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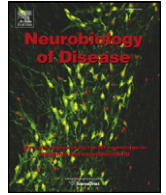
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A B S T R A C T

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive and ultimately fatal neurodegenerative disease, caused by the loss of motor neurons in the brain and spinal cord. Although 10% of ALS cases are familial (FALS), the majority are sporadic (SALS) and probably associated to a multifactorial etiology. Currently there is no cure or prevention for ALS. A prerequisite to formulating therapeutic strategies is gaining understanding of its etio-pathogenic mechanisms. In this study we analyzed whole-genome expression profiles of 41 motor cortex samples of control (10) and sporadic ALS (31) patients. Unsupervised hierarchical clustering was able to separate control from SALS patients. In addition, SALS patients were subdivided in two different groups that were associated to different deregulated pathways and genes, some of which were previously associated to familiar ALS. These experiments are the first to highlight the genomic heterogeneity of sporadic ALS and reveal new clues to its pathogenesis and potential therapeutic targets.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fast progressive and disabling neurodegenerative disease characterized by upper and lower motor neuron loss, leading to respiratory insufficiency and death after 3–5 years (Mitchell and Borasio, 2007). The incidence of ALS ranges from 1.7 to 2.3 cases per 100,000 population per year world-wide (Beghi et al., 2006). Despite intensive research, knowledge of the pathogenetic mechanisms and precise genetic causes of ALS remains incomplete. Although most cases of ALS are isolated or sporadic (SALS), about 10% are familial (FALS) and have been linked to the mutation of several genes (Abel et al., 2012; Andersen and Al-Chalabi, 2011; Lill et al., 2011; Simpson and Al Chalabi, 2006; Valdmanis and Rouleau, 2008; Yoshida et al., 2010), such as SOD1 (Rosen, 1993), ALSIN (Hadano et al., 2001), SETX (Chance et al., 1998; Chen et al., 2004), SPG11 (Orlacchio et al., 2010), FUS (Kwiatkowski et al., 2009; Vance et al., 2009), VAPB (Nishimura et al., 2004), ANG (Chen et al., 2010; Greenway et al., 2006), TARDBP (Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008), Fig. 4 (Chow et al., 2009), OPTN (van Es et al., 2009), ATXN2 (Elden et al., 2010), and C9ORF72 (DeJesus-Hernandez et al.,

2011; Renton et al., 2011). Although the etiology of SALS remains largely unknown, a number of observations suggest a role for genetic factors in SALS (Andersen and Al-Chalabi, 2011). While FALS genes may account for some cases of SALS, this is currently viewed as a multi-factorial complex disease, in which multiple genetic variants, each of small effect, combine with environmental triggers and risk factors (Andersen and Al-Chalabi, 2011; Armon, 2001; Majoor-Krakauer et al., 2003; Simpson and Al Chalabi, 2006).

The pathogenic processes underlying ALS are not fully determined. In the last few years, a number of transcriptome studies in peripheral cells or postmortem nervous tissue of ALS patients have started to decipher genes and pathways involved in disease pathogenesis (Cox et al., 2010; Dangond et al., 2004; Jiang et al., 2005; Lederer et al., 2007; Malaspina et al., 2001; Offen et al., 2009; Rabin et al., 2010; Wang et al., 2006). Although comparison of results is often difficult, because of different tissues and/or microarray platforms, common alterations implicated by these transcriptome studies were related to the cytoskeleton, inflammation, protein turnover and RNA splicing (Saris et al., 2013). Due to the inherent complexity of nervous tissue and the need for postmortem material, however, the existing genomics studies of ALS were restricted to a limited number of postmortem ALS samples (≤11 motor cortex, and 14 spinal cord) (Cox et al., 2010; Dangond et al., 2004; Jiang et al., 2005; Lederer et al., 2007; Malaspina et al., 2001; Offen et al., 2009; Rabin et al., 2010; Wang et al., 2006). To uncover the entire spectrum of genes and pathways involved in ALS pathology

we analyzed whole-genome expression profiles of motor cortex samples from control (10) and SALS (31) patients (Table 1). By unsupervised hierarchical clustering we separate control from ALS patients, and subdivide the latter in two different groups that are associated to differentially expressed genes and pathways.

Materials and methods

Characteristics of subjects

Two groups of patient samples were used in this study: 31 motor cortex from SALS patients, and 10 motor cortex from control individuals. Fresh-frozen samples were obtained from the department of Pathology of the Academic Medical Center (University of Amsterdam) and selected for post-mortem intervals (PMI) prior to freezing not exceeding 24 h (mean PMI: 7.07 h for controls and 6.62 h for SALS). All SALS patients (mean patient age of 57) fulfilled the El Escorial diagnostic criteria (Brooks et al., 2000) and underwent genetic screening for genes associated to ALS. Diagnosis was independently confirmed by two neuropathologists according to standard histopathological criteria (Ince et al., 1998; Piao et al., 2003). The control samples (mean patient age of 55 years) were obtained from patients who had died from a non-

neurological disease (cause of death: myocardial infarction, renal failure, pulmonary embolism). Both ALS and control patients included in the study displayed no signs of infection before death. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes and approval was obtained from the relevant local ethical committees for medical research.

Detailed information related to origin, source code, age, gender, race, disease state, survival time from diagnosis date and PMI of patient samples is given in Table 1.

Sample preparation

Individual slices of 10 μm were produced from tissue samples at −20 °C by a Leica CM1510S cryostat (Leica Microsystems) and stored at −80 °C until further processing. Two slices per sample were stained by hematoxylin/eosin staining (Bio Optica) and for Nissl substance (with a microfiltered solution of cresyl violet, Sigma-Aldrich), respectively, to assess integrity of cellular and tissue morphology. Ten adjacent slices per sample were pooled and used for RNA extraction with Trizol (Life Technologies) RNA integrity was confirmed by using a RNA chip and a 2100 Bioanalyzer (Agilent Technologies, Italy) with the protocol outlined by the manufacturer.

Table 1
Characteristics of subjects.

Patient code	Race	Gender	Age	PMI (hours)	Disease state	Survival time from diagnosis date (months)	Unsupervised cluster
1	Caucasian	Male	31	8	Control	n/a	Control
2	Caucasian	Male	59	7	Control	n/a	Control
3	Caucasian	Male	68	8	Control	n/a	Control
4	Caucasian	Female	71	9	Control	n/a	Control
5	Caucasian	Male	48	4	Control	n/a	Control
6	Caucasian	Male	58	7	Control	n/a	Control
7	Caucasian	Male	60	6.5	Control	n/a	Control
8	Caucasian	Male	44	9	Control	n/a	SALS 1
9	Caucasian	Male	73	10	Control	n/a	SALS 2
10	Caucasian	Male	39	8	Control	n/a	SALS 2
11	Caucasian	Male	67	8	SALS	90	SALS 1
12	Caucasian	Male	41	10	SALS	96	SALS 1
13	Caucasian	Male	65	6.5	SALS	38	SALS 1
14	Caucasian	Male	68	6	SALS	30	SALS 1
15	Caucasian	Female	67	8	SALS	27	SALS 1
16	Caucasian	Male	43	6	SALS	38	SALS 1
17	Caucasian	Male	54	3	SALS	31	SALS 1
18	Caucasian	Male	38	7	SALS	42	SALS 1
19	Caucasian	Male	45	6.5	SALS	38	SALS 1
20	Caucasian	Female	46	7	SALS	31	SALS 1
21	Caucasian	Female	65	7	SALS	52	SALS 1
22	Caucasian	Male	54	8	SALS	49	SALS 1
23	Caucasian	Male	51	4	SALS	60	SALS 1
24	Caucasian	Male	69	10	SALS	20	SALS 1
25	Caucasian	Male	68	7	SALS	18	SALS 1
26	Caucasian	Female	68	8	SALS	22	SALS 1
27	Caucasian	Male	61	3	SALS	11	SALS 1
28	Caucasian	Female	57	4	SALS	7	SALS 1
29	Caucasian	Female	40	5	SALS	130	SALS 2
30	Caucasian	Male	41	3	SALS	72	SALS 2
31	Caucasian	Female	61	6	SALS	43	SALS 2
32	Caucasian	Female	61	10	SALS	29	SALS 2
33	Caucasian	Female	51	7	SALS	29	SALS 2
34	Caucasian	Male	63	7.5	SALS	27	SALS 2
35	Caucasian	Female	70	4	SALS	30	SALS 2
36	Caucasian	Female	69	9	SALS	52	SALS 2
37	Caucasian	Female	64	5.3	SALS	71	SALS 2
38	Caucasian	Male	46	7.5	SALS	48	SALS 2
39	Caucasian	Male	55	6	SALS	18	SALS 2
40	Caucasian	Male	51	8	SALS	23	SALS 2
41	Caucasian	Male	59	8	SALS	13	SALS 2

Fresh-frozen motor cortex samples were obtained from the Department of Neuropathology of the Academic Medical Center, University of Amsterdam, The Netherlands. Control patients died from a non-neurological disease (myocardial infarction, renal failure, or pulmonary embolism). All patients included in the study displayed no signs of infection before death. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes, approval was obtained from the local ethical committees for medical research.

Microarray processing and data extraction

Complementary RNAs (cRNAs) labeled with Cy3-CTP (Perkin-Elmer) were synthesized from 1 μg of total RNA of each sample using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) following the manufacturer's protocol. Aliquots (750 ng) of Cy3 labeled cRNA targets were co-hybridized on 4x44K Whole Human Genome Oligo Microarrays (Agilent Technologies, Italy). Microarray hybridization and washing were performed using reagents and instruments (hybridization chambers and rotating oven) as indicated by the manufacturer (Agilent Technologies). Microarrays were scanned at 5-μm resolution using a GenePix Personal 4100A microarray scanner and the GenePix Pro 6.0 acquisition and data-extraction software (Molecular Devices, Corp.). Quality control analysis on all samples and parameters associated (Table 1) was performed using GeneSpringGX v.12.6.1 (Agilent Technologies, Italy). Raw signal values were thresholded to 1, log2 transformed, normalized to the 50th percentile, and baselined to the median of all samples using GeneSpringGX v.12.6.1 (Agilent Technologies, Italy). Genes with a corrected P value < 0.05 (one-way ANOVA followed by the Benjamini and Hochberg False Discovery Rate and the Tukey's Post Hoc test) were considered differentially expressed.

To analyze gene expression changes in the context of known biological pathways we used MetaCore (Nikolsky et al., 2005). P values were calculated using a basic formula for hypergeometric distribution where the P value essentially represents the probability of particular pathway arising by chance. To limit possible number of type I errors among the test results a False Discovery Rate (FDR) threshold of 0.05 was used to identify statistically significant pathways.

Raw data of the microarrays are available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with the accession number E-MTAB-2325.

Quantitative RT-PCR

To further confirm the reliability of the microarray data, the mRNA levels of ten differentially expressed genes (ANXA2, AQP1, ATP1A3, HMOX2, HPRT1, NGFR, NRGN, OLFM1, SERPINA3, VIP) were quantified by real-time quantitative RT-PCR. Genes were randomly selected by crossing differentially expressed genes with RT-PCR primers available in our repository. Aliquots of cDNA (0.1 and 0.2 μg) from individual samples, and known amounts of external standards (purified PCR product, 10^2 to 10^8 copies) were amplified in parallel reactions using primers shown in Table 2. PCR amplifications were performed as previously described (Lederer et al., 2007). Specificity of PCR products obtained was characterized by melting curve analysis followed by gel electrophoresis and DNA sequencing.

Immunohistochemistry

Paraffin-embedded tissue was sectioned at 6 μm and mounted on pre-coated glass slides (StarFrost, Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Germany). Representative sections of all specimens were processed for hematoxylin and eosin, Klüver-Barrera and Nissl stains. Antibodies against SERPINA3, major histocompatibility complex (MHC) class I (HLA A, B and C; mouse clone HC-10; 1:200; gift from Prof. J. Neefjes, NKI, The Netherlands), and MHC class II (HL-DR; mouse clone CR3/43; 1:400; DAKO, Glostrup, Denmark) were used for imunohistochemical analysis of ALS specimens. Target selection was based on the encoding genes strongly and differentially regulated, and on the availability of commercial antibodies that could be used on human formalin fixed paraffin embedded material. Single-label immunohistochemistry was performed as previously described (Aronica et al., 2003; Aronica et al., 2001) with the Powervision kit (Immunologic, Duiven, The Netherlands) and 3,3-diaminobenzidine as chromogen. The intensity of HLA-ABC, HLA-DR and serpina3 immunoreactive stainings was evaluated using a scale of 0–3 (0: no; 1: weak; 2: moderate; 3: strong staining). All areas of the stained specimen were examined and the score represents the predominant cell staining intensity found for each case. The frequency of positive cells (microglia/astrocytes) was also evaluated and scored (1: rare; 2: sparse; 3: high) to inform on the relative number of positive cells within the hippocampus. As proposed before (Aronica et al., 2005; Iyer et al., 2010), the product of these two values (intensity and frequency scores) was taken to give the overall score (total labeling score). Evaluation of albumin IR (extravasation, with uptake in astrocytes) was also performed.

Results

Patient segregation by unsupervised hierarchical clustering

By using oligonucleotide microarrays we monitored mRNA expression profiles of 41,059 genes in motor cortex control (10) and SALS (31) patients (Table 1). An unsupervised, hierarchical clustering algorithm allowed us to cluster the 41 motor cortex samples on the basis of their similarities measured over the most informative genes (9646 genes with a standard deviation >1.5; Supplementary Table 1). Similarly, the same genes were clustered on the basis of their similarities measured over the group of 41 motor cortex samples. The transcriptomic profiles obtained in cortex samples produced a good separation of controls and SALS patients, segregating these latter into two distinct groups (Fig. 1a). Control patients were mainly (7/10) grouped in a single cluster, whereas SALS patients segregated into cluster 1 (18/31) and cluster 2 (13/31). Thus, using unsupervised clustering we can distinguish

Table 2
Confirmation of microarray data by real time RT-PCR.

Fold change	ALS1/Control		ALS2/Control		P-value		Microarray probe ID	Forward primer	Reverse primer
Gene symbol	Microarray	RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR			
NGFR	−1.41	−1.50	2.68 *	3.42	8.27E−04	0.033	A_23_P389897	CGGCTACTACCAGGATGAGA	GTGCTGGCTATGAGGTCTTG
ATP1A3	2.44 *	3.66	−1.05	−1.38	9.36E−03	0.002	A_23_P27472	TCAAGAAGGAGGTGGCTATG	GAGAAGCAGCCAGTGATGAT
HPRT1	1.23	1.49	−2.46 *	−2.27	2.48E−04	0.013	A_23_P11372	CCTGCTGGATTACATTAAAGCACTG	CCTGAAGTACTCATTATAGTCAAGG
NRGN	1.70	2.10	−3.86 *	−3.56	4.02E−04	0.027	A_23_P116264	GACTAGGCCAGAACTGAGCA	AGTGGCACGGAGATGTAGG
HMOX2	1.77	2.11	−3.22 *	−4.41	3.42E−05	0.034	A_23_P100501	TCGTCTGACAGCATCCTCTC	GTGTGGAAGCCTGGAGTAGA
OLFM1	1.50	1.38	−3.90 *	−3.47	4.61E−05	0.041	A_24_P406601	ATTAGGAGGCGAGGAGAGAG	GATGAGGAAGAGGAGGCTGA
SERPINA3	13.40 *	11.03	3.07 *	4.35	1.88E−05*	0.008	A_23_P2920	CTGGCTGATGGTGTGAACTC	GCCAGGATGAAGTCGTAGAT
VIP	1.11	1.41	−2.51 *	−3.12	1.66E−03	0.015	A_23_P19650	TCCTTGTGCTCCTGACTCTT	AAGACTGCATCTGAGTGACG
AQP1	3.84 *	4.28	−1.56	−1.48	9.88E−05	0.022	A_23_P372834	GACACCTCCTGGCTATTGAC	TAAGAGGCTTGACCATGCAG
ANXA2	3.17	2.87	1.17	1.07	3.38E−05	0.032	A_23_P146644	GATCATCTGCTCCAGAACCA	GAGTCATACAGCCGATCAGC
Pearson coeff.	0.98		0.99						

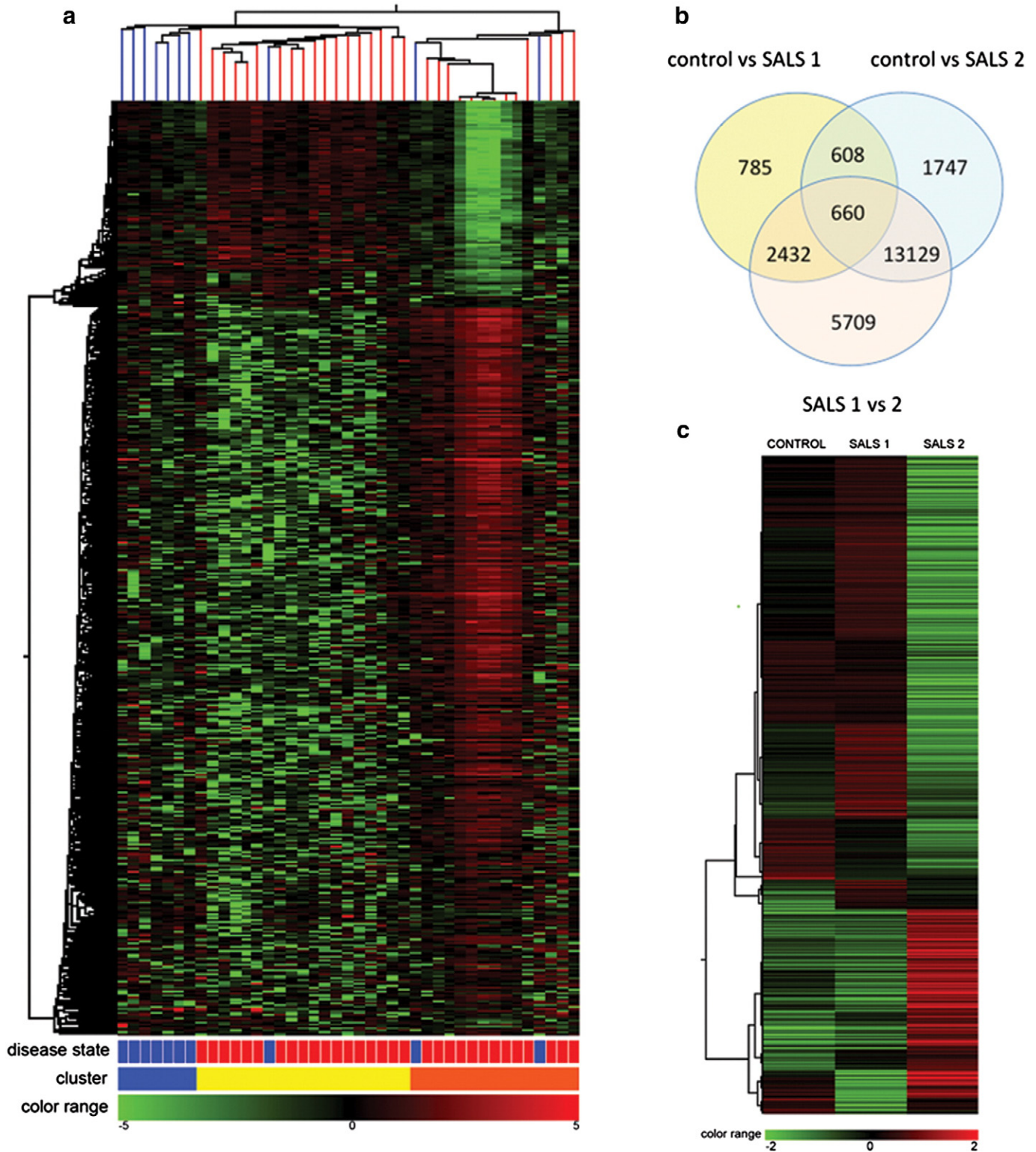Significant P-values refers to pairwise comparisons indicated by *.

Fig. 1. Panel a. Unsupervised hierarchical clustering of control and SALS patients. Unsupervised hierarchical clustering (similarity measure: Pearson centered; linkage rule: average) was used to cluster control and SALS patients on the basis of their similarities measured over the most informative genes expressed in motor cortex (9646 genes with a standard deviation >1.5). Similarly, the same genes were clustered on the basis of their similarities measured over the motor cortex of control and SALS patients. In this two-dimensional presentation, each row represents a single gene and each column a motor cortex from control or SALS patients. As shown in the color bar, red indicates up-regulation, green down-regulation, black no change. In the dendrograms shown (left and top), the length and the subdivision of the branches display the relatedness of the expression of the genes (left) and the motor cortex (top). Although SALS patients could be clearly distinguished on the basis of their motor cortex gene expression patterns, no significant association was found between their clinical characteristics and cluster assignment. In addition to the clinical characteristics listed in Table 1, our analysis included location of onset (arm, trunk, leg), occupation, the use of smoke or alcohol, the presence of other pathological conditions, therapy used, vaccinations, and family anamnesis (data not shown). Panel b. Venn diagrams of differentially expressed genes in motor cortex of control and SALS (clusters 1 and 2) patients (Supplementary Tables 2–4). Panel c. Hierarchical clustering of differentially expressed genes in motor cortex of control SALS1 and SALS2 patients. Genes are arranged in a hierarchical cluster based on their expression patterns, combined with a dendrograms (left) whose branch lengths reflect the relatedness of expression patterns. As shown in the color bar, red indicates up-regulation, green down-regulation, black no change.

control from SALS patients, and separate SALS patients in two greatly divergent groups. Patient stratification in these groups by unsupervised hierarchical clustering was not related to technical variation (arrays hybridization) or patient demographic (gender, age at onset, age at death, survival time from date of onset, PMI) (Supplementary Fig. 1).

Differentially expressed genes in motor cortex of control and SALS patients

When gene expression profiles in SALS1 and SALS2 were compared to controls, 4485 and 16,144 genes showed significant changes of gene expression, respectively (Fig. 1b) (Supplementary Tables 2–4). Although some of these genes (1268) were differentially expressed in both pairwise comparisons, the majority of differentially expressed genes were cluster specific. A larger number of genes (21,930) were differentially expressed between SALS1 and SALS2, indicating these clusters were greatly divergent at the genomic level. A comprehensive picture of transcriptional changes associated to these three clusters is shown in Fig. 1c where differentially expressed genes are grouped on the basis of similarity in their expression patterns in different patients with a hierarchical clustering method.

Confirmation by quantitative RT-PCR

To confirm the reliability of the array data, we quantitatively validated the differential expression of ten genes (ANXA2, AQP1, ATP1A3, HMOX2, HPRT1, NGFR, NRGN, OLFM1, SERPINA3, VIP) using real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) (Table 2).

Remarkably, the pattern of gene expression observed by microarrays precisely paralleled the pattern observed using real-time RT-PCR (correlation coefficient r = 0.98), confirming the up-regulation or down-regulation of these genes in the SALS motor cortex.

Confirmation by immunohistochemistry

To validate our findings for mRNAs at the protein level we performed immunohistochemistry analysis of SERPINA3, MHC class I (HLA-ABC) and II (HLA-DR) in motor cortex samples of control, SALS1 and SALS2 patients. In addition to showing a significant correlation between mRNA and protein levels, our analysis allowed to distinguish their cellular localization (Fig. 2 and Supplementary Fig. 1). Increased expression of SERPINA3 immunoreactivity was observed in reactive astrocytes of SALS1 patients. In control and SALS2 patients, MHC class I (HLA-ABC) immunoreactivity was mainly detected in endothelial cells, whereas an increased expression in SALS1 patients was observed in activated microglial cells. In control and SALS2 patients, MHC class II (HLA-DR) immunoreactivity was detected in cells of the microglia/macrophage lineage (weak expression in resting microglial cells), whereas in SALS1 patients an increased expression was observed in activated microglial cells.

Deregulated pathways in motor cortex of SALS patients

To identify deregulated pathways in motor cortex of SALS patients, the most informative genes (9646 genes with a standard deviation >1.5, depicted in Fig. 1a) were subjected to pathway analysis by using functional ontologies represented in the Metacore repository. Statistically significant canonical pathways (108) are indicated in Supplementary Table 5 and involve 7 different cellular processes: Apoptosis and survival, Cell adhesion, Cytoskeleton remodeling and axonal transport, Cell cycle, Immune response, Energy metabolism and Signal transduction.

To reduce redundancy of deregulated canonical pathways and simplify their comprehension, the most significant variations implicated in pathway analysis were summarized in Figs. 3–9 and super imposed to statistically significant changes of single genes.

Discussion

In the following paragraphs, we will discuss functional clusters of co-regulated genes and pathways. As described below and represented in Table 3, deregulation of these genes and pathways in SALS patients was cluster specific.

Apoptosis and survival

A number of genes previously associated to apoptosis and survival were deregulated in cortex of SALS patients (Fig. 3).

SALS2 patients showed increased expression of TNFSF6, FADD, RIPK1 and p38 MAPK, which are involved with the triggering of the extrinsic apoptotic signaling cascade. These findings are congruent with previous data found in degenerating spinal cord and cerebral cortex motor neurons of SOD1^{G93A} ALS mouse model (Holasek et al., 2005; Petri et al., 2006; Raoul et al., 2002; Raoul et al., 1999).

Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis and has been involved in motoneuron degeneration in ALS (Koyama et al., 2010; Rothstein, 2009). In accordance to these observations we found differential expression of caspase-4, 6 and 9 in SALS2 patients. One of the final targets of caspases is ICAD, the inhibitor of CAD, a DNase that fragments the DNA, causing the characteristic apoptotic DNA ladder (McIlroy et al., 1999; Wolf et al., 1999). In SALS2 patients we found the up regulation of ICAD and CAD.

SALS patients showed increased expression of BAD, a pro apoptotic regulator involved in the mitochondrial intrinsic signaling pathway, which has been extensively implicated in both ALS patients and transgenic SOD1^{G93A} mice (Gonzalez de Aguilar et al., 2000; Guegan et al., 2001; Vukosavic et al., 1999). SALS2 patients, however, showed down regulation of pro-apoptotic BID, BCL-2, BAX, and cytochrome c, and over expression of the anti-apoptotic protein BCL-XL. These changes may represent an adaptive response to death stimuli and antagonize to stress-induced apoptosis signaling.

The expression of Beclin1, a key autophagy-related gene previously linked to ALS neurodegeneration (Erlich et al., 2006; Sasaki, 2011), was increased in SALS patients.

Cell adhesion

Our analysis revealed differential expression of numerous genes involved in cell adhesion (Fig. 4), mainly in SALS2 patients. These genes encode for 12 integrin receptors, 3 extracellular matrix molecules (Collagen IV, Laminin 1 and Fibronectin), 7 components of tight junctions (Claudin-1, Claudin-3, Caludin-5, Jam1, Jam2, ZO-1 and ZO-2), and one component of Gap junctions (Connexin 43). Although such a vast deregulation of integrins was previously unknown, changes in plasma Fibronectin levels have previously been described in ALS patients and significantly correlated with the clinical progression of this disorder (Ono et al., 2000). A progressive decrease of Collagen IV has been demonstrated in serum (Ono et al., 1998) and the vascular structures of ALS spinal cord (Miyazaki et al., 2011). High levels of Laminin 1, previously observed in ALS spinal anterior horn, may represent a protective measure to aid neuronal survival (Wiksten et al., 2007). A significant decrease of both protein and mRNA levels of tight junction components has been described in ALS patients and animal models (Henkel et al., 2009; Nicaise et al., 2009; Zhong et al., 2008).

In SALS2 patients we also observed the differential expression of 4 extracellular matrix metalloproteinases, together with the metallopeptidase inhibitor TIMP1. MMPs are a family of zinc-dependent endoproteinases that regulate the extracellular matrix structure and play an important role in synaptic remodeling, neuronal regeneration, and remyelination, modulation of blood–brain and blood–cerebrospinal fluid barrier permeability and leukocytes invasion in neuroinflammatory diseases (Renaud and Leppert, 2007). Increased MMPs and TIMPs have been previously reported in post-mortem ALS brain tissue, as well as in plasma and
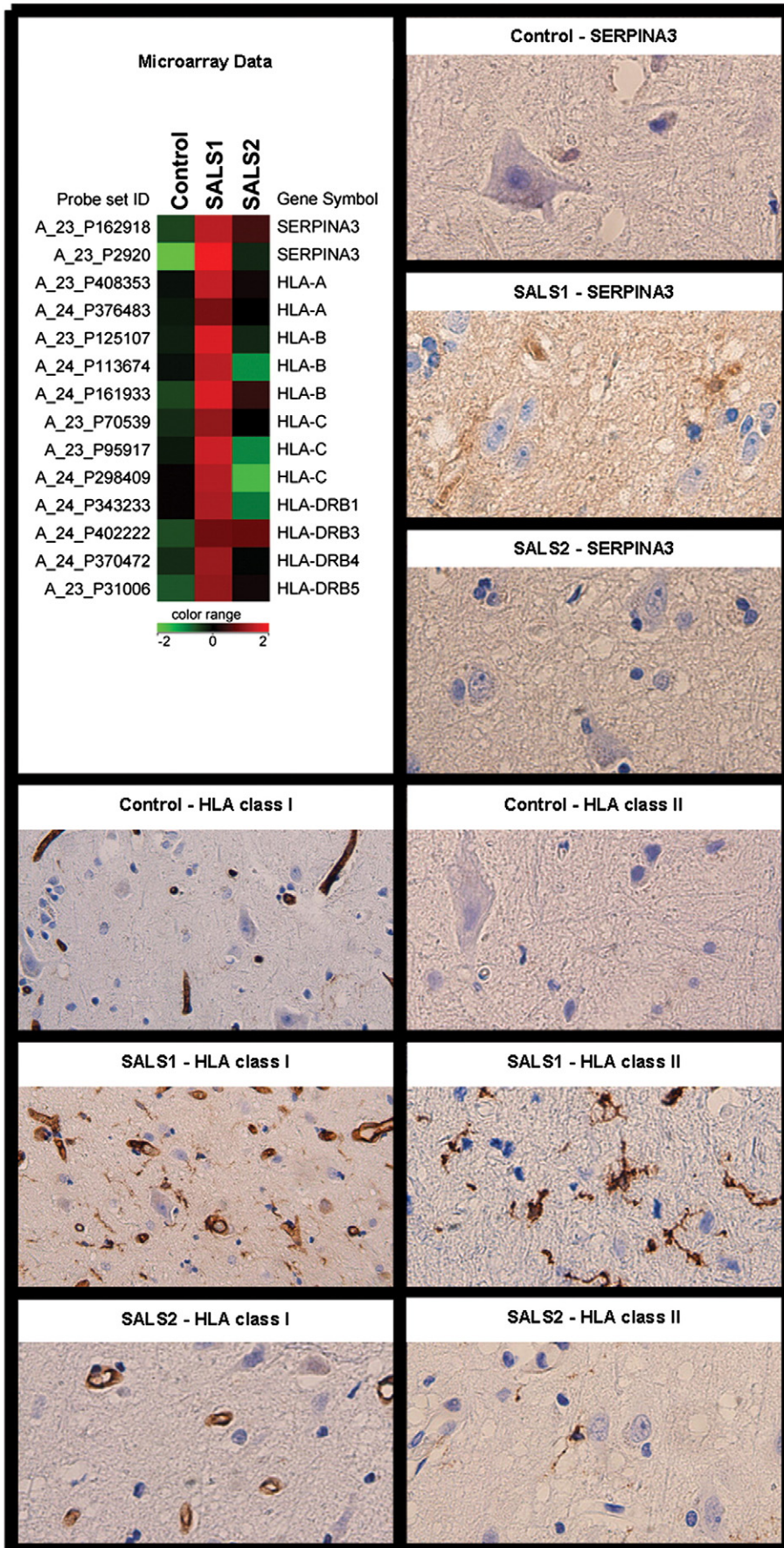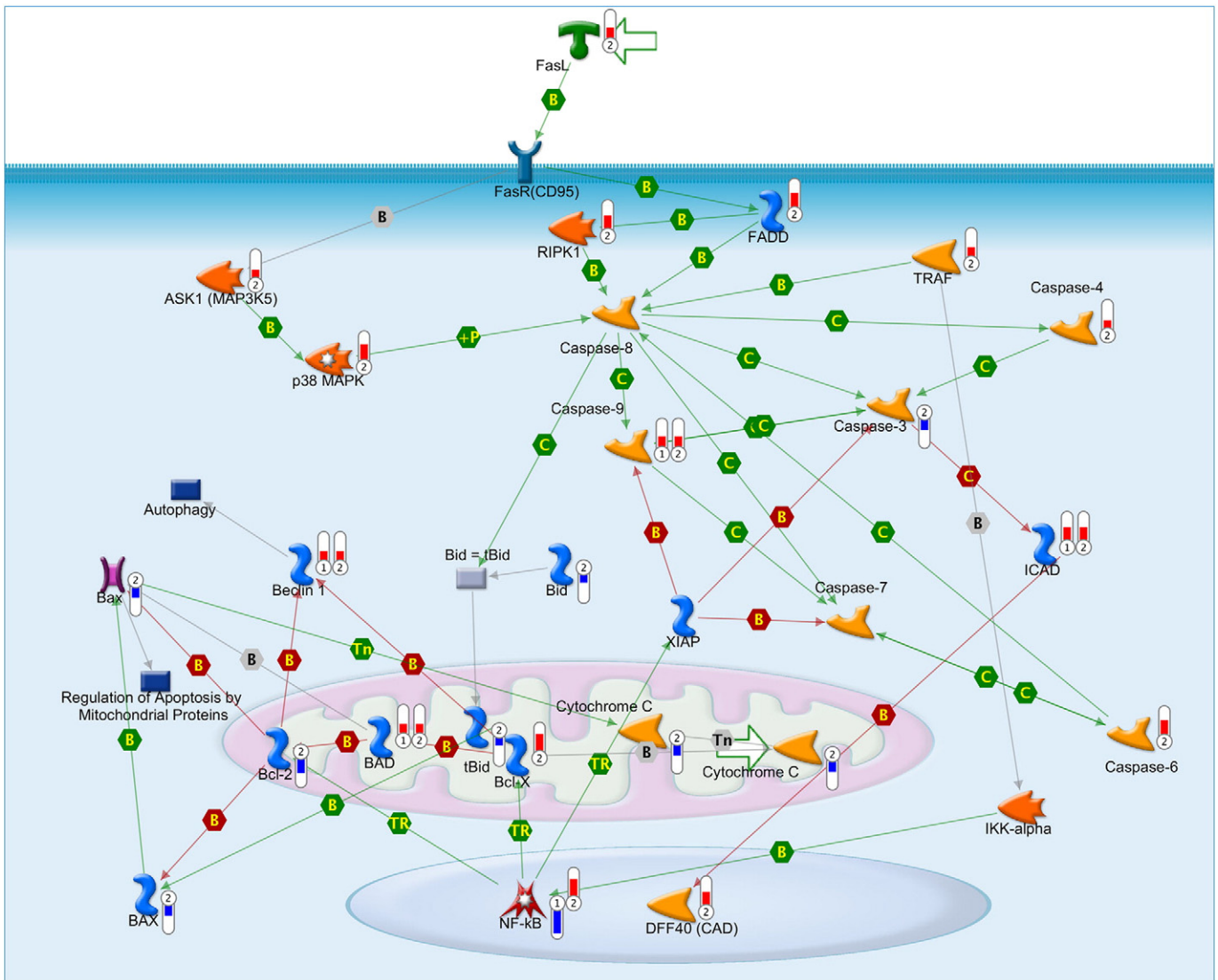
Fig. 3. Apoptosis and survival. The apoptotic response can be activated through either the intrinsic or the extrinsic pathway, depending on the origin of the death stimuli. The extrinsic apoptosis pathway is initiated by binding of an extracellular death ligand, such as FasL (TNFSF6) to its cell-surface death receptor FasR (CD95). Adaptor protein FADD, the serine–threonine kinase RIPK1 and protein kinase p38 MAPK transduce cell-death signals to effector caspases. The intrinsic apoptotic pathway is characterized by permeabilisation of the mitochondria and release of cytochrome c into the cytoplasm. Cytochrome c then forms a multi-protein complex known as the "apoptosome" and initiates activation of the caspase cascade through caspase 9. Sequential activation of caspases by the intrinsic or extrinsic signaling pathways plays a central role in the execution-phase of apoptosis. Bcl-2 proteins (Bcl-2, Bcl-X, Bax, Bad, Bid) play a fundamental role in the regulation of the intrinsic pathway by controlling mitochondrial membrane permeability and the release of the pro-apoptotic factor, cytochrome c. Thermometers labeled with (1) or (2) indicate expression levels in SALS cluster 1 and 2, respectively. Up-ward thermometers have red color and indicate up-regulated signals in SALS patients, down-ward (blue) ones indicate down-regulated signals. Pathway objects and links are described separately in the Supplementary Fig. 3.

cerebrospinal fluid (Lorenzl et al., 2003). A decrease of MMP-9 together with an increase of MMP-1, MMP-2 and TIMP-1 values, have been confirmed in cerebrospinal fluid of ALS patients (Niebroj-Dobosz et al., 2010). Furthermore, deletion of MMP-9 gene in mutant SOD-1 mice accelerates motor neuron disease and shortens survival (Dewil et al., 2005). Although the role of MMPs in ALS pathogenesis is currently unknown, altered levels of MMPs may reflect the degeneration process of motor neurons and tissues remodeling (Niebroj-Dobosz et al., 2010).

Cytoskeleton remodeling and axonal transport

The cytoskeleton is critical for neuronal maintenance and plasticity, neurite outgrowth, axonal caliber and transport. Our analysis uncovered modification of major components of cytoskeleton in SALS patients (Fig. 5). Among these are genes encoding intermediate filaments proteins (Nestin, GFAP, Desmin, Desmuslin, Vimentin, Peripherin, Keratins 5, 8, 14, and 18), Actin, Tubulin (alpha, beta and gamma), Myosin and all

Fig. 2. Immunohistochemistry. Differential expression of genes implicated by microarray was validated by immunohistochemistry. Differential expression of SERPINA3, MHC class I (HLA-ABC) and MHC class II (HLA-DRB1, DRB3, DRB4 and DRB5) mRNAs is shown in the left/upper panel with a color matrix. As shown in the color bar, red indicates up-regulation, green down-regulation, black no change. Some of these mRNAs were detected by more than one probe set on the microarray showing consistent changes. Immunohistochemistry analysis of SERPINA3, MHC class I antigens (HLA-ABC), and MHC class II antigens (HLA-DR) was performed in motor cortex samples of control, SALS1 and SALS2 patients (n = 5/group). Immunoreactivity of SERPINA3 was observed in reactive astrocytes of SALS1 patients. MHC class I immunoreactivity was mainly localized in endothelial cells of control patients and in activated microglial cells of SALS1 patients. Weak expression MHC class II immunoreactivity was detected in resting microglial cells of control and SALS2 patients, whereas in SALS1 patients an increased expression was observed in activated microglial cells. Quantitative data are reported in Supplementary Fig. 2.
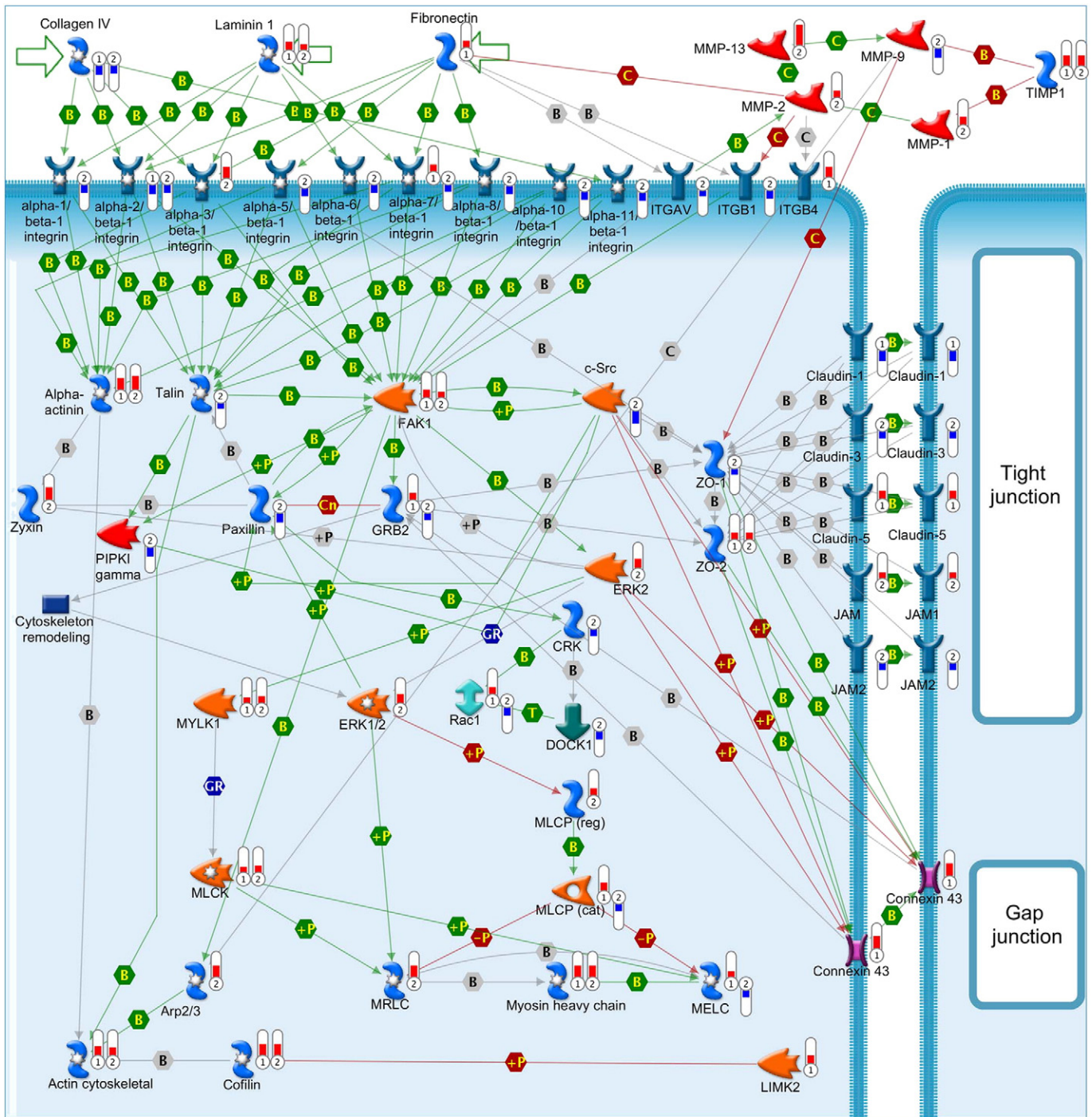
Fig. 4. Cell adhesion. Integrins are heterodimeric cell surface adhesion receptors formed by two noncovalently associated subunits, alpha and beta. Most integrins (ITGA1, ITGA2, ITGA3, ITGA5, ITGA6, ITGA7, ITGA8, ITGA10, ITGA11, ITGAV, ITGB1, ITGB4) recognize several ECM proteins, such as Laminin 1, Fibronectin and Collagen IV, whereas alpha-5/beta-1 integrin recognizes only Fibronectin. The ECM, integrins and the cell cytoskeleton interact at sites called focal contacts. The integrin-binding proteins Paxillin and Talin recruit Focal adhesion kinase (FAK1) to focal contacts. Alpha-actinin is a cytoskeletal protein that crosslinks Actin in actomyosin stress fibers and tethers them to focal contacts. Phosphorylation of Alpha-actinin by FAK1 reduces the crosslinking of stress fibers and prevents maturation of the focal contacts. The Actin-related protein complex (Arp2/3) nucleates new Actin filaments from the sides of preexisting filaments. Zyxin is an Alpha-actinin and stress-fiber-binding protein found in mature contacts. Integrin clustering promotes FAK1 autophosphorylation, thereby creating a binding site for c-Src. Phosphorylation of FAK1 by c-Src maximizes catalytic activity of FAK1 and creates a binding site for GRB2, thereby leading to the activation of ERK1/2. ERK2 phosphorylates FAK1 and decreases Paxillin binding to FAK1. Within focal contacts, FAK1-c-Src-mediated phosphorylation of Paxillin promotes ERK2 binding. ERK2-mediated phosphorylation of Paxillin can facilitate FAK1 binding to Paxillin and enhance FAK1 activation. ERK2-mediated phosphorylation and activation of MYLK1 together with inactivation of PAK1 contribute to cell-matrix adhesion dynamics. CRK facilitates activation of Rac1 by DOCK1. Rac1 leads to activation of MRLC and the Arp2/3 complex. Tight junctions are particularly expressed in endothelia of the blood–brain barrier and are composed by transmembrane constituents (Claudin 1, Claudin 3, Claudin 5, JAM1, JAM2) and cytoplasmic proteins (ZO-1, ZO-2). Gap junctions are clusters of transmembrane channels formed by connexins. The cytoplasmic domain of Connexin 43 binds ZO-1 and ZO-2, allowing close association between gap and tight junctions. Pathway objects and links are described separately in the Supplementary Fig. 3.

three neurofilament subunits (NEFL, NEFM, NEFH). Most of these cytoskeletal proteins were up-regulated in SALS1 patients, whereas some of them (Tubulin alpha and beta, Peripherin, NEFL, NEFM, NEFH, Dynein, Dynactin, MELC) were down-regulated in SALS2. These data are in agreement with previous findings showing differential expression of Peripherin, Tubulin and neurofilament subunits (Kudo et al., 2010;
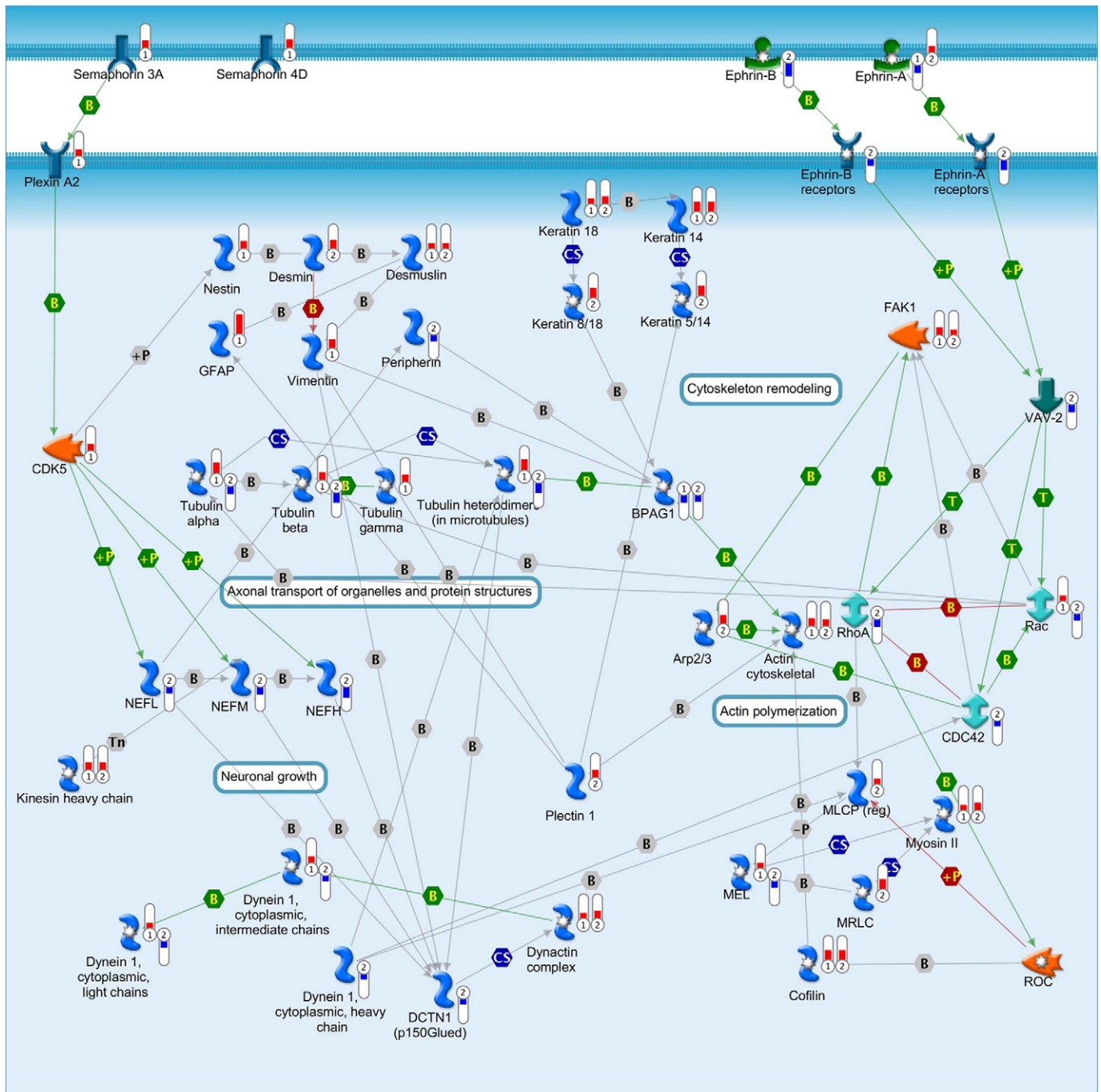
Fig. 5. Cytoskeleton remodeling and axonal transport. Cytoskeleton consists of three distinct, yet interconnected filament systems: microfilaments (actin filaments), intermediate filaments and microtubules. Microfilaments are the thinnest filaments of the cytoskeleton and are composed of linear polymers of actin subunits. They act as tracks for the movement of myosin molecules and are controlled by the Rho family of small GTP-binding proteins such as Rho, Rac and Cdc42. Intermediate filaments (Vimentin, Desmin, Desmusin, GFAP, Peripherin, Nestin, NEFL, NEFM, NEFH) organize the internal tridimensional structure of the cell, anchoring organelles and serving as structural components of the nuclear lamina and sarcomeres. Microtubules are polymers of alpha and beta tubulin and, in association to other proteins such as Dynein and Dynactin, are important for axonal transport. Semaphorins act as axonal guidance factors. Semaphorin 3A binds to Plexin A1 and induces repulsive responses. Ephrins and their receptor protein–tyrosine kinases regulate a variety of biological processes including the guidance of axon growth. Pathway objects and links are described separately in the Supplementary Fig. 3.

Lederer et al., 2007; Robertson et al., 2003). Although the role of neurofilaments (NFs) in ALS is still controversial (Shaw, 2005), aberrant accumulation of neurofilaments in the cell body and proximal axons of motor neurons is a hallmark of ALS. Deletion of NEFL subunits in the SOD1 G85R mouse model is accompanied by preferential increase of the NEFH and NEFM subunits in the motor neuron cell bodies and reduction of these subunits in the axons, with an overall significant delay in the onset and progression of clinical disease (Williamson et al., 1998). Over-expression of the NEFH subunit has similar effects

(Couillard-Despres et al., 1998) and has prompted the hypothesis that NFs may act as an abundant buffer for otherwise deleterious processes, for example offering phosphorylation sites for dysregulated intracellular kinases, or reducing the burden of axonal transport (Bruijn et al., 1997; Julien and Beaulieu, 2000; Shaw, 2005). Decreasing the axonal burden of neurofilaments may protect motor neurons, at least in part, by enhancing axonal transport, a hypothesis supported by the observation of defects in slow axonal transport in presymptomatic mutant SOD1 mice (Williamson and Cleveland, 1999). Consistent with the
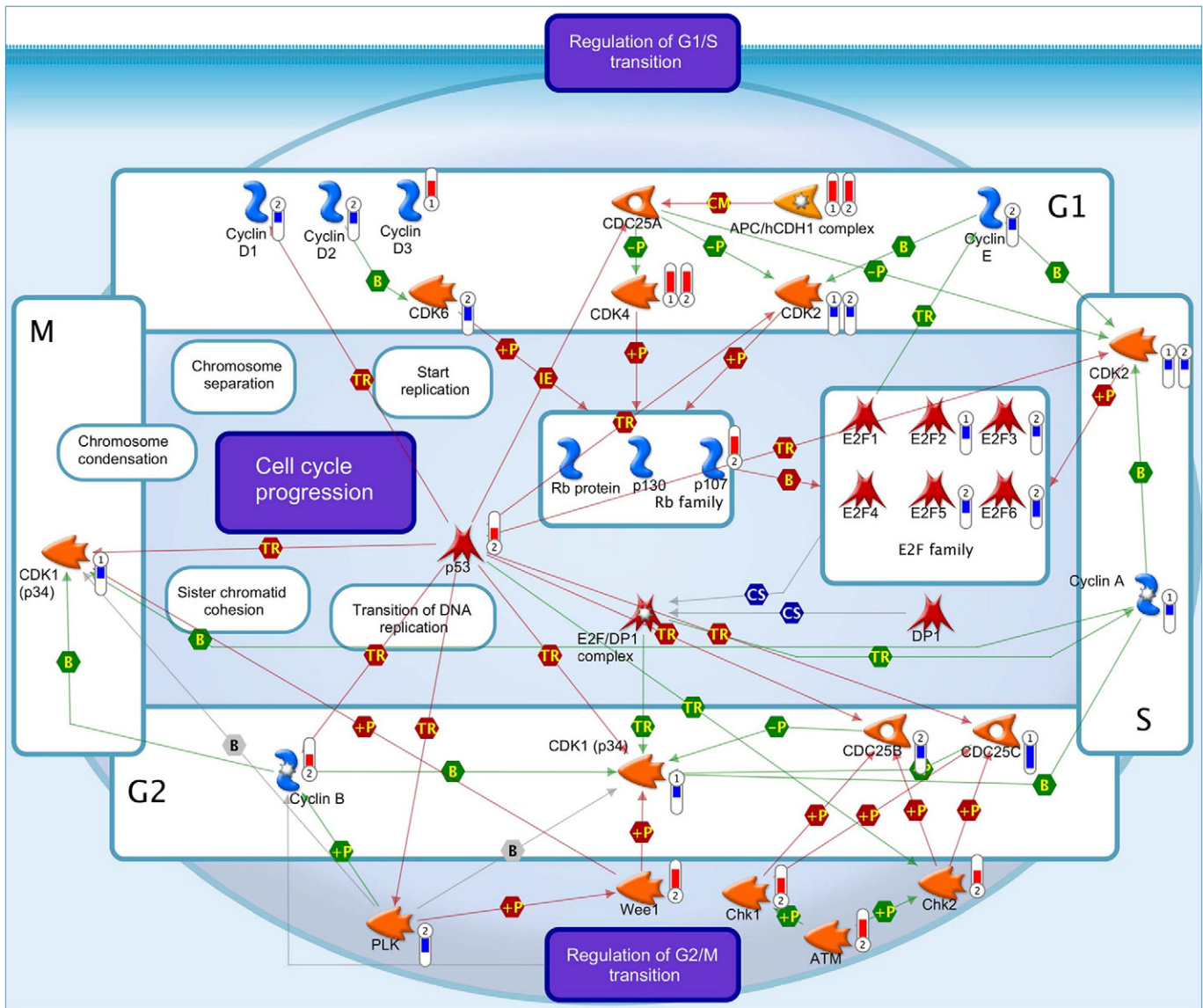
Fig. 6. Cell cycle. Cell cycle is characterized by four phases: G1 (gap phase 1) in which the cell prepares for the upcoming events of S-phase; S (synthesis phase), in which DNA is replicated; G2 (gap phase 2) in which the cell prepares for the upcoming events of M-phase; and M (mitosis), in which chromosomes are separated over two new nuclei. Progress in cell cycle is driven by oscillations in the activities of cyclin-dependent kinases (CDKs), which are controlled by periodic synthesis and degradation of Cyclins, as well as by other regulators. Cyclin/CDK complexes are involved in regulating different cell cycle transitions: Cyclin D/CDK4 (or CDK6) for G1 progression, Cyclin E/CDK2 for the G1-S transition, Cyclin A/CDK2 for S-phase progression, and Cyclin A/CDK1 and Cyclin B/CDK1 for entry into M-phase. Cyclin D/CDK4 (or CDK6) complexes, together with Cyclin E/CDK2 phosphorylate of the retinoblastoma family of tumor suppressor proteins (Rb family) (Rb protein, p107 and p130), thereby liberating the E2F transcription factors. These factors are associated with DP1 and together they drive expression of Cyclin E, Cyclin A, CDK1 and products that are necessary for the replication of DNA and beginning of the S phase. Checkpoint homologues (Chk1 and Chk2) inactivate by phosphorylation cell division cycle 25A phosphatases (CDC25A, CDC25B AND CDC25C). Lack of active Cdc25A, CDC25B and CDC25C results in the accumulation of the phosphorylated (inactive) form of CDK1, Cdk2 and Cdk4. Events controlling cell division are governed by the degradation of different regulatory proteins by the ubiquitin-dependent pathway. Phosphorylated CDC25A may be exposed to ubiquitination by Anaphase-promoting complex (APC). CDK1 phosphorylation by Wee1 inhibits its activity during the G2-phase of the cell cycle. Phosphorylation by PLK1 inhibits Wee1 and activates Cyclin B. Pathway objects and links are described separately in the Supplementary Fig. 3.

view that impaired axonal transport may be involved in the degeneration of motor neurons, in SALS2 patients we observed the down regulation of cytoplasmic Dynein intermediate, light and heavy chains, together with the p150Glued subunit of Dynactin (DCTN1), implicated in retrograde transport of cargoes, such as endosomes. Mutations in the cytoplasmic Dynein heavy chain gene have previously been found in two mouse models, Legs at odd angles (Loa) and Cramping 1 (Cra1), with late-onset motor neuron degeneration, while mutations of DCTN1 gene are responsible of a lower motor neuron disorder with vocal cord paresis (Hafezparast et al., 2003; Jiang et al., 2007; Puls et al., 2003). In addition to this, decreased expression of DCTN1 has been reported in motor neurons of patients with SALS suggesting that abnormalities in Dynactin may play a role in pathogenesis of ALS

(Jiang et al., 2007; Puls et al., 2003). Because axon transport is a tightly regulated process, our observation of deregulation in cargo adaptors has the potential to significantly disrupt transport in ALS motor neurons.

Aberration in axon guidance may also result from differential expression of (i) Semaphorin 3A, Semaphorin 4D, and Plexin A2 in SALS1 patients; (ii) Ephrin A, Ephrin B and their receptors, together with their downstream signaling factors (Cdc42, Rac, RhoA) (Fig. 5). The precise role of Semaphorins in the pathogenesis of ALS is unclear. Increased expression of Semaphorin 3A in terminal Schwann cell of SOD1G93A transgenic mice has previously been associated to de-adhesion or repulsion of motor axons away from the neuromuscular junction, eventually resulting in axonal denervation and motor neuron
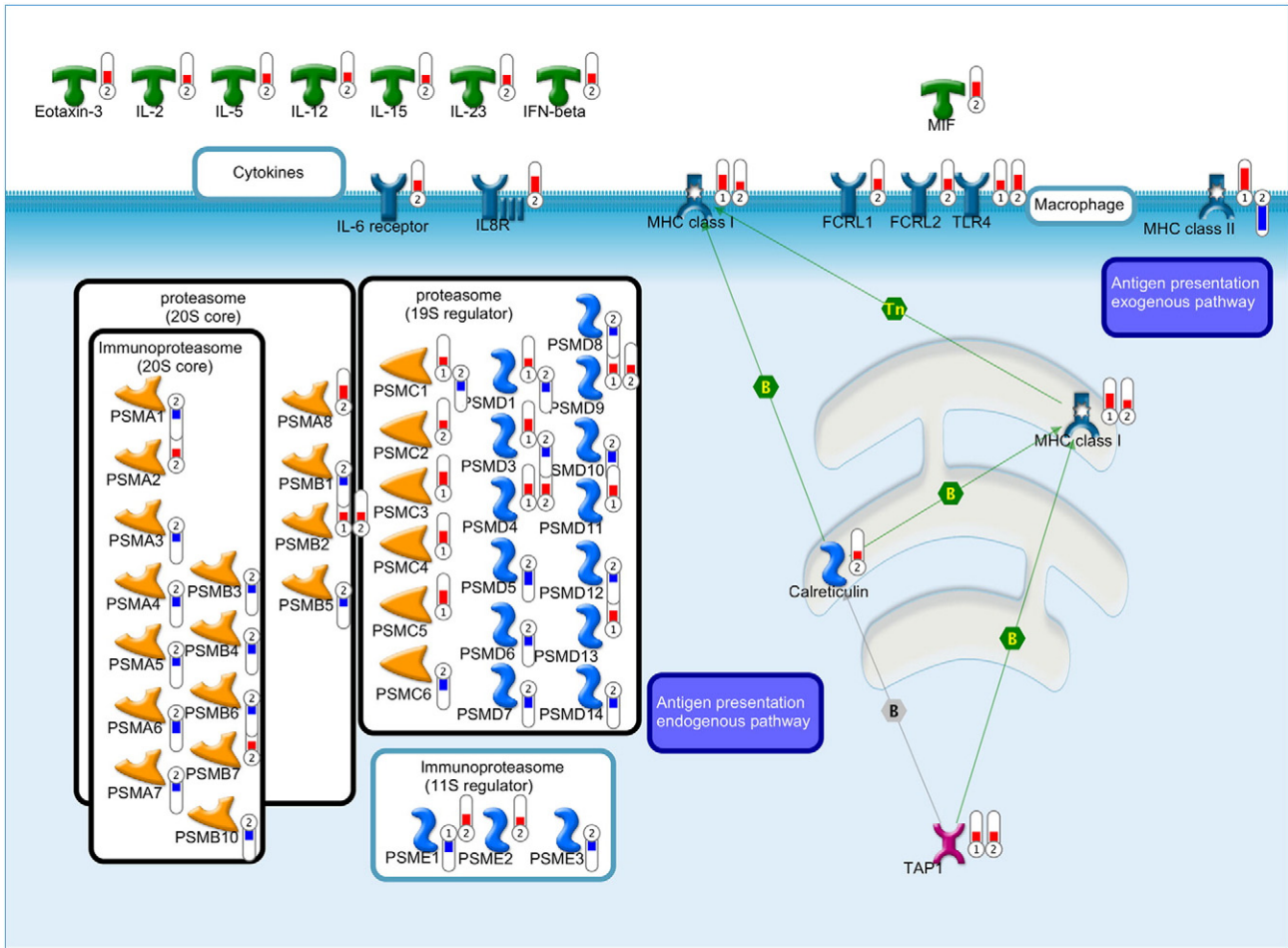
Fig. 7. Immune response. Intracellular proteins are degraded by the proteasome, a multicatalytic proteinase complex composed of two sub-complexes: the 20S core complex (PSMA1, PSMA2, PSMA3, PSMA4, PSMA5, PSMA6, PSMA7, PSMA8, PSMB1, PSMB2, PSMB3, PSMB4, PSMB5, PSMB6, PSMB7, PSMB10) that carries the catalytic activity, and the 19S regulatory complex (PSMC1, PSMC2, PSMC3, PSMC4, PSMC5, PSMC6, PSMD1, PSMD3, PSMD4, PSMD5, PSMD6, PSMD7, PSMD8, PSMD9, PSMD10, PSMD11, PSMD12, PSMD13, PSMD14) that receives the polyubiquitylated substrate, removes the ubiquitin and unfolds the protein for translocation into 20S complex. The immunoproteasome is a modified proteasome, which consists of 11S regulator (PSME1, PSME2, PSME3) and modified 20S core. Degraded proteins are transported into endoplasmic reticulum (ER) via TAP1 transporter. MHC class I molecules are specialized for presentation of endogenously synthesized proteins, bind ER chaperone calreticulin and then antigenic peptides. MHC class II molecules are found on antigen-presenting cells and present antigen derived from extracellular proteins (not cytosolic as in class I). TLR4, FCRL1, and FCRL2 are monocyte/macrophage specific genes. Pathway objects and links are described separately in the Supplementary Fig. 3.

degeneration (De Winter et al., 2006). Indeed, Semaphorin 3A plays a role during several stages of motor neuron circuitry formation, and its altered expression might lead to aberrant outgrowth of corticospinal tract fibers from the cortex, inappropriate guidance of cranial motor axons and hyperfasciculation or defasciculation of both cranial nerves and MMC and LMC motor axons. These changes in expression might be small and may not cause obvious defects during early life. However, minor changes in motor neuron circuitry as a result of altered Semaphorin 3A expression may result in motor connections, which are more vulnerable to additional genetic or environmental changes.

Similarly to Semaphorins, Ephrins have a variety of important functions including axonal outgrowth and cytoskeletal structure development, neuronal connectivity, neuronal apoptosis, synaptic maturation and plasticity (Huot, 2004; Klein, 2004). It is therefore plausible that variability in such molecules could contribute to the initiation and progression of neurodegenerative diseases. A marked increase of Ephrin A1 has previously been found in motor neurons of SALS patients (Jiang et al., 2005) and SNPs in several Ephrin and Eph receptor genes, including Ephrin B1, have been used to predict susceptibility, survival free and age at onset of ALS (Lesnick et al., 2008). Our findings in ALS motor cortex supports the hypothesis that aberrant expression or function of

Ephrins may induce pathological changes in motor neuron circuitry and contribute to ALS pathogenesis (Schmidt et al., 2009).

Cell cycle

Progress in cell cycle is driven by oscillations in the activities of cyclin-dependent kinases (CDKs), which are controlled by periodic synthesis and degradation of Cyclins, as well as by other regulators. Alterations in expression and cellular distribution of these proteins characterize several human neurodegenerative diseases (Currais et al., 2009; Greene et al., 2004; Herrup et al., 2004; Kruman, 2004; Nguyen et al., 2002; Vincent et al., 2003; Yang and Herrup, 2007; Zhang et al., 2007). Our results show de-regulated expression of genes encoding key regulators of G1, S, M and G2 phases (Fig. 6). With only few exceptions, observed changes are consistent with a cell cycle inhibition. Dysregulation of the cyclin system has been already proposed as a possible mechanism for ALS neurodegeneration, where cell death is the result of an unsuccessfully attempt by terminally differentiated neurons to re-entry into the cell cycle (Becker and Bonni, 2004). If so, drug inhibitors of the cell cycle might counteract neuronal degeneration in ALS, as suggested by in vitro studies on motor neurons (Appert-Collin et al., 2006). In SALS2 patients we also
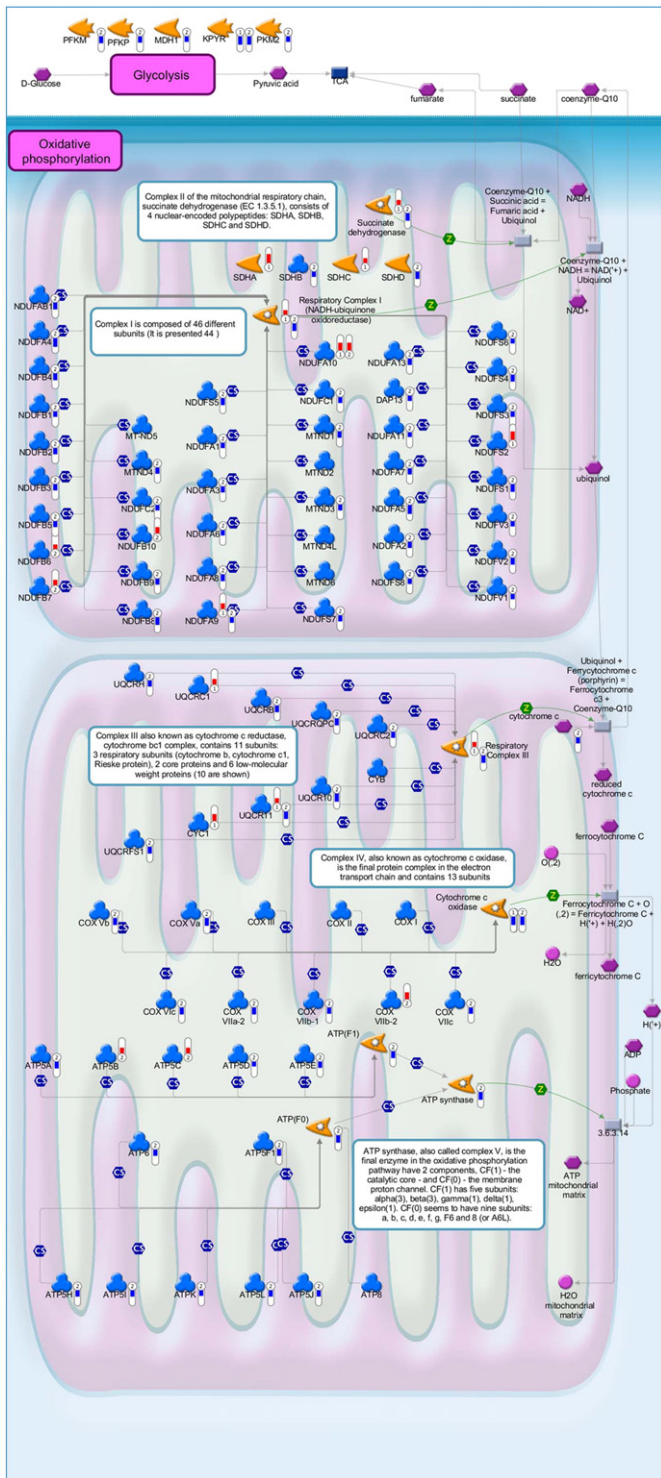
Fig. 8. Energy metabolism. Glycolysis (upper level) converts glucose into pyruvate. During oxidative phosphorylation (lower level) electrons are transferred from electron donors to electron acceptors such as oxygen, in redox reactions. These redox reactions, carried out by five main protein complexes, release energy that is used to form ATP. The energy released by electrons flowing through this electron transport chain is used to transport protons across the inner mitochondrial membrane, in a process called chemiosmosis. This generates potential energy in the form of a pH gradient and an electrical potential across this membrane. Protons flow back across the membrane and down this gradient through ATP synthase, an enzyme that generates ATP from adenosine diphosphate (ADP), in a phosphorylation reaction. Pathway objects and links are described separately in the Supplementary Fig. 3.

observed the up-regulation of Ataxia–telangiectasia-mutated (ATM), cell cycle checkpoint kinases (CHK1/CHK2), and the tumor suppressor gene P53, whose increased levels have been associated with arrest of cell cycle progression and were observed in both SOD1 transgenic mice and ALS patients (de la Monte et al., 1998; Gonzalez de Aguilar et al., 2000; Martin, 2000).

Immune response

The immune response has been implicated in ALS and may contribute to the pathogenesis of disease or represent a response to damage (McCombe and Henderson, 2011). While these positive and negative effects are beginning to be appreciated, their potential as drug targets is being explored (Calvo et al., 2010; Holmoy, 2008; Moisse and Strong, 2006). Reactive microglia and inflammatory processes coincide with ALS onset and disease progression in SOD1 transgenic mice (Hall et al., 1998), and postmortem examinations of neural tissues in ALS patients show both innate and adaptive immunity activation (Barbeito et al., 2010; Sta et al., 2011).

In our study we observed the differential expression of an extensive number of immune-related genes in the cortex of SALS patients (Fig. 7). Correlation of these with a compendium of immune related genes (Abbas et al., 2005) provided a qualitative assessment of the preponderance of immune cell types present (Supplementary Table 6). The 1188 immune related genes in SALS patients were specific for the presence of genes related to both the lymphoid and myeloid (such as macrophages and dendritic cells) lineages. Among these immune-cell specific genes is TLR4, a marker of monocyte/macrophage activation previously observed in spinal cords of ALS patients (Casula et al., 2011).

In SALS patients we observed deregulated expression of several genes encoding proteins involved with antigen processing and presentation. Most of these genes were increased in SALS1 and reduced in SALS2 patients. Proteins involved in antigen processing belong to the 26S proteasome/20S immunoproteasome, proteasome 19S regulator and immunoproteosome 11S regulator (Fig. 7). Increased expression of serpin peptidase inhibitor, clade A, member 3 (SERPINA3) was mainly observed in SALS1 patients (Fig. 2). The protein encoded by this gene is an acute phase reactant protein considered an important link between the immune/inflammatory response and proteosomal turnover. Up-regulation of SERPINA3 in ALS pathology was previously found in several mouse models and human studies (Saris et al., 2013).

Proteins involved in antigen presentation include major histocompatibility complex (MHC) class I molecules (HLA-A, HLA-B, HLA-C), TAP1 and calreticulin. The proteasome system is the major intracellular proteolytic mechanism controlling the degradation of misfolded/abnormal proteins and their accumulation into damaged neurons represents a common hallmark in ALS. Deregulation of the constitutive and inducible proteasome subunits may not only influence the ubiquitin-mediated protein degradation but also lead to generation of peptides that can be used by MHC I molecules for antigen presentation to the immune system, providing an interesting connection between the immune-responses and proteasome function (Bendotti et al., 2012). In addition to MHC class I, we found the deregulated expression of a series of genes, encoding MHC class II molecules, which are found on antigen-presenting cells and present antigen derived from extracellular proteins (Figs. 2 and 7). These MHC class II molecules were up-regulated in SALS1 patients and down-regulated in SALS2.

Up-regulation of several cytokines and IFNβ was observed in SALS2 patients. Although the pathogenic role of cytokines in ALS is still unknown, previous studies have associated their abnormal expression to the clinical status (Moreau et al., 2005; Rentzos et al., 2010; Shi et al., 2007). Similarly, enhanced expression of IFNβ has been demonstrated in spinal cord of SOD1 mice and may represent an early response to pathological changes in ALS (Wang et al., 2011).
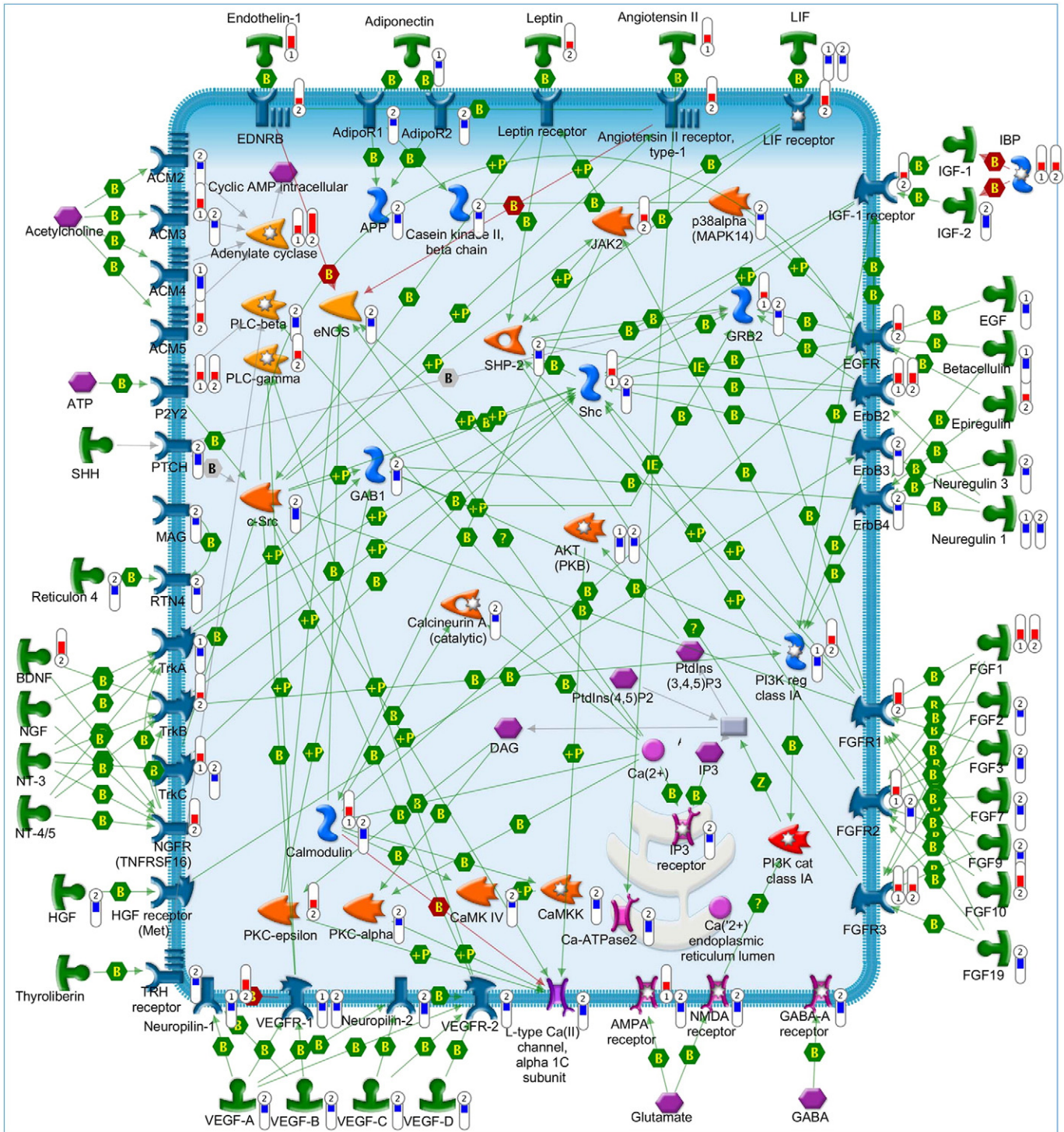
Fig. 9. Signal transduction. Several genes encoding ligands and receptors involved in signal transduction were differentially expressed in SALS patients. Signaling pathways include primary (such as Adenylate cyclase, Phospholipase C or tyrosine kinase receptors) and secondary (such as Ca²⁺ release, PKC or protein kinase cascades) effectors that are regulated at multiple levels with different mechanisms (such as binding or phosphorylation). Pathway objects and links are described separately in the Supplementary Fig. 3.

Energy metabolism

Genes implicated in energy metabolism included those involved in glycolysis and mitochondrial oxidative phosphorylation (Fig. 8). Among the first are those encoding two rate limiting step enzymes, 6-phosphofructokinase (PFKM and PFKP) and pyruvate kinase (KPYR and PKM2), and MDH1, which we previously linked to ALS pathology (Lederer et al., 2007). These genes were mainly down-regulated in

SALS2 patients. In the same patients we observed the coordinated decrease of several genes encoding proteins involved in the oxidative phosphorylation pathway, the major cellular energy supply system. In contrast, a limited but significant number (8) of these genes were increased in SALS1. Down-regulated genes in SALS2 encode proteins of respiratory complex I (36/46 subunits), II (SDHB and SDHD), III (7/11 subunits), IV (6/13 subunits) and V (10/14 subunits of the catalytic and membrane proton channel of ATP synthase) (Fig. 8). An increase

Table 3
Pathways differentially deregulated in cluster SALS patients.

Cellular processes	Sub-groups	SALS 1	SALS 2
Apoptosis and survival	Intrinsic/extrinsic apoptosis signaling		
	Caspase cascade		
	NF-kB signaling		
Cell adhesion	Remodeling of extracellular matrix		
	Chemokines and adhesion		
	Cell contacts by junctional mechanisms		
	Gap junctions		
	Integrin inside-out signaling		
	Tight junctions		
Cell cycle	Role of 14-3-3 proteins in cell cycle regulation		
	Cell-cycle control system (e.g., cyclins, CDKs)		
	S-phase/ DNA replication		
	ChK signaling/cell cycle arrest		
	Spindle assembly and chromosome separation		
Cytoskeleton remodeling and axonal transport	Cytoskeleton remodeling		
	Keratin filaments		
	Neurofilaments		
	Axonal transport		
	Ephrin B signaling		
	Neuronal growth		
	RhoA regulation pathway		
	CDK5 signaling		
Immune response	Antigen presentation		
	Antigen processing/proteosome system		
	Cytokines release		
Energy metabolism	Glycolysis		
	Mitochondrial oxidative phosphorylation		
Signal transduction	Activation of PKC via G-Protein coupled receptor		
	Angiotensin signaling		
	Calcium homeostasis		
	cAMP signaling		
	EGF signaling		
	FGF-family signaling		
	GABA-A receptor life cycle		
	IGF-1 receptor signaling		
	Leptin signaling via PI3K-dependent pathway		
	Regulation of eNOS activity		
	Adiponectin signaling		
	Calcium/calmodulin-dependent kinase (CaMK) activity		
	Thyroliberin signaling		
	BDNF signaling		
	Glutamate signaling		
	Acetylcholine-muscarinic receptor signaling		
	Endothelin signaling		
	HGF signaling		
	Shh signaling		
	VEGF-family signaling		

Red boxes represent cellular processes mainly up-regulated, green bars down-regulated, red/green bars indicate signal pathways both up- and down-regulated, gray bars indicate no significant change when compared to controls.

of complex I (Bowling et al., 1993), and a deficiency of complex IV was previously found in post mortem ALS tissue (Borthwick et al., 1999). This apparent discrepancy may be explained by the opposite behavior of SALS patients found in the present study (increase of complex I in cluster 1, and decrease of complex IV in both clusters). Mitochondrial dysfunction (mainly from morphological evidence) and disruption of energy metabolism have been proposed to play a major role in ALS pathology and treatments designed to improve respiratory chain function may ameliorate the progression of this disorder (Cozzolino and Carri, 2012; Lee et al., 2009; Pizzuti and Petrucci, 2011).

Signal transduction

Neuropeptides and receptors

The expression of genes encoding Adiponectin and its receptors R1 and R2 (Fig. 9) were down-regulated in SALS patients. Beyond their peripheral effects on fat metabolism and insulin sensitivity, Adiponectin

and its receptors are expressed in brain (Brown et al., 2007; Wilkinson et al., 2007), where they regulate neuronal excitability (Hoyda and Ferguson, 2010) and exert protective effects against neurotoxicity (Chan et al., 2012; Jeon et al., 2009; Qiu et al., 2011). In SALS2 patients we observed the up-regulation of another adipokine, Leptin, which is endowed with neurotrophic and neuroprotective properties (Tang, 2008). Induction of Leptin in brain (microglia/macrophage cells) has been described in ischemic cerebral cortex, suggesting the possibility that this peptide may act as an endogenous mediator of neuroprotection (Valerio et al., 2009). Decreased expression of PTCH1 is in agreement with the neuroprotective effects of Sonic hedgehog (Shh) signaling in SOD mice (Peterson and Turnbull, 2012). Three genes encoding proteins implicated in neurite outgrowth and ALS pathology, Myelin-associated glycoprotein (MAG), Reticulon-4 and its receptor were down-regulated in SALS2 patients. Anti-MAG antibodies have been observed in ALS patients (Antoine et al., 1993), whereas a number of studies have already implicated Reticulon-4 in ALS pathology (Teng and Tang, 2008), demonstrating a protective effect against ALS-like neurodegeneration (Yang et al., 2009).

Several trophic factors and their receptors were differentially expressed in SALS cortex. Among them are all the epidermal growth factor receptors and some of their ligands. Decreased levels of EGF were previously observed in liquor of ALS patients (Cieslak et al., 1986; Klimek et al., 1990) and treatment with this growth factor has been successfully evaluated in ALS animal models (Del Barco et al., 2011; Ohta et al., 2006). Consistent with our observations are reports showing decreased levels of Neuregulin in cerebral spinal fluid of ALS patients (Pankonin et al., 2009) and aberrant Neuregulin signaling in ALS patients and in SOD1 mice (Song et al., 2012). In SALS cortex samples we also observed the altered expression of genes encoding three fibroblast growth factor receptors and seven of their ligands. Increased FGF1 might activate astrocytes, which could in turn initiate motor neuron apoptosis in ALS (Cassina et al., 2005). Reduced expression of FGF9 is consistent with its role as an autocrine or paracrine survival factor for motoneurons (Kanda et al., 1999). Altered expression of genes encoding one member of the neurotrophin family of growth factors (BDNF) and their receptors (TrkA, TrkB, TrkC, NGFR) were observed in SALS patients. These data are in agreement with an extensive amount of data supporting their implication in human (Koliatsos et al., 1993; Seeburger and Springer, 1993) and animal models (Liao et al., 2012; Yanpallewar et al., 2012) of ALS pathology. The expression of vascular endothelial growth factors (VEGF-A, VEGF-B, VEGF-C, VEGF-D) and their receptors (VEGF-R1, VEGF-R2, Neuropilin 1, Neuropilin 2) was mainly down-regulated in SALS2 patients. There is extensive evidence linking this family of ligands and receptors to ALS pathology (Brockington et al., 2006; Lambrechts et al., 2003; Lunn et al., 2009; Nagara et al., 2013; Spliet et al., 2004) and treatment with VEGF was found to prevent neuronal death in different models of ALS (Hwang et al., 2009; Shimazawa et al., 2010; Storkebaum et al., 2005; Tovar-y-Romo and Tapia, 2012; Wang et al., 2007; Zheng et al., 2004).

Down regulation of hepatocyte growth factor (HGF), one of the most potent survival-promoting factors for motor neurons with potential therapeutic effects on ALS (Ishigaki et al., 2007; Kadoyama et al., 2007; Sun et al., 2002) was observed in SALS2. Decreased expression of Thyrotropin-releasing hormone receptor in SALS2 patients supports previous studies showing a reduction of this receptor in spinal cord of ALS patients (Manaker et al., 1988) and may explain the conflicting results obtained by TRH therapy (Brooks, 1989; Congia et al., 1991).

The expression of several genes encoding G-protein coupled receptors and/or their ligands is altered in SALS patients. Some of these, such as the Muscarinic acetylcholine receptors (Gillberg and Aquilonius, 1985; Whitehouse et al., 1983), the endothelin-1 and its receptor B, have been previously associated to ALS pathology (Lederer et al., 2007). Differential expression of Angiotensin II and its type-1 receptor may be linked to the altered levels of Angiotensin II found in liquor of ALS patients (Kawajiri et al., 2009), whereas that of Leukemia inhibitory
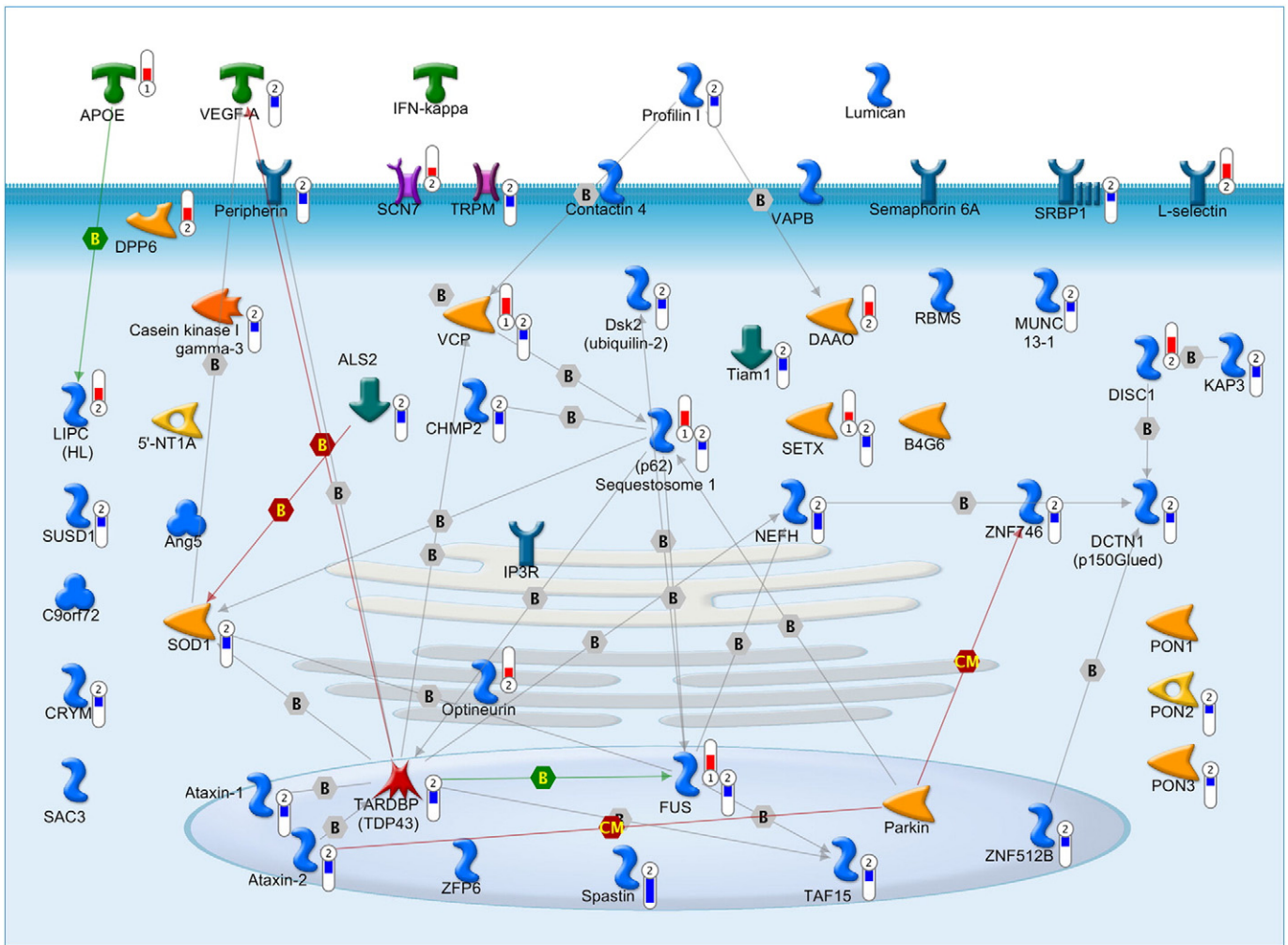
Fig. 10. Genes previously associated to FALS. To analyze changes in gene expression in the context of genes previously associated to FALS, we built up a pathway with 53 of these genes. A majority of these genes (37/53) were significantly deregulated in the cortex of SALS patients. Pathway objects and links are described separately in the Supplementary Fig. 3.

factor (LIF) and its receptor are consistent with a study proposing LIF as a modifier gene in ALS (Giess et al., 2000). The up regulation of IGF1 receptor is in agreement with previous studies (Chung et al., 2003; Wilczak et al., 2003), whereas that of purinergic receptor P2Y2 supports the role for P2 receptor signaling in ALS (D'Ambrosi et al., 2009).

Ion homeostasis

A number of genes encoding proteins regulation ion homeostasis were deregulated in motor cortex of SALS patients (Fig. 9). Decreased expression of CACNA1C is consistent with the presence of immunoglobulins against this L-type voltage-gated calcium channels in ALS patients, which correlate with disease progression and exert neurototoxicity (Kimura et al., 1994; Smith et al., 1994). Down regulation of three subunits of the N-methyl-D-aspartate (NMDA) receptor (GRIN1, GRIN2A, GRIN2D) in SALS2 patients is in agreement with previous studies in animal models (Spalloni et al., 2011) and with a large literature indicating that a dysfunction of these ligand-gated cation channels may be an underlying molecular mechanism in ALS (Spalloni et al., 2013). Three subunits of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) glutamate receptor were differentially expressed in SALS patients: GRIA1 increased in cluster 1 patients, whereas GRIA2 and GRIA3 were reduced in SALS2. Similar changes in expression of GRIA1 and GRIA2 have been reported in SOD1 mice (Spalloni et al., 2006), whereas in human a defect in the editing of the messenger RNA encoding GRIA2 has been previously reported (Kawahara et al., 2004). AMPA receptors lacking the GRIA2 subunit are permeable to Ca^{2+} and

the entrance of this cation might be responsible for the selective vulnerability of spinal motoneurons in ALS. Down regulation of six subunits of the gamma-aminobutyric acid (GABA) A receptor were found in SALS2 patients. Although impaired GABAergic signaling in ALS pathology has been previously proposed, little is known about its receptor composition. The few studies present in the literature, confirm the reduced expression of the alpha1 subunit in ALS patients (Petri et al., 2003).

Genes previously linked to ALS

As described in the introduction, a large number of genes have been associated to FALS and available in online databases, such as OMIM, ALSGene (Lill et al., 2011), ALS mutation database (Yoshida et al., 2010), and ALSoD (Abel et al., 2012). To analyze changes in gene expression in the context of these genes, we built up a pathway with 53 of these genes (Fig. 10). A majority of these genes (37/53) were differentially expressed in the cortex of SALS patients (some are up-regulated in SALS1, whereas most are down-regulated in SALS2). Deregulation of genes whose mutation has been previously associated to FALS provides a common pathogenic link between familiar and sporadic ALS that has not been previously appreciated.

Conclusion

ALS is a rare neurodegenerative disease affecting motor neurons. Although postmortem brain tissue reveals the end-stage pathogenic

mechanisms of this disorder, its use provides indispensable elements that cannot be obtained by other approaches or on a living person. In the last few years, a number of transcriptome studies have started to decipher genes and pathways involved in ALS pathogenesis but were restricted to a limited number of postmortem samples (≤11 motor cortex, and 14 spinal cord) (Cox et al., 2010; Dangond et al., 2004; Jiang et al., 2005; Lederer et al., 2007; Malaspina et al., 2001; Offen et al., 2009; Rabin et al., 2010; Wang et al., 2006). To uncover the entire spectrum of genes and pathways involved in ALS pathology we have now screened a large number of well-characterized (clinically and neuropathologically) motor cortex specimens from control and SALS patients. In addition to confirming the differential expression of 83% of those genes (Supplementary Table 7) that were reported in our previous study (Lederer et al., 2007), the results obtained in a larger cohort show that gene expression profiles of motor cortex samples can differentiate SALS pathology from controls and clearly distinguish two SALS groups, each associated to deregulation of different genes and pathways. Although the present study represents the largest and most comprehensive transcriptome study of SALS brains to date, the sample size lacked sufficient power to associate clinical characteristics of patients with cluster assignment. While this functional association will require a much larger sample size, the present study proposes the use of unsupervised hierarchical clustering as an objective method to identify different SALS subtypes and reveal new clues to the pathogenesis and potential therapeutic targets. Molecular classification based on gene expression is revolutionizing the way different pathologies, such as cancer, are diagnosed and opening the way to personalized therapies. Similarly, a molecular taxonomy of SALS patients may reveal etio-pathogenic mechanisms that may have been masked by considering SALS pathology as a single entity and help orienting them to personalized treatments.

The altered networks of biological molecules in SALS provide a number of potential therapeutic targets (Supplementary Table 8), which could be used to interfere with ALS pathogenesis. In view of the genomic heterogeneity of sporadic ALS, we may rethink our strategies for drug development, targeting ALS pathogenesis with personalized drugs and as a system rather than at the level of the single protein molecule.

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Author contributions

Conflict of interest

The author declares no competing financial interests.

Acknowledgments

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