A transcript profiling approach reveals the zinc finger transcription factor ZNF191 is a pleiotropic factor

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Abstract

**Background:** The human zinc finger protein 191 (ZNF191) is a member of the SCAN domain family of Krüppel-like zinc finger transcription factors. ZNF191 shows 94% identity to its mouse homologue zinc finger protein 191 (Zfp191), which is the most highly conserved among the human-mouse SCAN family member orthologues pairs. Zfp191 is widely expressed during early embryogenesis and in adult organs. Moreover, Zfp191−/− embryos have been shown to be severely retarded in development and die approximately at embryonic day E7.5. ZNF191 can specifically interact with the widespread TCAT motif which constitutes the HUMTH01 microsatellite in the tyrosine hydroxylase (TH) gene. Allelic variations of HUMTH01 have been stated to have a quantitative silencing effect on TH gene expression and to correlate with quantitative and qualitative changes in the binding by ZNF191. In addition, ZNF191 displays a suppressive effect on the transcription; however, little downstream targets have identified.

**Results:** We searched for ZNF191 target genes by using a transient overexpression and knockdown strategy in the human embryo kidney (HEK293) cells. Microarray analyses identified 6094 genes modulated by overexpression of ZNF191 and 3332 genes regulated by knockdown of ZNF191, using a threshold of 1.2-fold. Several interested candidate genes, validated by real time RT-PCR, were correlated well with the array data. Interestingly, 1456 genes were identified in both transient overexpression and transient knockdown strategies. The GenMAPP and MappFinder software packages were further used for pathway analysis of these significantly altered genes. Several gene pathways were found to be involved in processes of the regulation of kinase activity, transcription, angiogenesis, brain development and response to DNA damage.

**Conclusion:** Our analysis reveals for the first time that ZNF191 is a pleiotropic factor that has a role in hematopoiesis, brain development and cancers.
**Background**

The regulation of gene expression in response to intrinsic and extrinsic cues is a fundamental cellular process in the growth and development of organisms. Critical to the control of gene expression are transcription factors that bind to specific DNA sequences and subsequently modulate gene transcription [1]. A significant number of transcription factors use a conserved zinc finger domain to bind their target DNAs. Zinc finger factors participate in a variety of cellular activities, such as development and differentiation, and play a role in human disease. In fact, the human genome encompasses approximately 600 to 700 genes that contain a particular C$_2$H$_2$-type of zinc finger, which employs two cysteine and two histidine amino acid residues to coordinate the single zinc atom in the finger-like structure [2-4]. In the C$_2$H$_2$-type of zinc finger proteins, there is a highly conserved consensus sequence TGEKP(F/Y)X (X representing any amino acid) between adjacent zinc finger motifs. The zinc finger proteins containing this specific structure are termed Krüppel-like zinc finger proteins because the structure was first found in *Drosophila* Krüppel protein [5,6]. Many Krüppel-like factors exhibit diverse regulatory functions in cell growth, proliferation, differentiation, and embryogenesis [7-10]. The SCAN (SRE-ZBP, C$\alpha$fin 51, AW-1, and Number 18) [11,12], which is also known as the leucine rich region (LeR) [12], KRAB (Krüppel-associated box) [10,13], and POZ (poxvirus and zinc finger) [14] domains are highly conserved modules found in the N-terminus of C$_2$H$_2$ zinc finger transcription factors that have been demonstrated to mediate specific protein-protein interactions [15-18]. The SCAN domain is a conserved motif that appears to control the association of SCAN-containing proteins into non-covalent complexes, and may be the primary mechanism underlying partner choice in the dimerization of these transcription factors [16,19-22]. The genes encoding SCAN domains are clustered, often in tandem arrays, in both the human and mouse genomes and are capable of generating isoforms that may affect the function of family members. Although the function of most of the family members is not known, some of the SCAN domain family members play roles in cell survival and differentiation or development [16,23-25]. Like the KRAB motif, the SCAN domain-containing C$_2$H$_2$ zinc finger proteins are unique to the vertebrate lineage [2,3].

Human zinc finger protein 191 (ZNF191, also known as ZNF24 and KOX[17] [10]) is a member of the SCAN domain family of Krüppel-like zinc finger transcription factors [15]. This gene is initially named as RSG-A (for retinoic acid suppressed gene-A) because its mRNA can be amplified by homologous RT-PCR only in retinoic acid-un-treated but not in retinoic acid-treated acute promyelocytic leukemia NB4 cells[26]. ZNF191 shows 94% identity to its mouse homologue zinc finger protein 191(Zfp191, also called ZF-12), which is the most highly conserved among the human-mouse SCAN family member orthologues pairs[16]. Tissue mRNA analysis showed that ZNF191 gene was ubiquitously expressed [26,27]. The mouse Zfp191 has been previously isolated from chondrocytic and mesenchymal precursor cell lines using a subtractive hybridization screening. Zfp191 mRNA is expressed during embryonic development and in different organs in adult, including rib cartilage, suggesting that Zfp191 may have a role in cartilage differentiation and in basic cellular processes[28].

A study demonstrated that ZNF191 can specifically bind to the TCAT repeats (HUMTH01) in the first intron of the human tyrosine hydroxylase (TH) gene, which encodes the rate-limiting enzyme in the synthesis of catecholamines [27,29]. Allelic variations of HUMTH01 have a quantitative silencing effect on TH gene expression in vitro, and correlate with quantitative and qualitative changes in the binding by ZNF191 [27]. ZNF191 mRNA levels was present at high levels in catecholaminergic tissues including the substantia nigra, the hypothalamus and the olfactory bulb. In the rat embryo, the mRNA was present at high levels in total embryonic head, which may reflect a role in brain development [27]; however, the molecular mechanisms remain unknown.

The ZNF191 gene is located on chromosome 18q12.1[10,26], a region frequently deleted in colorectal carcinomas, suggesting a possible role in the negative regulation of tumor growth[30,31]. ZNF191 contains four continuous typical C$_2$H$_2$ zinc fingers in its C-terminus, and one SCAN domain in its N-terminus [10,26]. Biochemical binding study shows the SCAN domain of ZNF191 as a selective oligomerization domain[20]. The SCAN domain of ZNF191 displays a suppressive effect on the transcription in CHO and NIH3T3 cells[26]; however, little else is known regarding its role in gene expression.

In the present study, we transiently overexpressed and silenced ZNF191 gene in the human embryo kidney (HEK293) cells and then applied microarray assay to identify possible ZNF191 target genes. Expression of interested candidate genes was also examined by real time RT-PCR, and these data correlated well with the array data. In general, our data reveal that ZNF191 modulates the expression of a large number of genes that are implicated in regulation of kinase activity, regulation of transcription, angiogenesis, brain development and response to DNA damage. Our analysis reveals for the first time that ZNF191 is a pleiotropic factor that has a role in hematopoiesis, brain development, tumor growth.
Results
Data analysis: general features
To identify ZNF191 target genes, a combined approach of transient overexpression and transient knockdown (KD) are used to identify genes modified by the ZNF191 transcription factor with 44 K Whole-Genome 60-mer oligonucleotide microarrays, using a cellular model (HEK293). Plasmids overexpressing ZNF191 cDNA (pcDNA3-ZNF191) or control empty pcDNA3 vectors were transiently transfected into HEK293 cells for 24 hours. As expected, introduction of pcDNA3-ZNF191 in HEK293 cells resulted in higher expression of ZNF191 mRNA than in cells transfected with control empty plasmid or untreated cells (Figure 1A). Real-time PCR results showed that the ZNF191 expression level was increased > 30 times in cells transfected with pcDNA3-ZNF191 compared with control cells (Figure 1B). For the reduction of ZNF191 expression, three hairpin siRNA expression vectors against ZNF191 were generated [32] and the knock-down of ZNF191 was assessed 48 hours after transfection of the HEK293 cells. Figure 2 showed that the three siRNA expression vectors were found to efficiently eliminate the target mRNA. The reduction of ZNF191 mRNA levels in transfected cells appears to be approximately 60% at the best compared to the control level. Due to no greater inhibition of ZNF191 mRNA at longer times than 48 hours, transfection of the HEK293 cells with the most efficient vector for 48 h was used in subsequent experiments.

RNA samples were prepared from the transient overexpression and transient knockdown cells as well as control cells. Probe labelling, hybridization, and scanning were done by Shanghai Biochip Co., Ltd (China). The complete list of genes with differential expression can be found at the website http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14273. Microarray showed that 3273 genes were up-regulated more than 1.2-fold, while 3631 were down-regulated by the overexpression of the ZNF191 gene (see Additional file 1), while 2111 were up-regulated by the KD and 1221 were down-regulated, using the same threshold (see Additional file 2). Interestingly, 1456 genes were identified in both situations, and 1344 (92%) behaved consistently between the two experiments (i.e. induced by the overexpression and repressed by the KD, or the other way around) (see Additional file 3). These identified genes were subsequently used in pathway analysis with MappFinder.

Using a threshold of 2 for data analysis, 592 genes were found to be regulated by the overexpression with 143 genes up-regulated and 449 down-regulated (see Additional file 1, highlighted data) while 172 genes were regulated by the KD with 83 genes up-regulated and 89 down-regulated (see Additional file 2, highlighted data). The overlap was found between transient overexpression and transient knockdown with 69 shared genes (see Additional file 3, highlighted data). Thus, ZNF191 appears to modu-
late multiple transcriptional events, acting both as an activator and a repressor.

Pathway analysis of the ZNF191 transient overexpression and knockdown signature
To further evaluate our microarray data, GenMAPP and MAPPFinder software [http://www.genmapp.org] were used to organize gene expression data into MAPPs (microarray pathway profiles) that represent specific biological pathways and functionally grouped genes, based on the GO system. Three main classes of branches in the GO tree are biological processes, molecular functions and cellular components [33].

We focused on microarray gene expression data of 1456 genes identified in both transient overexpression and transient knockdown treatment. Using gene-association files from the GO Consortium, MAPPFinder assigns the thousands of genes in the dataset to numerous GO terms. Our analysis was limited to the GO terms that comprise more than eight genes in a class. Several significant (i.e., Z score > 2) functional MAPPs were revealed with MAPPFinder and were presented in Table 1. Gene pathways that are involved in the regulation of kinase activity and angiogenesis were significantly up-regulated. In contrast, processes associated with response to DNA damage stimulus and brain development were down-regulated. Many up-regulated processes are localized to cellular component (endoplasmic reticulum) while down-regulated processes belong mainly to the cellular components (ribosome, extracellular matrix and cytoplasmic membrane-bound vesicle). Figure 3, 4, 5 and 6 and Table 2 showed the ZNF191 overexpression and ZNF191 knockdown differentially expressed genes involved in these signaling pathways.

Quantitative Real-Time RT-PCR
Twenty-one interested genes were selected and subjected to real-time PCR analysis to confirm our microarrays results. As shown in Figure 7, the direction of regulation of ATP7A, RECK, PDGFRB, BMPR2, RB1, BRCA1, BRCA2, ATM, ATRX, IFI16, CCNB2, MYO6, GADD45B, SEMA5A, NRP2, CTGF, C5, VEGF, THBS1, KITLG and FOXP2 (expect for CCNB2) by the overexpression and knockdown of ZNF191 was consistent with the microarray results.

Discussion
In this study, we identify genes modified by the ZNF191 transcription factor with a combined strategy of transient overexpression and transient knockdown (KD) in a cellular model (HEK293), using oligonucleotide microarray technology. Several gene pathways were revealed by MAPPfinder to be involved in processes of the regulation of kinase activity, transcription, angiogenesis, brain development and response to DNA damage.

Pathway of regulation of kinase activity was significantly affected (Z-score 2.73). This pathway had a large number...
of expression changes, mostly due to the regulation of 12 genes (GADD45B, SPRY4, DUSP6, RGS4, SPRED2, NRG1, EDN1, CCNA1, CDKN2B, CKS1B, SERTAD1 and DUSP6), which were up-regulated in the ZNF191-overexpressed cells and down-regulated in the ZNF191 knockdown cells. In additional, 8 genes (KITLG, PKIA, RB1, ZAK, PRKD3, C1QTNF6, C5 and MAP4K5) were down-regulated in the ZNF191-overexpressed cells and up-regulated in the ZNF191 knockdown cells. A map of the genes involved in regulation of kinase activity was shown in Figure 3. GADD45B, originally termed MyD118, is first cloned as a myeloid differentiation primary response gene. It can be induced in the absence of protein synthesis following treatment of M1 myeloblastic leukemia cells with differentiation inducers[34], suggesting that GADD45B play a role in hematopoiesis. KITLG is a pleiotropic factor that acts in utero in germ cell and neural cell development, and hematopoiesis[35]. Accordingly,

| GOID         | Pathway Name/GO Term                                                                 | NO. genes | % Changed | Z-score |
|--------------|--------------------------------------------------------------------------------------|-----------|-----------|---------|
| 43549        | regulation of kinase activity                                                       | 12        | 60        | 2.73    |
| 45859        | regulation of protein kinase activity                                                | 12        | 60        | 2.73    |
| 165          | MAPKK cascade                                                                       | 8         | 66.7      | 2.60    |
| 51338        | regulation of transferase activity                                                   | 12        | 57.1      | 2.51    |
| 65008        | regulation of biological quality                                                     | 15        | 53.6      | 2.51    |
| 22402        | cell cycle process                                                                  | 21        | 47.7      | 2.32    |
| 1525         | angiogenesis                                                                        | 8         | 61.5      | 2.31    |
| 6952         | defense response                                                                    | 13        | 52        | 2.19    |
| 6508         | proteolysis                                                                         | 12        | 52.2      | 2.12    |
| 48514        | blood vessel morphogenesis                                                           | 8         | 57.1      | 2.04    |
| 7264         | small GTPase mediated signal transduction                                            | 10        | 52.6      | 1.96    |
| 5515         | protein binding                                                                     | 108       | 36.9      | 2.38    |
| 5525         | GTP binding                                                                         | 9         | 60        | 2.36    |
| 30246        | carbohydrate binding                                                                | 10        | 55.6      | 2.18    |
| 19001        | guanyl nucleotide binding                                                            | 9         | 56.3      | 2.11    |
| 5783         | endoplasmic reticulum                                                                | 15        | 48.4      | 2.01    |

| GOID         | Pathway Name/GO Term                                                                 | NO. genes | % Changed | Z-score |
|--------------|--------------------------------------------------------------------------------------|-----------|-----------|---------|
| 6066         | alcohol metabolic process                                                            | 14        | 100       | 2.56    |
| 45449        | regulation of transcription                                                          | 95        | 77.9      | 2.45    |
| 9719         | response to endogenous stimulus                                                      | 17        | 94.4      | 2.40    |
| 6974         | response to DNA damage stimulus                                                     | 17        | 94.4      | 2.40    |
| 19219        | regulation of nucleoside, nucleotide and nucleic acid metabolic process              | 95        | 77.2      | 2.30    |
| 7420         | brain development                                                                    | 11        | 100       | 2.27    |
| 6355         | regulation of transcription, DNA-dependent                                          | 89        | 77.4      | 2.25    |
| 6350         | transcription                                                                       | 96        | 76.8      | 2.21    |
| 3735         | structural constituent of ribosome                                                   | 15        | 100       | 2.65    |
| 46872        | metal ion binding                                                                   | 153       | 75.7      | 2.63    |
| 43167        | ion binding                                                                         | 154       | 75.1      | 2.43    |
| 43169        | cation binding                                                                      | 140       | 75.3      | 2.32    |
| 3676         | nucleic acid binding                                                                 | 129       | 75.4      | 2.25    |
| 46914        | transition metal ion binding                                                         | 103       | 76.3      | 2.17    |
| 35081        | phosphoinositide binding                                                             | 10        | 100       | 2.16    |
| 8270         | zinc ion binding                                                                    | 94        | 76.4      | 2.09    |
| 4930         | G-protein coupled receptor activity                                                  | 9         | 100       | 2.05    |
| 16023        | cytoplasmic membrane-bound vesicle                                                   | 11        | 100       | 2.27    |
| 5830         | cytosolic ribosome (sensu Eukaryota)                                                | 11        | 100       | 2.27    |
| 5578         | proteinaceous extracellular matrix                                                   | 23        | 88.5      | 2.23    |
| 31012        | extracellular matrix                                                                | 23        | 88.5      | 2.23    |
| 5840         | ribosome                                                                            | 15        | 93.8      | 2.20    |
| 5842         | cytosolic large ribosomal subunit (sensu Eukaryota)                                  | 10        | 100       | 2.16    |
| 15934        | large ribosomal subunit                                                              | 10        | 100       | 2.16    |
| 44420        | extracellular matrix part                                                            | 9         | 100       | 2.05    |
Table 2: List of genes involved in the signal pathways

| Gene symbol     | ProbeName   | GenBank  | Description                                      | Overexpression | Knockdown |
|-----------------|-------------|----------|--------------------------------------------------|----------------|-----------|
| **Regulation of kinase activity** |             |          |                                                  |                |           |
| RB1             | A_23_P204850 | NM_000321 | Retinoblastoma 1 (including osteosarcoma)        | 0.74           | 1.53      |
| GADD45B         | A_23_P142506 | NM_015675 | Growth arrest and DNA-damage-inducible, beta     | 1.45           | 0.68      |
| PKIA            | A_23_P31765  | NM_006823 | Protein kinase (cAMP-dependent, catalytic) inhibitor alpha | 0.68           | 1.41      |
| TIZ/ZNF675      | A_24_P931755 | D70835   | TRAF6-inhibitory zinc finger protein             | 0.71           | 1.38      |
| SPRY4           | A_23_P269062 | NM_030964 | Sprouty homolog 4 (Drosophila)                  | 2.12           | 0.57      |
| DUSP6           | A_23_P139704 | NM_001946 | dual specificity phosphatase 6                  | 1.56           | 0.74      |
| RGS4            | A_23_P200737 | BC000737  | Regulator of G-protein signalling 4              | 1.83           | 0.65      |
| SPRED2          | A_32_P225854 | NM_181784 | Sprouty-related, EVH1 domain containing 2        | 1.52           | 0.72      |
| NRG1            | A_23_P315815 | NM_004495 | Neuregulin 1                                     | 1.51           | 0.74      |
| ZAK/MLTK_HUMAN  | A_23_P318300 | NM_133646 | Sterile alpha motif and leucine zipper containing kinase AZK | 0.69           | 1.56      |
| **Angiogenesis**|             |          |                                                  |                |           |
| EDN1            | A_23_P214821 | NM_001955 | Endothelin 1                                     | 2.33           | 0.49      |
| PRKDC           | A_24_P165656 | NM_005813 | Protein kinase D3                                | 0.74           | 1.56      |
| KITLG           | A_24_P133253 | NM_000899 | KIT ligand                                      | 0.68           | 1.51      |
| C1QTNF6         | A_24_P211565 | NM_031910 | C1q and tumor necrosis factor related protein 6  | 0.47           | 1.54      |
| CS              | A_23_P71855  | NM_001735 | Complement component 5                          | 0.42           | 2.62      |
| MAP4K5          | A_23_P205646 | BC036013  | Mitogen-activated protein kinase kinase kinase 5 | 0.74           | 1.39      |
| CCNA1           | A_23_P48414  | NM_003914 | Cyclin A1                                       | 2.51           | 0.54      |
| CDKN2B          | A_24_P360674 | NM_078487 | Cyclin-dependent kinase inhibitor 2B             | 1.81           | 0.71      |
| C5              | A_24_P17855  | NM_013735 | Complement component 5                          | 0.42           | 2.62      |
| MAP4K5          | A_23_P205646 | BC036013  | Mitogen-activated protein kinase kinase kinase 5 | 0.74           | 1.39      |
| **Brain development** |         |          |                                                  |                |           |
| APAF1           | A_23_P36611  | NM_181861 | Apoptotic protease activating factor             | 0.58           | 1.36      |
| ATM              | A_23_P35916  | NM_000051 | Ataxia telangiectasia mutated                    | 0.63           | 1.35      |
| CXCL12          | A_23_P202448 | U19495    | Chemokine (C-X-C motif) ligand 12                | 0.73           | 1.37      |
| CYR61           | A_23_P46426  | Z90746    | Cysteine-rich, angiogenic inducer, 61            | 1.74           | 0.44      |
| EDN1            | A_23_P214821 | NM_001955 | Endothelin 1                                     | 2.33           | 0.49      |
| SEMASA          | A_23_P213415 | NM_003966 | Sena domain, seven thrombospondin repeats        | 0.28           | 2.26      |
| IL17F           | A_23_P167882 | NM_052872 | Interleukin 17F                                  | 1.78           | 2.01      |
| RUNX1           | A_24_P34155  | X90980    | Runt-related transcription factor 1              | 1.48           | 0.74      |
| THBS1           | A_23_P206212 | X04665    | Thrombospondin 1                                 | 1.97           | 0.59      |
| **SEPP1**       | A_23_P121926 | NM_005410 | Selenoprotein P, plasma, l                       | 0.66           | 1.36      |
| SMARCA1         | A_23_P44244  | NM_003069 | SWI/SNF related, matrix associated, actin        | 0.43           | 1.40      |
| ATRX            | A_24_P348660 | NM_000489 | Alpha thalassemia/mental retardation syndrome    | 0.60           | 1.49      |
| NOTCH3          | A_32_P42895  | A_32_P42895 | Notch homolog 3 (Drosophila)                    | 0.68           | 1.46      |
ZNF191 has been isolated from bone marrow and promyelocytic leukemia cell lines [26]. These data infer that ZNF191 may play a role in hematopoiesis.

Angiogenesis was another pathway markedly affected by ZNF191 (Z-score 2.31). As shown in Figure 4, CTGF, CYR61, EDN1, MYH9, NRP2, RUNX1, THBS1 were up-regulated in the ZNF191-overexpressed cells, and down-regulated in the knockdown cells. In addition, CEACAM1, PLXDC1, CXCL12, SEMA5A and VEGF were down-regulated in the ZNF191-overexpressed cells, and up-regulated in the knockdown cells. Angiogenesis, the growth of new blood vessels, is required for a variety of normal proliferative processes. Furthermore, angiogenesis is well established as also playing an important role in neoplastic growth and metastasis. VEGF is a potent stimulator of angiogenesis. ZNF191 has been reported to be up-regulated in angiogenic tumor nodules where VEGF expression is significantly decreased compared with preangiogenic nodules[36]. In this study, our result in HEK293 cells is consistent with the findings that in human breast carcinoma cells overexpression of ZNF191

Table 2: List of genes involved in the signal pathways (Continued)

| Gene | Accession | NM  | Z-score |
|------|-----------|-----|---------|
| PRKG1 | A_23_P136041 | 0.43 | 1.34 |
| ATP7A | A_23_P217737 | 0.48 | 1.52 |
| FOXF2 | A_24_P16559 | 0.67 | 1.40 |
| CEP290 | A_23_P36856 | 0.70 | 1.43 |
| ZAK/NP_598407.1 | A_23_P318300 | 0.69 | 1.56 |
| BRCA1 | A_23_P207400 | 0.71 | 1.3 |
| ATM | A_23_P35916 | 0.63 | 1.35 |
| ATRX | A_24_P348660 | 0.60 | 1.49 |
| BRCA2 | A_23_P99452 | 0.62 | 1.3 |
| BTG2 | A_23_P62901 | 0.72 | 1.35 |
| Q6P2G2_HUMAN | A_24_P361457 | 0.63 | 1.49 |
| RBM14 | A_23_P61555 | 0.62 | 1.47 |
| MLH3 | A_24_P61550 | 0.59 | 1.45 |
| N4BP2_HUMAN | A_24_P222114 | 0.52 | 1.47 |
| PMS1 | A_23_P40059 | 0.65 | 1.34 |

Figure 3
Gene list involved in the regulation of kinase activity pathway generated by GenMAPP. The color on the left side of gene box illustrates the gene changes by ZNF191 overexpression; the color on the right indicates the gene changes by siRNA-mediated knockdown of ZNF191. Red and green indicates up-regulated and down-regulated in ZNF191 overexpression experiment, respectively. Yellow and blue indicates up-regulated and down-regulated in siRNA experiment, respectively. Grey indicates that selection criteria were not met but the gene was represented in the array. White boxes indicate that the gene was not present in the chip.
results in a significant down-regulation of VEGF, whereas silencing of ZNF191 with small interfering RNA leads to increased VEGF expression as well as the same inverse correlation between ZNF191 and VEGF observed in malignant tissues from human colon and breast biopsies [36]. In addition, thrombospondin-1 (THBS1/TSP-1) has been shown to inhibit angiogenesis through direct effects on endothelial cell migration and survival, and through effects on vascular endothelial cell growth factor bioavailability. Aside from the inhibitory activity of angiogenesis, THBS1 also suppresses tumor growth by activating transforming growth factor beta and affects tumor cell function through interaction with cell surface receptors and regulation of extracellular proteases[37]. The data in this study revealed that overexpression of ZNF191 resulted in a significant up-regulation of THBS1, whereas silencing of ZNF191 led to decreased THBS1 expression in HEK293 cells. Taken together, these data suggest that ZNF191 may participate in negative regulation of angiogenesis. Furthermore, BMPR2 is overexpressed in the majority of human lung carcinomas independent of cell type[38,39]. PDGFR expression in colorectal cancer significantly correlates with lymphatic dissemination[40]. MYO6 (myosin VI) is critical in maintaining the malignant properties of the majority of human prostate cancers diagnosed today[41]. The finding that overexpression of ZNF191 significantly down-regulated BMPR2, PDGFR and MYO6, whereas silencing of ZNF191 increased BMPR2, PDGFR and MYO6 expression suggests a tumor suppressor role for ZNF191 in human cancers.

The response to DNA damage node was significantly affected (Z-score 2.4), mostly due to the regulation of BRCA1, BRCA2, ATM, BTK2 and ATRX (Figure 5). The most obvious explanation for this induction was that it was related to initiation of DNA replication. Indeed, DNA repair function is complementary to DNA replication, as the latter process is not error-free and produces mismatches that need to be repaired. However, we reported here that overexpression or elimination of ZNF191
Gene list involved in the response to DNA damage stimulus pathway generated by GenMAPP. The color on the left side of gene box illustrates the gene changes by ZNF191 overexpression; the color on the right indicates the gene changes by siRNA-mediated knockdown of ZNF191. Red and green indicates up-regulated and down-regulated in ZNF191 overexpression experiment, respectively. Yellow and blue indicates up-regulated and down-regulated in siRNA experiment, respectively. Grey indicates that selection criteria were not met but the gene was represented in the array. White boxes indicate that the gene was not present in the chip.

Gene list involved in the brain development pathway generated by GenMAPP. The color on the left side of gene box illustrates the gene changes by ZNF191 overexpression; the color on the right indicates the gene changes by siRNA-mediated knockdown of ZNF191. Red and green indicates up-regulated and down-regulated in ZNF191 overexpression experiment, respectively. Yellow and blue indicates up-regulated and down-regulated in siRNA experiment, respectively. Grey indicates that selection criteria were not met but the gene was represented in the array. White boxes indicate that the gene was not present in the chip.
affected the upstream regulators of the DNA damage pathway, such as ATM, ATRX, BRCA2 and BTG2. These data suggest that ZNF191 may be involved in the control of DNA damage response in addition to the regulation of DNA replication and concurrent DNA repair. Microarray and real-time PCR showed that RB1, ATM, ATRX and BRCA1 genes were down-regulated in the ZNF191-overexpressed cells (Figure 7). Accordingly, RB1 knockdown by siRNA caused the down-regulation of ATM, ATRX and BRCA1[42]. RB1 mRNA level was down-regulated in MCF-7 breast carcinoma cells by the overexpression of ZNF191 (data not published).

Mice Zfp191 transcript is detected early during embryogenesis in ectodermal, endodermal, mesodermal and extra-embryonic tissues. It is particularly observed in the developing central nervous system (CNS)[43]. In rat, ZNF191 mRNA is present at high levels in total embryonic head, and abundant in catecholaminergic tissues including substantia nigra, hypothalamus and olfactory bulb as well as peripheral catecholaminergic tissue adrenal medulla[27]. In human, ZNF191 transcript is also detected in brain[27]. These studies demonstrate an important role of ZNF191 in brain development. Interestingly, in support with previous studies, our results showed a node affected by ZNF191 was the brain development node (Z-score 2.27). 11 genes (ATP7A, FOXP2, APAF1, ATM, CXCL12, SEPP1, SMARCA1, ATRX, NOTCH3, PRKG1, and CEP290) down-regulated in the ZNF191-overexpressed cells and up-regulated in the ZNF191 knockdown cells (Figure 6). The human copper-transporting ATP7A is essential for dietary copper uptake, normal development and function of the CNS, and regulation of copper homeostasis in the body[44]. In addition, studies on Alzheimer’s disease (AD) suggest an important role for copper in the brain, with some AD therapies focusing on mobilising copper in AD brains[45]. The transport of copper into the brain is complex and involves numerous players, including amyloid precursor protein, A beta peptide and cholesterol[45,46]. Interestingly, in a yeast two-hybrid experiment using the SCAN domain of ZNF191 as bait, we identified that the zinc finger transcription factor ZNF191 can interact with NAE1(NEDD8 activating enzyme E1 subunit 1; amyloid beta precursor protein binding protein 1, 59 kDa) (unpublished data). FOXP2 mutations in humans are associated with a disorder that affects both the comprehension of language and its production, speech[47]. ZNF191 transcript was abundant in the mesencephalon, a structure that contains the major rat embryonic catecholaminergic tissues[27]. Accordingly,
ZNF191 can specifically interact with the widespread TCAT motif which constitutes the HUMTH01 microsatellite in the tyrosine hydroxylase (TH) gene (encoding the rate-limiting enzyme in the synthesis of catecholamines)[27]. Allelic variations of HUMTH01 are known to have a quantitative silencing effect on TH gene expression and to correlate with quantitative and qualitative changes in the binding by ZNF191[27]. In this study, overexpression of ZNF191 resulted in a significant down-regulation of TH, whereas knockdown of ZNF191 resulted in a non-significant change of TH level. The expression level of the TH gene is indistinguishable between the wild-type, Zfp191+/+, and Zfp191−/− mice[32]. These data suggest that other genes may compensate at least in part for the loss of ZNF191 activity, ameliorating the effect of the ZNF191 knockdown. Taken together, these data suggest that ZNF191 may be involved in the brain development and the neuropsychiatric diseases.

We have to point out that in the knockdown experiments we can only deduce the level of ZNF191 protein by the level of mRNA of ZNF191 because no antibody against the ZNF191 factor is commercially available and the turnover of the protein is still unknown. So there is no mean to ascertain whether and when the ZNF191 protein is reduced significantly. In addition, in this study HEK293 cells were used as a cellular model to identify genes modified by the ZNF191 transcription factor. Some other cells could give at least partially over transcriptome modification. Furthermore, the transcriptional response found upon alteration of ZNF191 levels does not necessarily imply that this transcription factor regulates the differentially expressed genes. Many of the observed changes could be downstream and not direct effects of ZNF191 regulation. A proof of direct regulation would require a promoter analysis or some kind of DNA-protein binding experiment or in silico analysis.

Conclusion
We applied overexpression and siRNA-mediated gene silencing coupled with microarray screening and systematic pathway analysis to obtain insights into the pathways controlled by the target genes. We found that ZNF191 regulated the expression of a large group of genes involved in regulation of kinase activity, angiogenesis, brain development and response to DNA damage. Our analysis reveals that ZNF191 is a pleiotropic factor in hematopoiesis, brain development, and human cancers. Our approach can be used in functional genomics to elucidate the role of other transcription factors.

Methods
Cell culture and transfection
HEK-293 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 95% air, 5% CO₂ at 37°C. Cells were plated into 60 mm dishes without antibiotics 24 h prior to transfection. The recombinant plasmids, or control vector, were transfected into HEK-293 cells using Lipofectamine 2000 reagents according to the manufacturer’s instructions. HEK-293 cells transfected with each cDNA or siRNA were lysed after 24 h or 48 h to isolate total RNA.

Plasmids
The cDNA clone of ZNF191 was provided by Prof. Long Y. (Genetics Institute, Fudan University, Shanghai, China). ZNF191 cDNA was cloned into pcDNA3 (Invitrogen) by using HindIII and XhoI restriction sites. The cloning was verified by DNA sequencing. The hairpin siRNA expression vectors against ZNF191 were previously generated [32].

RNA isolation
Total RNA was extracted using the Trizol reagent (Invitrogen) and purified on RNeasy columns (Qiagen). The amount and quality of RNA preparations were evaluated on the Agilent 2100 Bioanalyzer with RNA6000 Nano Reagents and Supplies (Agilent).

Microarray hybridization
Five hundred nanograms of RNA were reverse-transcribed to cDNA during which process a T7 sequences was introduced into cDNA. T7 RNA polymerase-driven RNA synthesis was used for the preparation and labeling of cRNA with Cy3 (the reference sample) and Cy5 (the treated sample), respectively. A Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA) was used to purify fluorescent cRNA probes. An equal amount (1 µg) of Cy3 and Cy5 labeled probes were mixed and used for hybridization on one Agilent 4 × 44 K Human Whole-Genome 60-mer oligonucleotide microarrays (Agilent Technologies Inc., Santa Clara, CA) following the protocol provided by the manufacturer.

Data acquisition and processing
Microarrays were scanned with a dynamic autofocus microarray scanner (Agilent dual laser DNA microarray scanner, Agilent technologies, Palo Alto, CA, USA), using Agilent-provided parameters (Red and Green PMT were each set at 100%, and scan resolution was set to 10 µm). The Feature Extraction Software v7.5 (Agilent Technologies) was used to extract and analyse the signals. Agilent-provided settings were used except for subtraction of the local background and adjustment of the global background. Poor quality features that were either saturated in the two channels (50% of pixels > saturation threshold) or non-uniform were flagged. Only those features with a signal-to-noise ratio (SNR) of up to 2.6 in at least one channel and significantly different from the local background (two sided Student’s t-test < 0.05) were used for...
further analysis. The mean signal ratio of the two fluorescent intensities (Cy-5 treated cRNA/Cy-3 reference cRNA) is expressed as a logarithm (base 2), providing a relative quantitative gene expression measurement between two samples. For subsequent analysis, we used mean-centered log2 of the normalized (linear & lowess method) sample: reference ratio. Microarray data meeting a criterion (> 1.2-fold change, FDR = 0, S/N > 2.6 and pValueLogRatio < 0.05) were considered to be significant and used for further analysis.

Pathway analysis

The GeneMapp and MAPPFinder software packages http://www.genmapp.org were used for pathway analysis of the significantly altered genes identified in both transient overexpression and transient knockdown of the ZNF191 gene. The MAPPFinder works with GenMAPP and uses the annotations from the Gene Ontology (GO) Consortium to identify global biological trends in gene expression data. MAPPFinder relates microarray data to each term in the GO hierarchy, calculates the percentage of genes changed within each GO term, and generates a gene expression profile at the level of biological processes, cellular components, and molecular functions that allows for identification of specific biological pathways. MAPPFinder then calculates the total number of genes changed within a "parent GO term" and all of its "children" (local MAPPs) using Fisher's exact test. The results are expressed as a "z score" for a particular pathway, and values greater than 2 were considered to be significant. GenMAPP was also used to view and analyze microarray data on biological pathways[33].

Quantitative real-time PCR

Twenty-one interested genes were selected for validation by real time PCR. cDNAs were prepared from 1 µg of total RNA using AMV reverse transcriptase (Promega) and random primers according to the manufacturer’s protocol. For each gene, PCR reactions were run three times on one sample. PCR was performed on Chromo4 Multicolor Real-Time Detection System (Bio-Rad, Inc.) in 20 µl reactions by using the SYBR™ Green PCR Master Mix (TaKaRa). The reaction was repeated for 40 cycles; each cycle consisted of denaturing at 95°C for 15 s, annealing and synthesis at 60°C for 1 min as per manufacturer’s instructions. The relative amounts of transcript of the tested genes were normalized by GAPDH endogenous control primers set. The average ratios of relative amounts of transcript in the ZNF191-overexpressed cells or ZNF191 knockdown cells versus empty vector cells from three replicate runs were calculated. Quantitative values are obtained from the cycle number (Ct value) using Opticon Monitor 3.1 (Bio-Rad) according to the manufacturer’s instructions. The threshold cycle (Ct) values were averaged from the values obtained from each reaction, and each gene was normalized to GAPDH level. Each gene of interest and GAPDH were tested in triplicates to determine the Ct-difference. These Ct values were averaged and the difference between the GAPDH Ct (Avg) and the gene of interest Ct (Avg) was calculated (Ct-diff). The fold change was calculated by the 2-ΔΔCT method [48]. Because SYBR Green binding is not sequence specific, careful design and validation of each primer pair, as well as cautious manipulation of RNA were undertaken to ensure that only target gene sequence-specific, non-genomic products were amplified by real-time PCR. To achieve this, primers were designed to either span or flank introns. PCR primers are listed in Additional file 4. A dissociation curve analysis was performed at the end of the amplification process in order to verify the specificity of the PCR products. The same PCR products were also evaluated by agarose gel electrophoresis. Data analysis and graphics were the results (mean ± SEM) of three different experiments.

Semi-quantitative PCR

Semi-quantitative PCRs were performed with Zfp191 specific primers (forward: 5’-ATCACCGTGCGCCACATCAA-3’ and reverse: 5’-GATGGCCCAACCCATATAT-3’), which yielded 481 bp products. To serve as an internal control, a 381 bp fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was co-amplified with primers (forward: 5’-AACITTTGATATGCCTG-3’; reverse: 5’-GGAGGATGGGTGTCGCTGT-3’). The PCRs were carried out for 25–40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min with a final extension of 5 min at 72°C. All RT-PCR experiments were repeated at least twice and the products were electrophoresed on 1.5% agarose gels containing ethidium bromide.

Authors’ contributions

JL was involved in all experiments, experimental design and bioinformatics analyses. XC and YL co-drafted the manuscript, performed Real RT-PCR. XG and HF assisted in bioinformatics analyses and uploaded data to Gene Expression Omnibus. LQ and ZH co-drafted the manuscript and participated in cell culture. JZ co-drafted the manuscript, made revisions critically for important intellectual content.
Additional material

Additional file 1
Regulated genes by ZNF191 overexpression. Table S1 consists of the regulated probes by ZNF191 overexpression, containing the assigned designations (Accession number and sequence name), the fold of changed expression and PValueLogRatio(smaller than 0.05) for these genes. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-10-241-S1.xls]

Additional file 2
Regulated genes by ZNF191 knockdown. Table S2 consists of the regulated probes by ZNF191 knockdown, containing the assigned designations (Accession number and sequence name), the fold of changed expression and PValueLogRatio (smaller than 0.05) for these genes. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-10-241-S2.xls]

Additional file 3
Commonly altered genes by ZNF191 overexpression and knockdown. Table S3 consists of the regulated probes by ZNF191 overexpression and knockdown, containing the assigned designations (Accession number and sequence name), the fold of changed expression and PValueLogRatio (smaller than 0.05) for these genes. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-10-241-S3.xls]

Additional file 4
List of primers used for RT-PCR. Abbreviations: see table 2. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-10-241-S4.doc]

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