseline. 3 independent experiments in which 15-20 neurons from multiple culture dishes were evaluated.

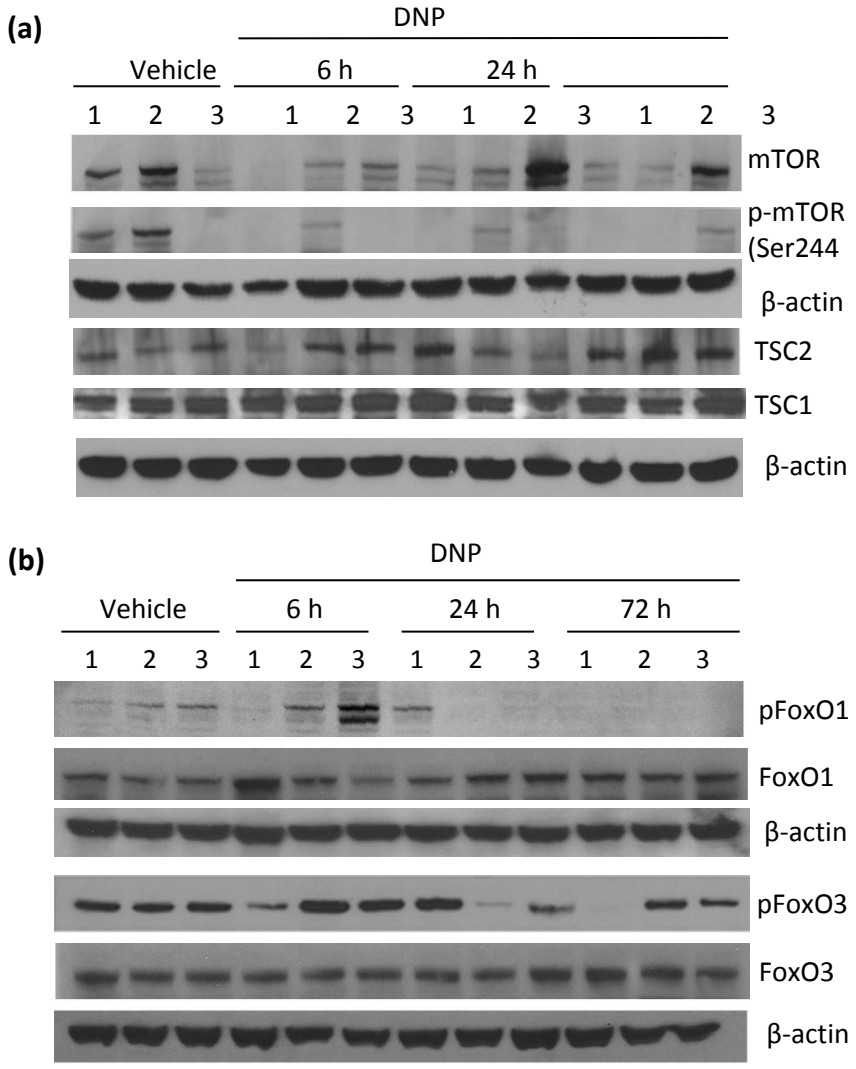


Figure S2 Mild mitochondrial uncoupling modulates mTOR signaling pathway in mouse brains. (**a**) Immunoblotes on the expression of indicated proteins in mTOR signaling pathway from brain striatum samples of DNP-treated and control mouse during a time course (n= 7 individual mouse/group/each time point; samples from 3 animals/group were presented). (**b**) Immunoblotes on the expression of indicated FoxO1, pFoxO and Foxo3a, pFoxO3a transcription factors proteins from brain cortical samples of vehicle or DNP-treated and control mouse during a time course (n= 7 individual mouse/group/each time point; samples from 3 animals/group were presented).

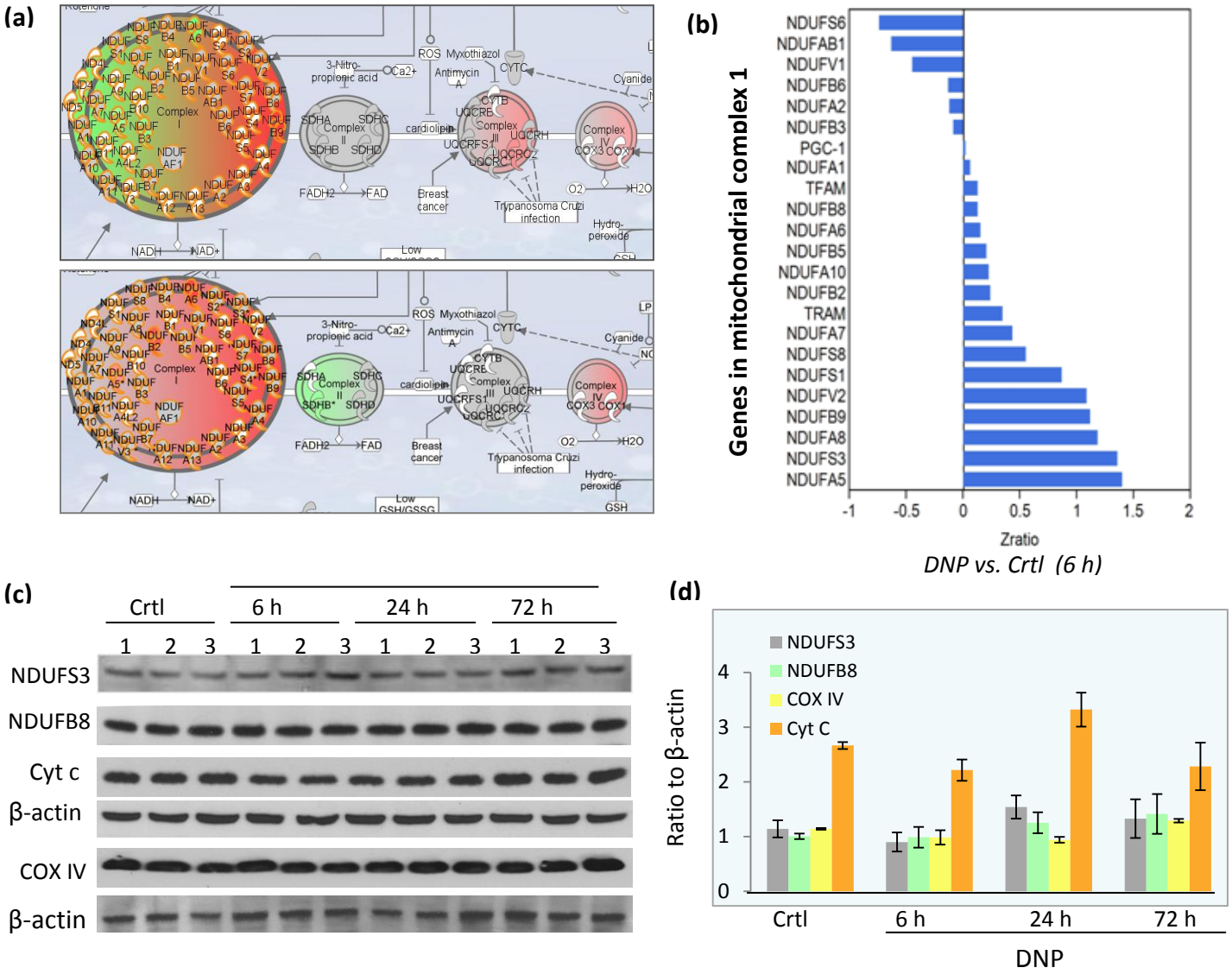
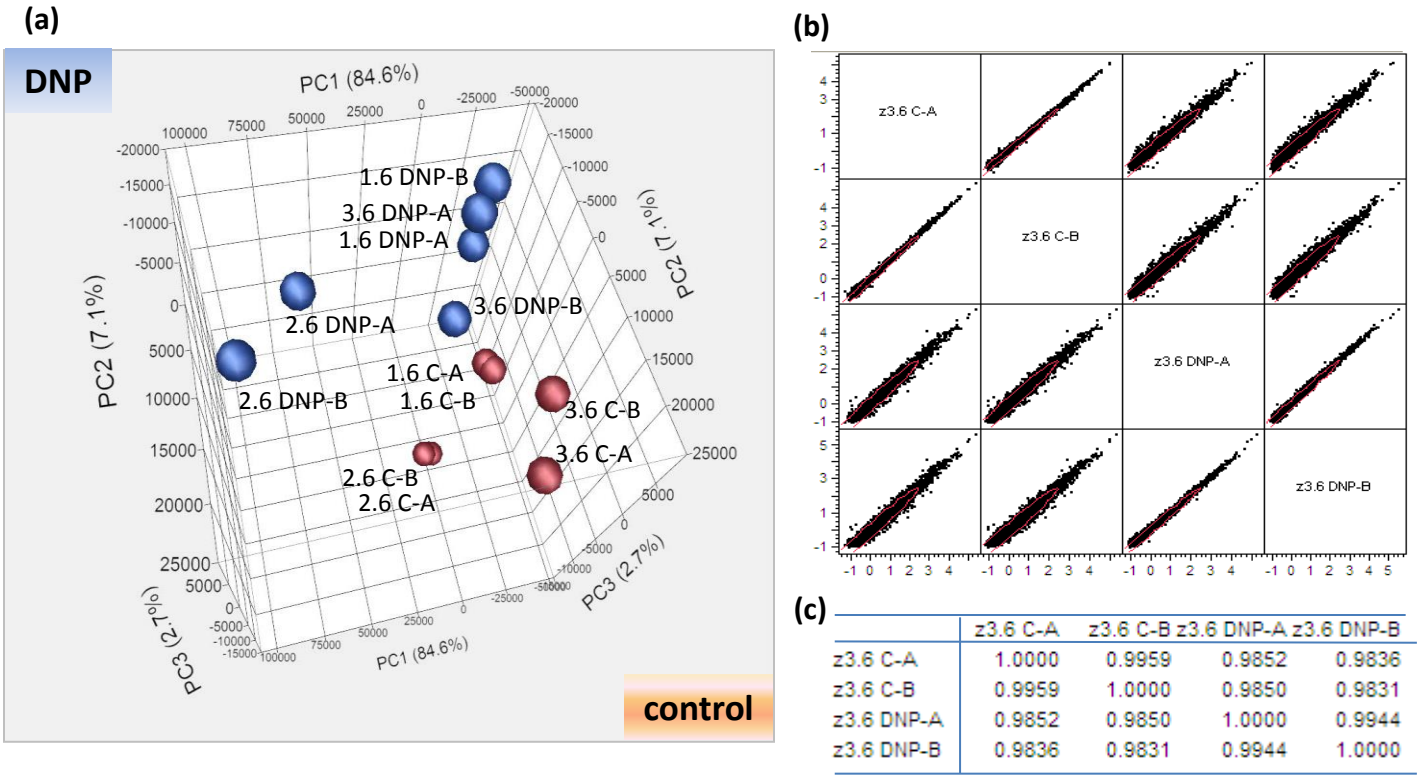


Figure S3. Mild mitochondrial uncoupling upregulates multiple genes encoding proteins of mitochondrial complex I. **(a**) Illustrations showing changes in the expression of genes encoding mitochondrial proteins in cerebral cortex of mice treated for 6 hours (A-a) or 72 hours (A-b) with DNP. (**b**) Genes upregulated or downregulated in the cerebral cortex in response to treatment with DNP for 6 hours (B-a) or 72 hours (B-b). (**c)** Immunoblot showing relative levels of the indicated mitochondrial proteins in the cerebral cortex of control mice and mice treated with DNP for the indicated time periods. (**d)** Densitometric quantification of protein band intensities normalized to β-actin. Values are mean ± SD (n = 3 mice/group).



	z3.6 C-A	z3.6 C-B	z3.6 DNP-A	z3.6 DNP-B
z3.6 C-A	1.0000	0.9959	0.9852	0.9836
z3.6 C-B	0.9959	1.0000	0.9850	0.9831
z3.6 DNP-A	0.9852	0.9850	1.0000	0.9944
z3.6 DNP-B	0.9836	0.9831	0.9944	1.0000

Figure S4. (**a**) Principal components analysis (PCA) is a mathematical exploratory technique used to find major variations in gene expression and reduce the dimensionality of data sets from microarray experiments. The current principal components (orthogonal eigenvectors) are selected for the top three greatest variances shared among samples. The figure shows that DNP-treated and control samples show different expression vectors by clustering together. (**b**) Comparison of correlation efficiency. Correlation efficiency on data distribution between individual animals from vehicle and DNP treated groups at 6 h (3 individual animals per group). (**c**) The correlation efficiency is higher between animals in the same group (vehicle control-A vs. Control-B or DNP-A vs. DNP-B) was higher than animals from different group (control vs. DNP), indicating the consistency of data distribution.

Supplementary Tables S1-S2

Table S1. Genes modified in mTORC1 signaling network

Symbol	Gene description	z-ratio	p-value
Akt	PKB	-1.35	<0.01
AMPK	AMP-activated kinase	0.4	<0.01
ATF4	activating transcription factor 4	4.03	<0.01
CCND1	cyclin D1	-2.08	<0.01
CREB3	cAMP responsive element binding protein 3	2.19	<0.01
DDR1	discoidin domainreceptor tyrosine kinase 1	-2.26	<0.01
FOXO1	forhead box O1	- 2.56	<0.01
FOXP3	forhead box P3	0.55	0.05
FOS	AP-1	2.69	<0.01
IGF1	insulin-like growth factor 1	0.03	>0.05
MAPK1	mitogen-activated protein kinase 1	-1.85	<0.05
MAPK11	mitogen-activated protein kinase 1	1.61	<0.01
MAPK9	mitogen-activated protein kinase 1	2.1	<0.01
Mek/Erk	Erk kinase	-1.85	<0.01
MLST8	mTOR associated proteinLST8 homolog	-0.95	<0.01
NTS	neurotensin	-4.22	<0.01
PDIA3	ER60	-3.59	<0.01
RhebL1	ras homolog enriched in brain like 1	-0.04	>0.05
SREBF2	sterl regulatory element binding factor 2	-1.07	<0.05
TSC2	tuberous sclerosis 2	3.47	<0.01
TSG101	tumor susceptibility gene 101	-1.51	<0.05
VCAM	V-ATPase	-1.56	<0.01

Microarray gene analysis on DNP vs. vehicle treated mouse cortex samples at 72 h.

Table S2. Genes modified in mTORC2 signaling network

Symbol	Gene description	z-ratio	p-value
CARHSP1	calcium regulated heat stablr protein 1	-2.03	<0.05
COMMD1	copper metabolism domain containing 1	-1.89	<0.01
CUL1	cullin 1	1.7	<0.01
FOXO1	forkhead box O1	-2.56	<0.01
EGR1	early growth response 1	3.15	<0.01
JUNB	jub B proto-oncogene	3.82	<0.01
MLST8	MTOR associated protein, LST8	-0.95	<0.01
MTOR	mechanistic target of rapamycin	-1.5	<0.01
PABPC1	poly(A) binding protein, cytoplasmic 1	1.56	<0.01
PDIA3	protein disulfide isomerase family A	-3.59	<0.01
PDPK1	3-phosphoinositide depend protein kinase 1	2.38	>0.05
PRKCA	protein kinase C, alpha	-0.57	>0.05
PRR5	proline rich 5	0.89	<0.01
PSMD6	proteasome, non-ATPaase, 6	1.5	>0.05
RICTOR	RPTOR independent companion of MTOR	-0.22	<0.01
RND2	Rho family GTPase 2	-4.78	<0.01
SATB1	SATB homeobox 1	1.61	<0.01
SGK1	serum/glucocorticoid regulated kinase 1	3.73	<0.01
TSC2	tuberous sclerosis 2	3.47	<0.01
WNK2	WNK lysine deficient protein kinase 2	2.75	<0.01

Microarray gene analysis on DNP vs. vehicle treated mouse cortex samples at 72 h.

Supplemental Methods

Data analysis

Microarray data were analyzed using DIANE 6.0, a spreadsheet-based microarray analysis program based on SAS JMP 7.0 system.
Raw microarray data were subjected to z-transformed and filtered by the detection p-value and Z normalization; the data are further tested for significant changes as previously described. The sample quality was first analysis by scatter plots, principal component analysis, and gene sample z-scores based hierarchy clustering to exclude possible outliners. ANOVA test were used to eliminate the genes with larger variances within each comparing group. Genes were determined to be differentially expressed after calculating the Z ratio, which indicates the fold-difference between experimental groups, and false discovery rate (FDR), which controls for the expected proportion of false rejected hypotheses. Individual genes with t-test p value <=0.05, absolute value of |Z ratio| >= 1.5, average z-scores over the comparison group is non-negative, and FDR <= 0.3 were considered significantly changed. Hierarchy clustering/K-means clustering and Principal Components Analysis (PCA) was performed to identify clustering within groups. Array data for each experimental animal was also originally hierarchically clustered in Ilumina Bead Studio version 2.0. All of the results were also presented graphically as well as by spreadsheets. The multiple factor interactions are further analysis by mean of n-way ANOVA to decide the integration effect. Logical Venn diagram analysis is done across the multiple comparison groups to find distinguished and common patterns and gene association profiles.

The Parameterized Analysis of Gene Enrichment (PAGE, Kim *et al.*, 2005) algorithm is employed for gene set enrichment analysis by using all of the genes in each sample as input against and the data sets supplied by Gene Ontology Institute, GAD pathology sets, Mouse phenotype gene sets, microRNA sets, and MIT Broad Institute. For each relevant comparison, the lists of differentially expressed genes and Z ratios were entered into the PAGE Pathway Analysis software to organize them according to known biological pathways. The Enrichment Zscores for each functional grouping were calculated based on the chance of mRNA abundance changes predicting these interactions and networks by z-test. The P-value was calculated by comparing the number of user-specified genes of interest participating in a given function or pathway relative to the total number of occurrences of these genes in all functional/pathway annotations stored in the knowledge base. All of the Pathways must at least have three genes found in the microarray gene set. The t-test or fisher test p value <=0.05 and FDR<=0.3 are the cutoff criteria for the significant Disease Gene Set/pathway/Gene Ontology selection. The pathology/pathway/Gene Ontology results are further presented by cluster heat map for their association relations and by bar plot for their change patterns. The related gene sets in each pathway plus their expression patterns and the related pathway for each gene are also supplied. The genes in the pathway/functional groups are supplied and their expression patterns are also indicated. Each gene's pathway/gene ontology information is also available.
Ingenuity Pathways Analysis IPA (Ingenuity Systems; Redwood City, CA) and/or Ariadne Pathway Studio 7 were used to identify top network functions involving differentially expressed genes. Network analysis utilizes a curetted knowledge base of known functional interactions and protein functions to algorithmically infer biochemical interactions.

Significance of functions and pathways was calculated using the right-tailed Fisher's Exact Test. With the request, the type of protein products and protein-protein interactions is also presented graphically by HTML linkage hot maps produced by Pathway Studio 6.1. Their literature references and cellular location are also supplied for each protein-protein interaction linkage.