various target RNAs and interacting proteins, exceeding the size of 500-kDa in human cells (Sephton et al., 2011). Previously, we found that substantial amounts of TDP-43 proteins form a dimer in human cells and brain tissues (Shiina et al., 2010), being consistent with the self-assembling capacity (Kuo et al., 2009) and the aggregation-prone propensity of TDP-43 (Johnson et al., 2009). Several recent studies identified numerous TDP-43 target RNAs and interacting proteins by deep sequencing with next-generation sequencers and mass spectrometric analysis (Freibaum et al., 2010; Sephton et al., 2011). The present study is conducted to characterize the comprehensive molecular network of TDP-43 target RNAs and interacting proteins derived from publicly available datasets by using pathway analysis tools of bioinformatics endowed with comprehensive knowledgebase.

METHODS

Dataset of TDP-43 Target RNAs

By RNA immunoprecipiation followed by deep sequencing (RIP-seq), a recent study identified thousands of TDP-43 targets RNAs in cultured rat cortical neurons (Sephton et al., 2011). The cell lysate of rat cortical neurons was processed for immunoprecipitation with anti-TDP-43 antibody or nonspecific rabbit IgG. Then, RNA was purified from the immunoprecipitates, and treated with DNAse I to remove genomic DNA contamination. cDNA libraries were constructed and sequenced on the Illumina GA IIx Genome Analyzer. The short reads were mapped onto the rat genome (build rn4) using the Bowtie software. The numbers of short sequence fragments enriched in the TDP-43 library versus the control library were counted. The study identified top 25% enriched 4,352 TDP-43 target RNAs as the most reliable set. We converted individual RefSeq accession numbers of the genes listed in this dataset into corresponding Entrez Gene IDs by using the Gene ID Conversion tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 (david.abcc.ncifcrf.gov) (Huang et al., 2009). Non-annotated IDs and overlapping IDs were deleted, resulting in the selection of 4,163 rat TDP-43 target RNAs.

Dataset of TDP-43 Interacting Proteins

By mass spectrometry analysis, a recent study identified 261 TDP-43-interacting proteins in cultured human cells (Freibaum et al., 2010). The cell lysate was prepared from HEK 293 cells following overexpression of FLAG-tagged TDP-43 by transfection of the expression vector, and purified by immunoprecipitation with anti-FLAG M2 affinity gel. Then, the immunoprecipitates were gel-separated, digested into peptides, and processed for LC-MS/MS analysis, followed by database search using the Mascot software. The cell lysate of nontransfected cells processed in parallel was used as a control to determine nonspecific interactions. Among 261 TDP-43

interacting proteins they identified, we selected 227 proteins that exhibit a two-fold or greater increase in TDP-43 immunoprecipitates versus the control as the most reliable set. We converted individual UniProt accession numbers listed in this dataset into corresponding Entrez Gene IDs by using the Gene ID Conversion tool of DAVID.

Molecular Network Analysis

Entrez Gene IDs of TDP-43 target RNAs (Sephton et al., 2011) and interacting proteins (Freibaum et al., 2010) described above were imported into three distinct pathway analysis tools of bioinformatics endowed with comprehensive knowledgebase, including Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.kegg.jp), Ingenuity Pathways Analysis (IPA) (Ingenuity Systems; www.ingenuity.com), and KeyMolnet (Institute of Medicinal Molecular Design; www.immd.co.jp). KEGG is a public database, while both IPA and KeyMolnet are commercial ones updated regularly.

KEGG systematically integrates genomic and chemical information to create the whole biological system *in silico*. KEGG includes manually curated reference pathways that cover a wide range of metabolic, genetic, environmental, and cellular processes, and human diseases and drugs. Currently, KEGG contains 148,769 pathways generated from 410 reference pathways. By importing the list of Entrez Gene IDs into the Functional Annotation tool of DAVID, it identifies KEGG pathways and Gene Ontology (GO) categories composed of the genes enriched in the given set with statistical significance evaluated by the modified Fisher's exact test.

IPA is a knowledgebase that contains approximately 2,500,000 biological and chemical interactions and functional annotations with definite scientific evidence, curated by expert biologists. By uploading the list of Entrez Gene IDs, the network-generation algorithm identifies focused genes integrated in a global molecular network. IPA calculates the score p-value, the statistical significance of association between the genes and the networks by the Fisher's exact test.

KeyMolnet contains knowledge-based contents on 137,300 relationships among human genes and proteins, small molecules, diseases, pathways and drugs, curated by expert biologists (Satoh, 2010). They are categorized into the core contents collected from selected review articles with the highest reliability or the secondary contents extracted from abstracts of PubMed and Human Reference Protein database (HPRD). By importing the list of Entrez Gene IDs, KeyMolnet automatically provides corresponding molecules as a node on networks. The "neighboring" network-search algorithm selects one or more molecules as starting points to generate the network of all kinds of molecular interactions around starting molecules, including direct activation/inactivation, transcriptional activation/repression, and the complex formation within the designated number of paths from starting points. The generated network was compared side by side with 459 human canonical pathways installed in the KeyMolnet library. The algorithm counting the number of overlapping molecular relations between the extracted network and the canonical pathway makes it possible to identify the canonical pathway showing the statistically significant contribution to the extracted network, as described previously (Satoh, 2010).

RESULTS

Molecular Network of TDP-43 Target RNAs

By importing Entrez Gene IDs of 4,163 TDP-43 target RNAs detected in cultured rat cortical neurons (Sephton et al., 2011) into the Functional Annotation tool of DAVID, it identified 43 KEGG pathways with statistically significant relevance under the Expression Analysis Systematic Explorer (EASE) Score threshold of 0.01, a modified Fisher Exact test p-value. The top three KEGG pathways included "Axon guidance" (p = 7.474E-13), "ErbB signaling pathway" (p = 2.507E-07), and "Spliceosome" (p = 3.495E-07) (Fig. 1; **Fig. 1. Molecular Network of TDP-43 Target RNAs Illustrated by KEGG.** By importing Entrez Gene IDs of 4,163 rat TDP-43 target RNAs (Sephton et al., 2011) into the Functional Annotation tool of DAVID, it identified KEGG pathways with statistically significant relevance. The top KEGG pathway termed "Axon guidance" is shown, where the genes highlighted by orange are included in the set of TDP-43 target RNAs.). The top three GO categories consisted of "GO:0000166~nucleotide binding " (p = 7.846E-29), "GO:0008104~protein localization" (p = 1.403E-25), and "GO:0005829~cytosol" (p = 1.942E-25) (Table 1). These results suggest that TDP-43 target RNAs are chiefly involved in regulation of neuronal signaling pathways via RNA-protein interactions.

By importing Entrez Gene IDs of 4,163 TDP-43 target RNAs into IPA, it extracted the top three molecular networks with statistical significance, composed of "RNA Post-Transcriptional Modification, Genetic Disorder, Neurological Disease" (p = 1E-104), "Genetic Disorder, Metabolic Disease, RNA Damage and Repair" (p = 1E-104), and "Gene Expression, Cancer, DNA Replication, Recombination, and Repair" (p = 1E-98). The category of "RNA Post-Transcriptional Modification" network includes modification, processing, and splicing of various classes of RNAs. These results suggest a key role of TDP-43 in RNA-dependent regulation of gene expression. By importing Entrez Gene IDs of 4,163 TDP-43 target RNAs into KeyMolnet, the neighboring network-search algorithm based on the core contents extracted

the highly complex molecular network composed of 4,430 molecules and 8,999 molecular relations, which exhibited the most significant relationship to the canonical pathways termed "Transcriptional regulation by p53" (p = 2.082E-256), "Transcriptional regulation by RB/E2F" (p = 1.041E-194), and "Integrin family" (p = 3.760E-171). It is of particular interest that p53, RB, and E2F act as a principal transcriptional factor that regulates cell cycle progression and apoptosis.

Molecular Network of TDP-43 Interacting Proteins

By importing Entrez Gene IDs of 227 TDP-43 interacting proteins detected in HEK293 cells overexpressing TDP-43 (Freibaum et al., 2010) into the Functional Annotation tool of DAVID, it identified only 2 KEGG pathways with statistically significant relevance named "Ribosome" (p = 7.438E-56) and "Spliceosome" (p = 2.182E-11) under the EASE Score threshold of 0.01 (Fig. 2; **Fig. 2. Molecular Network of TDP-43 Interacting Proteins Illustrated by KEGG.** By importing Entrez Gene IDs of 227 human TDP-43 interacting proteins (Freibaum et al., 2010) into the Functional Annotation tool of DAVID, it identified KEGG pathways with statistically significant relevance. The second rank KEGG pathway termed "Spliceosome" is shown, where the genes highlighted by orange are included in the set of TDP-43 interacting proteins.). The top three GO categories included "GO:0030529~ribonucleoprotein complex" (p = 9.329E-101), "GO:0003723~RNA binding" (p = 2.460E-98), and "GO:0006414~translational elongation" (1.252E-60) (Table 1). These results suggest that TDP-43 and its binding partners play a key role in regulation of spliceosome and ribosome functions.

By importing Entrez Gene IDs of 227 TDP-43-interacting proteins into IPA, it extracted the top three molecular networks with statistical significance, composed of "Protein Synthesis, RNA Post-Transcriptional Modification, Cell Cycle" (p = 1E-69), "RNA Post-Transcriptional Modification, Cell Morphology, Cellular Function and Maintenance" (p = 1E-69), and "RNA Post-Transcriptional Modification, Cancer, Gene Expression" (p = 1E-63), suggesting again a central role of TDP-43 in RNA-dependent regulation of gene expression. By importing Entrez

Gene IDs of 227 TDP-43 interacting proteins into KeyMolnet, the neighboring network-search algorithm based on the core contents extracted the complex molecular network comprised of 517 molecules and 552 molecular relations, which exhibited the most significant relationship to the canonical pathways, composed of "spliceosome assembly" (4.149E-086), being consistent with the results of KEGG, and in addition, "Kinesin family signaling pathway" (1.806E-081) and "14-3-3 signaling pathway" (3.042E-073) (Fig. 3; **Fig. 3. Molecular Network of TDP-43 Interacting Proteins Illustrated by KeyMolnet.** By importing Entrez Gene IDs of 227 human TDP-43 interacting proteins (Freibaum et al., 2010) into KeyMolnet, it extracted the complex molecular network composed of 517 molecules and 552 molecular relations, exhibiting the most significant relationship to the canonical pathways composed of "spliceosome assembly". Red nodes reflect the molecules included in the set of TDP-43 interacting proteins, while white nodes exhibit additional molecules extracted automatically from the core contents to establish molecular connections. The molecular relation is indicated by solid line with arrow (direct binding or activation), solid line with arrow and stop (direct inactivation), solid line without arrow (complex formation), dash line with arrow (transcriptional activation), and dash line with arrow and stop (transcriptional repression).).

The Integrated Molecular Network of TDP-43 Target RNAs and Interacting Proteins

Finally, we converted Entrez Gene IDs of 4,163 rat TDP-43 target RNAs (Sephton et al., 2011) into the corresponding human Entrez Gene IDs by using the Gene ID Conversion tool of DAVID, resulting in the identification of 4,063 presumptive human TDP-43 target RNAs. Among them, we identified the set of 106 genes shared between human TDP-43 target RNAs and interacting proteins (Table 2), in which the components of Ribosome and Splicesome pathways of KEGG were enriched significantly (data not shown). Then, we imported Entrez Gene IDs of the combined set of total 4,063 human TDP-43 RNA targets and 227 TDP43 interacting proteins into the Functional Annotation tool of DAVID, IPA and KeyMolnet.

DAVID identified 32 KEGG pathways with statistically significant relevance under the EASE Score threshold of 0.01. The top three KEGG pathways included "Ribosome" (4.249E-10), "Axon guidance" (p = 1.247E-09), and "Spliceosome" (p = 1.240E-08). The top three GO categories included "GO:0003723~RNA binding" (p = 3.995E-38), "GO:0005829~cytosol" (p = 5.094E-34), and "GO:0000166~nucleotide binding" (p = 1.365E-27) (Table 1). IPA extracted the top three molecular networks with statistical significance, composed of "RNA Post-Transcriptional Modification, Genetic Disorder, Neurological Disease" (p = 1E-104), "Protein Synthesis, Gene Expression, RNA Trafficking" (p = 1E-90), and "Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry" (p = 1E-88) (Fig. 4; **Fig. 4. The Integrated Molecular Network of Human TDP-43 RNA Targets and Interacting Proteins Illustrated by IPA.** Entrez Gene IDs of the combined set of total 4,063 human TDP-43 RNA targets and 227 TDP-43 interacting proteins were imported into IPA. The top molecular network termed "RNA Post-Transcriptional Modification, Genetic Disorder, Neurological Disease" is shown. Dark red nodes indicate the molecules that concurrently serve as both TDP-43 target RNAs and interacting proteins, while bright red nodes represent the molecues that act only as TDP-43 interacting proteins, and green nodes indicate the molecules that act only as TDP-43 target RNAs. The molecular relation is indicated by solid line (direct interaction), dash line (indirect interaction), line only (binding), line with arrowhead (acts on), line with stop (inhibits), and line with stop and arrowhead (inhibits and acts on).). These results validated a crucial role of TDP-43 in RNA-dependent regulation of gene expression. By the neighboring network-search algorithm based on the core contents, KeyMolnet extracted the highly complex molecular network comprised of 4,515 molecules and 9,362 molecular relations, which exhibited the most significant relationship to the canonical pathways, composed of "Transcriptional regulation by p53" (p = 1.383E-252), "Integrin family" (p = 2.121E-192), and "Transcriptional regulation by RB/E2F" (p = 1.823E-189), being almost consistent with the molecular network of rat TDP-43 target RNAs.

DISCUSSION

Abnormally phosphorylated, ubiquitinated, and aggregated TDP-43 proteins constitute a principal component of neuronal and glial cytoplasmic and nuclear inclusions in the brains of ALS and FTLD (Arai et al., 2006; Neumann et al., 2006). These intractable neurodegenerative diseases share substantial clinical and pathological features, establishing a novel clinical entity designated as TDP-43 proteinopathy (Geser et al., 2009). Although increasing evidence suggests that the neurodegenerative process underlying ALS and FTLD is attributable to a toxic gain of function or a loss of cellular function of TDP-43 (Kabashi et al., 2010; Janssens et al., 2011), the precise molecular mechanisms remain largely unknown. To investigate molecular mechansims responsible for development of TDP-43 proteinopathy, we attempted to characterize the comprehensive molecular network of TDP-43 target RNAs and interacting proteins, which are identified by recent studies using deep sequencing with next-generation sequencers and mass spectrometric analysis (Freibaum et al., 2010; Sephton et al., 2011).

By using RIP-seq, a recent study identified thousands of TDP-43 target RNAs in cultured rat cortical neurons (Sephton et al., 2011). The study showed that the binding sites consisting of (UG)n and (UG)nUA(UG)m are enriched in TDP-43 target RNAs. They are categorized into three groups composed of the genes having the binding sites in exons, introns, and both exons and introns. The study showed that functional categories related to synaptic function, RNA metabolism, and neuronal development are enriched in TDP-43 RNA targets. By importing their dataset into three different pathway analysis tools, we found that TDP-43 RNA targets are most closely associated with "Axon guidance" pathway of KEGG, "RNA Post-Transcriptional Modification, Genetic Disorder, Neurological Disease" network of IPA, and "Transcriptional regulation by p53" pathway of KeyMolnet. Our results suggest a pivotal role of TDP-43 in RNA-dependent regulation of neuronal gene expression. Most importantly, axonal guidance function is markedly disturbed in the brains of ALS patients and mouse models, characterized by accumulation of neurofilamentous swellings named spheroids within the axons of motor neurons (King et al., 2011). A recent genome-wide association study (GWAS) showed that

TMEM106B variants on chromosome 7p21 serve as a risk factor for development of FTLD-TDP (Van Deerlin et al., 2010). Interestingly, we identified TMEM106B as one of 4,163 TDP-43 target RNAs.

More recently, by using ultraviolet cross-linking and immunoprecipitation (CLIP) and deep sequencing (CLIP-seq), a different study identified 6,304 TDP-43 target RNAs in the adult mouse brain (Polymenidou et al., 2011). The study showed that the vast majority of TDP-43-binding sites are located in introns of the target genes regardless of the presence or absence of UG-rich repeats. Among them, 2,672 genes (42.4%) are identical to the previous results derived from rat cortical neurons (Sephton et al., 2011). By using RNA sequencing (RNA-seq) and splicing-sensitive microarrays, they found that depletion of TDP-43 by local administration of TDP-43-specific antisense oligonucelotides in the striatum of the mouse brain downregulated the expression of 239 genes involved chiefly in synaptic activity, and altered splicing events of 965 mRNAs (Polymenidou et al., 2011).

By using individual nucleotide resolution CLIP (iCLIP) and deep sequencing, another study characterized the global picture of TDP-43 target RNAs isolated from human brain tissues of healthy subjects, FTLD-U patients, and cultured cells (Tollervey et al., 2011). The study verified that the majority of TDP-43-binding sites are located in introns of target genes having long clusters of UG-rich repeats. Importantly, small populations of iCLIP cDNAs are mapped on long non-coding RNAs (ncRNAs), including small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), ribosomal RNAs (rRNAs), and telomeric ncRNAs. They found that TDP-43-binding sites located on NEAT1 and NEAT2 ncRNAs are overrepresented in FTLD-U brain samples. Knockdown of TDP-43 in SH-SY5Y cells affected 158 alternative splicing events related to regulation of the genes involved in neuronal development and survival (Tollervey et al., 2011). They also confirmed that TDP-43 binds RNA by forming a homodimer, supporting our previous observations (Shiina et al., 2010). These results suggest that TDP-43, by binding to various classes of RNAs, regulates diverse neuronal functions.

By using immunoaffinity purification and mass spectrometric analysis, a recent study identified over 200 TDP-43 interacting proteins in HEK293 cells (Freibaum et al., 2010). The

study showed that disease-causing TDP-43 mutations do not primarily affect the interacting capacity. They revealed that TDP-43 interacting proteins, functionally associated with RNA metabolism, forms two clusters composed of the nuclear splicing cluster and the cytoplasmic translation cluster. By importing their dataset into pathway analysis tools, we found that these proteins are most closely associated with "Ribosome" pathway of KEGG, "Protein Synthesis, RNA Post-Transcriptional Modification, Cell Cycle" network of IPA, and "spliceosome assembly" pathway of KeyMolnet, being consistent with a central role of TDP-43 in splicing and translation as reported previously (Freibaum et al., 2010). Based on these results, we concluded that TDP-43 and interacting proteins cooperatively regulate mRNA splicing on spliceosome and translation on ribosome, by dynamically translocating themselves from the nucleus to the cytoplasm. Previous studies indicate that TDP-43 continuously shuttles between the nucleus and the cytoplasm in a transcription-dependent manner (Ayala et al., 2008), supporting this view. Defective karyopherin-mediated nuclear import causes cytoplasmic accumulation of TDP-43 (Nishimura et al., 2010). TDP-43 proteins in the nucleus are concentrated in perichromatin fibrils (PFs), which act as nuclear sites for mRNA transcription and cotranscriptional splicing (Casafont et al., 2009).

The expression of approximately 95% of human multi-exon genes involves alternative splicing that contributes to protein diversity and tissue-specific gene expression (Chen and Manley, 2009). Both constitutive and alternative splicing events are carried on the spliceosome, composed of a battery of small nuclear ribonucleoproteins (snRNPs) and auxiliary proteins that cooperate to recognize the exact splice site and catalyse the precise splicing reaction (Chen and Manley, 2009). The cis-regulatory elements of mRNA precusors are classified into four groups, such as exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and intronic splicing silencers (ISSs). ESEs are recognized by the serine/arginine-rich (SR) protein family members, while ISSs and ESSs are bound by heterogeneous nuclear RNPs (hnRNPs). ISEs are recognized by several proteins, including neuro-oncological ventral antigen 1 (NOVA1), NOVA2, *Caenorhabditis elegans* fox-1 homolog 1 (FOX1), FOX2, hnRNPF, and hnRNPH (Chen and Manley, 2009). Binding of

TDP-43 to pre-mRNAs influences alternative splicing in a position-dependent manner similar to NOVA proteins (Tollervey et al., 2011).

Finally, we for the first time investigated the integrated molecular network of total 4,063 presumptive human TDP-43 target RNAs and 227 TDP-43 interacting proteins by using pathway analysis tools. We found that all of these molecules are most closely associated with "Ribosome" pathway of KEGG, "RNA Post-Transcriptional Modification, Genetic Disorder, Neurological Disease" network of IPA, and "Transcriptional regulation by p53" pathway of KeyMolnet. We identified the set of 106 genes concurrently serving as both TDP-43 target RNAs and interacting proteins (Table 2), which suggest the existence of an autoregulatory loop in the control of gene expression of TDP-43 interacting proteins. The list of 106 genes includes DDX17, DDX3, DHX9, ILF2, and ILF3, all of which were previously identified in HeLa cells as the components of the microRNA processing machinery (Ling et al., 2010). These results support an active role of TDP-43 in microRNA biogenesis (Buratti and Baralle, 2010). Actually, the levels of expression of let-7b are reduced, while those of miR-663 are elevated in TDP-43 knockdown Hep-3B cells (Buratti et al., 2010). The set of 106 genes also includes TDP-43 (TARDBP) itself. Importantly, TDP-43 negatively regulates its own protein expression level by binding to 3' untranslated region (UTR) of TDP-43 mRNA and increasing mRNA degradation (Ayala et al., 2011). Thus, cellular TDP-43 protein levels are tightly regulated autonomously, and overexpression of exogenous TDP-43 induces a dramatic depletion of normal nuclear TDP-43 in transgenic mouse brains *in vivo* (Igaz et al., 2011).

CONCLUSIONS

TDP-43 with RNA-binding and protein-interacting domains in its structure forms a functional complex with multiple target RNAs and interacting proteins *in vivo*. By using KEGG, IPA, and KeyMolnet, the present study characterized the comprehensive molecular network of TDP-43 target RNAs and interacting proteins recently identified. Although the three different tools did not illustrated perfectly matched molecular networks and pathways, the results consistently suggest that the complex network of TDP-43 target RNAs and interacting proteins plays a pivotal role in mRNA splicing on spliceosome and translation on ribosome, by dynamically translocating themselves from the nucleus to the cytoplasm. Based on these observations, the present study would propose the systems biological view that TDP-43 serves as a molecular scaffold that coordinates RNA-dependent regulation of gene transcription and translation essential for achievement of diverse neuronal functions, including axon guidance. Therefore, even a trivial perturbation that disturbs the TDP-43-mediated molecular coordination, possibly caused by genetic and environmental insults and stresses, could deregulate robustness of the molecular network, disturb normal neuronal function, and induce neurodegeneration in ALS and FTLD.

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KEY TERMS AND DEFINITIONS

ALS: Amyotrophic lateral sclerosis (ALS) is an adult-onset fatal neurodegenerative disease characterized by generalized skeletal and bulbar muscle atrophy owing to progressive loss of cortical and spinal motor neurons. Up to 10% of ALS cases are responsible for inheritable genetic mutations of the genes, such as SOD1, FUS/TLS, TDP-43/TARDBP, OPTN, UBQLN2, and ANG.

FTLD: Frontotemporal lobar degeneration (FTLD) constitutes a group of clinically, pathologically, and genetically heterogeneous disorders characterized by remarkable atrophy of the frontotemporal cortex in the brain. Up to 40% of FTLD cases are responsible for inheritable genetic mutations of the genes, such as TAU, TDP-43/TARDBP, FUS/TLS, GRN, VCP, and CHMP2B. Substantial populations of ALS and FTLD share clinical and pathological manifestations, categorized into TDP-43 proteinopathy.

KEGG: Kyoto Encyclopedia of Genes and Genomes (KEGG) is a public database that systematically integrates genomic and chemical information to create the whole biological system *in silico*. KEGG includes 148,769 manually curated reference pathways that cover a wide range of metabolic, genetic, environmental, and cellular processes, and human diseases and drugs.

KeyMolnet: KeyMolnet is a pathway analysis tool of bioinformatics composed of knowledge-based contents on 137,300 relationships among human genes and proteins, small molecules, diseases, pathways and drugs, curated by expert biologists. By importing the list of Entrez Gene IDs, KeyMolnet automatically provides corresponding molecules as a node on networks.

IPA: Ingenuity Pathways Analysis (IPA) is a pathway analysis tool of bioinformatics composed