Bilateral gene interaction hierarchy analysis of the cell death gene response emphasizes the significance of cell cycle genes following unilateral traumatic brain injury

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Abstract

Background: Delayed or secondary cell death that is caused by a cascade of cellular and molecular processes initiated by traumatic brain injury (TBI) may be reduced or prevented if an effective neuroprotective strategy is employed. Microarray and subsequent bioinformatic analyses were used to determine which genes, pathways and networks were significantly altered 24 h after unilateral TBI in the rat. Ipsilateral hemi-brain, the corresponding contralateral hemi-brain, and naïve (control) brain tissue were used for microarray analysis.

Results: Ingenuity Pathway Analysis showed cell death and survival (CD) to be a top molecular and cellular function associated with TBI on both sides of the brain. One major finding was that the overall gene expression pattern suggested an increase in CD genes in ipsilateral brain tissue and suppression of CD genes contralateral to the injury which may indicate an endogenous protective mechanism. We created networks of genes of interest (GOI) and ranked the genes by the number of direct connections each had in the GOI networks, creating gene interaction hierarchies (GIHs). Cell cycle was determined from the resultant GIHs to be a significant molecular and cellular function in post-TBI CD gene response.

Conclusions: Cell cycle and apoptosis signalling genes that were highly ranked in the GIHs and exhibited either the inverse ipsilateral/contralateral expression pattern or contralateral suppression were identified and included STAT3, CCND1, CCND2, and BAX. Additional exploration into the remote suppression of CD genes may provide insight into neuroprotective mechanisms that could be used to develop therapies to prevent cell death following TBI.

Keywords: Traumatic brain injury, Cell death, Microarray, Bioinformatics, Gene interaction hierarchy

Background

Traumatic brain injury (TBI) is a major public health problem in both the civilian and military populations as TBI has now become a prominent injury in war zones. Of the 1.7 million new TBIs that are sustained annually in the United States [1], 53,000 result in death [2] while an additional 125,000 leave the affected people with long-term behavioral deficits [3]. Overall, about 3 million Americans are currently suffering with chronic effects of TBI [4]. Additionally, it is estimated that 17–30 % of soldiers returning for Iraq and Afghanistan have suffered TBIs [5, 6]. Development of more effective clinical treatments is necessary to reduce the healthcare and financial burden of TBI. Such development requires basic experimentation into the mechanisms underlying TBI.

Primary damage to cells by TBI may be irreversible and lead to immediate cell death, however, delayed or secondary cell death that is caused by a cascade of cellular and molecular processes may be amenable to neuroprotective strategies.
molecular processes initiated by the trauma [7–10] may be reduced or prevented if an effective neuroprotective strategy is employed. Development of such a strategy requires an understanding of the molecular environment in the injured brain so that deleterious molecules and processes can be identified and inhibited. A step towards understanding the molecular response to TBI is examining gene expression profiles following the injury.

Microarray technology allows for examination of thousands of genes in one assay. The key to using this technology is interpreting the resulting gene expression patterns and using the interpreted data to guide further study. The development of advanced bioinformatic analysis tools have aided in deciphering microarray data. One such tool is the Ingenuity Pathway Analysis (IPA) software program which uses a database built from published scientific literature to draw direct and indirect interactions between genes and to assign genes to specific biological functions, canonical pathways, and networks [11]. IPA also features a strong network building component that allows for the creation and analysis of networks composed of any genes of interest (GOI). We have previously devised a method for using the initial information that IPA provides and subsequent network analysis to determine which genes are most significant to the inflammatory response following neuronal injury unilateral controlled cortical impact (CCI) in the rat [12]. This analysis results in a gene interaction hierarchy (GIH) where genes of interest are ranked based on the number of interactions they have with each other. The theory behind the analysis is that a gene that interacts with more genes in a particular set of genes has the potential to influence that set of genes the most.

The current study uses gene expression profiling and bioinformatic analysis to examine the cell death gene response 24 h following unilateral CCI. One significant finding of our previous study was that while inflammatory gene expression was induced on the ipsilateral side of the brain following TBI, there was a suppression of inflammatory genes contralateral to the injury [12]. We believe that this endogenous anti-inflammatory response may hold clues for the development of anti-inflammatory treatments for TBI and other acute brain injuries. Inflammation resulting from many different types of acute brain injuries, including TBI and ischemic stroke, has been linked to subsequent neuronal cell death [13–16]. By extension, we believe that understanding the post-TBI expression of genes involved in acute cell death will provide clues for the development of neuroprotective strategies.

Methods

Animals

All animals used in these studies were treated humanely and with regard for alleviation of suffering and pain and all protocols involving animals were approved by the IACUCs of Morehouse School of Medicine and/or The Georgia Institute of Technology prior to the initiation of experimentation. Adult male Sprague–Dawley rats (290–300 g; Charles River Laboratories International, Inc., USA) were housed individually in standard plastic cages in a temperature-controlled room (22 ± 2 °C) on a 12 h reverse light–dark cycle. Food and water were provided ad libitum.

Controlled cortical impact

Under isoflurane anesthesia, rats received a unilateral controlled cortical impact (CCI/TBI) using the Pittsburgh Precision Instruments, Inc. device. A craniotomy was made with the center 4 mm posterior and 3–4 mm lateral to bregma using a 6 mm diameter trephine drill bit. The impact was done at an angle of 15° from vertical with a velocity of 3 m/s to a depth of 2 mm using a 5 mm diameter impact tip. These parameters were chosen to produce a moderate injury [17]. The rats were sacrificed 24 h post-injury and the brains were removed for RNA isolation or histology.

RNA preparation and GeneChip analysis

The ipsilateral hemi-brain tissue at the site of the injury, the corresponding contralateral hemi-brain tissue, and naïve (control) brain tissue (n = 3 for each) were used for RNA isolation. Total RNA was extracted with TRIzol Reagent (Life Technologies, Rockville, MD, USA) and cleaned (RNAqueous Kit, Ambion, Austin, TX, USA). The RNA was prepared for microarray hybridization with the GeneChip® 3′ IVT Express Kit (Affymetrix Inc., Santa Clara, CA, USA) aRNA amplification procedure. Briefly, total RNA was reverse transcribed to synthesize first-strand cDNA containing a T7 promoter sequence. The single-stranded CDNA was converted into a double-stranded DNA template for transcription. The reaction employed DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second-strand cDNA. In vitro transcription generated multiple copies of biotin-modified aRNA from the double-stranded cDNA templates (this was the amplification step). aRNA Purification removed unincorporated NTPs, salts, enzymes, and inorganic phosphate to improve the stability of the biotin-modified aRNA. Finally, the labeled aRNA was fragmented to prepare the sample for hybridization to GeneChip® 3′ expression arrays [18]. Following fragmentation, 15 pg of the biotinylated cRNA was hybridized to an Affymetrix Rat Genome 230 2.0 GeneChip. The chips were hybridized at 45 °C for 16 h, and then washed, stained with streptavidin–phycoerythrin and scanned according to manufacturing guidelines.

Microarray data analysis

Data analysis was performed using Affymetrix Expression Console™ software that supports probe set summarization
and CHP file generation of 3′ expression using the MAS5 Statistical algorithm. Affymetrix microarrays contain the hybridization, labeling and housekeeping controls that help determine the success of the hybridizations. The Affymetrix Expression Analysis algorithm uses the Tukey's biweight estimator to provide a robust mean Signal value and the Wilcoxon's rank test to calculate a significance or p-value and Detection call (present, marginal or absent) for each probe set. The Detection p-value is calculated using a Discrimination Score [R] for all probes. The Discrimination Score is a basic property of a probe pair that describes its ability to detect its intended target. It measures the target-specific intensity differences of the probe pair (perfect match (PM) – mismatch (MM)) relative to

![Graphs](https://via.placeholder.com/150)

**Fig. 1** Overall functional analysis. Analysis of the top 15 molecular and cellular functions determined by IPA for the TBI-I (ipsilateral vs. naïve) dataset (**a**) and the TBI-C (contralateral vs. naïve) dataset (**b**) showed that cell death and survival was a top ranked function on both sides of the brain.
its overall hybridization intensity (PM + MM). Background estimation is provided by a weighted average of the lowest 2% of the feature intensities. Mismatch probes are utilized to adjust the perfect match (PM) intensity. Linear scaling of the feature level intensity values, using the trimmed mean, is the default to make the means equal for all arrays being analyzed. False-negative and false-positive rates are minimized by subtracting nonspecific signal from the PM probe intensities and performing an intensity-dependent normalization at the probe set level. Three chips were used for each experimental group: ipsilateral, contralateral and naïve control. The dataset produced by the Affymetrix software contains gene identifiers, corresponding expression values, and determination of whether genes are confirmed as present, marginal or absent. Previous principle component analysis of the raw datasets demonstrated that ipsilateral, contralateral and naïve clustered together by injury status and each group was well isolated from the other two groups [12]. The data were analyzed in Microsoft Excel for calculation of fold change and whether the genes were confirmed as present in the tissue sample. Genes in the injured brain that increased or decreased in expression by 2-fold or more compared to controls and were present in either all 3 ipsilateral samples or all 3 contralateral samples were identified. The gene datasets that were generated were ipsilateral vs. naïve (TBI-I) and contralateral vs. naïve (TBI-C) fold changes.

**Ingenuity pathway analysis**

The gene datasets were analyzed between December 3, 2014 and January 8, 2015 using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com) and overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. The right-tailed Fisher’s Exact Test was used to determine the likelihood that the association between a set of experimental genes and a given biological function or pathway is not due to random chance [19]. In general, p-values less than 0.05 indicate a statistically significant, non-random association. The functions, canonical pathways, and gene networks that were most significant to the dataset were identified. Gene expression profiles were overlaid on the canonical pathway and gene network figures to reveal similarities and dissimilarities in their gene expression patterns. Gene networks were also created using Ingenuity Knowledge Base to further understand specific interactions between our genes of interest.

**TBI-I/TBI-C ratio**

We used the following formulas to calculate the ratio of TBI-I to TBI-C fold changes: (1) Gene increased on both sides (TBI-I > TBI-C): ratio = (TBI-I)/(TBI-C); (2) Gene decreased on both sides (TBI-I > TBI-C): ratio = 1/[(TBI-I)/(TBI-C)]; (3) Gene decreased on both sides (TBI-I < TBI-C): ratio = −1/[(TBI-C)/(TBI-I)]; (4) Gene

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**Fig. 2** Fluoro-Jade® B staining of the cortex and hippocampus. Fluoro-Jade® B (FJB) staining showed a dense distribution of damaged neurons throughout all layers of the cortex near the sight of impact (a, b). Damaged neurons were also detected in the hippocampus ipsilateral to the injury (d). These neurons were sparsely distributed in the hippocampal CA regions. No FJB staining was detected in either brain region contralateral to the injury (C: cortex; E: hippocampus). FJB: green; Scale bars: 200 μm (a–e), 100 μm (b)
Fig. 3 TUNEL staining of the cortex and hippocampus. TUNEL staining showed distribution of injured cells in the cortex similar to FJB as they were distributed throughout all layers of the cortex (a, b). However, no TUNEL staining was detected in the ipsilateral hippocampus (d). No TUNEL was observed on the contralateral side of the brain (C: cortex; E: hippocampus). TUNEL: red; Scale bars: 200 μm (a, c-e), 100 μm (b).

Fig. 4 Breakdown of CD genes based on increased and decreased expression. a 361 CD genes changed uniquely on the ipsilateral side of the brain and 88% (317 genes) of those increased in expression. b 136 CD genes changed uniquely on the contralateral side of the brain and 75% (102 genes) of those decreased in expression. c There were 405 genes that changed more than 2-fold on both sides of the brain. Eighty-two percent of them (332 genes) changed similarly while the remaining 18% (73 genes) changed differently (TBI-I/TBI-C ratio >2; see text).
| Gene symbol | Entrez gene name | TBI-I fold change | TBI-C fold change | TBI-I/TBI-C ratio | Molecular type |
|-------------|------------------|-------------------|-------------------|------------------|----------------|
| Extracellular Space | | | | | |
| SPP1 | secreted phosphoprotein 1 | 37.905 | 2.370 | 15.994 | cytokine |
| TIMP1 | TIMP metallopeptidase inhibitor 1 | 38.486 | 2.101 | 18.318 | cytokine |
| CP | ceruloplasmin (ferroxidase) | 27.838 | 8.477 | 3.284 | enzyme |
| FGL2 | fibrinogen-like 2 | 16.793 | 4.017 | 4.180 | peptidase |
| LCN2 | lipocalin 2 | 71.824 | 3.895 | 18.440 | transporter |
| SERPINA3 | serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3 | 58.488 | 2.509 | 23.311 | other |
| Plasma Membrane | | | | | |
| CD44 | CD44 molecule (Indian blood group) | 15.558 | 2.399 | 6.485 | enzyme |
| EHD4 | EH-domain containing 4 | 2.361 | –2.056 | 4.854 | enzyme |
| SDC1 | syndecan 1 | 13.681 | 2.566 | 5.332 | enzyme |
| KCND2 | potassium voltage-gated channel, Shal-related subfamily, member 2 | –2.792 | –7.585 | 2.717 | ion channel |
| KCNQ4 | potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 | 3.088 | –9.429 | 29.117 | ion channel |
| CAMK2N1 | calcium/calmodulin-dependent protein kinase II inhibitor 1 | –11.813 | –23.824 | 2.017 | kinase |
| EGFR | epidermal growth factor receptor | 6.773 | 2.374 | 2.853 | kinase |
| PTPRF | protein tyrosine phosphatase, receptor type, F | –6.365 | –20.492 | 3.219 | phosphatase |
| IL6ST | interleukin 6 signal transducer | 2.307 | –3.283 | 7.574 | transmembrane receptor |
| CD68 | CD68 molecule | 4.365 | 2.007 | 2.175 | other |
| HLA-A | major histocompatibility complex, class I, A | 9.296 | 3.657 | 2.542 | other |
| PMEPA1 | prostate transmembrane protein, androgen induced 1 | 2.682 | –2.937 | 7.877 | other |
| Cytoplasm | | | | | |
| CYP1B1 | cytochrome P450, family 1, subfamily B, polypeptide 1 | 10.998 | 4.808 | 2.287 | enzyme |
| KIF3A | kinesin family member 3A | –5.083 | –11.754 | 2.312 | enzyme |
| MX1 | MX dynamin-like GTPase 1 | 28.177 | 7.326 | 3.846 | enzyme |
| PDE4B | phosphodiesterase 4B, cAMP-specific | 5.602 | 2.359 | 2.375 | enzyme |
| RND3 | Rho family GTPase 3 | 2.864 | –2.971 | 8.509 | enzyme |
| SRXN1 | sulfiredoxin 1 | 6.306 | 2.402 | 2.625 | enzyme |
| CARD11 | caspase recruitment domain family, member 11 | 7.343 | 2.892 | 2.539 | kinase |
| CSNQA1 | casein kinase 2, alpha 1 polypeptide | 2.992 | –2.750 | 8.228 | kinase |
| EIF5B | eukaryotic translation initiation factor 5B | –3.044 | –8.766 | 2.880 | translation regulator |
| RASA1 | RAS p21 protein activator (GTPase activating protein) 1 | 2.392 | –2.105 | 5.035 | transporter |
| AHI1 | Abelson helper integration site 1 | 2.243 | –2.897 | 6.498 | other |
| CSD2 | CDDGSH iron sulfur domain 2 | –7.833 | –19.012 | 2.427 | other |
| CMIP | c-Maf inducing protein | –3.778 | –13.763 | 3.643 | other |
| Ctdsp1 | CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like | –7.271 | –36.886 | 5.073 | other |
| HSPB1 | heat shock 27 kDa protein 1 | 46.922 | 2.639 | 17.780 | other |
| KIFAP3 | kinesin-associated protein 3 | –2.281 | –7.831 | 3.433 | other |
| LCP1 | lymphocyte cytosolic protein 1 (L-plastin) | 6.082 | 2.799 | 2.173 | other |
| LSP1 | lymphocyte-specific protein 1 | 11.716 | 2.140 | 5.475 | other |
| PHLDA1 | pleckstrin homology-like domain, family A, member 1 | 5.129 | 2.160 | 2.375 | other |
| RDX | radixin | 4.828 | –5.274 | 25.463 | other |
increased ipsilaterally and decreased contralaterally: ratio $= (TBI-I)/(1/(TBI-C))$; (5) Gene decreased ipsilaterally and increased contralaterally: ratio $= (TBI-C)/[1/(TBI-I)]$.

### Histology

At 24 h post injury, rats were anesthetized with an intraperitoneal injection of a ketamine/xylazine/acetylpromazine cocktail (50:10:1.67 mg/kg respectively) and perfused

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**Table 1** Genes that change differently on each side of the brain (Continued)

| Gene          | TBI-I/TBI-C Ratio | $TBI-I$ $TBI-C$ | $TBI-I$ $TBI-C$ | $TBI-I$ $TBI-C$ | $TBI-I$ $TBI-C$ |
|---------------|-------------------|----------------|----------------|----------------|----------------|
| **Slpi**      | secretory leukocyte peptidase inhibitor | 82.908          | 3.119          | 26.582         | other          |
| **Tpm3**      | tropomyosin 3     | 2.592           | 2.715          | 7.037          | other          |
| **TRIM54**    | tripartite motif containing 54 | $-4.426$ | $-2.032$ | $-2.178$ | other          |

**Nucleus**

| Gene          | TBI-I/TBI-C Ratio | $TBI-I$ $TBI-C$ | $TBI-I$ $TBI-C$ | $TBI-I$ $TBI-C$ | $TBI-I$ $TBI-C$ |
|---------------|-------------------|----------------|----------------|----------------|----------------|
| **SETD8**     | SET domain containing (lysine methyltransferase) 8 | 2.029           | $-3.930$       | 7.974          | enzyme         |
| **TOP2A**     | topoisomerase (DNA) II alpha 170 kDa     | 2.260           | $-2.406$       | 5.438          | enzyme         |
| **CDK11A**    | cyclin-dependent kinase 11A               | $-4.290$        | $-14.872$      | 3.467          | kinase         |
| **GSK3B**     | glycogen synthase kinase 3 beta           | $-2.733$        | $-6.635$       | 2.428          | kinase         |
| **SRPK2**     | SRPK protein kinase 2                      | $-5.614$        | $-23.589$      | 4.020          | kinase         |
| **THRA**      | thyroid hormone receptor, alpha            | $-2.799$        | $-11.518$      | 4.115          | ligand-dependent nuclear receptor |
| **ATRX**      | alpha thalassemia/mental retardation syndrome X-linked | 2.091           | $-5.964$       | 12.471         | transcription regulator |
| **BTG2**      | BTG family, member 2                        | $-2.220$        | $-5.803$       | 2.614          | transcription regulator |
| **CCAR1**     | cell division cycle and apoptosis regulator 1 | $-2.943$ | $-11.648$ | 3.958          | transcription regulator |
| **CCND1**     | cyclin D1                                  | 2.152           | $-2.027$       | 4.362          | transcription regulator |
| **CEBPD**     | CCAAT/enhancer binding protein (C/EBP), delta | 11.271         | 2.037          | 5.533          | transcription regulator |
| **DEK**       | DEK proto-oncogene                           | $-3.006$        | $-7.352$       | 2.446          | transcription regulator |
| **DNAJB6**    | Dna1 (Hsp40) homolog, subfamily B, member 6 | $-4.383$        | 5.614          | $-24.606$      | transcription regulator |
| **KLF13**     | Kruppel-like factor 13                      | $-2.006$        | $-4.582$       | 2.284          | transcription regulator |
| **KLF6**      | Kruppel-like factor 6                       | 6.003           | 2.865          | 2.095          | transcription regulator |
| **NAA15**     | N(alpha)-acetyltransferase 15, NatA auxiliary subunit | 3.605           | $-3.751$       | 13.522         | transcription regulator |
| **NFX1**      | nuclear factor X (CCAAT-binding transcription factor) | $-2.548$ | $-8.112$ | 3.184          | transcription regulator |
| **PA2G4**     | proliferation-associated 2G4, 38 kDa        | $-2.702$        | $-5.783$       | 2.140          | transcription regulator |
| **SMARCA4**   | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 | 2.521           | $-7.712$       | 19.442         | transcription regulator |
| **STAT3**     | signal transducer and activator of transcription 3 (acute-phase response factor) | 4.219           | $-3.771$       | 15.910         | transcription regulator |
| **TBL1XR1**   | transducin (beta)-like 1 X-linked receptor 1 | 2.587           | $-2.134$       | 5.521          | transcription regulator |
| **TCF4**      | transcription factor 4                      | $-2.216$        | $-4.625$       | 2.087          | transcription regulator |
| **TPR**       | translocated promoter region, nuclear basket protein | 2.212           | $-2.728$       | 6.034          | transporter |
| **Brd4**      | bromodomain containing 4                    | $-3.528$        | $-15.202$      | 4.309          | other          |
| **CDT1**      | chromatin licensing and DNA replication factor 1 | 3.098           | $-2.295$       | 7.110          | other          |
| **GADD45G**   | growth arrest and DNA-damage-inducible, gamma | 3.191           | $-2.384$       | 7.607          | other          |
| **PSIP1**     | PC4 and SFRS1 interacting protein 1         | $-2.663$        | 2.113          | $-5.627$       | other          |
| **Rbm25**     | RNA binding motif protein 25               | $-5.547$        | $-16.213$      | 2.923          | other          |
| **THOC2**     | THO complex 2                              | 2.119           | $-4.886$       | 10.353         | other          |

**Unknown**

| Gene          | TBI-I/TBI-C Ratio | $TBI-I$ $TBI-C$ | $TBI-I$ $TBI-C$ | $TBI-I$ $TBI-C$ | $TBI-I$ $TBI-C$ |
|---------------|-------------------|----------------|----------------|----------------|----------------|
| **EIF3C**     | eukaryotic translation initiation factor 3, subunit C | $-4.369$ | $-9.072$ | 2.076          | translation regulator |
| **Nos1ap**    | nitric oxide synthase 1 (neuronal) adaptor protein | $-2.698$ | $-5.717$ | 2.119          | other          |
| **RASSF4**    | Ras association (RalGDS/AF-6) domain family member 4 | 4.289           | 2.106          | 2.037          | other          |
transcardially with saline followed by cold 4 % paraformaldehyde solution in PBS for 30 min. Brains were then frozen in OCT mounting medium and stored until sectioning. Coronal sections of 20 μm thickness were cryosectioned from the perilesional brain area of each animal. Sections were mounted on slides which were stored at −80 °C until further processed. Fluoro-Jade® B (AG310, Millipore, Billerica, MA) labeling was performed as previously described [20]. TUNEL staining was performed using the TUNEL reaction mixture from the In Situ Cell Death Detection Kit, TMR red (12 156 792 910, Roche Diagnostics, Mannheim, Germany). Briefly, slide-mounted sections were post-fixed with 4 % paraformaldehyde for 15 min followed by a 10 min incubation in a 20 μg/mL proteinase K solution in 100 mM Tris HCl (pH 8.0) and 50 mM EDTA. The sections were then incubated for 60 min at 37 °C in the TUNEL reaction mixture. Phosphate buffered saline was used to rinse the sections after each step. A Zeiss fluorescence microscope equipped with a CCD camera (Carl Zeiss Microimaging, Inc., Thornwood, NY) was used to capture digital images of the sections.

Real-time polymerase chain reaction (PCR)
RNA was extracted as above and quantified using the Nanodrop 2000c (Thermo Scientific, Waltham, MA). Equal amounts of ipsilateral, contralateral, and naïve RNA (n = 2 for each) were converted to cDNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (170–8840, Bio-Rad Laboratories, Inc., Hercules, CA). The resulting product was diluted 1:100 with RNase-free sterile water. The diluted product was used in the real-time PCR analysis using the Quantitect SYBR® Green PCR Kit (204143, Qiagen, Hilden, Germany), custom oligo primers for SPP1, HSPB1, STAT3, CCND1, and GAPDH (reference gene) (Life Technologies, Rockville, MD), and a Bio-Rad CFX96™ Real-Time System mounted on a C1000™ Thermal Cycler. All steps were carried out according to manufacturer’s protocols. The real-time PCR results were analyzed using the ΔΔCt method where ΔCt1 = Ct (Target A-exp) − Ct (GAPDH-exp); ΔCt2 = Ct (Target A-naïve) − Ct (GAPDH-naïve); and ΔΔCt = ΔCt1 − ΔCt2. The normalized target gene expression level was given by 2^−ΔΔCt. The results were compared pairwise using a one-tail T-test assuming equal variance. Differences were considered significant when p < 0.05.

Results
Functional analysis
To begin understanding the cell death gene response following TBI, we first looked at the biological functions associated with our datasets. Analysis of the top
15 molecular and cellular functions associated with the TBI-I (ipsilateral vs. naïve) and TBI-C (contralateral vs. naïve) datasets in IPA showed that cell death and survival (CD) was the second ranked TBI-I function that is also ranked in the top 7 functions for TBI-C (Fig. 1a, b). Also ranked in the top 7 molecular and cellular functions for both datasets are cellular growth and proliferation, cellular assembly and organization, cellular function and maintenance, cellular development, and cell morphology. Cellular movement and cell-to-cell signaling and interaction are ranked in the top 7 only for TBI-I and TBI-C, respectively.

**Histology**

To examine cell death histologically, we chose to look at the cortical area adjacent to the impact site so we could observe the cellular response to the injury in all layers of the cortex. This is not possible at the impact site because of the resulting injury cavity. Fluoro-Jade B (FJB) staining showed a dense distribution of damaged neurons throughout all layers of the cortex near the site of impact (Fig. 2a, b). Damaged neurons were also detected in the hippocampus ipsilateral to the injury (Fig. 2d). These neurons were sparsely distributed in the hippocampal CA regions. No FJB staining was detected in the cortex (Fig. 2c) or hippocampus (Fig. 2e) contralateral to the injury.

**Cell death gene expression patterns**

Focusing on the CD genes in our datasets, we determined that 902 CD genes had a greater than 2-fold change in expression. Of these genes, 361 CD genes changed uniquely on the ipsilateral side of the brain. 317 of those genes (88 %) increased while 44 genes (12 %) decreased in expression (Fig. 4a). 136 CD genes changed uniquely on the contralateral side of the brain and, in contrast to what we observed on the ipsilateral side, only 34 genes (25 %) increased while 102 genes (75 %) decreased in expression (Fig. 4b).

There were 405 CD genes that changed on both the ipsilateral and contralateral sides of the brain. In order to determine whether these common genes changed differently on one side of the brain compared to the other, we

| Network ID | Molecules in network | Score | Focus molecules | Top diseases and functions |
|------------|----------------------|-------|------------------|---------------------------|
| 1          | CADM1, CALB1, CBFB, CDC2, CMIP, Cytochrome bc1, cytochrome-c oxidase, DAB2, DEDD, FGFR, FN, GCLC, GCM, GFA, GFRA1, ITG, JDP2, MDA, MED14, MGEA5, MFE2L1, NFE2L2, NPTX1, NRP1, PDHA1, PDLIM7, Rsr, RPS24, RTN4, SLCA1, Sox, STK17B, TAF4B | 46    | 31               | Cell Death and Survival, Drug Metabolism, Molecular Transport |
| 2          | AMOT, ANXA1, AP15, ATF3, ATG12, BAG3, CCNA2, Cdc2, CDK1, CDK2, CKDN1B, ET5, FGFR3, FN1, GJA1, Hedgehog, LAT5, MCL1, MCM2, MCM8, MMTL4, MMS2L, NAA15, Patched, PIG3CA2, PKP2, PMSA7, RAB35, RPS1, SPIN1, TALG2, THOC2, TJP2, UNC5B, XPO1 | 46    | 32               | Cell Death and Survival, Cell Cycle, Reproductive System Development and Function |
| 3          | AHCF1, AKAP12, amyase, BCL11A, CA4, CACNA1G, CCND1, CLCN7, CREB1, CREBBP, CSF1, CSRNP1, CTNNB1, DES, Histone h3, IKK (complex), ITPR2, KLF6, KPNB1, MITF, MTMR1, NFIX, PRKD3, PGR1, RA14, RNA polymerase II, RRM2, SENP2, SMAD4, SMARCA4, SUSD5, TBL1XR1, TGM2, THRA, ZBTB18 | 43    | 31               | Cell Death and Survival, Organismal Survival, Gene Expression |
| 4          | ABCA1, ALB, ALDH1A2, BTG2, Ccl2, Ccl7, CD36, CREBP, CREM, CXL3, DUSP5, EGR2, FGFR2, FGL2, FOSS1, FSR, Hmgb2 (includes others), HMOX1, IL1, IL12 (family), IL6R, ITGB2, KLF4, MAPK9, NEK6, NEK7, PDE4B, Pik, PPRK1, PGTS2, SPP1, THBD, TLR4, WNT5A | 42    | 30               | Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking |
| 5          | ACS5L, AGTR2, AMFR, AVP, CAMK2N1, CAPRIN1, CH51, CUL5, DCK, ELAV1, Endothelin, GMCL1, GNH, Insulin, MAP4K4, MS22, MTC1, NEO1, OPAL, Proinsulin, PTGER3, Relaxin, RNF2, SLCA3, SMAD7, STAG1, TACR1, TEC8, TEMEM23, TRAF6, WAPAL, WFS1, WATAP, ZMYM2, ZNF280B | 41    | 29               | Cell Death and Survival, Cardiovascular System Development and Function, Hereditary Disorder |
| 6          | 26 s Proteasome, ARL11, BCL2L1, CAMK1G, CAMK2D, CASP3, CAV1, CDS1, CLASP1, CLN5, DLG4, EN2, ENC1, Esr1-Esr1-estrogen-estrogen, FBOX9, G2E3, Hsp70, Hsp90, HSP90A1, IDE, KIF18, MDM2, PCDH15, PGR, PI4K2A, PRDM2, PSEN1, SGLP1, SNCA, SPTBN1, SRC (family), SRPK2, TEMEM109, TRIM2, VPS41 | 40    | 30               | Cell Death and Survival, Cancer, Neurological Disease |

Bold= > Gene included in the dataset

Note: Some of the nodes in the original networks represent gene groups, complexes or families that, when expanded, contain more potential GOI.
Table 3: The top 6 gene networks associated with the TBI-C dataset

| Network ID | Molecules in network | Score | Focus molecules | Top diseases and functions |
|------------|----------------------|-------|-----------------|---------------------------|
| 1          | ACER2, ACIN1, ACVR1C, ALDH1A2, ARHGEF7, BCL11B, caspase, CBFB, CD38, CD44, CLCN3, CUL5, DPYD, EEF1A2, FGL2, Fibrinogen, ITGB1, MAP3K1, MAP3K8, MAPK8, MAPK9, MIF, MTDH, PAK1, PRDX6, Rac, RAD23B, SPARC, Srebp, TNKS2, TITL1, VCL, WNT5A, ZBTB18, ZYX | 50    | 31              | Cell Death and Survival, Cellular Movement, Ophthalmic Disease |
| 2          | ABCA1, AURKAIP1, BRINP1, BTG2, CACNA1G, CAV1, CCND1, CDK2, DCK, GCLC, Histone h3, Histone h4, Insulin, IRAK1, KMT2A, LCN2, MAFG, MTMR1, PIT10, PIA51, PPARGC1B, Pro-inflammatory Cytokine, Ras homolog, RBM5, RNA polymerase II, SBF1, SETD8, SLC18A2, SMARCA2, SOX2, STAT1, TRPM7, ZBTB7A, ZMYND11, ZNF148 | 44    | 28              | Cell Death and Survival, Gene Expression, Cellular Growth and Proliferation |
| 3          | ADNP, AHI1, ANKS1B, ARL6IP1, CDK11A, CXCL12, DNAJb6, ENC1, estrogen receptor, FBXO9, FBXW7, FGFRI, G2E3, Hdac, HSP, Hsp90, HSP90AA1, HSP90B1, IKB, KLF9, KLF13, LING01, MED1, MED14, mediator, PA2G4, PGR, PPP3CB, RNF4, STUB1, THRA, TRAP/Media, TUFM, Ubiquitin, VPS41 | 43    | 28              | Cell Death and Survival, Post-Translational Modification, Protein Folding |
| 4          | A2M, ACACA, AKT2, ALDH1A1, Alp, AMPK, ATG12, ATP1A1, BSG, CA3, EIF5B, ENTPD5, FGF9, FGFRI, Focal adhesion kinase, FOXO1, KRAS, MAP1B, MEF2A, Mi2, NLK, NTRK3, PALLD, PDK1, PITX1, PPP3R1, PRKAA1, PRKCD, PSMA7, RASSF4, RPS24, Srebpl, Sfr, STK17B, TAO1 | 41    | 30              | Cell Death and Survival, Carbohydrate Metabolism, Cellular Development |
| 5          | ACAC, AP2B1, APAF1, APC-AXIN-GSK3β, ATP2A2, ATP2B1, ATP2B2, BAX, Ca2 ATPase, calpain, CAST, CDH13, Cytochrome bc1, cytochrome C, cytochrome-c oxidase, DDIT4, DNM1L, GBX2, glutathione peroxidase, GSK3b, ITP3, KDRN2, LMO4, MAVb, MAFb, Mfn1, Mitochondrial complex 1, MTF2, NC51, NDUFA61, NFE2L1, OPA1, PACS2, PEX11B, PRKAA2 | 39    | 26              | Cell Death and Survival, Cell Cycle, Cellular Compromise |
| 6          | Ap1, ARHGAP1, ARL6IP5, CDC26, CCND2, CEBPD, Cg, COL1A1, DACH1, FSH, Growth hormone, Gsk3, IGF1BP3, Lh, MGEA5, NEO1, PDHA1, PPP2R1A, PRLR, PSIP1, PRU, RAB27A, RPRM, RS1, SMAD4, SMAD7, Smad1/5/8, Smad2/3, Sp1, SPP1, TAF4B, Tgf beta, TIMP1, TNRC6A, ZMYM2 | 39    | 26              | Cell Death and Survival, Tissue Development, Cellular Growth and Proliferation |

Bold => Gene included in the dataset

Note: Some of the nodes in the original networks represent gene groups, complexes or families that, when expanded, contain more potential GOI
calculated the ratio of the TBI-I fold change to the TBI-C fold change. Those genes that had a TBI-I/TBI-C ratio greater than 2 were determined to have changed differently. We observed that 332 of the common CD genes (82 %) changed similarly (TBI-I/TBI-C ratio < 2; Fig. 4c). Of the genes that changed similarly, 242 genes (60 %) increased in expression and 90 genes (22 %) decreased in expression. The remaining 73 common CD genes (18 %) changed differently (TBI-I/TBI-C ratio > 2) (Fig. 4c). Table 1 shows the 73 common CD genes that changed differently. These genes span all cellular compartments (extracellular space, plasma membrane, cytoplasm, and nucleus) with diverse molecule types. The expression of all these genes was lower on the contralateral side of the brain with the exception of 3 genes, DNAJB6, TRIM54 and PSIP1 (negative TBI-I/TBI-C ratio). Because of their different expression patterns, these 73 genes became our first group of genes of interest (GOI; Table 1). Notable genes given their high TBI-I/TBI-C ratio included SPP1, TIMP1, LCN2, SERPINA3, KCNN4, SERPINA3, KCNN4, HSPB1, RDX, Slpi, ATRX, DNAJB6, NAA15, SMARCA4, STAT3, and THOC2.

Canonical pathway analysis
We used canonical pathway and network analysis in IPA to identify genes in our datasets that were potentially most relevant to the observed CD gene response. We defined

| Network ID | GOI found                                                                 | Total # of GOI | Overlap with previous analyses | Net # of GOI | Top molecular types                        |
|------------|---------------------------------------------------------------------------|----------------|-------------------------------|--------------|---------------------------------------------|
| 1          | CALB1, CDCA7L, CMIP, DAB2, FLNA, GCLM, GFAP, NFE2L2, PDLIM7                | 9              | 1                             | 8            | undefined                                   |
| 2          | ANXA1, ATF3, BAG3, CCNA2, CDK1, CDKN1B, ETV5, FN1, LAT5, MCL1, MCM2, MCM8, MMS22L, NAA15, RAB35, SPN1, TAGLN2, THOC2, TJP2, UNCSB | 20             | 4                             | 16           | undefined, enzymes, and kinases             |
| 3          | BCL11A, CCND1, CREB1, CREBBP, CSRP1, DES, JKBK8, JPR1, KLF6, KP1B1, MIF, NF1, PTGR1, RAI14, RMM2, SENP2, SMARCA4, SUDS3, TBL1X, TGM2, THRA | 21             | 7                             | 14           | transcription regulators and enzymes        |
| 4          | ALB, Btg2, Ccl2, CCL3L3, CCL4, Cdc6, Cdc7, CD36, CEBPB, CREM, CXC1L1, CCL3, Cxcl9, Dusps5, EGR2, FGF2, FGL2, FOSS1, HMOX1, IL1B, IL2, ITGB2, KLF4, NEK6, P2RSE, PTGS2, SPP1, TLR4 | 28             | 4                             | 24           | cytokines, transcription regulators, and transmembrane receptors |
| 5          | ACSL5, CAMK1N, CH51Y, ELAV1, MAPK4, MS12, PTGER3, TCEB3, TME123, TAF6, WFS1 | 11             | 2                             | 9            | undefined and kinases                       |
| 6          | ARL11, CAMK1G, CASP3, CSD2, CLN5, DNAJB6, DNAJB9, FGR, HCK, HSPA1A/HSPA1B, HSPA2, HSPA9, HSPB8, MDM2, PCDH15, PA2, PRDM2, SGFL1, SNCA, SRPK2, TME109 | 21             | 4                             | 17           | undefined, kinases, and transcription regulators |

Italics= > gene of interest also found in a previous analysis; Bold= > GOI unique to this analysis
potential GOI, in this context, as those genes that either changed in expression uniquely on one side of the brain, or were one of the 73 common genes that changed differently (Table 1). GOI were identified by comparing the genes in the canonical pathway and gene networks to the list of unique TBI-I or TBI-C CD genes with the genes from Table 1 added to each list and identifying the overlapping genes. Canonical pathways in IPA are well-characterized metabolic and cell-signaling pathways derived from information found in specific journal articles, review articles, text books, and KEGG Ligand [21]. Fig. 5 shows the apoptosis signaling canonical pathway with all relevant gene families, groups and complexes expanded to show the member genes. This pathway was chosen because apoptosis is a key process in cell death following TBI [22–24]. By overlaying the relative expression values of potential GOI for TBI-I (Fig. 5a) and TBI-C (Fig. 5b), we were able to identify 9 GOI that were increased (BCL2A1 (Bfl-1 in pathway), CASP3, CASP7, CDK1 (Cdc2), IKBKB, MAP4K4, MCL1, NFkB2, and TNFRSF1A) in the TBI-I dataset, 3 GOI that decreased (ACIN1 (Acinus), BAX and KRAS) and 1 GOI that increased (MAPK8 (JNK1)) in the TBI-C dataset.

Gene network analysis
In contrast to canonical pathways, which are relatively immutable in IPA, gene networks are generated de novo in IPA based on the list of genes that are imported. IPA takes “seed” molecules from the gene list, searches the Ingenuity Knowledge Base, and uses a network algorithm to draw connections between molecules based on biological function [25]. In order to generate the networks, we performed an IPA core analysis on the TBI-I and TBI-C CD datasets.

IPA scores the networks in order to rank them according to their degree of relevance to the network eligible molecules in the dataset [25]. The top 6 scoring networks for each dataset were used to identify GOI. Five of the top 6 networks for TBI-I and all 6 networks for TBI-C have cell death and survival as their top associated biological function (Tables 2 and 3). Only TBI-I network 4 does not have cell death and survival as one of the top 3 associated biological functions. Figure 6 shows networks 2 and 4 (Table 2) as examples of the TBI-I analysis. Figure 7 shows networks 2 and 4 (Table 3) as examples of the TBI-C analysis. (The other networks are available as supplemental materials (Additional files 1 and 2).) Like the canonical pathway analysis, all relevant gene families, groups and complexes were expanded to show the member genes. The relative TBI-I (Fig. 6) and TBI-C (Fig. 7) gene expression values of potential GOI were overlaid on these networks and additional GOI were identified. Tables 4 and 5 show the resulting GOI that were identified through this analysis. For TBI-I, a total of 110 GOI were found in these networks, 22 of which were previously identified (Table 4). Thus, 88 additional GOI were identified for TBI-I. For TBI-C, 38 additional GOI were identified as 28 of the 66 GOI found had been previously identified (Table 5). The most prevalent molecular types for TBI-I were transcription regulators, unspecified enzymes, kinases, and undefined molecules. Kinases transcription regulators, unspecified enzymes, and undefined molecules were most prevalent in the TBI-C analysis.

Compiling the gene interaction hierarchy (GIH)
TBI-I: By combining the GOI identified through canonical pathway and network analysis with those in Table 1, we identified a total of 170 GOI. In order to determine which genes might be most relevant to CD, we ranked these genes relative to each other by the number of direct interactions each had with the other GOI. Our analysis showed that 145 of the GOI formed an interconnected network, leaving 25 “orphan” genes (see Additional file 3). Genes having 1st order connections with more than 10 % of the other genes within the main

| Network ID | GOI found | Total # of GOI | Overlap with previous analyses | Net # of GOI | Top molecular types |
|-----------|-----------|---------------|-----------------------------|-------------|---------------------|
| 1         | ACIN1, ACOVR1C, CD44, DPYD, FGL2, MAPK8, MTDH, RAD23B, TALL1 | 9               | 4                           | 5           | enzymes and kinases |
| 2         | AURKAIP1, BTG2, CCND1, LCN2, MAFG, PIK3CD, PIK3R2, RND3, SETD8, SOX2, TRPM7, ZMYND11, ZNF148 | 13              | 5                           | 8           | transcription regulators, kinases, and enzymes |
| 3         | AKR1B1, CDX11A, CDX19, DNAJb6, HSP90AA1, HSPB1, KLF13, LINGO1, MED1, P264, PPP3CB, THRA, TUFM | 13              | 7                           | 6           | transcription regulators, undefined, and kinases |
| 4         | EEF3B, ENTPDS, FOXO1, KRAS, MEF2A, PALLD, PRKAA2, PTK2B, RASSF4, Serbp | 10              | 3                           | 7           | undefined, transcription regulators, enzymes, and kinases |
| 5         | ATP2A2, ATP2B2, BAX, CDH13, GBX2, GSK3B, KCND2, MAFB, MFN1, NDUFAB1, PRKAA2 | 11              | 4                           | 7           | transporters, kinases, enzymes, and undefined |
| 6         | Ccdc86, CCND2, CEBPD, GSK3B, PRR, PSIP1, RSF1, SP1, SPP1, TIMP1 | 10              | 5                           | 5           | transcription regulators, undefined, and cytokines |

Italics = gene of interest also found in a previous analysis; Bold = GOI unique to this analysis
GOI network (>14 connections) were considered “primary” in this analysis (see Fig. 8 for an example). Genes having connections with 5 %–10 % of the other genes (8–14 connections) were considered “secondary” (see Additional file 4 for an example) and those with connections with less than 5 % of the other genes (<8 connections) were considered “peripheral”. The resultant GIH is displayed in Table 6.

![TBI-I GOI Network](image)

**Fig. 8** An example of calculating the number of direct connections for the TBI-I GOI network. In IPA, the gene in question was selected (MDM2 in this example). Then, its direct connections were selected by right clicking on MDM2 and using the “select nearest neighbors” option (highlighted in purple). A list of the selected genes was exported and MDM2 was removed from the list (upper right corner). The remaining genes were counted (26 in this example) and MDM2 was ranked in the TBI-I gene interaction hierarchy (primary tier) by this number.
Table 6 TBI-I Gene interaction hierarchy (GIH)

| Gene symbol | Entrez gene name                                                                 | Fold change | Cellular compartment | Molecular type               |
|-------------|----------------------------------------------------------------------------------|-------------|----------------------|------------------------------|
| **Primary** |                                                                                  |             |                      |                              |
| ATF3        | activating transcription factor 3                                                | 12.027      | Nucleus              | transcription regulator      |
| CCND1       | cyclin D1                                                                        | 2.152       | Nucleus              | transcription regulator      |
| CEBPB       | CCAAT/enhancer binding protein (C/EBP), beta                                     | 3.366       | Nucleus              | transcription regulator      |
| CREB1       | cAMP responsive element binding protein 1                                       | 2.666       | Nucleus              | transcription regulator      |
| CREBBP      | CREB binding protein                                                             | 2.421       | Nucleus              | transcription regulator      |
| MDM2        | MDM2 proto-oncogene, E3 ubiquitin protein ligase                                 | 2.01        | Nucleus              | transcription regulator      |
| NFE2L2      | nuclear factor, erythroid 2-like                                                 | 2.452       | Nucleus              | transcription regulator      |
| SMARCA4     | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 | 2.521       | Nucleus              | transcription regulator      |
| STAT3       | signal transducer and activator of transcription 3 (acute-phase response factor) | 4.219       | Nucleus              | transcription regulator      |
| CDK1        | cyclin-dependent kinase 1                                                         | 2.105       | Nucleus              | kinase                       |
| CSNK2A1     | casein kinase 2, alpha 1 polypeptide                                             | 2.992       | Cytoplasm            | kinase                       |
| EGFR        | epidermal growth factor receptor                                                 | 6.773       | Plasma Membrane      | kinase                       |
| GSK3B       | glycogen synthase kinase 3 beta                                                  | −2.733      | Nucleus              | kinase                       |
| CD44        | CD44 molecule (Indian blood group)                                               | 15.558      | Plasma Membrane      | enzyme                       |
| FN1         | fibronectin 1                                                                    | 3.97        | Extracellular Space  | enzyme                       |
| TRAF6       | TNF receptor-associated factor 6, E3 ubiquitin protein ligase                     | 2.163       | Cytoplasm            | enzyme                       |
| CASP3       | caspase 3, apoptosis-related cysteine peptidase                                   | 2.535       | Cytoplasm            | peptidase                    |
| ELAVL1      | ELAV like RNA binding protein 1                                                  | 3.275       | Cytoplasm            | other                        |
| **Secondary** |                                                                                  |             |                      |                              |
| CEBPD       | CCAAT/enhancer binding protein (C/EBP), delta                                     | 11.271      | Nucleus              | transcription regulator      |
| CREM        | cAMP responsive element modulator                                                | 2.165       | Nucleus              | transcription regulator      |
| EGR2        | early growth response 2                                                           | 2.271       | Nucleus              | transcription regulator      |
| FOSL1       | FOS-like antigen 1                                                               | 5.875       | Nucleus              | transcription regulator      |
| KLF4        | Kruppel-like factor 4 (gut)                                                      | 2.057       | Nucleus              | transcription regulator      |
| MITF        | microphthalmia-associated transcription factor                                   | 4.755       | Nucleus              | transcription regulator      |
| TCF4        | transcription factor 4                                                            | −2.216      | Nucleus              | transcription regulator      |
| HSPA1A/HSPA1B | heat shock 70 kDa protein 1A                                              | 3.137       | Cytoplasm            | enzyme                       |
| MCM2        | minichromosome maintenance complex component 2                                   | 2.57        | Nucleus              | enzyme                       |
| PTGS2       | prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) | 3.106       | Cytoplasm            | enzyme                       |
| IL1B        | interleukin 1, beta                                                              | 5.166       | Extracellular Space  | cytokine                     |
| SPP1        | secreted phosphoprotein 1                                                         | 37.905      | Extracellular Space  | cytokine                     |
| CDKN1B      | cyclin-dependent kinase inhibitor 18 (p27, Kip1)                                  | 3.732       | Nucleus              | kinase                       |
| IKBKB       | inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta       | 2.127       | Cytoplasm            | kinase                       |
| KPNB1       | karyopherin (importin) beta                                                      | 3.173       | Nucleus              | transporter                  |
| MCL1        | myeloid cell leukemia 1                                                           | 3.25        | Cytoplasm            | transporter                  |
| THRA        | thyroid hormone receptor, alpha                                                   | −2.799      | Nucleus              | ligand-dependent nuclear receptor |
| CASP7       | caspase 7, apoptosis-related cysteine peptidase                                   | 2.579       | Cytoplasm            | peptidase                    |
| RAG3        | BCL2-associated athanogene 3                                                     | 4.045       | Cytoplasm            | other                        |
| Gene                                           | Description                                                                 | GIH   | Compartment       | GIH   |
|------------------------------------------------|------------------------------------------------------------------------------|-------|-------------------|-------|
| **Peripheral**                                 |                                                                              |       |                   |       |
| **ACSL5**                                      | acyl-CoA synthetase long-chain family member 5                              | −2.361| Cytoplasm enzyme  |       |
| **ANXA1**                                      | annexin A1                                                                   | 3.535 | Plasma Membrane   |       |
| **CHSY1**                                      | chondroitin sulfate synthase 1                                              | 2.873 | Cytoplasm enzyme  |       |
| **CP**                                         | ceruloplasmin (ferroxidase)                                                  | 27.838| Extracellular Space| enzyme|
| **EHD4**                                       | EH-domain containing 4                                                       | 2.361 | Plasma Membrane   |       |
| **GCLM**                                       | glutamate-cysteine ligase, modifier subunit                                 | 2.019 | Cytoplasm enzyme  |       |
| **HMOX1**                                      | heme oxygenase (decycling) 1                                                | 9.778 | Cytoplasm enzyme  |       |
| **HMC8**                                       | minichromosome maintenance complex component 8                             | 2.027 | Nucleus enzyme    |       |
| **MX1**                                        | MX dynamin-like GTPase 1                                                    | 28.177| Cytoplasm enzyme  |       |
| **PDE4B**                                      | phosphodiesterase 4B, cAMP-specific                                         | 5.602 | Cytoplasm enzyme  |       |
| **RAB35**                                      | RAB35, member RAS oncogene family                                          | 2.086 | Cytoplasm enzyme  |       |
| **RRM2**                                       | ribonucleotide reductase M2                                                 | 3.34  | Nucleus enzyme    |       |
| **SDC1**                                       | syndecan 1                                                                  | 13.681| Plasma Membrane   | enzyme|
| **SETD8**                                      | SET domain containing (lysine methyltransferase) 8                          | 2.029 | Nucleus enzyme    |       |
| **SGPL1**                                      | sphingosine-1-phosphate lyase 1                                             | 3.108 | Cytoplasm enzyme  |       |
| **SRXN1**                                      | sulfiredoxin 1                                                              | 6.306 | Cytoplasm enzyme  |       |
| **TGM2**                                       | transglutaminase 2                                                           | 3.574 | Cytoplasm enzyme  |       |
| **TOP2A**                                      | topoisomerase (DNA) II alpha 170 kDa                                        | 2.26  | Nucleus enzyme    |       |
| **BCL11A**                                     | B-cell CLL/lymphoma 11A (zinc finger protein)                               | −2.38 | Nuclear           |       |
| **BTG2**                                       | BTG family, member 2                                                         | −2.22 | Nucleus transcription regulator |       |
| **CCAR1**                                      | cell division cycle and apoptosis regulator 1                               | −2.943| Nuclear transcription regulator |       |
| **CSRN1**                                      | cysteine-serine-rich nuclear protein 1                                      | 2.821 | Nuclear transcription regulator |       |
| **DEK**                                        | DEK proto-oncogene                                                           | −3.006| Nuclear transcription regulator |       |
| **DNAJB6**                                     | DnaJ (Hsp40) homolog, subfamily B, member 6                                 | −4.383| Nuclear transcription regulator |       |
| **ETV5**                                       | ets variant 5                                                               | −2.163| Nuclear transcription regulator |       |
| **KLF13**                                      | Kruppel-like factor 13                                                      | −2.006| Nuclear transcription regulator |       |
| **KLF6**                                       | Kruppel-like factor 6                                                       | 6.003 | Nucleus transcription regulator |       |
| **NAA15**                                      | N(alpha)-acyetyltransferase 15, NatA auxiliary subunit                      | 3.605 | Nuclear transcription regulator |       |
| **NFIX**                                       | nuclear factor I/X (CCAAAT-binding transcription factor)                    | −2.548| Nuclear transcription regulator |       |
| **NFIB2**                                      | nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100) | 2.768 | Nuclear transcription regulator |       |
| **PA2G4**                                      | proliferation-associated 2G4, 38 kDa                                        | −2.702| Nuclear transcription regulator |       |
| **PRDM2**                                      | PR domain containing 2, with ZNF domain                                     | 3.677 | Nuclear transcription regulator |       |
| **TBL1XR1**                                    | transducin (beta)-like 1 X-linked receptor 1                               | 2.587 | Nuclear transcription regulator |       |
| **TCEB3**                                      | transcription elongation factor B (SII), polypeptide 3 (110 kDa, elongin A) | 3.053 | Nuclear transcription regulator |       |
| **CAMK1G**                                     | calcium/calmodulin-dependent protein kinase 1                              | −2.271| Cytoplasm kinase  |       |
| **CAMK2N1**                                    | calcium/calmodulin-dependent protein kinase II inhibitor 1                 | −11.813| Plasma Membrane  | kinase|
| **CARD11**                                     | caspase recruitment domain family, member 11                                | 7.343 | Cytoplasm kinase  |       |
| **CDK11A**                                     | cyclin-dependent kinase 11A                                                 | −4.29 | Nucleus kinase    |       |
Table 6 TBI-I Gene interaction hierarchy (GH) (Continued)

| Gene Symbol | Gene Name | GIH | Location | Function |
|-------------|-----------|-----|----------|----------|
| FGR         | FGR proto-oncogene, Src family tyrosine kinase | 3.915 | Nucleus | kinase |
| HCK         | HCK proto-oncogene, Src family tyrosine kinase | 3.887 | Cytoplasm | kinase |
| HSPB8       | heat shock 22 kDa protein 8 | 4.112 | Cytoplasm | kinase |
| LAT51       | large tumor suppressor kinase | 2.003 | Nucleus | kinase |
| MAP4K4      | mitogen-activated protein kinase kinase kinase kinase | 2.258 | Cytoplasm | kinase |
| NEK6        | NIMA-related kinase 6 | 2.322 | Nucleus | kinase |
| SRPK2       | SRSF protein kinase 2 | −5.614 | Nucleus | kinase |
| TJF2        | tight junction protein 2 | 2.552 | Plasma Membrane | kinase |
| Ccl2        | chemokine (C-C motif) ligand 2 | 195.455 | Extracellular Space | cytokine |
| CCL3L3      | chemokine (C-C motif) ligand 3-like 3 | 5.269 | Extracellular Space | cytokine |
| CCL4        | chemokine (C-C motif) ligand 4 | 2.162 | Extracellular Space | cytokine |
| Ccl6        | chemokine (C-C motif) ligand 6 | 10.291 | Extracellular Space | cytokine |
| Ccl7        | chemokine (C-C motif) ligand 7 | 124.78 | Extracellular Space | cytokine |
| CXCL3       | chemokine (C-X-C motif) ligand 3 | 13.211 | Extracellular Space | cytokine |
| Cxcl9       | chemokine (C-X-C motif) ligand 9 | 2.846 | Extracellular Space | cytokine |
| TIMP1       | TIMP metalloproteinase inhibitor 1 | 38.486 | Extracellular Space | cytokine |
| IL6R        | interleukin 6 receptor | 2.315 | Plasma Membrane | transmembrane receptor |
| IL6ST       | interleukin 6 signal transducer | 2.307 | Plasma Membrane | transmembrane receptor |
| ITGB2       | integrin, beta 2 (complement component 3 receptor 3 and 4 subunit) | 2.675 | Plasma Membrane | transmembrane receptor |
| TLR4        | toll-like receptor 4 | 2.699 | Plasma Membrane | transmembrane receptor |
| TNFRSF1A    | tumor necrosis factor receptor superfamily, member 1A | 3.555 | Plasma Membrane | transmembrane receptor |
| UNC5B       | unc-5 homolog B (C. elegans) | 2.067 | Plasma Membrane | transmembrane receptor |
| ALB         | albumin | −3.125 | Extracellular Space | transporter |
| LCN2        | lipocalin 2 | 71.824 | Extracellular Space | transporter |
| RASA1       | RAS p21 protein activator (GTPase activating protein) 1 | 2.392 | Cytoplasm | transporter |
| TPR         | translocated promoter region, nuclear basket protein | 2.212 | Nucleus | transporter |
| FGL2        | fibrinogen-like 2 | 16.793 | Extracellular Space | peptidase |
| SENP2       | SUMO1/sentrin/SMT3 specific peptidase 2 | 2.051 | Nucleus | peptidase |
| DUSP5       | dual specificity phosphatase 5 | 3.285 | Nucleus | phosphatase |
| PTPRF       | protein tyrosine phosphatase, receptor type, F | −6.365 | Plasma Membrane | phosphatase |
| EIF3C       | eukaryotic translation initiation factor 3, subunit C | −4.369 | Other | translation regulator |
| EIF5B       | eukaryotic translation initiation factor SB | −3.044 | Cytoplasm | translation regulator |
| FGF2        | fibroblast growth factor 2 (basic) | 2.387 | Extracellular Space | growth factor |
| KCND2       | potassium voltage-gated channel, Shal-related subfamily, member 2 | −2.792 | Plasma Membrane | ion channel |
| AHI1        | Abelson helper integration site 1 | 2.243 | Cytoplasm | other |
| BCL2A1      | BCL2-related protein A1 | 3.055 | Cytoplasm | other |
| CALB1       | calbindin 1, 28 kDa | −2.091 | Cytoplasm | other |
| CD68        | CD68 molecule | 4.365 | Plasma Membrane | other |
| CDC247L     | cell division cycle associated 7-like | 2.648 | Nucleus | other |
| CDT1        | chromatin licensing and DNA replication factor 1 | 3.098 | Nucleus | other |
| CSF2D2      | CDGSH iron sulfur domain 2 | −7.833 | Cytoplasm | other |
| CMPC        | c-Maf inducing protein | −3.778 | Cytoplasm | other |
| DAB2        | Dab, mitogen-responsive phosphoprotein, homolog 2 (Drosophila) | 3.053 | Plasma Membrane | other |
| Gene                  | Description                                              | GIH   | Location          | Classification       |
|-----------------------|-----------------------------------------------------------|-------|-------------------|----------------------|
| DES                   | desmin                                                   | 2.857 | Cytoplasm         | other                |
| DNAJB9                | DnaJ (Hsp40) homolog, subfamily B, member 9              | 2.128 | Nucleus           | other                |
| FLNA                  | filamin A, alpha                                         | 3.45  | Cytoplasm         | other                |
| GADD45G               | growth arrest and DNA-damage-inducible, gamma            | 3.191 | Nucleus           | other                |
| HLA-A                 | major histocompatibility complex, class I, A             | 9.296 | Plasma Membrane   | other                |
| HSPA2                 | heat shock 70 kDa protein 2                              | 3.51  | Cytoplasm         | other                |
| LCP1                  | lymphocyte cytosolic protein 1 (L-plastin)               | 6.082 | Cytoplasm         | other                |
| LSP1                  | lymphocyte-specific protein 1                            | 11.716| Cytoplasm         | other                |
| MMS22L                | MMS22-like, DNA repair protein 2                         | 2.918 | Nucleus           | other                |
| MSI2                  | musashi RNA-binding protein 2                            | 2.288 | Cytoplasm         | other                |
| PDLIM7                | PDZ and LIM domain 7 (enigma)                            | 4.695 | Cytoplasm         | other                |
| PHLD1A                | pleckstrin homology-like domain, family A, member 1      | 5.129 | Cytoplasm         | other                |
| PMEPA1                | prostate transmembrane protein, androgen induced 1       | 2.682 | Plasma Membrane   | other                |
| PSIP1                 | PC4 and SFRS1 interacting protein 1                      | −2.663| Nucleus           | other                |
| RDX                   | radixin                                                  | 4.828 | Cytoplasm         | other                |
| SERPINA3              | serpineptide inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3 | 58.488| Extracellular Space| other                |
| SPIN1                 | spindlin 1                                               | 2.178 | Nucleus           | other                |
| SUDS3                 | suppressor of defective silencing 3 homolog (S. cerevisiae) | 2.228 | Nucleus           | other                |
| TAGLN2                | transgelin 2                                             | 3.891 | Cytoplasm         | other                |
| THOC2                 | THO complex 2                                            | 2.119 | Nucleus           | other                |
| TMEM109               | transmembrane protein 109                                | 2.106 | Cytoplasm         | other                |
| TMEM123               | transmembrane protein 123                                | 2.348 | Plasma Membrane   | other                |

**Orphan**

| Gene                  | Description                                              | GIH   | Location          | Classification       |
|-----------------------|-----------------------------------------------------------|-------|-------------------|----------------------|
| CYP1B1                | cytochrome P450, family 1, subfamily B, polypeptide 1     | 10.998| Cytoplasm         | enzyme               |
| KIF3A                 | kinesin family member 3A                                  | −5.083| Cytoplasm         | enzyme               |
| PTGR1                 | prostaglandin reductase 1                                 | 2.258 | Cytoplasm         | enzyme               |
| RND3                  | Rho family GTPase 3                                       | 2.864 | Cytoplasm         | enzyme               |
| WFS1                  | Wolfram syndrome 1 (wolfamin)                             | 2.083 | Cytoplasm         | enzyme               |
| ITPR2                 | inositol 1,4,5-trisphosphate receptor, type 2             | 2.489 | Cytoplasm         | ion channel          |
| KCNIN4                | potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 | 3.088 | Plasma Membrane   | ion channel          |
| ATRX                  | alpha thalassemia/mental retardation syndrome X-linked    | 2.091 | Nucleus           | transcription regulator |
| RAI14                 | retinoic acid induced 1                                   | 3.284 | Nucleus           | transcription regulator |
| CX3CL1                | chemokine (C-X3-C motif) ligand 1                         | −2.044| Extracellular Space | cytokine             |
| PTGER3                | prostaglandin E receptor 3 (subtype EP3)                  | 2.425 | Plasma Membrane   | G-protein coupled receptor |
| P44K2A                | phosphatidylinositol 4-kinase type 2 alpha                | 2.96  | Cytoplasm         | kinase               |
| CD36                  | CD36 molecule (thrombospondin receptor)                   | 5.08  | Plasma Membrane   | transmembrane receptor |
| ARL11                 | ADP-ribosylation factor-like 11                           | 3.143 | Other             | other                |
| Brd4                  | bromodomain containing 4                                  | −3.528| Nucleus           | other                |
| CLN5                  | ceroid-lipofuscinosis, neuronal 5                         | 2.041 | Cytoplasm         | other                |
| Ctdspl                | CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like | −7.271| Cytoplasm         | other                |
| KIFAP3                | kinesin-associated protein 3                              | −2.281| Cytoplasm         | other                |
| Nos1tap               | nitric oxide synthase 1 (neuronal) adaptor protein        | −2.698| Other             | other                |
| PCDH15                | protocadherin-related 15                                  | 2.147 | Plasma Membrane   | other                |
Table 6 TBI Gene interaction hierarchy (GIH) (Continued)

| Gene   | Description                                      | Rank | TBI-I | Rank | TBI-C | Rank |
|--------|--------------------------------------------------|------|-------|------|-------|------|
| RASSF4 | Ras association (RalGDS/AF-6) domain family member 4 | 4.289| Other | other|       |       |
| Rbm25  | RNA binding motif protein 25                      | −5.547| Nucleus | other|       |       |
| Slpi   | secretory leukocyte peptidase inhibitor           | 82.908| Cytoplasm | other|       |       |
| Tpm3   | tropomyosin 3                                    | 2.592| Cytoplasm | other|       |       |
| TRIM54 | tripartite motif containing 54                    | −4.426| Cytoplasm | other|       |       |

Primary: >14 connections in GOI network (see text); Secondary: 8–14 connections in GOI network; Peripheral: <8 connections in GOI network; Orphan: No connections in GOI network; Italics: => Gene changes on both sides of the brain

TBI-C: A total of 115 GOI were identified. Our analysis showed that 78 of the GOI formed an interconnected network, leaving 37 “orphan” genes (see Additional file 5). Genes having 1st order connections with more than 10 % of the other genes within the main GOI network (>8 connections) were considered “primary” in this analysis (see Fig. 9 for an example). Genes having connections with 5 %–10 % of the other genes (4–8 connections) were considered “secondary” (see Additional file 6 for an example) and those with connections with less than 5 % of the other genes (<4 connections) were considered “peripheral”. The resultant GIH is displayed in Table 7.

Cell cycle genes included in the GIHs
We performed an IPA molecular and cellular functional analysis on the unranked GOI for both datasets and the top 2 tiers (most significant by our definition) of our resultant GIHs to further elucidate the most significant biological functions post-TBI (Fig. 10). The cell death and survival category was removed from this analysis since all genes were initially selected from this functional category. When analyzing the top 2 tiers of the GIHs, cell cycle was ranked second for TBI-I and first for TBI-C. Is was also the highest ranked molecular and cellular function common to both sides (Fig. 10 b, d). The cell cycle moved up 5 functional ranking spots on both sides of the brain from where it was ranked prior to the GIH analysis. This result was intriguing because aberrant attempts to reactivate the cell cycle by post-mitotic neurons have been implicated as a trigger for apoptosis [26, 27]. By cross-referencing our GIHs with genes that IPA includes in the cell cycle upper level biological function, we determined that 74 genes in the TBI-I GIH and 47 genes in the TBI-C GIH were associated with the cell cycle (Tables 8 and 9). Just over 85 % of the cell cycle genes increased in expression ipsilaterally compared to controls. The relative inverse is true contralaterally with nearly 79 % of the cell cycle genes decreasing in expression. Remarkably, 83 % of TBI-I and 70 % of TBI-C primary and secondary tier genes were classified as cell cycle genes (TBI-I: 35 of 42 genes; TBI-C: 19 of 27 genes).

Real-time PCR
As expected, ipsilateral expression was significantly increased compared to naïve for all genes tested following TBI (Fig. 11). However, ipsilateral expression was only significantly different from contralateral expression for SPP1 and HSPB1 while this comparison for STAT3 (p = 0.088) and CCND1 (p = 0.063) fell short of statistical significance. Contralateral expression was not significantly different from naïve for any of the genes tested.

Discussion
We used microarray technology and subsequent bioinformatic analysis in this study to examine molecular and functional alterations following TBI. Not surprisingly, cell death and survival was determined to be a significant molecular and cellular function associated with the genes expressed ipsi- lateral to the injury. Interestingly, while cell death was not observed on the contralateral side of the brain, there was significant modulation of cell death and survival genes and this molecular and cellular function is very highly associated with the gene expression pattern.

Our histology results using markers for cell damage (FJB) and DNA fragmentation (TUNEL) suggest a potential opportunity for therapeutic intervention. At 24 h post-injury, there is a developing cortical cavity at the site of impact surrounded with FJB and TUNEL-positive cells. Therapy aimed at preserving cortical tissue should be administered in the acute period to exert maximal neuroprotective effects. However, while there is significant correlation between FJB and TUNEL staining in the cortex at this time point, no TUNEL-positive cells were detected in the hippocampus where FJB detected some neuronal damage on the ipsilateral side. Similar histology results were recently seen with our model of nerve agent exposure [20] and a neuroprotective agent was able to rescue the hippocampal neurons [28]. This suggests that these hippocampal neurons have not yet progressed to the point of apoptosis and an extended therapeutic window may exist for subcortical brain areas.

Our microarray data showed that TBI resulted in significant alterations in CD gene expression on both sides of the brain. Nearly 45 % of the differentially expressed CD genes were common to both sides of the brain and 82 % of those genes changed similarly. However, a distinct expression pattern was exhibited by the balance of the common CD genes and those that change in expression
uniquely on one side of the brain. The vast majority of these ipsilateral CD genes increased in expression compared to controls, while the majority of these contralateral CD genes decreased in expression compared to controls or were reduced compared to ipsilateral expression. Notable was the expression of key apoptosis-related genes. BCL2A1, caspases 3 and 7, CDK1, cyclins A2 and D1, and NFKB2 showed increased expression ipsilaterally, while BAX, cyclins D1 and D2, KRAS and PIK3CD showed decreased expression contralaterally.

It is important to note here that the real-time PCR results for the genes selected did not agree totally with the microarray results. This was especially true for the contralateral samples. However, it has been shown that the correlation between microarray and real-time PCR results is lower for genes showing

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**Fig. 9** An example of calculating the number of direct connections for the TBI-C GOI network. In IPA, the gene in question was selected (SOX2 in this example). Then, its direct connections were selected by right clicking on SOX2 and using the "select nearest neighbors" option (highlighted in blue). A list of the selected genes was exported and SOX2 was removed from the list (upper right corner). The remaining genes were counted (13 in this example) and SOX2 was ranked in the TBI-C gene interaction hierarchy (primary tier) by this number.
| Gene symbol | Entrez gene name                                                                 | Fold change | Cellular compartment | Molecular type            |
|-------------|---------------------------------------------------------------------------------|-------------|----------------------|----------------------------|
| **Primary** |                                                                                  |             |                      |                            |
| CCND1       | cyclin D1                                                                        | −2.027      | Nucleus              | transcription regulator    |
| MED1        | mediator complex subunit 1                                                       | −4.011      | Nucleus              | transcription regulator    |
| SMARCA4     | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 | −7.712      | Nucleus              | transcription regulator    |
| SOX2        | SRY (sex determining region Y)-box 2                                             | −4.791      | Nucleus              | transcription regulator    |
| SP1         | Sp1 transcription factor                                                         | −2.076      | Nucleus              | transcription regulator    |
| STAT3       | signal transducer and activator of transcription 3 (acute-phase response factor) | −3.771      | Nucleus              | transcription regulator    |
| CSNK2A1     | casein kinase 2, alpha 1 polypeptide                                             | −2.75       | Cytoplasm            | kinase                     |
| EGFR        | epidermal growth factor receptor                                                 | 2.374       | Plasma Membrane      | kinase                     |
| GSK3B       | glycosynthase kinase 3 beta                                                      | −6.635      | Nucleus              | kinase                     |
| CD44        | CD44 molecule (Indian blood group)                                               | 2.399       | Plasma Membrane      | enzyme                     |
| HSP90AA1    | heat shock protein 90 kDa alpha (cytosolic), class A member 1                    | −4.843      | Cytoplasm            | enzyme                     |
| **Secondary** |                                                                              |             |                      |                            |
| FOXO1       | forkhead box O1                                                                  | −3.329      | Nucleus              | transcription regulator    |
| MEF2A       | myocyte enhancer factor 2A                                                       | −6.31       | Nucleus              | transcription regulator    |
| NFIX        | nuclear factor 1/X (CCAAT-binding transcription factor)                         | −8.112      | Nucleus              | transcription regulator    |
| TCF4        | transcription factor 4                                                            | −4.625      | Nucleus              | transcription regulator    |
| MAPK8       | mitogen-activated protein kinase 8                                               | 2.102       | Cytoplasm            | kinase                     |
| PKR3R2      | phosphoinosotide-3-kinase 2 (beta)                                              | 2.332       | Cytoplasm            | kinase                     |
| PTK2B       | protein tyrosine kinase 2 beta                                                   | 2.15        | Cytoplasm            | kinase                     |
| KRAS        | Kirsten rat sarcoma viral oncogene homolog                                       | −2.027      | Cytoplasm            | enzyme                     |
| TOP2A       | topoisomerase (DNA) II alpha 170 kDa                                            | −2.406      | Nucleus              | enzyme                     |
| ATP2A2      | ATPase, Ca++ transporting, cardiac muscle, slow twitch 2                         | −2.607      | Cytoplasm            | transporter                |
| BAX         | BCL2-associated X protein                                                        | −3.306      | Cytoplasm            | transporter                |
| SPP1        | secreted phosphoprotein 1                                                        | 2.37        | Extracellular Space  | cytokine                   |
| THRA        | thyroid hormone receptor, alpha                                                  | −11.518     | Nucleus              | ligand-dependent nuclear receptor |
| TUFM        | Tu translation elongation factor, mitochondrial                                 | −2.109      | Cytoplasm            | translation regulator      |
| CCND2       | cyclin D2                                                                       | −3.617      | Nucleus              | other                      |
| HSPB1       | heat shock 27 kDa protein 1                                                      | 2.639       | Cytoplasm            | other                      |
| **Peripheral** |                                                                              |             |                      |                            |
| BTG2        | BTG family, member 2                                                             | −5.803      | Nucleus              | transcription regulator    |
| CCAR1       | cell division cycle and apoptosis regulator 1                                     | −11.648     | Nucleus              | transcription regulator    |
| CEBPD       | CCAAT/enhancer binding protein (C/EBP), delta                                    | 2.037       | Nucleus              | transcription regulator    |
| DEK         | DEK proto-oncogene                                                               | −7.352      | Nucleus              | transcription regulator    |
| DNAJB6      | DnaJ (Hsp40) homolog, subfamily B, member 6                                       | 5.614       | Nucleus              | transcription regulator    |
| GBX2        | gastrulation brain homeobox 2                                                    | 2.59        | Nucleus              | transcription regulator    |
| KLF6        | Kruppel-like factor 6                                                            | 2.865       | Nucleus              | transcription regulator    |
| MAFG        | v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog G                   | −2.632      | Nucleus              | transcription regulator    |
| MTDH        | metallothionein                                                                | −2.544      | Cytoplasm            | transcription regulator    |
| PAG4        | proliferation-associated 2G4, 38 kDa                                             | −5.783      | Nucleus              | transcription regulator    |
| RSF1        | remodeling and spacing factor 1                                                  | −2.618      | Nucleus              | transcription regulator    |
| Gene        | Description                                                                 | Log2 Fold Change | Location          | Function                  |
|-------------|------------------------------------------------------------------------------|------------------|-------------------|---------------------------|
| TBL1XR1     | transducin (beta)-like 1 X-linked receptor 1                                 | −2.134           | Nucleus           | transcription regulator   |
| ZMYND11     | zinc finger, MYND-type containing 11                                         | −2.211           | Nucleus           | transcription regulator   |
| ZNF148      | zinc finger protein 148                                                      | 2.114            | Nucleus           | transcription regulator   |
| ACIN1       | apoptotic chromatin condensation inducer 1                                   | −2.515           | Nucleus           | enzyme                    |
| CYP1B1      | cytochrome P450, family 1, subfamily B, polypeptide 1                        | 4.808            | Cytoplasm         | enzyme                    |
| DPYD        | dihydropyrimidine dehydrogenase                                              | 2.292            | Cytoplasm         | enzyme                    |
| MFN1        | mitofusin 1                                                                  | 2.304            | Cytoplasm         | enzyme                    |
| MX1         | MX dynamin-like GTPase 1                                                    | 7.326            | Cytoplasm         | enzyme                    |
| SETD8       | SET domain containing (lysine methyltransferase) 8                           | −3.93            | Nucleus           | enzyme                    |
| TICL1       | tubulin tyrosine ligase-like family, member 1                                | 2.284            | Extracellular Space| enzyme                   |
| ACVR1C      | activin A receptor, type IC                                                  | −9.107           | Plasma Membrane   | kinase                    |
| CARD11      | caspase recruitment domain family member 11                                  | 2.892            | Cytoplasm         | kinase                    |
| CDK11A      | cyclin-dependent kinase 11A                                                  | −14.872          | Nucleus           | kinase                    |
| CDK19       | cyclin-dependent kinase 19                                                  | −2.191           | Nucleus           | kinase                    |
| PKC3CD      | phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta     | −2.113           | Cytoplasm         | kinase                    |
| PRKAA2      | protein kinase, AMP-activated, alpha 2 catalytic subunit                     | −2.546           | Cytoplasm         | kinase                    |
| SPP2        | SRF domain kinase 2                                                          | −23.589          | Nucleus           | kinase                    |
| PPP3CB      | protein phosphatase 3, catalytic subunit, beta isozyme                       | 2.1              | Plasma Membrane   | phosphatase               |
| PTPRF       | protein tyrosine phosphatase, receptor type, F                              | −20.492          | Plasma Membrane   | phosphatase               |
| IL6ST       | interleukin 6 signal transducer                                              | −3.283           | Plasma Membrane   | transmembrane receptor    |
| PRLR        | prolactin receptor                                                          | −3.192           | Plasma Membrane   | transmembrane receptor    |
| LCN2        | lipocalin 2                                                                  | 3.895            | Extracellular Space| transporter               |
| RASA1       | RAS p21 protein activator (GTPase activating protein) 1                     | −2.105           | Cytoplasm         | transporter               |
| TIMP1       | TIMP metallopeptidase inhibitor 1                                            | 2.101            | Extracellular Space| cytokine                 |
| EIF5B       | eukaryotic translation initiation factor 5B                                  | −8.766           | Cytoplasm         | translation regulator     |
| AH1         | Abelson helper integration site 1                                            | −2.897           | Cytoplasm         | other                     |
| CDT1        | chromatin licensing and DNA replication factor 1                             | −2.295           | Nucleus           | other                     |
| CISO        | CDGSH iron sulfur domain 2                                                   | −19.012          | Cytoplasm         | other                     |
| GADD45G     | growth arrest and DNA-damage-inducible, gamma                               | −2.384           | Nucleus           | other                     |
| HLA-A       | major histocompatibility complex, class I, A                                | 3.657            | Plasma Membrane   | other                     |
| LINGO1      | leucine rich repeat and Ig domain containing 1                              | −2.173           | Plasma Membrane   | other                     |
| MAFB        | v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B               | −2.018           | Nucleus           | other                     |
| PHLD1A      | pleckstrin homology-like domain, family A, member 1                         | 2.16             | Cytoplasm         | other                     |
| PMEP4A1     | prostate transmembrane protein, androgen induced 1                           | −2.937           | Plasma Membrane   | other                     |
| PSIP1       | PC4 and SFRS1 interacting protein 1                                           | 2.113            | Nucleus           | other                     |
| RAD23B      | RAD23 homolog B (S. cerevisiae)                                              | −2.217           | Nucleus           | other                     |
| RASSF4      | Ras association (RalGDS/AF-6) domain family member 4                         | 2.106            | Other             | other                     |
| RXD         | radixin                                                                     | −5.274           | Cytoplasm         | other                     |
| Serpb1      | Serpine1 mRNA binding protein 1                                              | −2.059           | Cytoplasm         | other                     |
| SERPINA3    | serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3 | 2.509            | Extracellular Space| other                     |

**Orphan**

| Gene        | Description                                                                 | Log2 Fold Change | Location          | Function                  |
|-------------|------------------------------------------------------------------------------|------------------|-------------------|---------------------------|
| AURKAIP1    | aurora kinase A interacting protein 1                                        | −2.023           | Nucleus           | enzyme                    |
| CP          | ceruloplasmin (ferroxidase)                                                  | 8.477            | Extracellular Space| enzyme                   |
decreased expression and having lower fold changes [29]. Our results do show better correlation with the large TBI-I fold change genes SPP1 (37.9 fold) and HSPB1 (46.9 fold). The remaining fold changes for the selected genes are less than ± 4.22 with most in the 2.0-2.6 range. While further validation including more genes and a larger sample size may be needed for subsequent studies, these PCR results are consistent with expression of these genes being higher for TBI-I and lower for TBI-C. It is in this context that the discussion of the microarray results continues.

As stated above, this contralateral expression pattern in our model may indicate an endogenous effort to suppress

### Table 7 TBI-C Gene interaction hierarchy (GIH) (Continued)

| Gene       | Description                          | Fold Change | Location       | Function                         |
|------------|--------------------------------------|-------------|----------------|----------------------------------|
| EHD4       | EH-domain containing 4               | –2.056      | Plasma Membrane | enzyme                           |
| ENTPDS     | ectonucleoside triphosphate diphosphohydrolase 5 | –2.055      | Cytoplasm      | enzyme                           |
| KIF3A      | kinesin family member 3A             | –11.754     | Cytoplasm      | enzyme                           |
| NDUFA1     | NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8 kDa | –2.028      | Cytoplasm      | enzyme                           |
| PDE4B      | phosphodiesterase 4B, CAMP-specific  | 2.359       | Cytoplasm      | enzyme                           |
| RND3       | Rho family GTPase 3                 | –2.971      | Cytoplasm      | enzyme                           |
| SDC1       | syndecan 1                           | 2.566       | Plasma Membrane | enzyme                           |
| SPINK1     | sulfiredoxin 1                       | 2.402       | Cytoplasm      | enzyme                           |
| ATRX       | alpha thalassemia/mental retardation syndrome X-linked | –5.964      | Nucleus        | transcription regulator          |
| KLF13      | Kruppel-like factor 13               | –4.582      | Nucleus        | transcription regulator          |
| NAA15      | N(alpha)-acetyltransferase 15, NatA auxiliary subunit | –3.751      | Nucleus        | transcription regulator          |
| KCN2       | potassium voltage-gated channel, Shal-related subfamily, member 2 | –7.585      | Plasma Membrane | ion channel                      |
| KCN4       | potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 | –9.429      | Plasma Membrane | ion channel                      |
| CAMK2N1    | calcium/calmodulin-dependent protein kinase II inhibitor 1 | –23.824     | Plasma Membrane | kinase                           |
| TRPM7      | transient receptor potential cation channel, subfamily M, member 7 | 2.226       | Plasma Membrane | kinase                           |
| ATP2B2     | ATPase, Ca++ transporting, plasma membrane 2 | 2.276       | Plasma Membrane | transporter                       |
| TPR        | translated promoter region, nuclear basket protein | –2.728      | Nucleus        | transporter                       |
| FGL2       | fibrinogen-like 2                    | 4.017       | Extracellular Space | peptidase                      |
| EIF3C      | eukaryotic translation initiation factor 3, subunit C | –9.072      | Other           | translation regulator             |
| Brd4       | bromodomain containing 4             | –15.202     | Nucleus        | other                            |
| CCDC86     | coiled-coil domain containing 86     | –2.149      | Nucleus        | other                            |
| CD68       | CD68 molecule                        | 2.007       | Plasma Membrane | other                            |
| CDH13      | cadherin 13                          | –2.692      | Plasma Membrane | other                            |
| CMP        | c-Maf inducing protein               | –13.763     | Cytoplasm      | other                            |
| Ctdsp1     | CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like | –36.886     | Cytoplasm      | other                            |
| KIFAP3     | kinesin-associated protein 3         | –7.831      | Cytoplasm      | other                            |
| LCP1       | lymphocyte cytosolic protein 1 (L-plastin) | 2.799       | Cytoplasm      | other                            |
| LSP1       | lymphocyte-specific protein 1        | 2.14        | Cytoplasm      | other                            |
| Nos1ap     | nitric oxide synthase 1 (neuronal) adaptor protein | –5.717      | Other           | other                            |
| PALLD      | palladin, cytoskeletal associated protein | –5.086     | Plasma Membrane | other                            |
| Rbm25      | RNA binding motif protein 25         | –16.213     | Nucleus        | other                            |
| Slpi       | secretory leukocyte peptidase inhibitor | 3.119       | Cytoplasm      | other                            |
| THOC2      | THO complex 2                        | –4.886      | Nucleus        | other                            |
| Tpm3       | tropomysin 3                         | –2.715      | Cytoplasm      | other                            |
| TRIM54     | tripartite motif containing 54       | –2.032      | Cytoplasm      | other                            |

Primary: >8 connections in GOI network (see text); Secondary: 4–8 connections in GOI network; Peripheral: <4 connections in GOI network; Orphan: No connections in GOI network; Italics= > Gene changes on both sides of the brain
cell death promoting genes remote from the injury in order to prevent spreading of the injury and offer additional protection from additional insults, similar to gene expression changes in ischemic preconditioning [30, 31]. An analogous and potentially neuroprotective gene expression pattern was observed in an in vitro model of mild TBI where the modulation of genes reflected an endogenous effort to prevent oxidative/nitrosative stress and apoptosis during a transient period of mitochondrial malfunctioning [32]. We have previously reported a similar gene expression pattern for inflammatory response genes following TBI [12]. In that previous study, genes from both sides of the brain were pooled for analysis. Because we now believe that analyzing gene expression on the contra-lateral side is critical to understanding endogenous protective mechanisms, the full GIH analysis [33] was performed on each side of the brain separately. By determining the key molecules involved in the endogenous effort to suppress cell death, it may be possible to develop molecular strategies to provide neuroprotection for the injured brain as well as augment the endogenous neuroprotective process.

We identified 170 TBI-I and 115 TBI-C GOI through canonical pathway and network analysis combined with the common genes that change differently on each side of the brain. Many of these genes have been previously associated with acute brain injuries (i.e., TBI, stroke) but not all of them have been connected to the cell death caused by these injuries. These genes include BAX, CASP3, CCNA2, CCND1, CD44, CD68, CEBPD, GSK3B, HSPB1, IL1B, LCN2, NFKB2, SERPINA3, SPP1, STAT3, TIMP1, TNFRSF1A, and TOP2A [14, 34–42]. This supported the idea that our methods for identifying genes of interest targets important genes in the post-injury response. Several genes which have been linked to cell death in cancer, epilepsy, or psychological disorders but not yet associated with brain injury, including CSNK2A1, ELAVL1, MITF, and SMARCA4, were also identified which may provide additional therapeutic targets for prevention of cell death following TBI. We next
wanted to determine which genes were central to cell death processes. We approached this by creating a network of our GOI within IPA and determining how many 1st order connections each gene had with the other genes in the network. A GIH was created based on these numbers and distinct patterns in terms of molecular type were found.

For TBI-I, transcription regulators were the predominant molecular type in the top 2 tiers of the GIH. This result was expected from our previous GIH analyses [12]. After the transcription regulators, kinases and unspecified enzymes were prominent in the top 2 tiers of this GIH. In the peripheral tier, unspecified enzymes, transcription regulators and kinases were most represented. Cytokines, transmembrane receptors, and transporters also had notable numbers in the peripheral tier. Remarkably, only 2 cytokines, IL1B and SPP1, are included in the top 2 tiers of this GIH. This result is not unexpected as previous GIH analysis had shown that the near 1-to-1 relationship that cytokines have with their receptors limits the 1st order connections these molecules have in the GOI network [12].

Transcription regulators were also predominant in the top 2 tiers of the TBI-C GIH followed by kinases and unspecified enzymes. These same molecular types headed the peripheral tier as well with transcription regulators ahead of enzymes and kinases. Other notable molecular types in the peripheral tier were phosphatases, transmembrane receptors, and transporters. Again, cytokines do not have significant numbers in this GIH. Our analysis strongly suggests that other molecular types, transcription regulators, kinases, and other enzymes in this case, may be better therapeutic targets because they have the potential to impact the overall cell death process to a greater extent.

Very intriguing in our cell death analysis was how cell cycle moved up significantly in functional ranking on both sides of the brain when comparing the functional analysis for unranked GOI to that for the top 2 tiers of our GIHs. Cell cycle molecules have been implicated as apoptotic mediators for post-mitotic cells under stress due to trauma or neurological disease. It is believed that there is an aberrant attempt the re-enter the cell cycle that causes the cells to eventually undergo apoptosis [26, 43–48]. Much attention has been given to the cyclin-dependent kinases (CDKs), cyclins, which activate the CDKs [27, 48, 49], and CDK inhibitors. Significant evidence for CDK involvement in cell cycle-related apoptosis has come from the experimental use of exogenous CDK inhibitors that prevented apoptosis [47, 50–56].

Pertinent to this discussion, evidence has shown that CDK1, when activated by cyclin A [57], and CDK4 and CDK6, when activated by cyclin D in post-mitotic neurons, can lead to cell death via caspase-dependent apoptosis [26, 27, 44, 49]. Additionally, ablation of cyclin D1 reduces neurodegeneration caused by TBI [58]. CDK11 has been shown to initiate apoptosis by interacting with either cyclin D3 [59] or eukaryotic translation initiation factor 3 subunit F (EIF3F) [60]. In our model, cyclins A2 and D1 are increased ipsilaterally, consistent with other studies [27, 47, 50], while both cyclins D1 and D2 are decreased contralaterally. CDK1 and the CDK4 inhibitors, CDKN1A (p21Cip1 (not in GIH)) and CDKN1B (p27Kip1), are all increased ipsilaterally. CDK11 (CDK11A (both sides); CDK19 (TBI-C

### Table 8 Cell cycle genes in the TBI-I gene interaction hierarchy by tier

| Primary | Secondary | Peripheral | Orphan |
|---------|-----------|------------|--------|
| ATF3    | BAG3      | BCL2A1     | NEK6   | ATRX   |
| CASP3   | CASP7     | BTG2       | NFIX   | Brd4    |
| CCND1   | CCNA2     | CAMK2N1    | PA2G4  | CYP1B1  |
| CD44    | CDKN1B    | Cc12       | PDLIM7 |         |
| CDK1    | CEBPD     | CDK11A     | PMEPA1 |         |
| CEBPB   | CREM      | CDT1       | PRDM2  |         |
| CHER1   | FOSL1     | DEK        | PTPrF  |         |
| CREEB1  | HSPP1A1/H5PA1 | ETV5 | RAB35  |         |
| CSNK2A1 | HSPP1     | FGF2       | SETD8  |         |
| EGFR    | IKBKB     | FLNA       | SRPK2  |         |
| ELAV1   | IL1B      | GADD45G    | SUSD3  |         |
| FN1     | KLF4      | HMOX1      | TBLXR1 |         |
| GSK3B   | KPNB1     | HSPP2      | TCEB3  |         |
| MDM2    | MCL1      | IL6R       | THOC2  |         |
| NFE2L2  | MCM2      | KLF6       | TIMP1  |         |
| SMARCA4 | MITF      | LAT5       | TNP5F1A|         |
| STAT3   | PTG52     | MCM8       | TOP2A  |         |
| SPP1    | MMP32L    |            | TPR    |         |

### Table 9 Cell cycle genes in the TBI-C gene interaction hierarchy by tier

| Primary | Secondary | Peripheral | Orphan |
|---------|-----------|------------|--------|
| CCND1   | BAX       | ACIN1      | MTDH   | ATRX   |
| CD44    | CCND2     | BTG2       | PA2G4  | Brd4    |
| CSNK2A1 | FOXO1     | CDK11A     | PMEPA1 | CAMK2N1 |
| EGFR    | HSPP1     | CDK19      | PRKAA2 | CDH13   |
| GSK3B   | KRB5      | CDT1       | PTPRF  | ENTPD5  |
| SMARCA4 | MAPK8     | CEBPD      | RSF1   | THOC2   |
| SOX2    | NFIX      | CYP1B1     | SETD8  | TPR     |
| SPI     | PTK2B     | DEK        | SRPK2  |         |
| STAT3   | SPP1      | GADD45G    | TBLXR1 |         |
| TOP2A   | KLF6      | TIMP1      |         |         |
|         | MAFB      |            |         |         |
(only)) decreases in expression on both sides of the brain. While not found in our analysis, EIF3F is part of the functional core of EIF along with EIF3A (TBI-C only (not in GIH)) and EIF3C (both sides) which decrease in expression following TBI [61]. It is plausible that apoptosis would occur in this injury state because these molecules are not being expressed in the tightly controlled manner necessary to properly navigate the cell cycle [46, 55]. Other CDKs have also been implicated in apoptosis and excitotoxic cell death [26, 49, 51, 52, 62, 63] but our GIH does not point to those as major players.

In addition to 4 TBI-I and 2 TBI-C CDK-related genes, IPA classified 31 other TBI-I genes and 17 other TBI-C genes in the top 2 tiers of their respective GIHs as cell cycle genes. It should be noted that cell cycle is an upper level function in IPA. That means these genes, while associated with the cell cycle, are not necessarily integral to its progression. These genes fell into 3 general categories. The first category included those genes that have been experimentally linked to a model of TBI. Genes in this category were ATF3, BAG3, CASP3, CASP7, CD44, CEBPB, CEBPD, CREB1, CREM, EGFR, FN1, FOSL1, GSK3B, HSPA1A/HSPA1B, HSPB1, IKBKB, IL1B, KLF4, MCL1, MDM2, NFE2L2, PTGS2, SPP1, and STAT3 for TBI-I [22, 36, 40–42, 64–78] and BAX, CD44, EGFR, FOXL1, GSK3B, HSPB1, MAPK8, SOX2, SPP1, and STAT3 for TBI-C [22, 36, 40, 41, 67, 71, 78–81]. The second category included genes that had been observed in models of hypoxia/ischemia, chemical brain lesions, or spinal cord injury. Genes in this category were CREBBP and KPNB1 for TBI-I [82, 83] and KRAS, PTK2B, SP1, and TOP2A for TBI-C [84–87]. The third category included genes that were previously linked only to the progression of cancers or psychotic disorders and, therefore, novel to a discussion of cell death following TBI. Genes in this category were CSNK2A1, ELAVL1, MCM2, MITF, and SMARCA4 for TBI-I and CSNK2A1, NFIX, and SMARCA4 for TBI-C. The specifics of how these genes are associated with the cell cycle and affect cell death are beyond the scope of this analysis. However, our GIH analysis would suggest that these genes would be intriguing targets for further study in relation to post-TBI cell death. Specifically, CCND1, CSNK2A1, SMARCA4, and STAT3 were included in the top 2 tiers for both datasets and exhibit increased

![Fig. 11 Real-time PCR results for selected genes. SPP1, HSPB1, STAT3, and CCND1 were chosen for real-time PCR studies. Using the ΔΔCt method, the normalized target gene expression level was given by 2^ΔΔCt. For all genes, ipsilateral (IPSI) expression was significantly different from naive (a–d). Ipsilateral expression was also significantly different from contralateral (CONTRA) expression for SPP1 (a) and HSPB1 (b). The comparison of ipsilateral to contralateral expression for STAT3 (c; p = 0.088) and CCND1 (d; p = 0.063) fell short of statistical significance. Contralateral expression was not significantly different from naive for any genes. The results are shown as mean ± SE. * p < 0.05, ** p < 0.01, *** p < 0.005](image-url)
expression in TBI-I and decreased expression in TBI-C. Additionally, cyclin D2 and 2 apoptosis signaling genes, BAX and KRAS, are in the secondary tier of the TBI-C GIH and show decreased expression. Targeting these key molecules showing contralateral suppression for potential therapies may prove effective because their expression correlates to the observed absence of cell death.

Conclusions
Unilateral TBI results in significant gene expression changes on both sides of the brain. The overall gene expression pattern in the brain suggests a suppression of CD genes contralateral to the injury which may be an endogenous protective mechanism. Using canonical pathways and IPA generated networks as a guide, we were able to identify genes that were central to the post-TBI CD gene response. Further network analysis allowed for the ranking of these genes into GIHs. The GIH ranking then led to the identification of cell cycle as a key molecular and cellular function on both sides of the brain. Significantly, several cell cycle molecules were identified in this analysis that exhibit increased expression ipsilaterally and decreased expression contralaterally. GIH analysis relies on connections in a virtual network. Future experiments will use discrete microdissected portions of the brain (cortex, hippocampus, striatum) in order to increase the likelihood that the molecular interactions described in the network actually do occur in vivo. This will increase the power of the GIH analysis. Further real-time PCR confirmation will be necessary with an emphasis on contralateral and decreased gene expression. Also, proteomic confirmation will be necessary to show that in vivo protein levels match our microarray results [88, 89]. Once confirmed, the key CD molecules suggested by our GIH can be further explored. Additional exploration into the remote suppression of CD genes may provide insight into neuroprotective mechanisms that could be used to develop therapies to prevent cell death following TBI.

Additional files

**Additional file 1: Examples of TBI-I networks.** TBI-I CD networks 1 (A), 3 (B), 5 (C), and 6 (D) (see Table 2) with all gene families, groups and complexes expanded to show the member genes and showing the relative expression values of potential GOI for TBI-I; red: relative increase in expression; green: relative decrease in expression; white: no change in expression; gold: connections and outlines: expansion of gene families, groups and complexes in the original network. (TIF 4.06 mb)

**Additional file 2: Examples of TBI-C networks.** TBI-C CD networks 1 (A), 3 (B), 5 (C), and 6 (D) (see Table 3) with all gene families, groups and complexes expanded to show the member genes and showing the relative expression values of potential GOI for TBI-C; red: relative increase in expression; green: relative decrease in expression; white: no change in expression; gold: connections and outlines: expansion of gene families, groups and complexes in the original network. (TIF 4.30 mb)

**Additional file 3: The TBI-I GOI network.** This is the resultant network when IPA connected our 170 TBI-I GOI using only direct (1st order) connections between the genes. 145 of the GOI formed an interconnected network, leaving 25 “orphan” genes. (TIF 4.20 mb)

**Additional file 4: An example of calculating the number of direct connections for the TBI-I GOI network.** In IPA, the gene in question was selected (HSPB1 in this example). Then, its direct connections were selected by right clicking on HSPB1 and using the “select nearest neighbors” option (highlighted in purple). A list of the selected genes was exported and HSPB1 was removed from the list (upper right corner). The remaining genes were counted (11 in this example) and HSPB1 was ranked in the TBI-I gene interaction hierarchy (secondary tier) by this number. (TIF 3.99 mb)

**Additional file 5: The TBI-C GOI network.** This is the resultant network when IPA connected our 115 TBI-C GOI using only direct (1st order) connections between the genes. 78 of the GOI formed an interconnected network, leaving 37 “orphan” genes. (TIF 4.84 mb)

**Additional file 6: An example of calculating the number of direct connections for the TBI-C GOI network.** In IPA, the gene in question was selected (CCND2 in this example). Then, its direct connections were selected by right clicking on CCND2 and using the “select nearest neighbors” option (highlighted in purple). A list of the selected genes was exported and CCND2 was removed from the list (upper right corner). The remaining genes were counted (6 in this example) and CCND2 was ranked in the TBI-C gene interaction hierarchy (secondary tier) by this number. (TIF 4.69 mb)
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