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(54) Title: NEUROTHERAPEUTIC AGENTS

(57) Abstract: The invention relates to diseases, disorders and conditions of the brain, and agents for treatment of the same.



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Neurotherapeutic Agents

Field of the Invention

The present invention relates to diseases, disorders and conditions of the brain and particularly, although not exclusively, to the treatment and prevention of
5 neuroinflammation, especially epilepsy. The invention extends to agents that inhibit a particular gene implicated in the promotion of behavioural seizures.

Background of the Invention

10 Epilepsy is a serious neurological disorder affecting about 1% of the world's population. Recently, a growing body of experimental and clinical data has implicated Toll-like receptor (TLR) signaling¹ and release of proconvulsant inflammatory molecules (i.e., IL-1 β) in both seizure generation and epileptogenesis^{2,3}. However, the pathogenetic mechanisms linking these inflammatory processes with the development (and
15 recurrence) of epileptic seizures in humans are unclear. Despite high heritability of epilepsy⁴⁻⁶, both genome-wide association studies (GWAS) and exome sequencing approaches have so far provided limited insights into the genetic regulatory mechanisms underlying inflammatory pathways in epilepsy etiology⁷⁻¹¹, and traditional single-variant association approaches are likely to be underpowered to detect complex
20 gene network interactions that underlie disease susceptibility¹².

As the molecular processes driving complex disease usually affect sets of genes acting in concert, the inventors' have used systems-level approaches to investigate transcriptional networks and pathways within pathologically relevant cells and
25 tissues^{13,14}. Integrated analysis of transcriptional networks with genetic susceptibility data and phenotypic information allows specific transcriptional programmes to be connected to disease states, and thereby can identify disease pathways and their genetic regulators as new targets for therapeutic intervention¹⁵.

30 Almost uniquely among disorders of the human brain, epilepsy surgery offers opportunities for gene expression profiling in ante-mortem brain tissue from pathophysiologically relevant brain structures such as the hippocampus¹⁶. This allows direct investigation of transcriptional programmes in brain tissue from living epilepsy patients.

35

Summary of the Invention

The inventors have now integrated unsupervised network analysis of global gene expression in the hippocampi of patients with temporal lobe epilepsy (TLE) with GWAS data in a systems-genetics approach¹⁷. They have uncovered pathways and
5 transcriptional programmes associated with epilepsy that are conserved in mouse epileptic hippocampus, including a proconvulsant gene network encoding IL-1 β ³ and TLR-signaling genes¹ previously implicated in epilepsy. Using genome-wide Bayesian expression QTL mapping¹⁸ the inventors have probed the genome for key genetic regulators of the network in human brain. They have pinpointed an unexpected gene,
10 *Sestrin 3* (*SESN3*) whose protein product controls the intracellular response to reactive oxygen species¹⁹⁻²², as a *trans*-acting genetic regulator of the proconvulsant gene network in human epileptic hippocampus. The inventors have carried out validation experiments in independent *in vitro* and *in vivo* systems, which have confirmed the genetic regulation of the proconvulsant transcriptional program in epilepsy by *Sestrin*
15 *3*, therefore providing a first evidence of a function for *SESN3* gene in disorders of the human brain.

Detailed Description

The invention described herein is based upon the inventors' surprising discovery, in
20 surgically acquired hippocampi from TLE patients, of a specialised, highly expressed transcriptional module encoding proconvulsive cytokines and TLR-signaling genes. RNA-Seq analysis in a mouse model of TLE using epileptic and control hippocampi showed that the proconvulsive module is preserved across-species, specific to the epileptic hippocampus and upregulated in chronic epilepsy. In the TLE patients, the
25 inventors have mapped the *trans*-acting genetic control of this proconvulsive module to *SESN3*, and have demonstrated that *SESN3* positively regulates the module in macrophages, microglia and neurons. Morpholino-mediated *sesn3* knockdown in zebrafish confirmed the regulation of the transcriptional module, and attenuated chemically-induced behavioural seizures *in vivo*.

30 As explained further herein, *SESN3* positively regulates a proconvulsive gene co-expression module (Module 1 as described herein) and so inhibiting, reducing, knocking down or knocking out *SESN3* gene expression or protein activity can ameliorate seizures. This means that an inhibitor of *SESN3* gene expression or *SESN3*
35 protein activity can hence be used in therapy to treat neuroinflammation, and diseases, disorders and conditions in which neuroinflammation plays a part, such as epilepsy.

Both the *SESN3* gene and its protein product are known in the art, and their nucleic acid and amino acid sequences are thus publically available.

- 5 Accordingly, in a first aspect, the invention provides an inhibitor of *SESN3* gene expression for use in therapy.

In an embodiment, the inhibitor of *SESN3* gene expression is for use in a method of treating a disease, disorder or condition of the brain. In a preferred embodiment, the
10 inhibitor is for use in a method of treating neuroinflammation, or a disease, disorder or condition involving neuroinflammation. In a very preferred embodiment, the inhibitor is for use in a method of treating epilepsy.

In a second aspect, the invention provides an inhibitor of *SESN3* protein activity for use
15 in therapy.

In an embodiment, the inhibitor of *SESN3* protein activity is for use in a method of treating a disease, disorder or condition of the brain. In a preferred embodiment, the inhibitor of *SESN3* protein activity is for use in a method of treating
20 neuroinflammation, or a disease, disorder or condition involving neuroinflammation. In a very preferred embodiment, the inhibitor of *SESN3* protein activity is for use in a method of treating epilepsy.

The inhibitor may be any agent capable of inhibiting *SESN3* gene expression or *SESN3*
25 protein activity. The agent may be a competitive or non-competitive antagonist of *SESN3*. The agent may be a biological agent, such as a protein or a nucleic acid, such as siRNA, or it may be a pharmaceutical agent.

“Inhibiting” can mean reducing the normal level of *SESN3* gene expression or *SESN3*
30 protein activity by any amount. For example, the inhibitor may be an agent capable of reducing the level of *SESN3* gene expression or protein activity by up to 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100%.

“Treating” includes both preventing and ameliorating a disease, disorder or condition.
35 Methods of prophylaxis and any and all methods that treat, reduce or help alleviate

symptoms associated with said disease, disorder or condition are therefore expressly included.

5 The inhibitor may be administered to the subject to be treated on its own. It may be administered in a pharmaceutically acceptable vehicle. The inhibitor according to the invention may be combined in compositions having a number of different forms depending, in particular, on the manner in which the composition is to be used. Thus, for example, the composition may be in the form of a powder, tablet, capsule, liquid, ointment, cream, gel, hydrogel, aerosol, spray, micellar solution, transdermal patch,
10 liposome suspension or any other suitable form that may be administered to a person or animal in need of treatment. It will be appreciated that the vehicle of medicaments according to the invention should be one which is well-tolerated by the subject to whom it is given, and preferably enables delivery of the agents across the blood-brain barrier.

15 Medicaments comprising agents of the invention may be used in a number of ways. For instance, oral administration may be required, in which case the agents may be contained within a composition that may, for example, be ingested orally in the form of a tablet, capsule or liquid. Compositions comprising agents of the invention may be administered by inhalation (e.g. intranasally). Compositions may also be formulated
20 for topical use. For instance, creams or ointments may be applied to the skin.

Agents according to the invention may also be incorporated within a slow- or delayed-release device. Such devices may, for example, be inserted on or under the skin, and the medicament may be released over weeks or even months. The device may be
25 located at least adjacent the treatment site. Such devices may be particularly advantageous when long-term treatment with agents used according to the invention is required and which would normally require frequent administration (e.g. at least daily injection).

30 In a preferred embodiment, agents and medicaments according to the invention may be administered to a subject by injection into the blood stream or directly into a site requiring treatment. Injections may be intravenous (bolus or infusion) or subcutaneous (bolus or infusion), or intradermal (bolus or infusion).

35 It will be appreciated that the amount of the agent or medicament that is required is determined by its biological activity and bioavailability, which in turn depends on the

mode of administration, the physiochemical properties of the agent, vaccine and medicament, and whether it is being used as a monotherapy or in a combined therapy.

The frequency of administration will also be influenced by the half-life of the agent within the subject being treated. Optimal dosages to be administered may be

5 determined by those skilled in the art, and will vary with the particular agent in use, the strength of the pharmaceutical composition, the mode of administration, and the advancement of the disease, disorder or condition. Additional factors depending on the particular subject being treated will result in a need to adjust dosages, including subject age, weight, gender, diet, and time of administration.

10

Generally, a daily dose of between 0.001 µg/kg of body weight and 10 mg/kg of body weight of agent or medicament according to the invention may be used for treating a disease, disorder or condition of the brain, depending upon which agent or medicament is used. More preferably, the daily dose is between 0.01 µg/kg of body weight and 1

15 mg/kg of body weight, more preferably between 0.1 µg/kg and 100 µg/kg body weight, and most preferably between approximately 0.1 µg/kg and 10 µg/kg body weight.

The agent or medicament may be administered before, during or after onset of the disease, disorder or condition of the brain. Daily doses may be given as a single

20 administration (e.g. a single daily injection). Alternatively, the agent or medicament may require administration twice or more times during a day. As an example, agents and medicaments may be administered as two (or more depending upon the severity of the disease, disorder or condition being treated) daily doses of between 0.07 µg and 700 mg (i.e. assuming a body weight of 70 kg). A patient receiving treatment may take

25 a first dose upon waking and then a second dose in the evening (if on a two dose regime) or at 3- or 4-hourly intervals thereafter. Alternatively, a slow release device may be used to provide optimal doses of agents, vaccines and medicaments according to the invention to a patient without the need to administer repeated doses. Known procedures, such as those conventionally employed by the pharmaceutical industry
30 (e.g. *in vivo* experimentation, clinical trials, etc.), may be used to form specific formulations of the agents and medicaments according to the invention and precise therapeutic regimes (such as daily doses of the agents and the frequency of administration).

A “disease, disorder or condition of the brain” includes any and all diseases, disorders and conditions that affect the brain. In a preferred embodiment, the disease, disorder or condition involves neuroinflammation. In a very preferred embodiment, the disease, disorder or condition is epilepsy.

5

A “subject” may be a vertebrate, mammal, or domestic animal. Hence, medicaments according to the invention may be used to treat any mammal, for example livestock (e.g. a horse), pets, or may be used in other veterinary applications. Most preferably, the subject is a human being.

10

A “therapeutically effective amount” of agent is any amount which, when administered to a subject, is the amount of drug that is needed to treat the disease, disorder or condition, or produce the desired effect. For example, the therapeutically effective amount of agent used may be from about 0.001 ng to about 1 mg, and preferably from about 0.01 ng to about 100 ng. It is preferred that the amount of agent is an amount from about 0.1 ng to about 10 ng, and most preferably from about 0.5 ng to about 5 ng.

15

A “pharmaceutically acceptable vehicle” as referred to herein, is any known compound or combination of known compounds that are known to those skilled in the art to be useful in formulating pharmaceutical compositions.

20

In one embodiment, the pharmaceutically acceptable vehicle may be a solid, and the composition may be in the form of a powder or tablet. A solid pharmaceutically acceptable vehicle may include one or more substances which may also act as
25 flavouring agents, lubricants, solubilisers, suspending agents, dyes, fillers, glidants, compression aids, inert binders, sweeteners, preservatives, dyes, coatings, or tablet-disintegrating agents. The vehicle may also be an encapsulating material. In powders, the vehicle is a finely divided solid that is in admixture with the finely divided active agents according to the invention. In tablets, the active agent may be mixed with a
30 vehicle having the necessary compression properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active agents. Suitable solid vehicles include, for example calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, polyvinylpyrrolidone, low melting waxes and ion exchange resins. In another
35 embodiment, the pharmaceutical vehicle may be a gel and the composition may be in the form of a cream or the like.

However, the pharmaceutical vehicle may be a liquid, and the pharmaceutical composition is in the form of a solution. Liquid vehicles are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The active agent according to the invention may be dissolved or suspended in a pharmaceutically acceptable liquid vehicle such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid vehicle can contain other suitable pharmaceutical additives such as solubilisers, emulsifiers, buffers, preservatives, sweeteners, flavouring agents, suspending agents, thickening agents, colours, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid vehicles for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the vehicle can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid vehicles are useful in sterile liquid form compositions for parenteral administration. The liquid vehicle for pressurized compositions can be a halogenated hydrocarbon or other pharmaceutically acceptable propellant.

Liquid pharmaceutical compositions, which are sterile solutions or suspensions, can be utilized by, for example, intramuscular, intrathecal, epidural, intraperitoneal, intravenous and particularly subcutaneous injection. The agent may be prepared as a sterile solid composition that may be dissolved or suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium.

The agents and compositions of the invention may be administered orally in the form of a sterile solution or suspension containing other solutes or suspending agents (for example, enough saline or glucose to make the solution isotonic), bile salts, acacia, gelatin, sorbitan monooleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the like. The agents used according to the invention can also be administered orally either in liquid or solid composition form. Compositions suitable for oral administration include solid forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups,

elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

5 It will be appreciated that administration, into a subject to be treated, of an agent or medicament according to the invention will result in the inhibition of *SESN3* gene expression or *SESN3* protein activity, and that this inhibition will aid in treating a disease, disorder or condition of the brain, such as neuroinflammation, or a disease, disorder or condition involving neuroinflammation, and preferably epilepsy.

10 In a third aspect, the invention provides a method of treating a disease, disorder or condition of the brain in a subject comprising administering an inhibitor of *SESN3* gene expression to the subject.

15 In a fourth aspect, the invention provides a method of treating a disease, disorder or condition of the brain in a subject comprising administering an inhibitor of *SESN3* protein activity to the subject.

20 In these aspects of the invention, the disease, disorder or condition of the brain, the subject, and the inhibitor may be as defined for the first and second aspects of the invention.

All of the features described herein (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined with any of the above aspects in any combination, except combinations
25 where at least some of such features and/or steps are mutually exclusive.

For a better understanding of the invention, and to show how embodiments of the same may be carried into effect, reference will now be made, by way of example, to the accompanying diagrammatic drawings, in which:

30 Figure 1 shows an identification of the TLE-network and functionally specialised transcriptional modules in human epileptic hippocampus. (a) Gene co-expression network identified in the hippocampus of TLE patients (TLE-network). Nodes represent genes and edges represent partial correlations between their expression
35 profiles (FDR<5%). Node colour indicates the best GWAS *P*-value of association with focal epilepsy for SNPs within 100kb of each gene (Supplementary Table 2). Boxes

mark two transcriptional modules within the network. (b) KEGG pathways significantly enriched in the TLE-network (FDR<5%). The fold enrichment for each KEGG pathway is reported on the side of each bar. (c) Module-1 and Module-2 details. The size of each node is proportional to its degree of inter-connectivity within each module. Light blue indicates genes showing nominal association with susceptibility to focal epilepsy. Numbers in parenthesis indicate multiple microarray probes representing the same gene. (d) KEGG pathways significantly enriched in Module-1 (top) and Module-2 (bottom) (FDR<5%). (e) Module-1 is significantly highly expressed in the hippocampus of TLE patients. mRNA expression of Module-1 (n=80 probes, representing 69 unique annotated genes) as compared with Module-2 (n=60 probes, representing 54 unique annotated genes), other network genes (n=371 probes, representing 319 unique annotated genes) and all other probes represented on the microarray (n=48,256). ** $P=3.8 \times 10^{-4}$; *** $P < 10^{-10}$, Mann-Whitney test, two-tailed.

Figure 2 shows TLE-network conservation in mouse epileptic hippocampus. (a) Human TLE-network genes that are conserved and co-expressed (84%) in the mouse hippocampus. Each node in the network represents a transcript that had partial correlation with at least another transcript in the network (5% FDR). Conserved Module-1 and Module-2 genes are indicated in blue and green, respectively. (b) Distribution of partial correlations (FDR<5%) between pairs of transcripts from 10,000 bootstrap permutation samples in epileptic (top) and control (bottom) mouse hippocampus. In each case, the red line indicates the actual number of significant partial correlations (FDR<5%) between all genes in the network. The number of significant partial correlations observed in control hippocampus was no different from chance expectation ($P=0.659$). In contrast, the number of significant partial correlations detected in epileptic hippocampus was significantly higher than expected by chance ($P=0.001$). (c) Differential expression of Module-1 genes between control and epileptic mouse hippocampus shows specific enrichment for TLR-signaling and cytokine genes amongst the upregulated genes. Stars denote significant fold changes between epileptic and control mouse hippocampus (FDR<5%); blue bars indicate TLR-signaling and cytokine genes.

Figure 3 shows that *SESN3* is a trans-acting genetic regulator of Module-1 in epileptic hippocampus. (a) Genome-wide mapping of genetic regulation of Module-1. For each autosome (horizontal axis), the strength of evidence for each SNP (filled dot) being a regulatory locus for the first PC of Module-1 expression is measured by the $\log_{10}(\text{Bayes})$

Factor) (vertical axis). The Bayes Factor quantifies evidence in favor of genetic regulation versus no genetic control of module expression, and is reported as a ratio between the strengths of these models. At 5% FDR (i.e., $\log_{10}(\text{Bayes Factor}) > 6$, dashed line), SNP rs10501829 (11q21, highlighted in red) was significantly correlated with
5 Module-1 expression. (b) Joint mRNA levels-SNPs analysis within the 1Mbp region centered on SNP rs10501829, comprising 178 SNPs genotyped in the TLE-patient cohort. The inventors carried out multivariate Bayesian regression modeling¹⁸ of all Module-1 probes (n=89) and all SNPs (n=178) to identify the most informative SNPs in the region predicting Module-1 expression. For each SNP the inventors report the
10 proportion of associated genes in Module-1 (vertical axes): four SNPs (rs10501829, rs530190, rs7107661 and rs6483435) that are individually associated with 58% to 74% of Module-1 genes are highlighted. The grey box indicates the boundaries of the associated regulatory region (delimited by SNPs rs530190 and rs6483435), spanning 383kb. (c) For each candidate gene at the 1Mbp regulatory locus the inventors report
15 the average Pearson correlation (r ; $\pm$ s.e.m.) with the expression of Module-1 genes (y-axes) and its statistical significance for deviation from $r=0$ (x-axes). Two-tailed P -values are reported on a negative log scale and were calculated using one sample Wilcoxon Signed Rank test. Two genes (*ENDOD1* and *MTMR2*) were represented by two microarray probes and were analysed separately. (d) Association between
20 increased *Sesn3* mRNA expression and upregulation of Module-1 genes in epileptic mouse hippocampus. For each gene the inventors report its $\log_{10}(\text{fold change})$ in epilepsy *vs* control (y-axes) and its correlation with *Sesn3* mRNA expression (x-axes). The 95% confidence interval of the slope of the regression line is indicated. TLR-signaling and cytokines genes are highlighted in blue.

25

Figure 4 shows that *SESN3* regulates expression of Module-1 genes in macrophages, microglial cells and neurons. Effect of siRNA-mediated knockdown of *Sesn3* as compared to control siRNA (siControl), showing significant inhibition of *Sesn3* mRNA expression and downregulation of Module-1 genes in murine LPS-stimulated (1hr)
30 BMDM (a) and BV2 microglial cells (b), as well as in unstimulated BV2 microglial cells (c). Five independent biological replicates were used for BMDM experiments and at least three replicates in the BV2 microglia cells experiments. Data normalised to β -*actin* levels are shown as means relative to control $\pm$ s.e.m. (d) *SESN3* immunofluorescence of human hippocampal slices from TLE patients: co-
35 immunostainings with NeuN (green) antibody showed that *SESN3* (red) is localised in neurons. Scale bar=100 μ m. (e) Quantification of *SESN3* expression in human

hippocampal tissue by immunofluorescence analysis. Maximum intensity projections of confocal z-stack images of immunohistochemical stainings with antibody against SESN3 were used. For determination of SESN3 cell fluorescence as a measure of SESN3 expression level, SESN3 expressing cells in the CA2 region of the hippocampus in both TLE patients samples (n=7) and autopsy samples (n=8) were measured using ImageJ software. Cell fluorescence was assessed as follows: integrated density – (area of selected cell x mean fluorescence of background readings). SESN3 total cell fluorescence in TLE patients is significantly increased as compared to the SESN3 total cell fluorescence in autopsy samples (two-tailed Mann-Whitney test $P < 0.001$).

Fluorescence intensity data are reported as means $\pm$ s.e.m. (f) Effect of lentiviral-mediated *Sesn3* overexpression on Module-1 genes in primary hippocampal neurons. *Left*, relative levels of *Sesn3* mRNA in transduced neurons (LV-CMV-*Sesn3*) compared with the levels in mock transduction (Mock). *Right*, relative mRNA levels of Module-1 genes and a control gene not in the network (*Hprt*) in transduced neurons compared with levels in mock transduction. Data normalised to *Gapdh* levels are shown as means relative to control $\pm$ s.e.m. Four (Mock group) and twelve (LV-CMV-*Sesn3* group) replicates were used in neuronal cell experiments. Statistical significance of the differences (*P-value*) between siSESN3 (or LV-CMV-*Sesn3*) and siControl (or Mock) was assessed by *t*-test (2-tailed) and adjusting for unequal variances across different groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant ($P > 0.05$).

Figure 5 shows that *Sesn3* modulates PTZ-induced *c-fos* expression, locomotor convulsions and Module-1 genes in zebrafish. (a) *Left*, *sesn3* promotes convulsive locomotor response of zebrafish larvae exposed to PTZ. Three dpf zebrafish larvae were incubated with and without 20mM PTZ for 1-hr, during which locomotor activity was monitored continuously. Larvae microinjected with *sesn3* morpholinos exhibited a sustained reduction in locomotor activity throughout the period of incubation with PTZ, in comparison with control morphant larvae. Both *sesn3* morphant and control morphant larvae (n=12) exhibited similarly low levels of locomotor activity in the absence of PTZ. *Right*, *sesn3* morpholinos reduced the cumulative locomotor activity of zebrafish exposed to 20mM PTZ (black columns) without appreciably affecting basal locomotor activity of larvae incubated in the absence of PTZ (white columns). (b) Co-injecting *sesn3* morpholinos with synthetic *sesn3* mRNA showed that *sesn3* mRNA rescued the locomotor activity phenotype (total distance swam, y-axis). For each group, 16-18 larvae were analysed. Black bars, 1-hr PTZ treatment (20mM). (c) *Sesn3* morpholinos attenuate seizure-induced expression of the synaptic activity-regulated

gene *c-fos*. Left and central images, dorsal views of the brains of 3 dpf control morphant (top) and *sesn3* morphant larvae (bottom) maintained for 1-hr in the absence and presence of 20mM PTZ, after which larvae were fixed and analysed for *c-fos* expression by whole mount *in situ* hybridisation. Red arrowheads, position of the transverse sections of the brains. Following PTZ treatment (20mM, 1-hr), qPCR analysis revealed that *sesn3* morphant larvae exhibited significantly lower mRNA expression of *c-fos* in the brain than control morphant larvae (c, right panel). (d) PTZ-induced transcriptional response of Module-1 genes was significantly lower in *sesn3* morphants as compared with uninjected larvae; six samples were used in the qPCR experiments (one sample=15-20 pooled larvae). (e) Up-regulation of Module-1 genes upon injection of synthetic mRNA (1ng) in zebrafish embryos (n=30) as compared with uninjected control embryos (n=30). Total RNA was extracted 28 hours post fertilisation and qPCR experiments were performed for the 2 pools of embryos using 6 technical replicates. Data reported as means $\pm$ s.e.m. were determined by the $2^{-\Delta\Delta Ct}$ method and normalised to the housekeeping gene *beta-actin*. *P*-values calculated by *t*-test (2-tailed) adjusting for unequal variances across different groups. ns, not significant ($P>0.05$).

Examples

The materials and methods employed in the studies described in the Examples were as follows, unless where otherwise indicated:

Gene expression profiling in the human hippocampus

All 129 patients considered in this study had mesial TLE and all tissue samples were from indistinguishable hippocampal tissue portions. Sample preparation and microarray analysis of human hippocampi are detailed in Supplementary Methods. Expression data were analysed using Illumina's GenomeStudio Gene Expression Module and normalised by quantile normalisation with background subtraction. Microarray probes were annotated using either the Human HT-12 v3 annotation file or Ensembl (release 72). All patients gave informed consent for use of their tissue and all procedures were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Bonn Medical Center.

Gene co-expression network analysis

Gene co-expression networks were reconstructed using GGMs, which use partial correlations to infer co-expression relationships between any microarray probe pair in

the dataset, removing the effect of other probes²³. The inventors used the empirical Bayes local FDR statistic⁵⁴ to extract significant partial correlations (Supplementary Fig. 13), and which identified a large set of 2,124 inter-connected nodes belonging to the same connected component (TLE-network, Supplementary Data 2). Network
5 extraction and identification of transcriptional modules are described in the Supplementary Methods.

Mapping the genetic control of networks

The inventors used Bayesian variable selection models^{18,29} to identify the genetic
10 control points (regulatory ‘hotspots’) of transcriptional modules in the TLE patient cohort. First, the inventors combined PC analysis⁵⁵ with multivariate regression approaches to prioritise genome-wide genomic regions associated with the module expression. The inventors then analysed all genes of the module with all SNPs in the regulatory region using the hierarchical evolutionary stochastic search (HESS)
15 algorithm¹⁸, where the module genes’ expression are jointly considered. Further details are reported in Supplementary Methods.

Genetic association of the TLE-network with epilepsy

To test if TLE-network genes are likely to be causally involved in the disease process,
20 the inventors assessed whether network genes are enriched for SNP variants associated to focal epilepsy by GWAS^{6,25}. Full details on the GWAS of focal epilepsy are reported in Supplementary Methods. Briefly, in the GWAS-enrichment analysis each gene of the TLE-network was assigned a GWAS significance value consisting of the smallest *P*-value of all SNPs mapped to it. The inventors used the hypergeometric distribution test
25 to assess whether SNPs close to (<100 kb from) any network gene were more likely to associate with epilepsy by GWAS than SNPs close to genes not in the TLE-network. Empirical GWAS-enrichment *P*-values were generated by 1,000,000 randomly selected gene-sets and are reported in Supplementary Table 2 (see Supplementary Methods for additional details).

30

RNA-Seq analysis in the mouse hippocampus

RNA-Seq analysis in whole hippocampus from 100 epileptic (pilocarpine model)²⁷ and 100 control naïve mice (NMRI) is detailed in Supplementary Methods. Briefly, raw reads were mapped to the reference mouse genome (mm 10) using TopHat version
35 2.0.8⁵⁶ and read counts per gene were normalised across all samples using the ‘trimmed mean of M-value’ (TMM) approach⁵⁷. Differential expression analysis was

performed using the edgeR⁵⁷ and a threshold of 5% FDR was used to identify significant gene expression changes. Experimental animals were used only once for each study. All experimental procedures complied with the guidelines of the European Union Directive 2010/63/EU. A local ethical committee approved the experimental protocol.

5

In vitro studies

Details on all cell cultures used in these studies are reported in Supplementary Methods. SiRNA knockdown experiments were performed in murine BMDMs and BV2 microglia cell lines using a mouse *Sesn3* ON-TARGETplus SMARTpool siRNA (100 nM, 10 ThermoFisher Scientific) and Dharmafect 1 (ThermoFisher Scientific) as transfection reagent, according to the manufacturer's recommendations. For LPS stimulation experiments, the transfected cells were washed twice in DMEM and stimulated with LPS (Sigma, 100 ng/ml) for an hour. For overexpression experiments, a third-generation Lentiviral Vectors (LV) was used to transduce murine primary hippocampal 15 neuronal culture. Additional information and details on *Sesn3* siRNA target sequences, the real-time quantitative PCR for Module-1 genes and primer sequences are given in Supplementary Methods and Supplementary Table 4. The relative expression levels normalised to *Beta-actin* (or *Gapdh* as indicated) gene expression were then determined by the $2^{-\Delta\Delta Ct}$ method.

20

In vivo studies

To study the function of sestring3 in response to PTZ-induced seizures, two different morpholinos were designed to block the normal splicing of the zebrafish *sesn3* primary transcript (see Supplementary Fig. 12 and Supplementary Methods). Embryos that 25 were to be analysed by whole-mount *in situ* hybridisation were first treated with 1-phenyl-2 thiourea (PTU) at 23 h post-fertilisation (h.p.f.) to inhibit melanogenesis. At 3 days post-fertilisation (d.p.f.), larvae were treated for 1 h with 20 mM PTZ or left untreated, and all larvae were then fixed with paraformaldehyde immediately after the treatment period. RNA *in situ* hybridisation analysis was carried out using a *c-fos* 30 digoxigenin-labelled probe, which was prepared as recommended by the manufacturer of the *in situ* hybridisation reagents (Roche). Whole-mount *in situ* hybridisation was performed using standard procedures⁵⁸. Analysis of zebrafish locomotor activity was carried out using the Viewpoint Zebrabox system (Viewpoint) as previously reported³⁴. Rescue experiments were performed by co-injection of synthetic *sesn3* RNA into one- 35 cell stage AB wild type zebrafish embryos alone (2 nl of 0.3 ng/nl *sesn3* mRNA) or in combination with *sesn3* morpholinos. Additional details, including the quantitative

PCR analyses of *c-fos* and Module-1 genes, primer sequences are reported in Supplementary Methods. All experimental procedures involving zebrafish were performed in compliance with the UK Animal (Scientific Procedures) Act and approved by the University of Sheffield Animal Welfare Ethical Review Board.

5

Example 1 – Identification of a gene network associated with epilepsy

The inventors first assessed the degree of variation in gene expression between hippocampal subfields in TLE patients with hippocampal sclerosis (HS), and compared this with the total variation in gene expression measured both across subjects and
10 between subfields (Supplementary Fig. 1). The inventors found higher variability in gene expression across TLE subjects than between the hippocampal subfields alone, suggesting that variation in whole hippocampus expression can be used to infer co-expression networks in the hippocampus of TLE patients (Supplementary Fig. 1). After excluding subjects with incomplete clinical data or non-HS pathology, whole-genome
15 expression profiles in surgically resected hippocampi from 129 TLE patients (median age at surgery 35 years, range 1-64 years, male:female ratio of 1.2:1; Supplementary Table 1) were available for gene co-expression network analysis.

The inventors then investigated whether the transcriptome in the hippocampus of these
20 129 TLE patients was organised into discrete gene co-expression networks, and if these had functional implications for susceptibility to epilepsy. Gene co-expression networks were reconstructed using Graphical Gaussian Models (GGMs)²³, which identified a large co-expression network comprising 442 annotated genes (false discovery rate (FDR)<5%, Fig. 1a and Supplementary Data 1). To investigate whether the protein
25 products of the TLE-hippocampus derived transcriptional network (TLE-network) genes had a shared function at the protein level, the inventors used the DAPPLE algorithm²⁴. This method interrogated high-confidence protein-protein interactions (PPI) to assess the physical connections among proteins encoded by the genes in the network. Genes comprising the TLE-network were found to have increased protein-
30 protein interconnectivity as compared with random PPI networks ($P = 9.9 \times 10^{-5}$, Supplementary Fig. 2). This provided evidence that proteins encoded by the co-expressed genes in the TLE-network interacted physically, supporting the validity of the gene network topography.

35 As gene expression may vary both as a cause and a consequence of disease, the inventors investigated the causal relationship between the TLE-network and epilepsy

by integration with genetic susceptibility data. Here, DNA variation was used to infer causal relationships between the network and epilepsy by assessing whether the network as a whole was genetically associated with epilepsy. To this aim, the inventors used focal epilepsy GWAS data^{6,25} from a separate cohort of 1,429 cases (consisting
5 mainly of patients with TLE) and 7,358 healthy controls. Although no Single Nucleotide Polymorphism (SNP) achieved genome-wide significance in the epilepsy GWAS (Supplementary Fig. 3), the inventors found that the TLE-network as a whole was highly enriched for genetic associations to focal epilepsy compared with genes not in the network ($P = 2 \times 10^{-7}$; Fig. 1a and Supplementary Table 2). These integrated
10 analyses of co-expression network and genetic susceptibility data from a focal epilepsy GWAS provided independent evidence to support the causal involvement of the TLE-network in epilepsy aetiology.

Example 2 - Conservation and functional specialisation of the network

15 The TLE-network was significantly enriched for genes belonging to several biological pathways involving cell-to-extracellular matrix adhesion including 'extracellular matrix-receptor interaction' ($P = 3.9 \times 10^{-5}$), 'focal adhesion' ($P = 1.4 \times 10^{-4}$), and inflammation, such as the 'cytokine-cytokine receptor interaction' ($P = 2.4 \times 10^{-5}$) and 'TLR signaling' ($P = 3.9 \times 10^{-5}$; Fig. 1b). The observation that the TLE-network was
20 enriched for multiple pathways led the inventors to investigate whether the network contained functionally homogenous transcriptional modules (i.e., sub-networks of highly correlated genes) with implications for epilepsy etiology. Using unsupervised agglomerate clustering approaches (see Supplementary Methods), the inventors identified two transcriptional modules comprising 69 (Module-1) and 54 (Module-2)
25 unique genes, respectively (Fig. 1c and Supplementary Data 1). Module-1 was specifically enriched for gene ontology categories related to inflammatory mechanisms, whereas Module-2 was enriched for cell-to-extracellular matrix adhesion processes (Fig. 1d and Supplementary Table 3), indicating functional sub-specialisation within the larger TLE-network. The inventors observed that Module-1 genes were significantly
30 upregulated as compared with genes in the larger TLE-network, Module-2, or with respect to all other genes profiled in the hippocampus of TLE patients (Fig. 1e). This increased hippocampal expression of Module-1 genes in TLE patients was not observed in separate gene expression data sets from the hippocampus of healthy subjects (i.e., individuals clinically classified as neurologically normal) (Supplementary Fig. 4).
35 Notably, Module-1 was enriched for highly expressed inflammatory cytokines (16-fold enrichment, $P = 6.6 \times 10^{-13}$) many of which belong to the IL-1 signaling cascade (*IL-1 β* ,

IL-1RN, *IL-1α*, *TNFα*), and the TLR-signaling pathway (11-fold enrichment, $P = 1.4 \times 10^{-6}$), previously implicated in epileptogenesis¹ and brain inflammation^{2,3} (Supplementary Fig. 5). Taken together these data indicated the presence of a coordinated transcriptional program (Module-1), encompassing TLR activation and release of proinflammatory cytokines (including *IL-1β*), in chronic human epileptic hippocampus as previously hypothesised^{3,26}.

The inventors then investigated whether the TLE-network and Module-1 genes, in particular, were conserved across-species and to this aim they carried out high-throughput sequencing of mRNA (RNA-Seq) in whole hippocampus from 100 epileptic (pilocarpine model)²⁷ and 100 control naïve mice (full details of this model are reported in the Supplementary Methods). The inventors employed GGMs to assess the co-expression relationships between the 371 mouse orthologues of the human TLE-network genes, and found that 312 genes (84%) had significant co-expression (FDR<5%) with at least another network gene in mouse epileptic hippocampus (Fig. 2a). The conserved TLE-network genes formed 1,119 significant partial correlations in mouse epileptic hippocampus, which is significantly higher than expected by chance ($P=0.001$ by 10,000 bootstrap permutations; Fig. 2b). In contrast, only 615 significant partial correlations between the same 312 genes were detected in healthy hippocampus ($P=0.659$ by 10,000 bootstrap permutations), suggesting that the TLE-network is specifically conserved in the epileptic mouse hippocampus (Fig. 2b). In keeping with the high expression of proinflammatory genes observed in the hippocampus of TLE patients (Supplementary Fig. 5), the mouse orthologues of Module-1 genes that were significantly upregulated in epileptic hippocampus were enriched for TLR-signaling and cytokines (gene set enrichment analysis²⁸, $P = 9.03 \times 10^{-4}$, Fig. 2c). These comparative genomics analyses revealed that, to a large extent, the hippocampal TLE-network is conserved across-species, and confirm that genes for TLR-signaling and pro-inflammatory cytokines within the TLE-network are upregulated in chronic epileptic hippocampus.

Example 3 - SESN3 is a genetic regulator of the pro-inflammatory network

The inventors set out to identify genetic variants that regulate the gene co-expression modules (i.e., regulatory ‘hotspots’) by employing genome-wide Bayesian expression QTL mapping approaches^{18,29}. To this aim, the inventors have developed a multi-step strategy to identify SNPs that regulate the expression of a transcriptional module (or network) as a whole. As first step, the inventors summarised the expression of the

genes in each module using principal component (PC) analysis and detected regulatory 'hotspots' using a Bayesian regression model at the genome-wide level²⁹. This analysis prioritised genomic regions associated with variation in mRNA expression of the genes in each module. As a second step, to refine the genetic mapping results, the inventors
5 regressed jointly the mRNA levels of module genes to all SNPs within the regulatory locus identified in the first step¹⁸. Given the functional specialisation within the large TLE-network (Fig. 1d), the inventors investigated the genetic regulation of both Module-1 and Module-2, by analysing 527,684 genome-wide SNPs in the TLE patient cohort. In the first step, they identified a single locus on chromosome 11q21 centered
10 on SNP rs10501829, which was significantly associated with the first PC of Module-1 expression (FDR<5%; Fig. 3a). Module-2 showed no significant genome-wide associations (Supplementary Fig. 6). In the second step, the inventors investigated in detail the locus on chromosome 11q21 regulating Module-1 and carried out joint mRNA levels-SNPs analysis of all genes in Module-1 and all SNPs genotyped within a 1-Mbp
15 region centered on SNP rs10501829. This analysis identified three additional SNPs (rs530190, rs7107661 and rs6483435) in the Best Model Visited (i.e., the best combination of SNPs predicting mRNA level of module genes, see Supplementary Methods) that were associated with the majority of genes of Module-1 (58-74% of Module-1 genes are predicted by individual SNPs, Fig. 3b). The set of SNPs regulating
20 *in trans* the expression of Module-1 genes defined the boundaries of a minimal regulatory region spanning ~383kb (Fig. 3b).

The larger 1-Mbp region centered on SNP rs10501829 contained eight annotated protein-coding genes (Fig. 3b). To further prioritise candidate genes, the inventors
25 carried out co-expression analysis between each of these genes and all genes in Module-1, and found that *Sestrin 3* (*SESN3*) was, on average, most strongly and positively correlated with Module-1 gene expression ($P = 1.7 \times 10^{-13}$, Fig. 3c). The positive association between *SESN3* and Module-1 gene expression remained significant following genome-wide correlation analysis in human hippocampus ($P < 0.00001$,
30 Supplementary Fig. 7). In summary, *SESN3* is the only gene within the minimal regulatory region and, when compared with all genes within a 1-Mbp window around SNP rs10501829, showed the strongest correlation with Module-1 gene expression. Similarly, in the epileptic mouse hippocampus, the inventors found that increased *Sesn3* mRNA expression was also significantly associated with upregulation of Module-
35 1 genes ($P = 5.4 \times 10^{-6}$, Fig. 3d), therefore providing independent, cross-species

evidence supporting *SESN3* as a positive regulator of Module-1 genes in epileptic hippocampus.

Taken together, these data prioritise *SESN3* as a candidate gene for the *trans*-acting genetic regulation of Module-1. To test this hypothesis, the inventors first carried out gene knockdown experiments followed by transcriptional analysis of Module-1 genes by means of RNA interference using short interfering RNA (siRNA). Initially, they used murine bone-marrow-derived macrophages (BMDMs) and BV2 microglia cell line as an *in vitro* system as Module-1 recapitulates the ATF3/AP1 transcriptional complex and IL-1 signaling (Supplementary Fig. 8), known to be highly expressed in lipopolysaccharide (LPS)-stimulated macrophages³⁰. Consistent with the positive correlation of Module-1 genes with *SESN3* mRNA expression (Fig. 3c-d and Supplementary Fig. 7), the inventors observed decreased expression of Module-1 genes after siRNA-mediated knockdown of *SESN3* in both LPS-stimulated BMDMs and BV2 microglia cells (Fig. 4a-b). Similar results were found in unstimulated BV2 microglial cells, suggesting that *SESN3* can modulate expression of pro-inflammatory genes (e.g., *IL-1 β* , *IL-1RN*, *IL-1 α* , *TNFA*) even in the absence of a strong inflammatory stimulus (Fig. 4c). Within the human brain the inventors localised *SESN3* expression to neurons by immunohistochemistry (Fig. 4d), and found that it is highly expressed in the hippocampus of TLE patients as compared with hippocampus from control autopsy samples (Fig. 4e and Supplementary Fig. 9). In keeping with this, the inventors found increased *Sesn3* mRNA expression in the mouse hippocampus after pilocarpine-induced status epilepticus (Supplementary Fig. 10), suggesting an association between *SESN3* gene expression and epilepsy that is conserved across species. The inventors then tested whether Module-1 genes are upregulated when *SESN3* is overexpressed in neurons. To address this aim the inventors used an integrating lentiviral vector for gene overexpression in primary murine neurons (see Supplementary Methods) and quantitative PCR analysis showed that the relative levels of *Sesn3* mRNA were markedly increased in transduced neurons compared with the levels observed in mock transduction (Fig. 4f). Consistent with the observed positive correlation between increased *Sesn3* expression and Module-1 genes in the hippocampus (Fig. 3c-d), lentiviral-mediated overexpression of *Sesn3* resulted in significant upregulation of Module-1 genes in hippocampal neurons (Fig. 4f). These *in vitro* experiments show that *Sesn3* is capable of regulating Module-1 gene expression in different cell types and in particular of inducing upregulation of proinflammatory genes in hippocampal neuronal cells. The inventors' findings in primary neurons are in keeping with previous

data reporting the activity of several inflammatory molecules in neuronal cells under pathological conditions³¹, including IL-1 β and its receptor³². Furthermore, the upregulation of proinflammatory genes in neurons supports the 'neurogenic inflammation' hypothesis, wherein neurons are proposed as triggers of innate and adaptive immune-cell activation in the central nervous system (CNS) (reviewed in Xanthos and Sandkuhler³³).

Example 4 - SESN3 regulates chemically-induced behavioural seizures

The *in vitro* data, combined with the positive association between *SESN3* and Module-1 gene expression in human and mouse epileptic hippocampus, indicated that *SESN3* is a positive regulator of Module-1. The inventors hypothesised that inhibiting *SESN3* would reduce the activity of genes in functional pathways enriched in Module-1, including proconvulsant signaling molecules, and thus by extension could have seizure-suppressing effects. To test this hypothesis *in vivo*, the inventors investigated the role of *SESN3* in a zebrafish model of convulsant-induced seizures^{34,35}. In this model, exposure of 2- or 3-day-old zebrafish larvae to the convulsant agent pentylenetetrazole (PTZ) rapidly induces the expression of synaptic activity-regulated genes in the central nervous system and causes vigorous episodes of calcium flux in muscle cells as well as intense locomotor activity characteristic of epileptic seizures^{34,35}. This acute seizure model has been primarily used to investigate the anti-/proconvulsant activity of compounds³⁶ and for *in vivo* drug discovery³⁴. In particular, molecular and behavioral phenotypes in the zebrafish PTZ-induced seizure model have been employed to identify compounds that attenuate seizure activity³⁷. The inventors employed this model to correlate the locomotor responses with gene network dynamics, i.e., transcriptional activation of the neuronal activity-regulated gene *c-fos*³⁸ and an additional subset of Module-1 genes in response to PTZ treatment in *sesn3* morphant and control morphant larvae. *Sesn3* showed widespread expression in the brain of 3 and 4 days post fertilisation (d.p.f.) zebrafish larvae (Supplementary Fig. 11) and, following PTZ-treatment, the inventors found that *sesn3* morphant zebrafish larvae exhibited significantly reduced locomotor activity as compared with control morphant larvae (Fig. 5a). To test the specificity of the morpholino effect, the inventors co-injected the *sesn3* morpholinos (Supplementary Fig. 12) along with synthetic *sesn3* mRNA, which cannot be targeted by either of the splice-blocking morpholinos (see Supplementary Methods), and assessed whether the *sesn3* mRNA could rescue the morphant phenotype. The inventors observed an almost complete rescue of the locomotor activity phenotype (only 10% difference between uninjected larvae and larvae co-injected with

sesn3 morpholinos and *sesn3* mRNA), with no significant differences in the locomotor activities between the uninjected larvae and the larvae injected with synthetic *sesn3* mRNA alone (Fig. 5b).

5 To further confirm *sesn3*-dependent modification of neuronal response to PTZ, the inventors measured transcriptional activation of the neuronal activity-regulated gene *c-fos*, an important regulator for cellular mechanisms mediating neuronal excitability and survival³⁸. Consistent with the behavioral assay (Fig. 5a), there was decreased expression of *c-fos* in the brain (mostly in forebrain and midbrain) following PTZ
10 exposure in *sesn3* morphant larvae as compared with control morphant larvae (Fig. 5c and Supplementary Fig. 12). As silencing of *Sesn3* resulted in downregulation of Module-1 genes *in vitro* (Fig. 4a-c), the inventors tested whether inhibiting *sesn3* could similarly reduce the activity of Module-1 genes following PTZ exposure *in vivo*. The inventors observed significant reduction in the PTZ-induced mRNA expression of
15 Module-1 genes in the *sesn3* morphant as compared with control morphant larvae (Fig. 5d), and they also found that transient overexpression of *sesn3* in zebrafish larvae increased expression of Module-1 genes independently of PTZ treatment (Fig. 5e). Taken together, these data show that *sesn3* knockdown attenuates both PTZ-induced locomotor convulsive behaviour and the transcriptional responses of *c-fos* and Module-
20 1 genes to treatment with PTZ. These findings in the zebrafish model supported the evidence from the inventors' studies of human and mouse epileptic hippocampus and primary murine neurons that *SESN3* positively regulates expression of proconvulsive molecules (Module-1 genes).

25 **Discussion & Concluding Remarks**

Deciphering the complex regulatory processes of pathophysiological pathways in human brain has previously remained a challenge due to the inaccessibility of ante-mortem tissue, but it could have important mechanistic and therapeutic implications³⁹. In the study reported herein, the inventors used surgical hippocampal tissue samples
30 and employed systems-genetics approaches¹⁷ to investigate transcriptional networks for epilepsy and their genetic regulation. The inventors identified a large gene co-expression network in the human epileptic hippocampus that was conserved in mouse epileptic hippocampus and was enriched for GWAS genetic signals of focal epilepsy. In keeping with similar network-based studies of complex disease such as Type-1
35 diabetes⁴⁰ and autism spectrum disorder^{41,42}, the inventors' approach leverages the combined evidence from genetic susceptibility variants across multiple genes¹⁵ to link

the TLE-hippocampus network with susceptibility to focal epilepsy. Within the TLE-network the inventors identified a functionally coherent and coordinated transcriptional program (Module-1), which was over-expressed in the hippocampus of TLE patients, and which encoded epileptogenic IL1^{2,3,43} and TLR-signaling pathways¹.

5 The inventors confirmed the upregulation TLR-signaling genes and proinflammatory cytokines in chronic epilepsy by RNA-Seq analysis in 200 mouse hippocampi. Preclinical studies in experimental models of epilepsy have consistently shown that individual pro-inflammatory cytokines such as IL-1 β or TNF- α are over-expressed in brain areas of seizure generation and propagation⁴⁴. Therefore, targeting TLR and IL1

10 signaling has been proposed as a possible avenue for therapeutic intervention in epilepsy and antiepileptogenesis^{1,3}, including reduction of acute seizures⁴⁵ and drug resistant chronic epileptic activity⁴⁶. The identification of genetic regulators of these pathways in the human epileptic brain might suggest opportunities for novel targets for disease modification. To investigate the genetic regulation of the TLE-network and the

15 proinflammatory module therein, the inventors employed Bayesian expression QTL mapping approaches¹⁸, which identified *SESN3* as a *trans*-acting genetic regulator of a proinflammatory transcriptional programme in the epileptic human hippocampus. The positive regulation of this network by *SESN3* was confirmed *in vitro* across different cell types by gene silencing (resulting in ~50% reduction of Module-1 gene expression)

20 and overexpression experiments (resulting in ~2-7 fold activation of Module-1 genes, Fig. 4), and *in vivo* using a zebrafish model of chemically-induced seizures (Fig. 5e).

SESN3 is a member of the Sestrin family of proteins that have been shown to decrease intracellular reactive oxygen species and to confer resistance to oxidative stress¹⁹.

25 Intrinsic antioxidant defenses are important for neuronal longevity and the genes that regulate these processes might well influence pathological processes associated with oxidative damage in the brain, a common feature of many neurodegenerative diseases including epilepsy^{47,48}. Therefore, the inventors hypothesised that *SESN3* might regulate neuro-inflammatory molecules, previously implicated in epilepsy^{1,33,43,49},

30 through modulation of oxidative stress in the brain.

The inventors' systems-genetics analysis in the human hippocampus, combined with *in vitro* and *in vivo* data, revealed *SESN3*-dependent regulation of epileptogenic IL-1 β ³ and TLR-signaling genes¹. The upstream genetic control of the proconvulsant

35 transcriptional program by *SESN3* in human TLE-hippocampus suggested a role for this gene in modulating seizures. To test the potential functional role of *SESN3* *in vivo*

the inventors used an experimental model of acute epileptic seizures^{34,37} and found that knockdown of *sesn3* attenuated chemical convulsant-induced locomotor activity and *c-fos* expression, as well as modulating Module-1 gene expression (Fig. 5). The inventors' *in vitro* data in macrophages, BV2 microglial cells and primary neurons showed that

5 *SESN3* is a positive regulator of proinflammatory molecules (Fig. 4), including IL-1 β and TNF- α , major mediators of inflammation, which are capable of inducing changes in neuronal excitability⁵⁰. The finding of reduced severity of PTZ-induced seizures upon knockdown of *sesn3* in the zebrafish model is consistent with previous studies in rodents describing the effects of pro-inflammatory cytokines on seizures. In the

10 context of pre-existing brain inflammation, antibody-mediated antagonism of TNF- α function inhibited susceptibility to PTZ-induced seizures in rats⁵¹, whereas administration of exogenous TNF- α increased susceptibility to PTZ-induced seizures⁵². The inventors' findings in zebrafish are therefore in keeping with a role for *SESN3* in regulating pro-inflammatory cytokines and their downstream effect on CNS excitability

15 and seizure susceptibility.

Taken together, the inventors' data provide the first evidence of a function for *SESN3* in regulating proconvulsant agents (e.g., TNF- α , IL1 and TLR-signaling genes) in human epileptic hippocampus, and support the use of *SESN3* as a new target for modulating

20 brain inflammation^{3,44} and CNS excitability⁵³. The inventors' systems genetics approach builds on and extends previous methods correlating individual genetic variation with disease susceptibility by identifying disease-associated gene networks, pathophysiological pathways and their upstream genetic regulators in human brain.

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Claims

1. An inhibitor of *SESN3* gene expression for use in therapy.
- 5 2. An inhibitor of *SESN3* gene expression for use in a method of treating a disease, disorder or condition of the brain.
3. An inhibitor of *SESN3* gene expression as claimed in claim 2, wherein the disease, disorder or condition of the brain involves neuroinflammation.
- 10 4. An inhibitor of *SESN3* gene expression as claimed in claim 2 or claim 3, wherein the disease, disorder or condition of the brain is epilepsy.
5. An inhibitor of *SESN3* protein activity for use in therapy.
- 15 6. An inhibitor of *SESN3* protein activity for use in a method of treating a disease, disorder or condition of the brain.
7. An inhibitor of *SESN3* protein activity as claimed in claim 6, wherein the disease, disorder or condition of the brain involves neuroinflammation.
- 20 8. An inhibitor of *SESN3* protein activity as claimed in claim 6 or claim 7, wherein the disease, disorder or condition of the brain is epilepsy.
- 25 9. A method of treating a disease, disorder or condition of the brain in a subject comprising administering an inhibitor of *SESN3* gene expression to the subject.
10. A method of treating a disease, disorder or condition of the brain in a subject comprising administering an inhibitor of *SESN3* protein activity to the subject.
- 30 11. An inhibitor as claimed in any one of claims 1-8, or a method as claimed in claim 9 or claim 10, wherein said inhibitor is a biological agent, a protein, a nucleic acid, an siRNA, or a pharmaceutical agent.

35

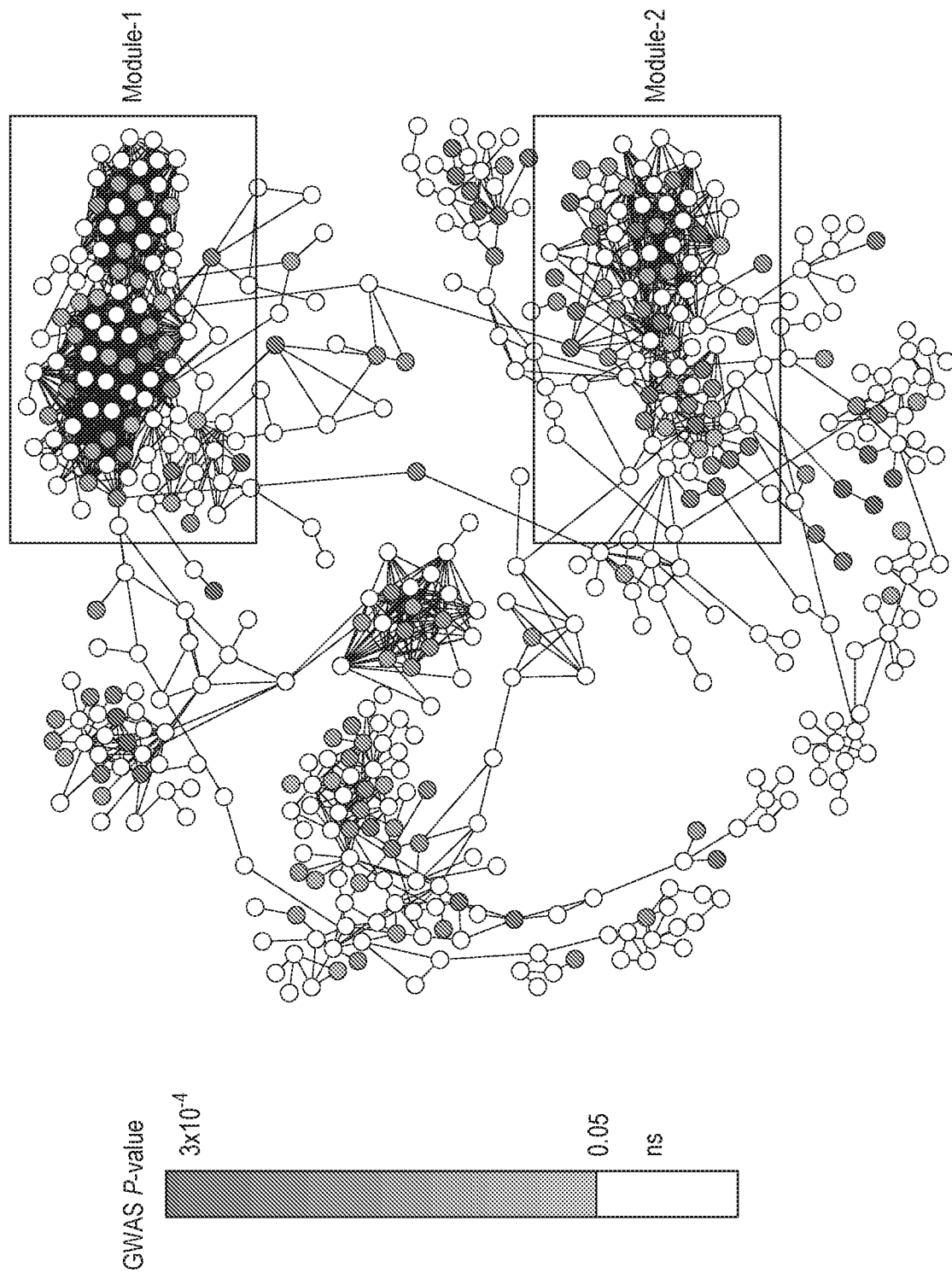


FIG. 1a

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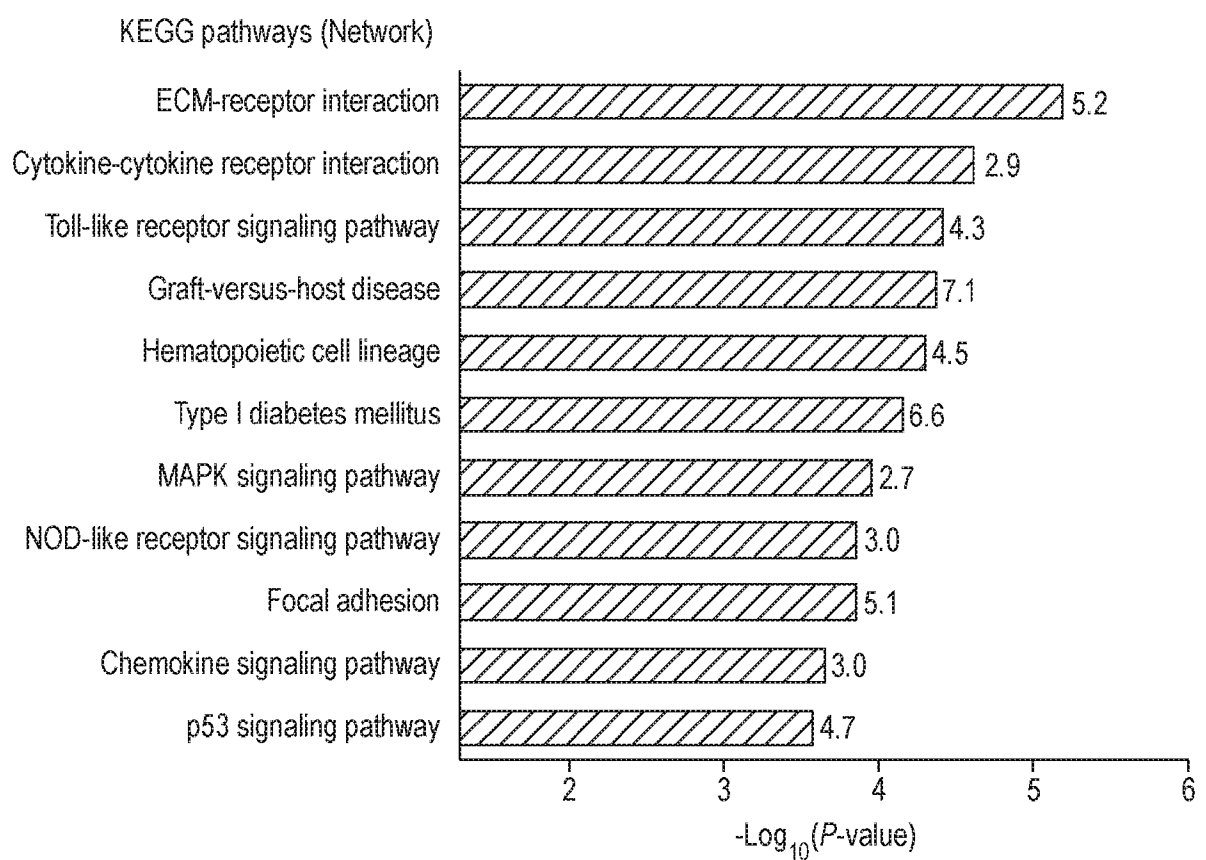
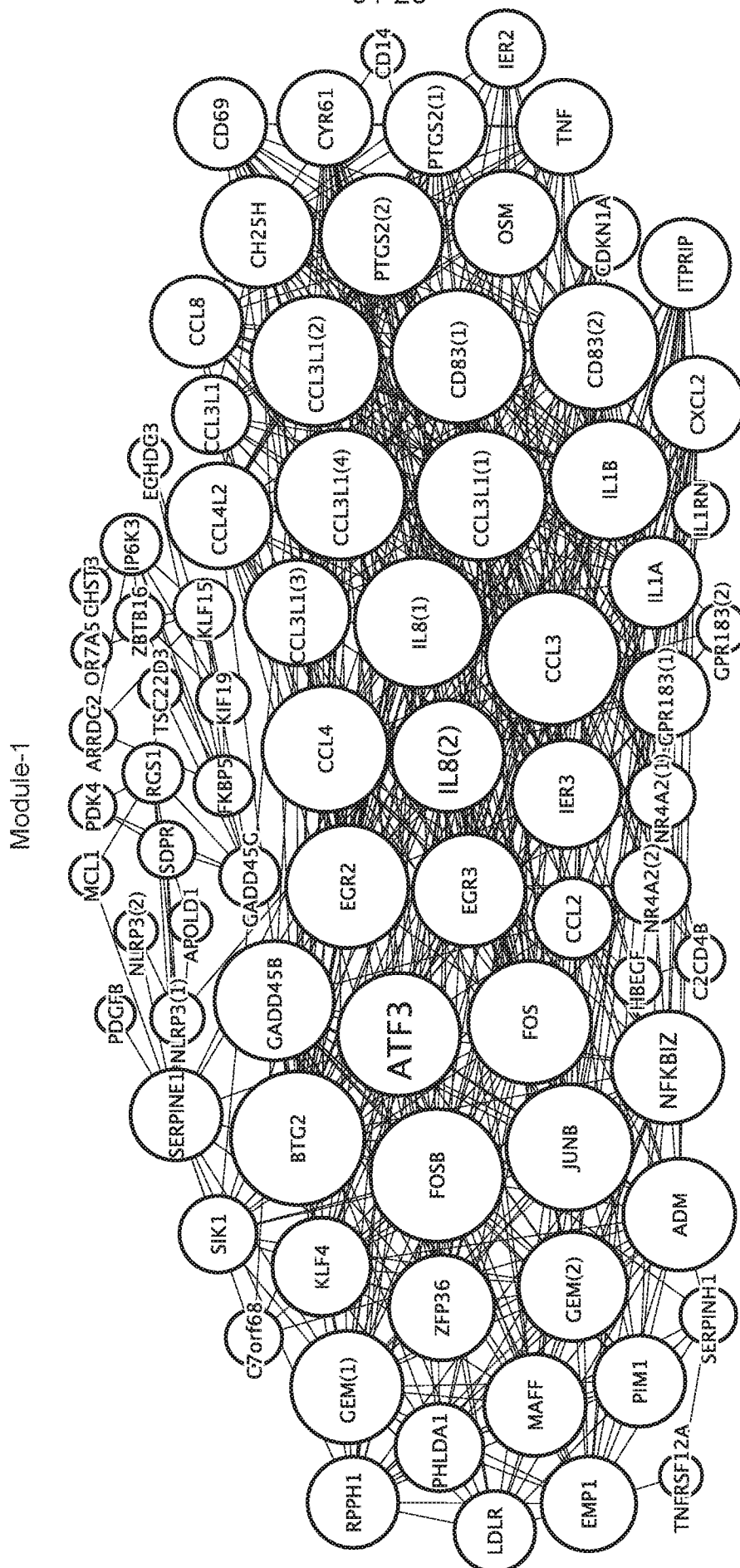


FIG. 1b



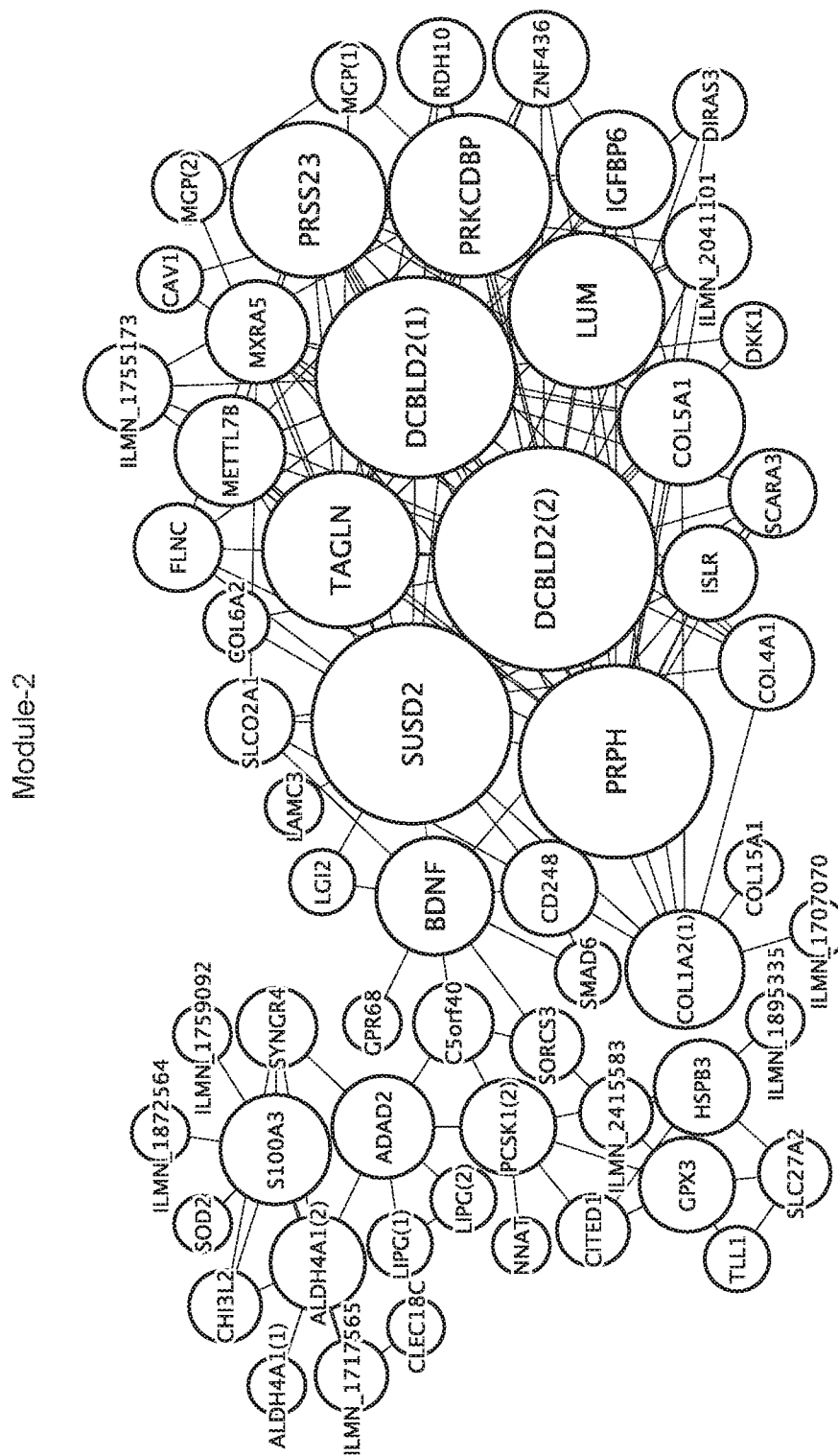


Fig. 1c control

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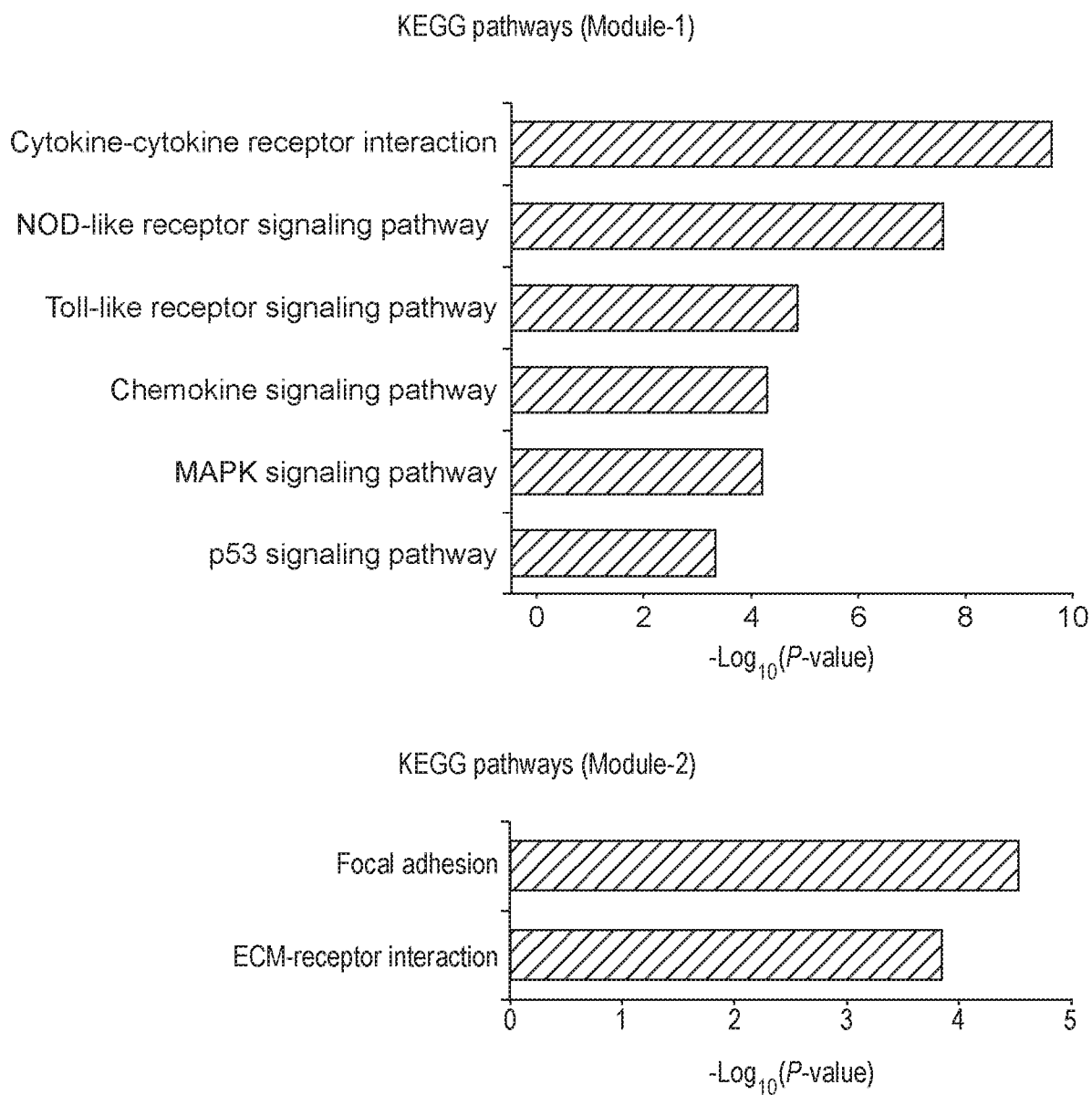


FIG. 1d

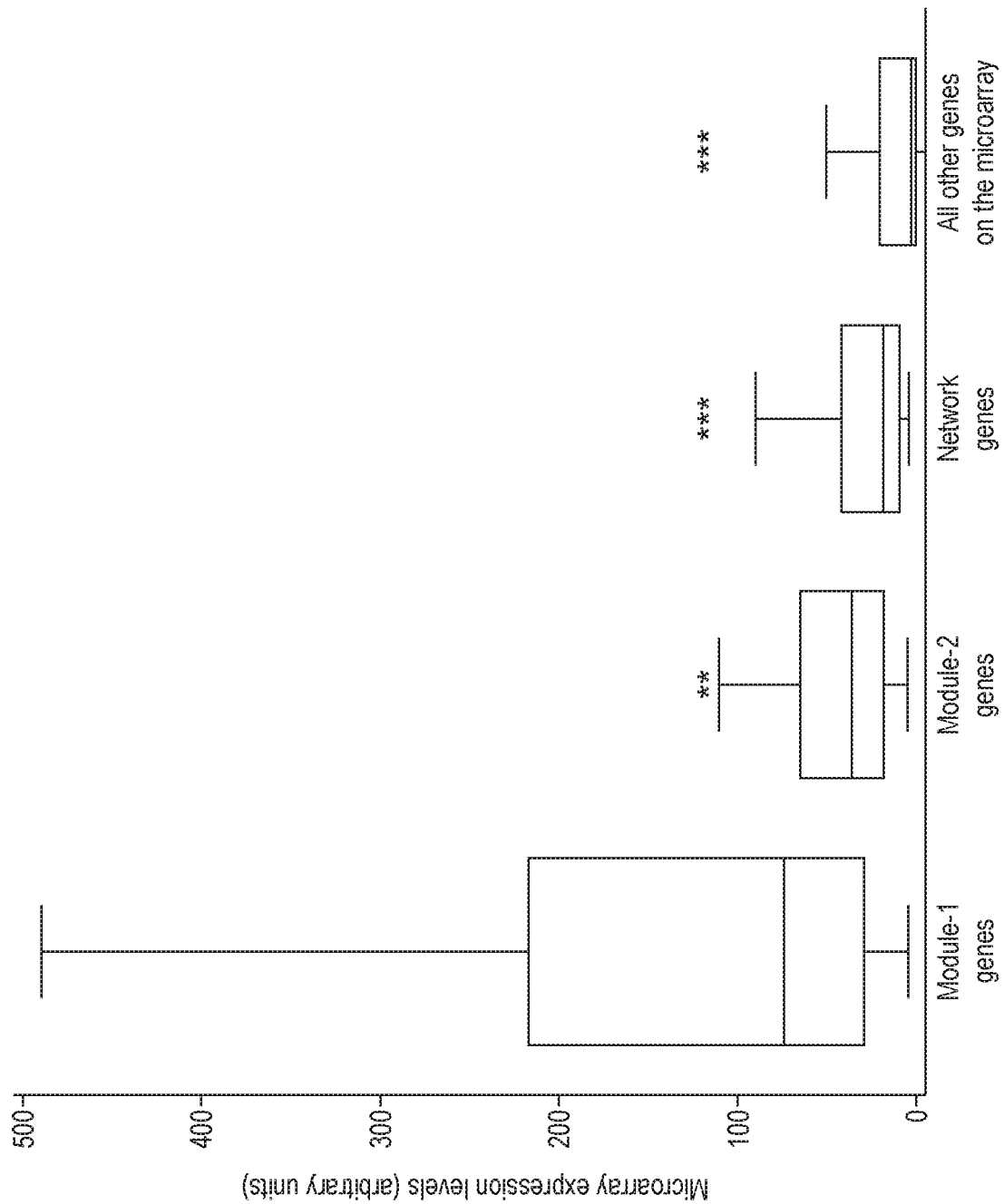


FIG. 1e

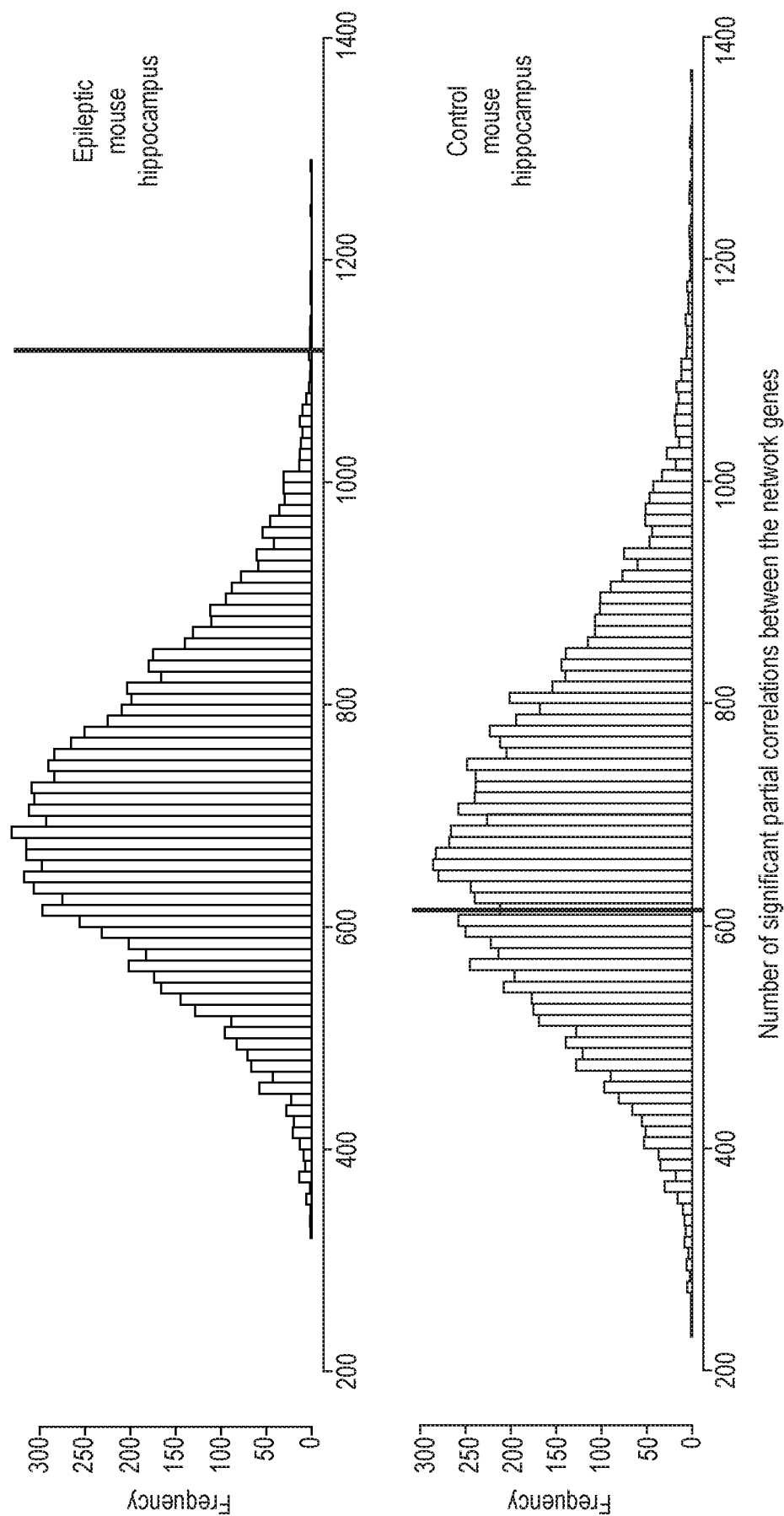


FIG. 2b

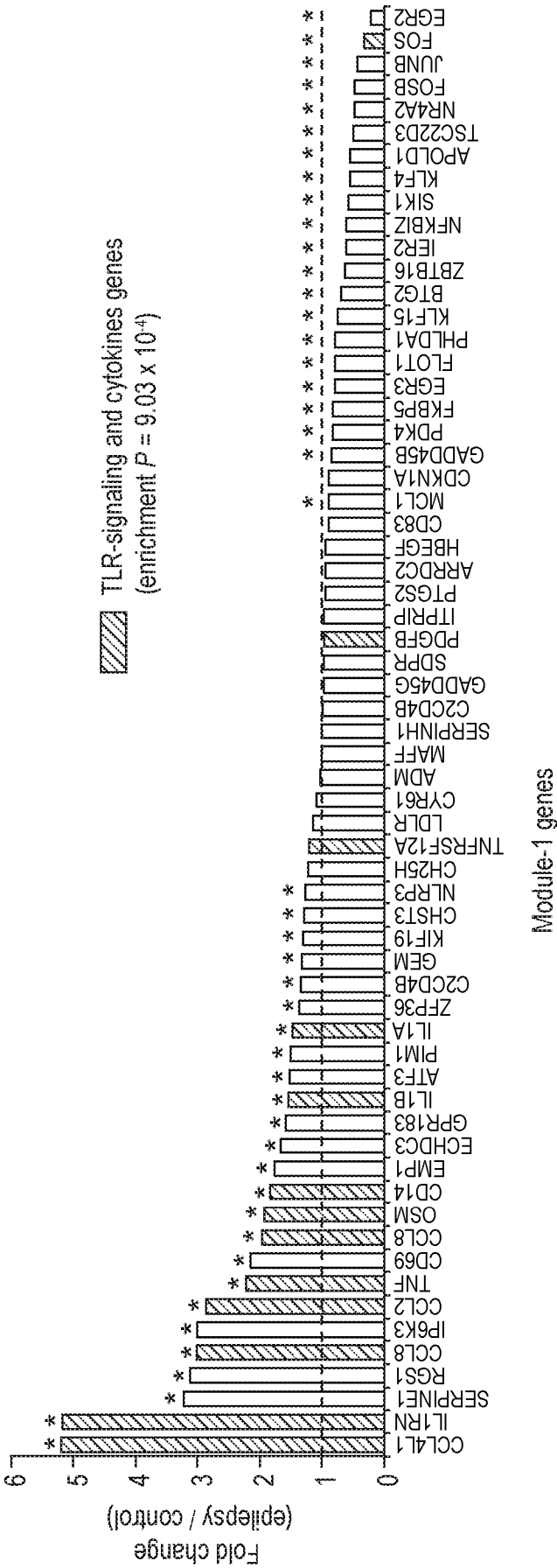


FIG. 2c

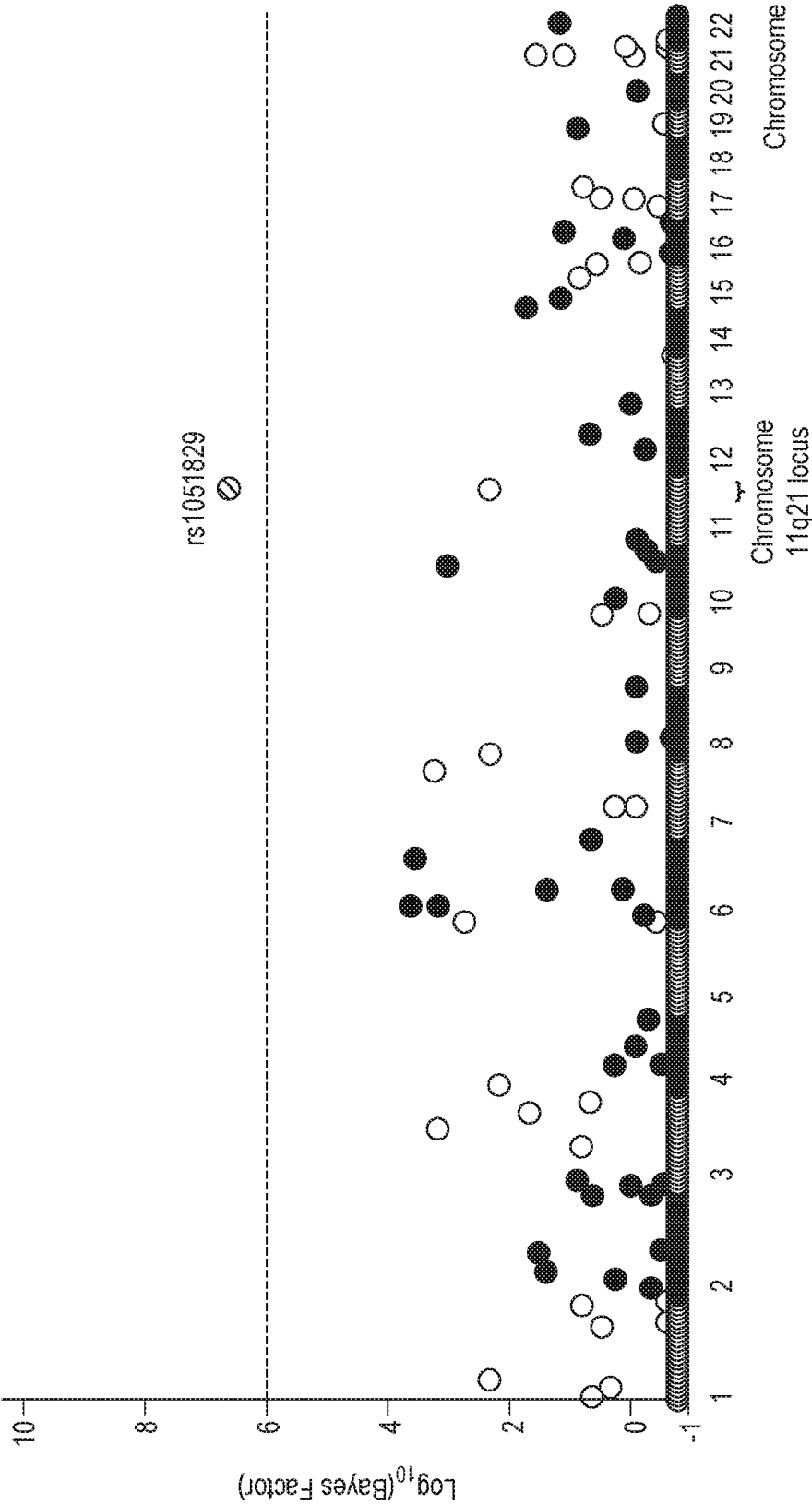
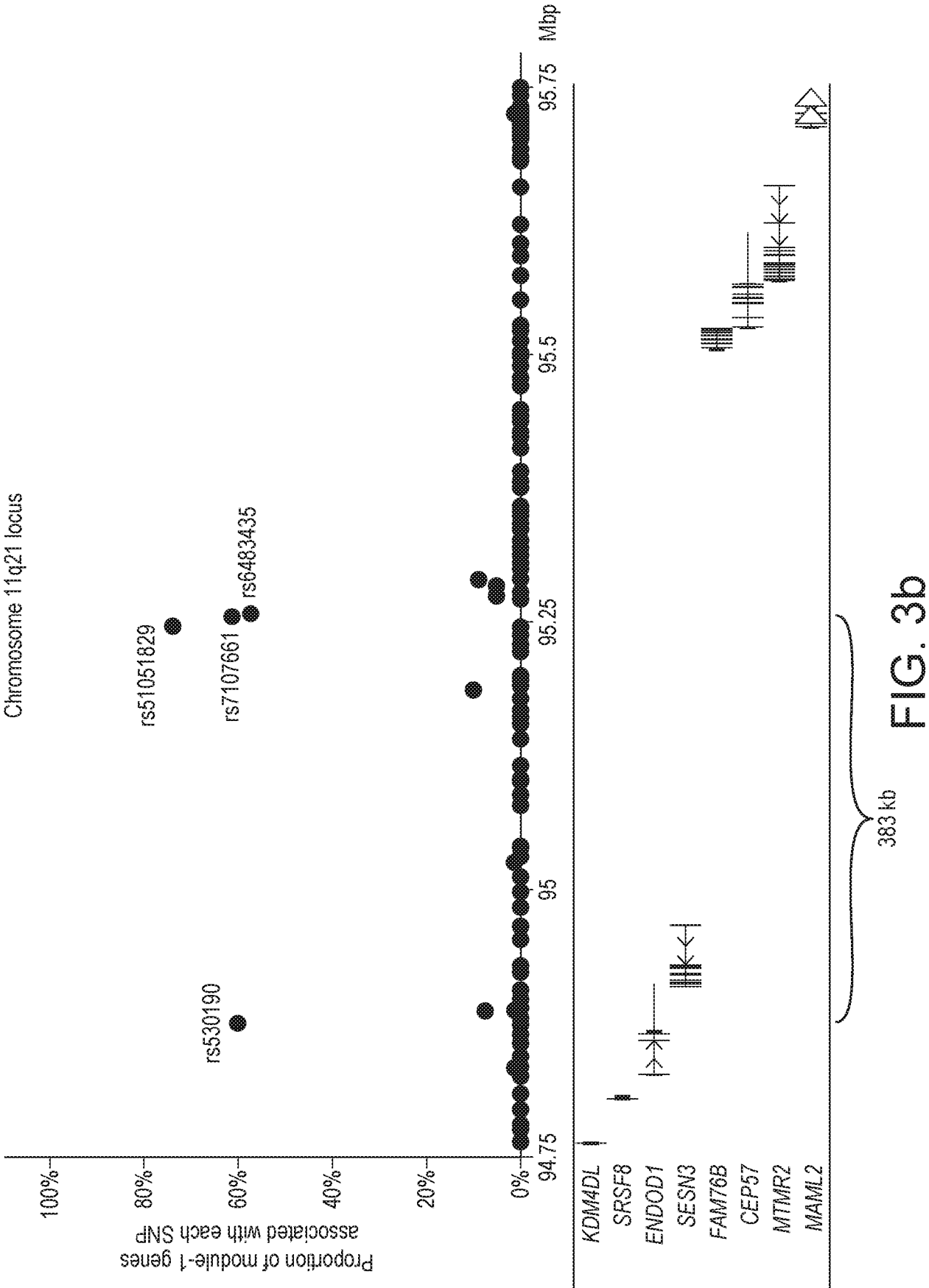


FIG. 3a



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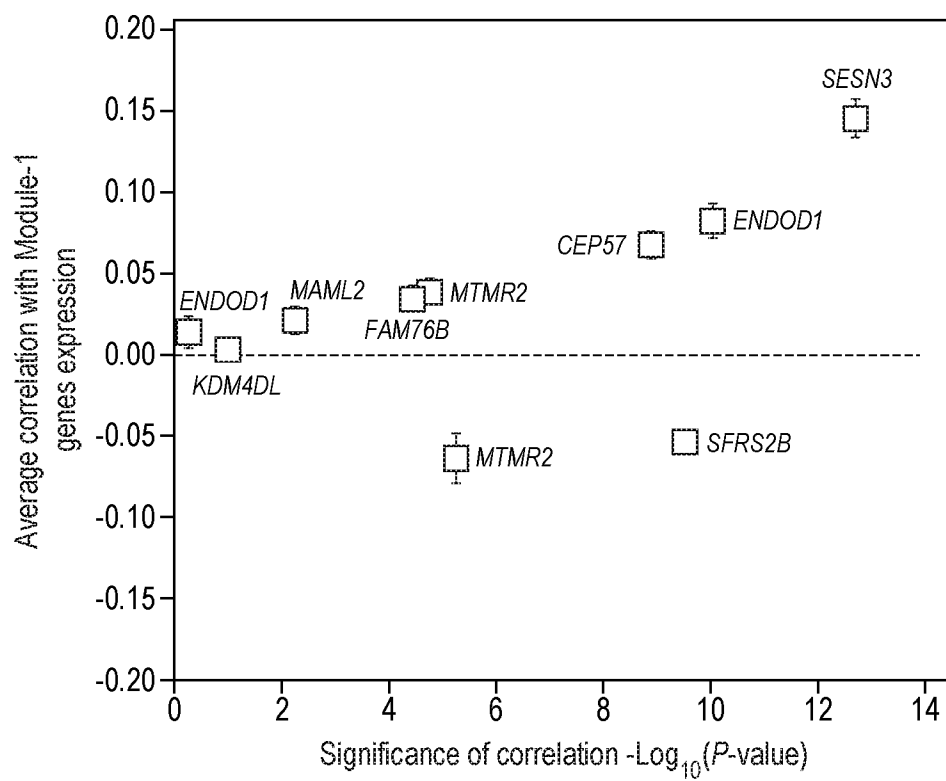


FIG. 3c

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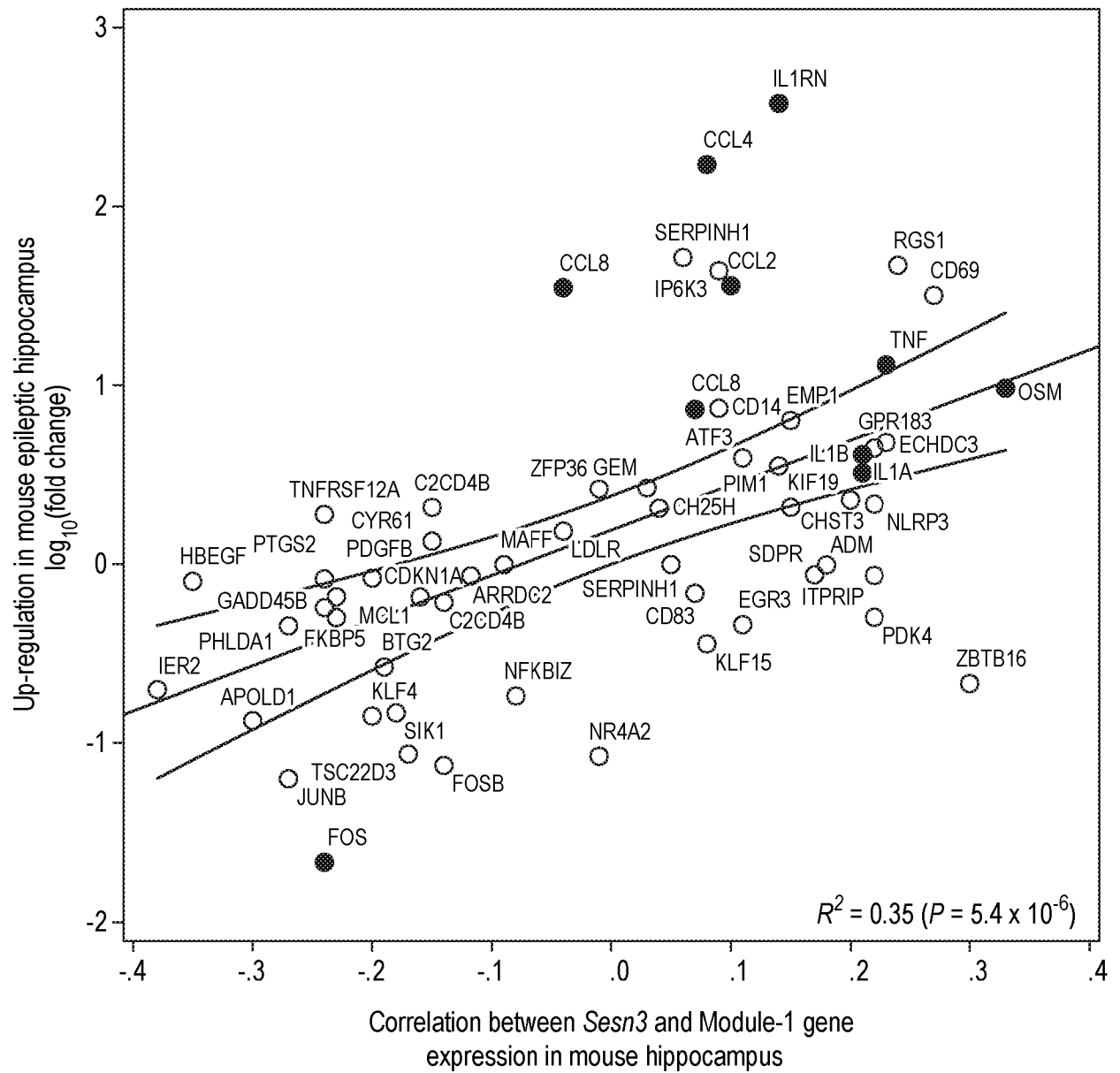


FIG. 3d

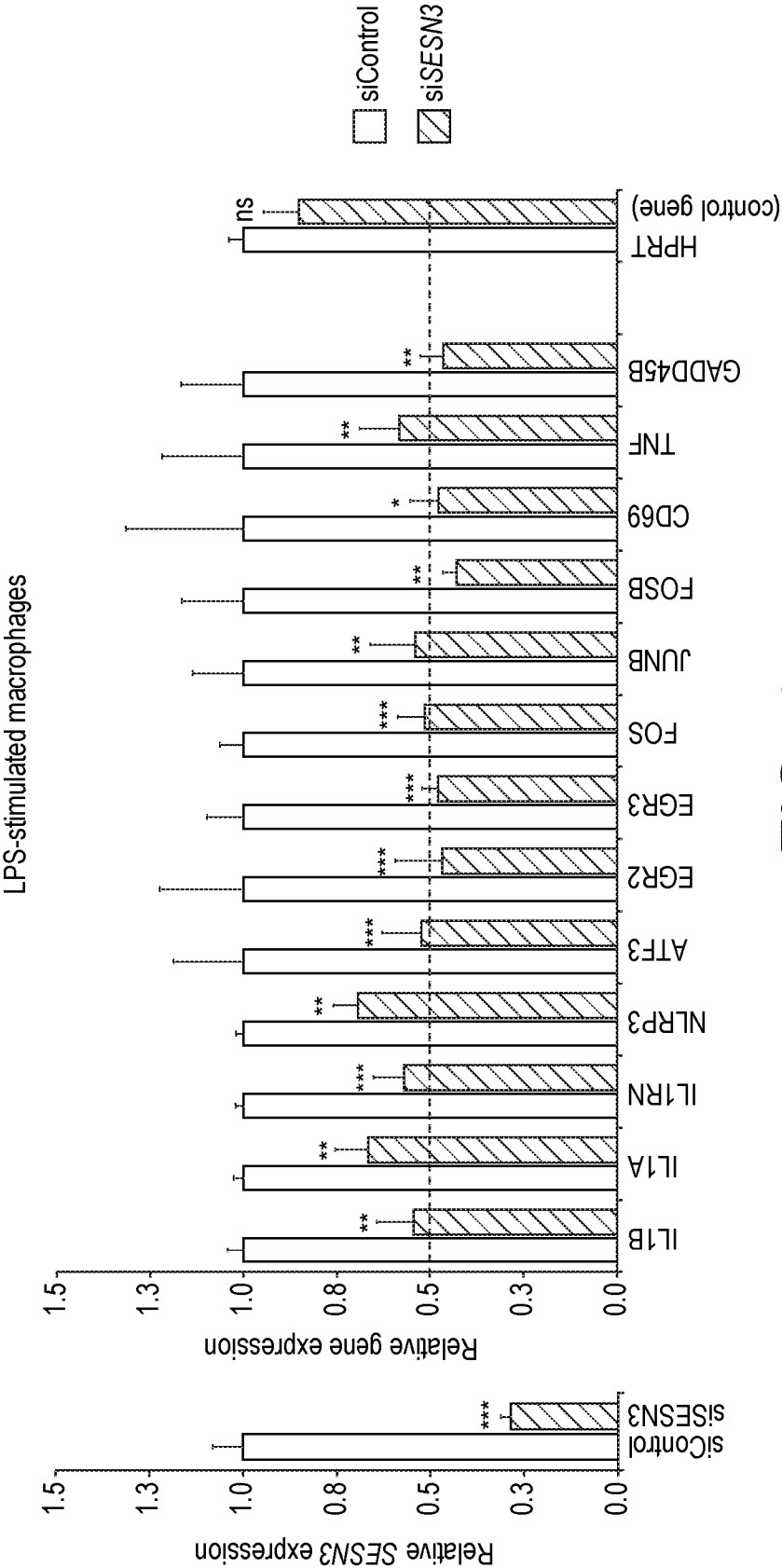


FIG. 4a

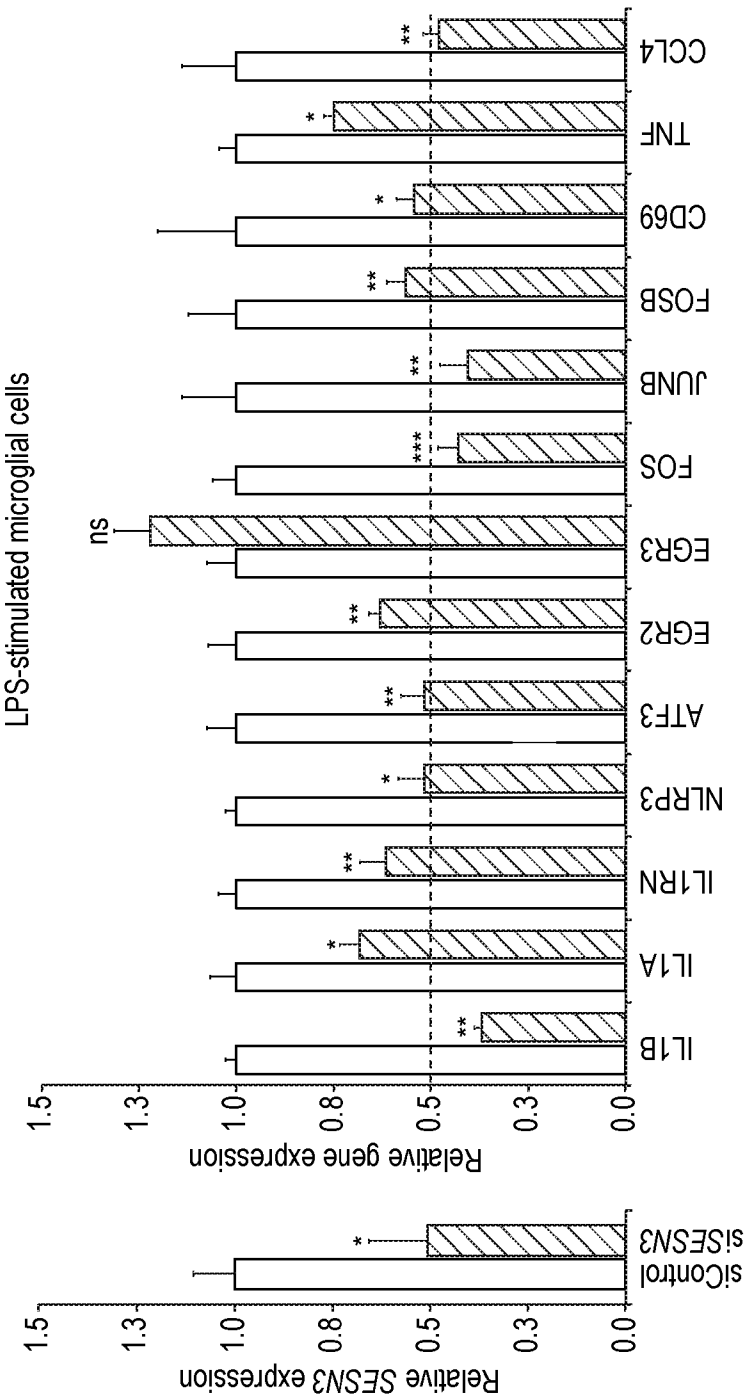


FIG. 4b

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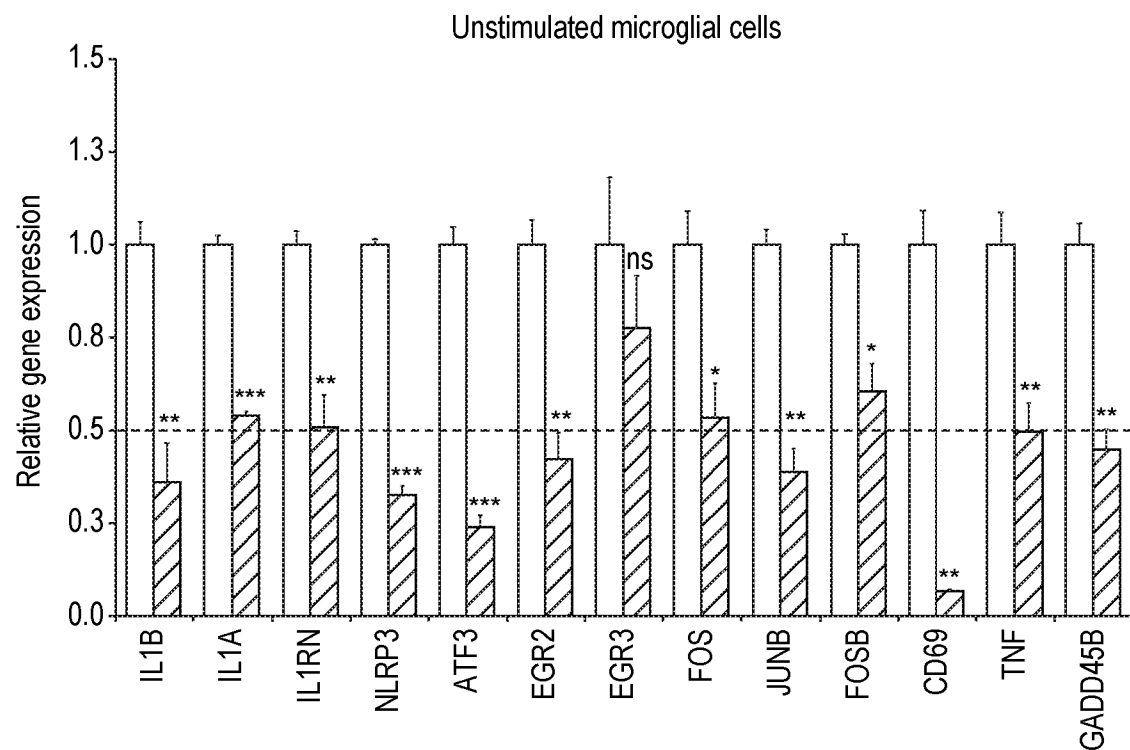


FIG. 4c

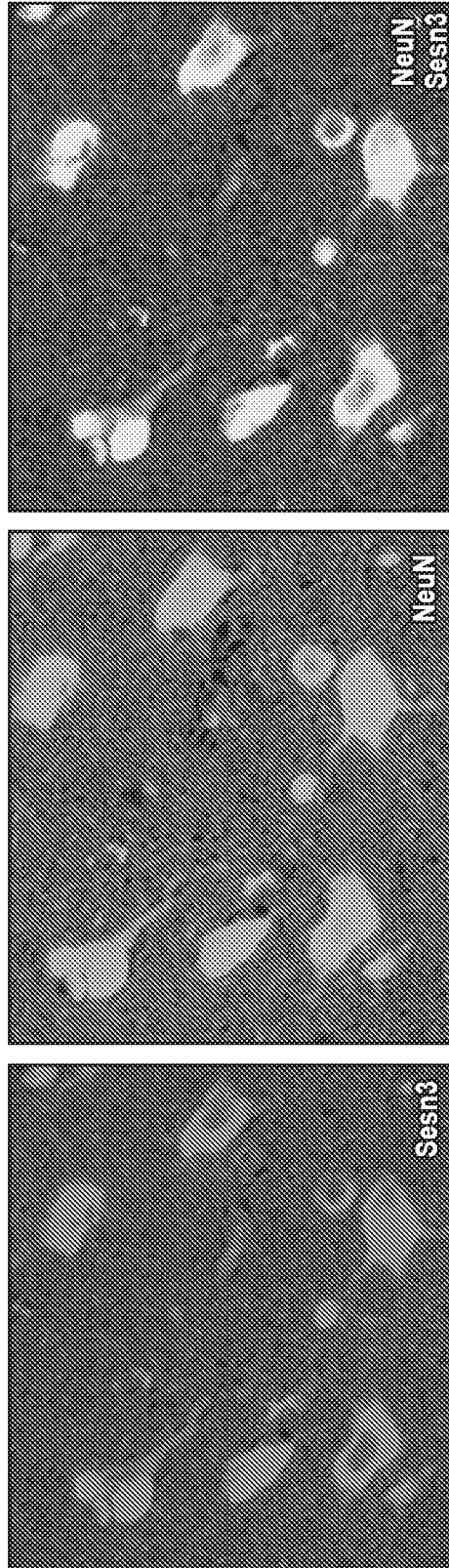


FIG. 4d

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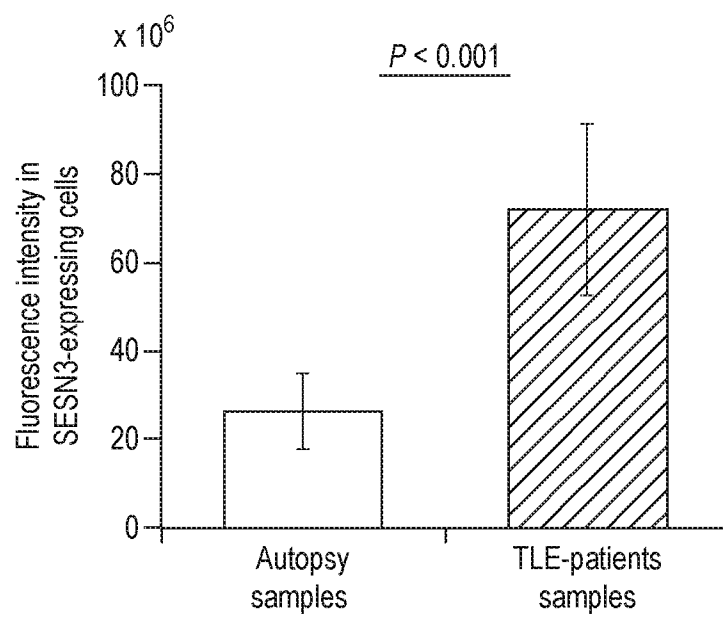
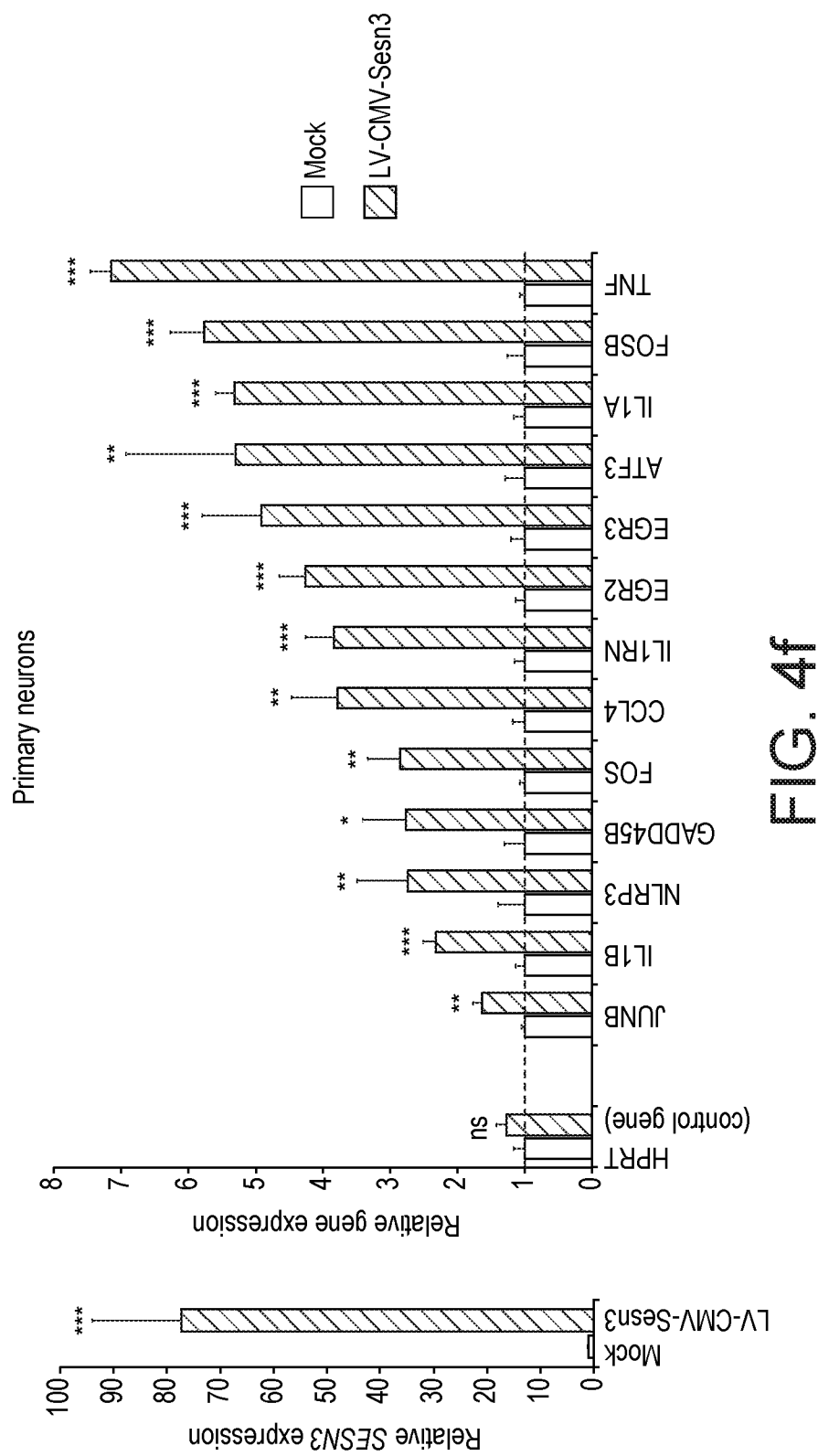


FIG. 4e



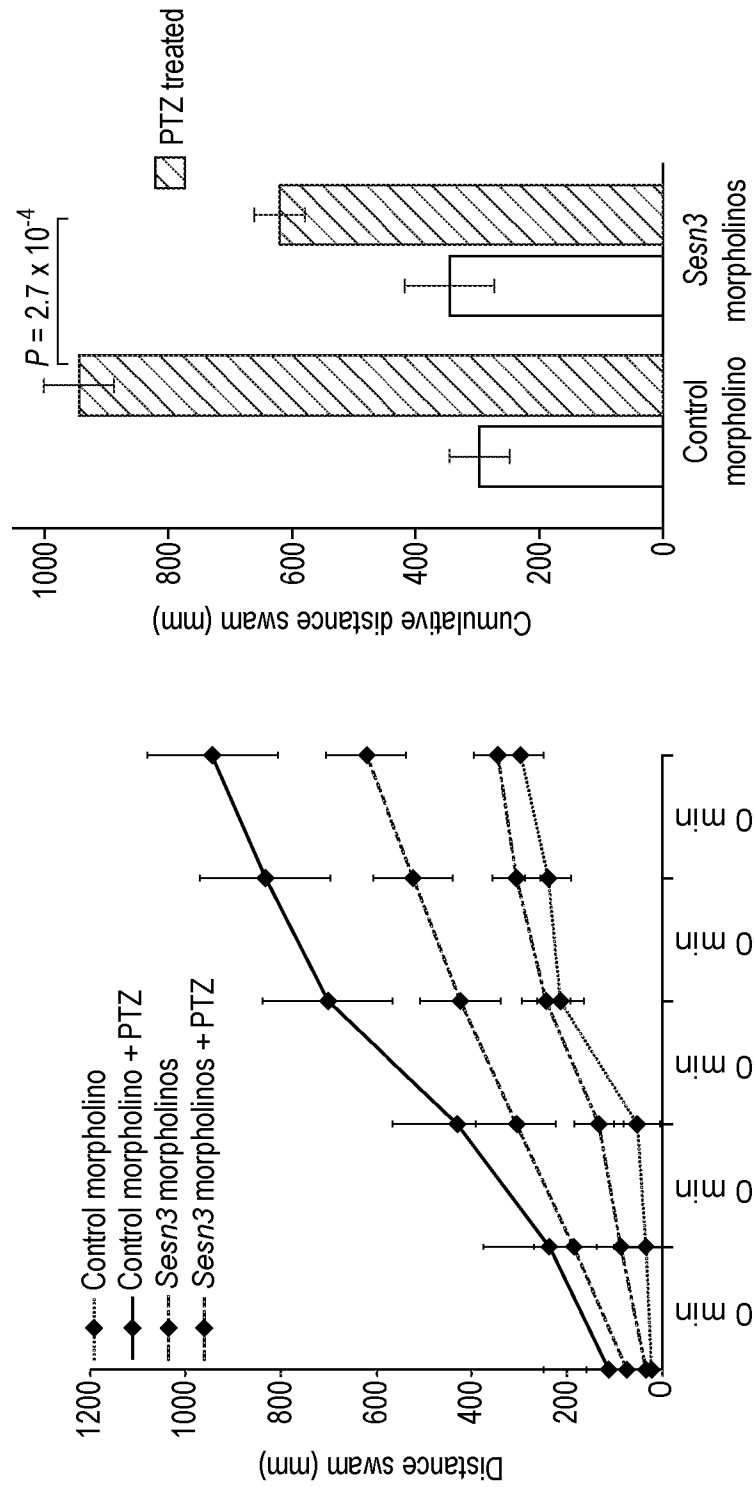


FIG. 5a

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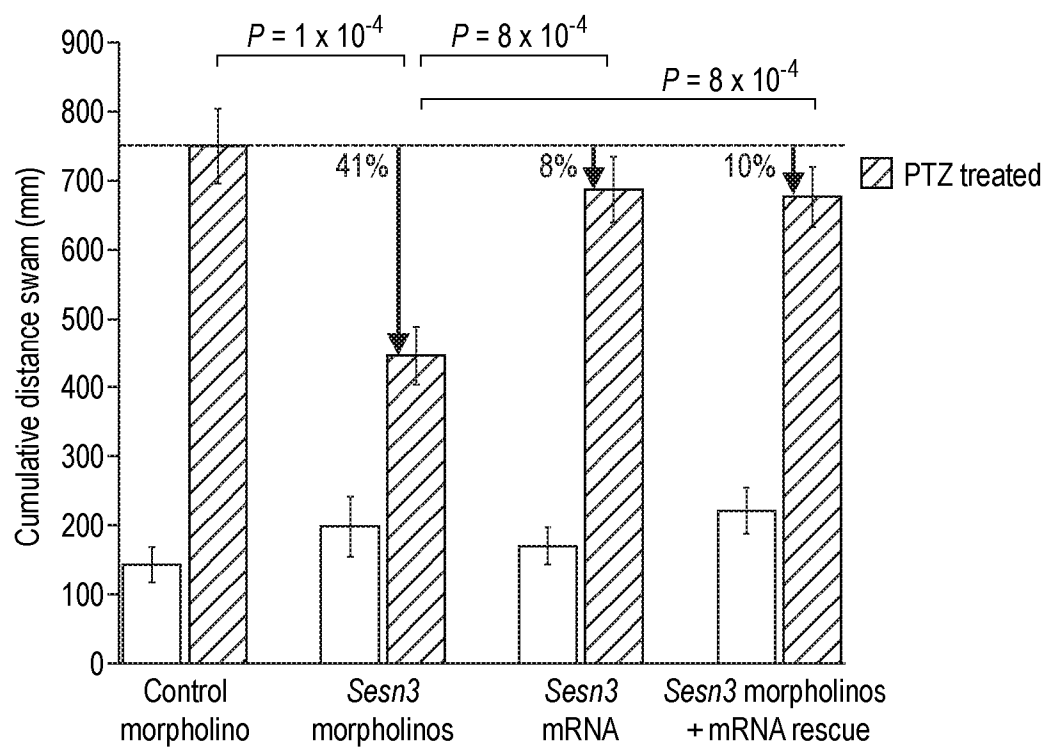


FIG. 5b

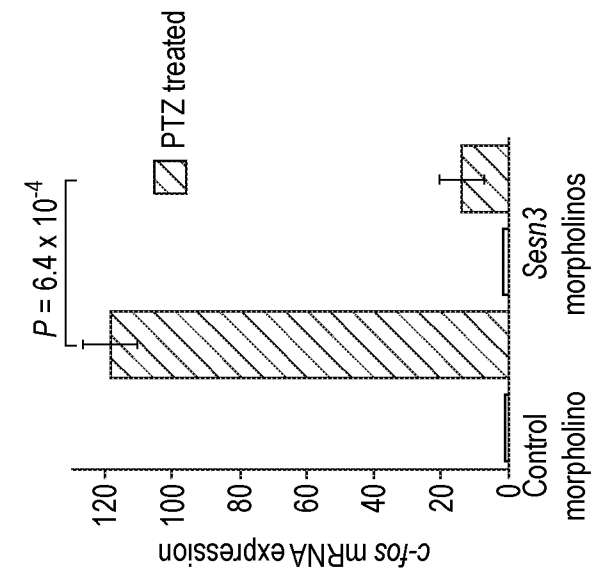


FIG. 5c

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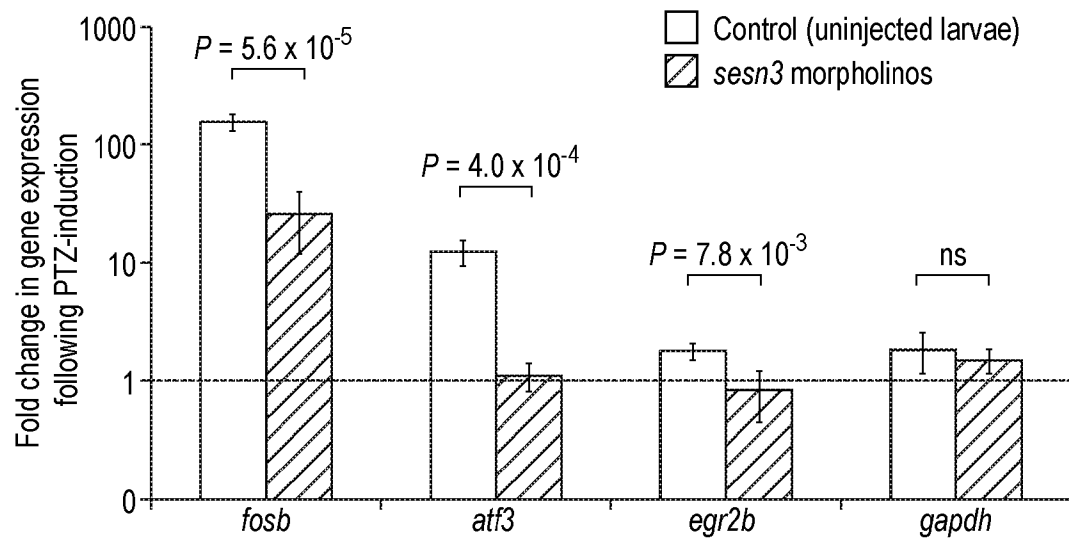


FIG. 5d

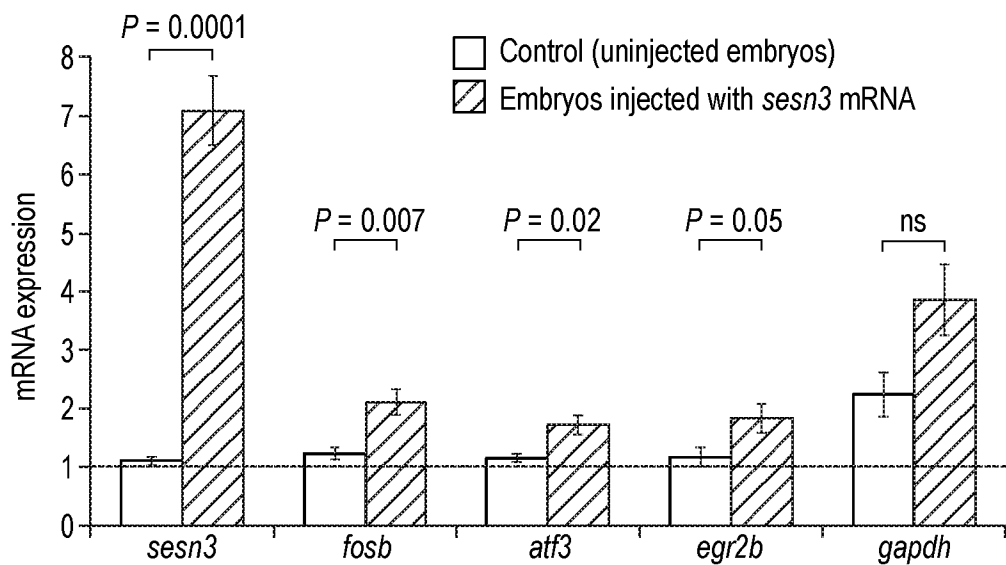


FIG. 5e

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/050145

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/713 A61K39/395 A61K48/00 C12N15/11 C12N15/86
A61P25/08

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C12N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, EMBASE, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	MICHAEL R. JOHNSON ET AL: "Systems genetics identifies Sestrin 3 as a regulator of a proconvulsant gene network in human epileptic hippocampus", NATURE COMMUNICATIONS, vol. 6, 23 January 2015 (2015-01-23), page 6031, XP055257202, DOI: 10.1038/ncomms7031 the whole document ----- -/--	1-11



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 March 2016

Date of mailing of the international search report

22/04/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Escolar Blasco, P

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/050145

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. HAGENBUCHNER ET AL: "FOXO3-induced reactive oxygen species are regulated by BCL2L11 (Bim) and SESN3", JOURNAL OF CELL SCIENCE, vol. 125, no. 5, 1 March 2012 (2012-03-01), pages 1191-1203, XP055257251, GB ISSN: 0021-9533, DOI: 10.1242/jcs.092098 page 1197, left-hand column, paragraph 1 page 1200	1,5,11
X	----- IGNACIO DOLADO ET AL: "AKT and Oxidative Stress Team Up to Kill Cancer Cells", CANCER CELL, vol. 14, no. 6, 1 December 2008 (2008-12-01), pages 427-429, XP055257404, US ISSN: 1535-6108, DOI: 10.1016/j.ccr.2008.11.006 page 427, right-hand column, paragraph 2; figure 1	1,5,11
X	----- E. B. NASCIMENTO ET AL: "Sestrin 3 regulation in type 2 diabetic patients and its influence on metabolism and differentiation in skeletal muscle", AMERICAN JOURNAL OF PHYSIOLOGY: ENDOCRINOLOGY AND METABOLISM., vol. 305, no. 11, 1 December 2013 (2013-12-01), pages E1408-E1414, XP055257254, US ISSN: 0193-1849, DOI: 10.1152/ajpendo.00212.2013 abstract	1,5,11
A	----- ARISTEA S. GALANOPOULOU ET AL: "Finding a better drug for epilepsy: The mTOR pathway as an antiepileptogenic target", EPILEPSIA, vol. 53, no. 7, 11 May 2012 (2012-05-11), pages 1119-1130, XP055257306, NEW YORK, US ISSN: 0013-9580, DOI: 10.1111/j.1528-1167.2012.03506.x page 1125, left-hand column - page 1126, left-hand column ----- -/--	1-11

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2016/050145

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	anonymous: "Polyclonal Antibody to Sestrin-3 (SESN3) (N-term) - Aff -Purified", , 15 January 2014 (2014-01-15), pages 1-2, XP002755386, Retrieved from the Internet: URL:https://ml.acris-antibodies.com/pdf/AP09871PU-N.pdf [retrieved on 2016-05-10] Background -----	1-11