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1 **Multi-influential interactions alters behaviour and cognition through six** 2 **main biological cascades in Down syndrome mouse models**

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23 **Keywords:** Down syndrome, mouse model, partial trisomy 21, Down syndrome critical
24 region, genotype–phenotype maps, learning, memory, transcriptomic.

25

26 **Short title:** Neuronal altered pathways in Down syndrome mouse models

27

28 **Highlights**

- 29 • Brain function and morphology changes in DS mouse models result from multiple genetic
30 loci
- 31 • Each combination of loci induces specific alteration of gene expression profile in mouse
32 models
- 33 • Altered gene expression converges to a few functional pathways in DS mouse hippocampi
- 34 • The synaptic pathway analysis leads to six connected biological cascades and defines a
35 specific DS disease network

36

37 **Abstract**

38 Down syndrome (DS) is the most common genetic form of intellectual disability caused by the
39 presence of an additional copy of human chromosome 21. To provide novel insights into
40 genotype–phenotype correlations, we screened the *in vivo* DS mouse library with standardized
41 behavioural tests, magnetic resonance imaging (MRI) and hippocampal gene expression.
42 Altogether this approach brings novel insights into the field. First, we unravelled several genetic
43 interactions between different regions of the chromosome 21 and how they importantly
44 contribute in altering the outcome of the phenotypes in brain function and structure. Then, in
45 depth analysis of misregulated expressed genes involved in synaptic dysfunction highlighted 6
46 biological cascades centered around DYRK1A, GSK3 β , NPY, SNARE, RHOA and NPAS4.
47 Finally, we provide a novel vision of the existing altered gene-gene crosstalk and molecular
48 mechanisms targeting specific hubs in DS models that should become central to advance in our
49 understanding of DS and therapies development.

50

51 **Introduction**

52 Down syndrome (DS) is the most common genetic form of intellectual disability and
53 was first described as a disease by John Langdon Down in 1866. One century later, genetic
54 studies demonstrated that DS is caused by trisomy of human chromosome 21 (Hsa21) (1).
55 People with DS have a wide range of phenotypic and physiological features with some
56 phenotypic variability but always present several disabling features like mental retardation or
57 Alzheimer disease (2). The leading cause of DS is non-disjunction of chromosome 21 (3).
58 However, in rare cases, people with partial Hsa21 duplications are observed with a smaller
59 spectrum of DS features. Studying these rare conditions increased our understanding of the
60 genotype–phenotype correlations in DS (4-10). The understanding of DS has been substantially
61 improved over the years. Recently, two human genetic studies showed that not a single trisomic
62 region can be responsible for all DS features but rather several susceptibility regions when

63 presented in 3 copies, in people with partial duplication of Hsa21, can induce a large variety of
64 features (5, 7). Nevertheless, several patients displayed complex rearrangement like contiguous
65 or non-contiguous deletions or duplications, copy number variations of other regions or
66 duplication of genes located in the short arm of Hsa21, that can potentially impact the
67 phenotypic outcome of the Hsa21 duplication and add noise to the genetic dissection of human
68 trisomy 21 clinical manifestations.

69 To circumvent the difficulties in studying DS in human, several efforts have been made
70 to generate DS mouse models (11). Indeed, there are three independent mouse homologous
71 regions to Hsa21, carrying altogether 158 protein-coding homologous genes of the 218 protein-
72 coding genes identified on the Hsa21 (12). The largest region is found on the mouse
73 chromosome 16 (*Mus musculus* 16, noted as Mmu16) in a fragment of 22.42 Mb with 119
74 orthologous genes between *Lipi* and *Zbtb21* (13). The most telomeric part is splitted into two
75 parts. The first part is found on the mouse chromosome 17 (noted as Mmu17) with 19
76 homologous genes in 1.1 Mb interval between *Umod11* and *Hsf2bp*. Then, the second part is on
77 the mouse chromosome 10 (Mmu10) with 37 genes included in the 2.3 Mb *Cstb-Prmt2* genetic
78 interval (11, 12, 14). Several DS mouse models have been generated over the years, most of
79 them were carrying trisomy of the largest genetic region located on Mmu16, and have built the
80 *in vivo* DS mouse library (11, 14). The Ts(17¹⁶)65Dn (noted Ts65Dn) is the most widely used
81 DS model and it is quite unique with a supplementary mini-chromosome obtained by x-ray
82 irradiation of the male germline and containing the centromeric region of Mmu17, with genes
83 from *Psid-ps2* to *Pde10a*, and the 13.5 Mb telomeric fragment of Mmu16 containing genes
84 between *Mrpl39* and *Zbtb21* (15-18). Several models were made by chromosomal engineering
85 (11) and carry segmental duplication of Mmu16. The Dp(16*Lipi-Zbtb21*)1Yey (noted Dp1Yey)
86 corresponds to the duplication of the entire Mmu16 region syntenic to Hsa21 (19). The
87 Dp(16*Cbr1-Fam3b*)1Rhr (noted Dp1Rhr) model carries a duplication from *Cbr1* to *Fam3b* and
88 demonstrate the contribution of the DS critical region (DCR) (20-23). All the DS mouse models

89 displayed defects in behaviour and cognition which had been investigated in different
90 laboratories with different protocols and environmental condition making difficult inter-model
91 comparison (11).

92 In order to improve our knowledge on DS, we analysed six DS mouse models, that
93 carries either large segmental duplication, like the Dp1Yey, or transgenic line overexpressing
94 *Dyrk1a*, the main driver gene of DS phenotype found on the Mmu16 (24-28). We used a unique
95 and in depth behaviour, morphological and transcriptomics pipeline in adult animals to dissect
96 the contribution of the genes located on Mmu16 to DS mouse features. The behaviour pipeline
97 relied on assessing specific hippocampal-dependent brain functions found altered in people
98 with DS (29, 30). Thus, we performed standardized tests for the Y-maze, Open field (OF),
99 Novel Object Recognition (NOR), Morris Water Maze (MWM) and contextual and cue Fear
100 Conditioning (FC). All procedures were carried in similar environmental conditions to reduce
101 any impact (31, 32). Besides, variations in specific brain regions have been observed in people
102 with DS and mouse models (33-36). Neuroanatomical changes affect the whole brain volume
103 or specific regions like the frontal region of the limbic lobe or the hippocampus in people with
104 DS. Thus, we performed an in depth morphological investigation of the brain by magnetic
105 resonance imaging (MRI). Finally, whole gene expression was performed on hippocampi
106 isolated from the six models to decipher the genes, functional pathways and biological cascades
107 affected in the different DS mouse models.

108

109 **Material and Methods**

110 **Mouse lines**

111 The duplications of different Mmu 16 regions (Dp(16*Lipi-Zbtb21*)1Yey (or Dp1Yey),
112 the Dp(16*Hspa13-App*)3Yah (noted Dp3Yah), the Dp(16*Cbr1-Fam3b*)1Rhr (or Dp1Rhr)) and
113 the BAC transgenic for *Dyrk1a* (Tg(*Dyrk1a*)) models were previously described (19, 20, 24,
114 37). The genetic background of the DS lines carrying each duplication was pushed toward the

115 C57BL/6J (B6J) genetic background for more than 7 generation of backcross. The only
116 exception is the trisomic Ts65Dn (Ts(17¹⁶)65Dn) mice, initially obtained from the Jax, that was
117 kept on a F1 B6C3B genetic background (with the C3B line as a C₃H/HeH congenic line for
118 the BALB/c allele at the *Pde6b* locus (38)). The Dp(16C*yyr1-Clic6*)5Yah (noted Dp5Yah) was
119 generated by the *in vivo* TAMERE technology inserting loxP sites in *App* and *Runx1* (see
120 Supplementary information). In the Dp3Yah and Dp5Yah models, only 2 complete copies of
121 *App* and *Runx1* genes are expressed. The Dp5Yah line was crossed with the Dp1Rhr in order
122 to generate the Dp5Yah/Dp1Rhr (also noted Dp5/Dp1) compound transheterozygote carrying
123 a similar trisomic Mmu16 gene content to that of the Ts65Dn. Indeed, only 15 Hsa21
124 homologous genes (*Mrpl39*, *Jam2*, *Atp5j*, *Gabpa*, *App*, *Cyyr1*, *Runx1*, *Setd4*, *Mx2*, *Tmprss2*,
125 *Ripk4*, *Prdm15*, *C2cd2* and *Zbpb21*) over 174 are not in 3 copies in Dp5/Dp1 compared to
126 Ts65Dn. In addition 46 protein-coding genes located on the Mmu17 centromeric region in the
127 Ts65Dn minichromosome (13, 16) are not trisomic in the Dp5/Dp1. The Dp5Yah model was
128 also combined with the Tg(*Dyrk1a*) by crossing Dp5Yah/+ and Tg(*Dyrk1a*)/0 animals and
129 generating the four genotypes (Dp1Yah, Dp5Yah, Tg(*Dyrk1a*) and [Dp5Yah; Tg(*Dyrk1a*)]
130 noted here Dp5-Tg), to test specific interaction between Dp5Yah and *Dyrk1a* overdosage.

131 All the lines were maintained under specific pathogen-free (SPF) conditions and were
132 treated in compliance with the animal welfare policies of the French Ministry of Agriculture
133 (law 87 848) and the phenotyping procedures were approved by our local ethical committee
134 (Com'Eth, n°17, APAFIS n°2012-069).

135 **Behaviour pipeline**

136 A serie of behavioural experiments were conducted in mice with a range of age starting
137 at 2,5 up to 7 months for the last test, as described in the Supplementary information. The tests
138 were administered in the following order: Y-maze, open field, novel object recognition (24h),
139 Morris water maze and fear conditioning (contextual and cue). Behavioural experimenters were
140 blinded to the genetic status of the animals. Separate groups of animals were composed for each

141 line (as indicated in S1 table). Several mouse models found defective for the NOR performed
142 with 24h of retention memory were also tested after 1h of retention. The Dp5Yah crossed with
143 Tg(*Dyrk1a*) was tested for Y-maze and NOR at 24h. All the standard operating procedures for
144 behavioural phenotyping have been already described (39-43) and are detailed in the
145 supplementary information.

146

147 **Magnetic Resonance Imaging**

148 A dedicated cohort of animals at the age 102 +/- 7 days was anesthetized and perfused
149 with 30 ml of room temperature 1X Phosphate Buffer Saline (PBS) complemented with 10%
150 (% w/v) heparine and 2mM of ProHance Gadoteridol (Bracco Imaging, Courcouronnes,
151 France) followed by 30ml of 4% PFA complemented with 2mM of ProHance Gadoteridol.
152 Then the brain structure was dissected and kept in PFA 4% 2mM ProHance over night at 4°C.
153 The next day, each specimen was transferred into 1X PBS 2mM ProHance until imaging.

154 Just prior to imaging, the brains were removed from the fixative and placed into plastic
155 tubes (internal diameter 1 cm, volume 13 mL) filled with a proton-free susceptibility-matching
156 fluid (Fluorinert® FC-770, Sigma-Aldrich, St. Louis, MO). Images of excised brains were
157 acquired on a 7T BioSpec animal MRI system (Bruker Biospin MRI GmbH, Ettlingen,
158 Germany). Images were reconstructed using ParaVision 6.0. An actively decoupled quadrature-
159 mode mouse brain surface coil was used for signal reception and a 72-mm birdcage coil was
160 used for transmission, both supplied by Bruker. The first protocol consisted of a 3D T2-
161 weighted rapid-acquisition with relaxation enhancement (RARE). The second imaging protocol
162 consisted of a 3D T2*-weighted Fast Low Angle (FLASH) sequence. The image matrix for
163 both sequences was 195 x 140 x 90 over a field of view 19.5 x 14.0 x 9.0 mm³ yielding an
164 isotropic resolution of 100 µm and was treated and analysed for anatomical parameters as
165 detailed in the supplementary information.

166 **Gene expression assay**

167 Hippocampus were isolated from DS trisomic models and their littermate controls (N =
168 5 per group) and flash frozen in liquid nitrogen. Total RNA was prepared using the RNA
169 extraction kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. The
170 samples quality was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa
171 Clara, California, USA). Gene expression analysis was carried out using GeneChip® Mouse
172 Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA). All the procedure and the analysis are
173 detailed in the supplementary information. Raw microarray data and re-analysed data have been
174 deposited in GEO (Accession No. GSE149470).

175

176 **Bioinformatic analysis**

177 The gene expression profile of the mouse hippocampi isolated from Dp1Yey, Dp3Yah,
178 Ts65Dn, Dp5/Dp1, Dp5Yah, Dp1Rhr and Tg(*Dyrk1a*) trisomic mouse models was analysed
179 with a specific bioinformatic pipeline and controlled for quality prior and after the data pre-
180 processing and normalization (see supplementary information in the detailed material and
181 methods section). The identification of the differentially expressed genes (DEGs) was
182 performed using a method based on fold change rank ordering statistics (FCROS)(44). In the
183 FCROS method, k pairs of test/control samples are used to compute fold changes (FC). For
184 each pair of test/control samples, obtained FCs for all genes are ranked in increasing order.
185 Resulting ranks are associated to genes. Then, the k ranks of each gene are used to calculate a
186 statistic and resulting probability (f-value) used to identify the DEGs after fixing the error level
187 at 5% False Discovery Rate (FDR).

188 We performed the functional differential analysis using GAGE (45) and grouped all the
189 pathways into 8 functional categories (noted meta-pathways). The functional intermodel meta-
190 pathway connectivity was studied identifying the genes shared between pathways and models
191 inside the same meta-pathway. Then, to assess the gene connectivity we build a minimum fully

192 connected protein-protein interaction (PPI) network (noted MinPPINet) of genes known to be
193 involved in synaptic function as they were associated with synaptic pathways via GO (46) and
194 KEGG databases (47) and furthermore added regulatory information to build the final
195 RegPPINet. We used the betweenness centrality analysis to identify hubs, keys for maintaining
196 the network communication flow. The relevance of the connecting nodes was further predicted
197 by the machine learning algorithm Quack (48). Finally, we computed 100000 random networks
198 with a similar degree, to assess if the likelihood of observing such connectivity in the DS
199 network was more than one can expect by chance using statnet and sna R packages
200 (<https://cran.r-project.org/web/packages/statnet/index.html>;
201 [https://cran.r-](https://cran.r-project.org/web/packages/sna/index.html)
202 [project.org/web/packages/sna/index.html](https://cran.r-project.org/web/packages/sna/index.html)).

203 **Western blot**

204 The expression levels of the RHOA protein and Myosin Light Chain phosphorylation
205 by Myosin Light Chain Kinase part of the RHOA pathway were analysed using Western Blot
206 in 5 animals Dp1Yey and 5 control (wt) littermates (See Fig 4G, Supplementary fig 16, and
207 supplementary information). We used the following primary antibodies: anti-RHOA (2117,
208 Cell Signaling, USA, 1:1.000), anti-pMLC (Thr18/Ser19 #3674, Cell signalling, Boston, MA,
209 USA, 1:1.000) and mouse monoclonal Anti- β -Actin–Peroxidase antibody (A3854 Sigma,
210 1:150.000); and HRP conjugated Goat anti-Rabbit IgG secondary antibody (A16096,
211 Invitrogen, France). Protein signals were visualized with Amersham™ Imager 600 and were
212 quantified using ImageJ and the statistical analysis using Sigma Plot. The relative amount of
213 RHOA and p-MLC proteins was calculated as the ratio of the signal detected for each proteins
214 of interest compared to the β -actin signal detected and normalized by the mean signal of the wt
215 samples.

216

217

218 **Visual stimulation**

219 Mice raised in a standard light cycle were housed in constant darkness for two weeks.
220 Then, animals in the light-exposed condition group, were consecutively exposed to light for 0,
221 1, 3, or 7.5 h before being sacrificed. Instead, animals part of the dark-housed condition group
222 were sacrificed in the dark. After euthanasia, their eyes were enucleated before the visual cortex
223 dissection in the light and flash frozen in liquid nitrogen. cDNA and quantitative PCR were
224 performed as indicated in the supplementary information. The Ct values were transformed to
225 quantities by using the comparative Ct method. Hence, all data were expressed relative to the
226 expression of the most expressed gene. This relative expression levels, were normalized with
227 Genorm by keeping the more stable reference genes (49). To calculate fold-induction, relative
228 quantity of gene expression at each time point was divided by the mean of relative level of gene
229 expression of dark housed mice for corresponding genotype. The mean and standard error were
230 calculated at each time point from these fold-induction values.

231 **Statistical analysis**

232 All data are expressed as mean group value \pm standard error of the mean (SEM) or as
233 box plots with the median and quartiles. For each data set, we analysed if the data was normally
234 distributed by Shapiro–Wilk test and Quantile-Quantile plots (S2 Fig) and the homogeneity of
235 variances by the Brown-Forsy test. Differences between groups were inferred by one-way
236 ANOVA (Open field) and ANOVA for repeated measures, or in case of datasets where the
237 assumptions of normality or homogeneity of variances were not fulfilled we performed the
238 Kruskal-Wallis non-parametric test. The post hoc tests (Fisher LSD Method) were conducted
239 only if the F parameter in ANOVA achieved 0.05 level. All the behavioral analysis results are
240 found in S1 table. For MRI data, intergroup comparisons on region-based data were conducted
241 on the normalized volumes (i.e., ratio between the volume of the structure and the whole brain
242 volume) of each segmented structure using the Student t-test while correcting by multiple
243 testing setting up a FDR correction.

244

245 **Results**

246

247 **Dissecting the contribution of Mmu16 subregions to the DS-related behavioral** 248 **phenotypes in mouse models**

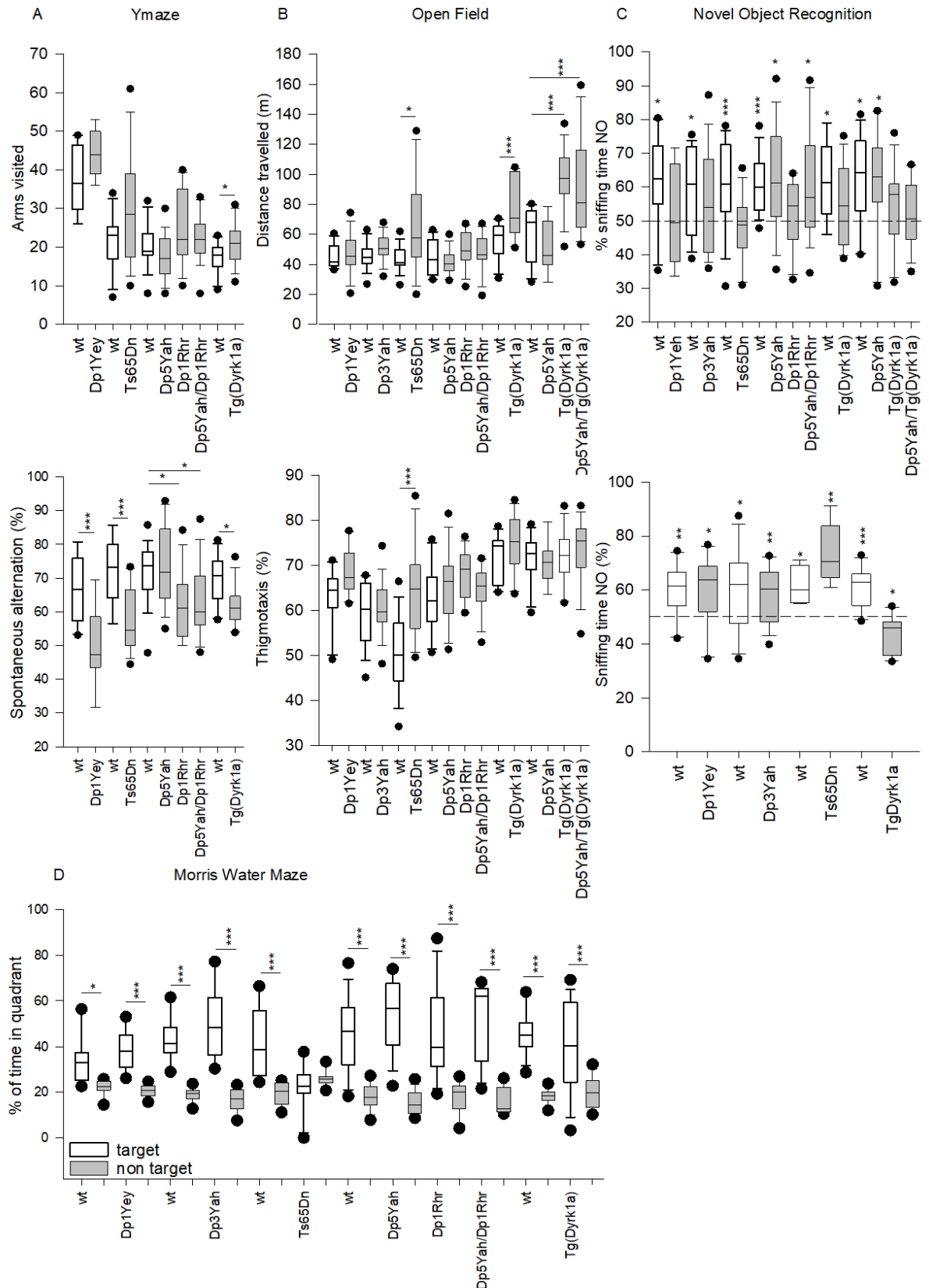
249 We wanted to dissect the contribution of sub-regions located in the telomeric part of
250 *Mus musculus* chromosome 16 (Mmu16), homologous to Hsa21 (50), to the DS-related
251 cognitive phenotypes. First, we selected 4 DS mouse models: the Ts65Dn, the most commonly
252 used DS model (15), and three additional models that are carrying segmental duplications of
253 well-defined sub-regions located on the Mmu16, the Dp1Yey (19), Dp3Yah (37) and Dp1Rhr
254 (20). In addition, we engineered a new one, the Dp5Yah, corresponding to three functional
255 copies of the genes included in the genetic interval between *Cyrr1* to *Clic6*. This model was
256 crossed with the Dp1Rhr one in order to generate the Dp5Yah/Dp1Rhr (noted Dp5/Dp1)
257 compound transheterozygote carrying a trisomic gene content similar to the Ts65Dn for the
258 genes located on the Mmu16. We also included a model carrying an additional copy of *Dyrk1a*,
259 one of the driver genes for DS-related phenotypes (25, 35), and the Tg(*Dyrk1a*) combined with
260 Dp5Yah model (noted Dp5-Tg) (S1 Fig). We used standardized behavioral tests to study several
261 aspects of learning and memory in mice including the Y-maze (working memory), the open
262 field (exploration memory), the novel object recognition (recognition memory), the Morris
263 water maze (spatial memory) and the fear conditioning (associative memory). For all the lines,
264 independent cohorts of control and trisomic mouse littermates went through the pipeline at
265 similar ages, then the resulting data were processed using standard statistical analyses (See
266 supplementary information for details). First, we assessed the potential existence of a
267 background effect over the distribution of the measurements taken in the different tests. In our
268 condition the Q-Q plots with the cumulative frequency were linear and thus, no notable

269 difference between B6J or hybrid B6JC3B wild-type controls was observed (see Fig 1, S1 table,
270 S2 Fig).

271 Mice activity and working memory were evaluated in the Y-maze (Fig 1A). The number
272 of arm entries in the Y-maze showed that only Tg(*Dyrk1a*) mutant line was hyperactive in this
273 test while deficit in spontaneous alternation was found in Dp1Yey (51), Ts65Dn (52), Dp1Rhr
274 and Tg(*Dyrk1a*) (53). The Dp5/Dp1 also showed a clear deficit in the percentage of spontaneous
275 alternation as compared to littermate controls while Dp5Yah trisomic animals showed normal
276 performance.

277 The patterns of exploratory activity and anxiety were assessed in the open field (Fig
278 1B). Ts65Dn, Tg(*Dyrk1a*) and Dp5-Tg presented hyperactivity with an increased travelled
279 distance compared to wild-type littermates, and support, for Tg(Dyrk1A), results obtained in
280 the Ymaze. In addition, Ts65Dn mice displayed thigmotaxic behavior with increased distance
281 travelled at the periphery.

282 The spatial reference memory was tested in the standard Morris water maze (MWM)
283 task, in which mice have to escape from a circular pool of opaque water by localising a hidden
284 platform at a single fixed location using distal spatial cues. We analysed the velocity, the
285 distance travelled by the mice to reach the platform and the thigmotaxis over training (S3 Fig).
286 The velocity of Dp1Yey and Dp5Yah, was slightly lower than the wild-type mice (S3 FigB).
287 As previously described (22, 54-60), Ts65Dn mice displayed a longer distance travelled to find
288 the platform during all the sessions, compared to the wild type (S3 FigA). Although Tg(*Dyrk1a*)
289 were able to locate the platform, they also showed delayed acquisition compared to the control
290 mice. Surprisingly, the Dp1Yey, Dp1Rhr, Dp5Yah and Dp3Yah completed this test without
291 any difference with the wild type group. Retention of place location was evaluated during a
292 single probe trial (PT) with no platform available, 24h after the last training session (Fig 1D).
293 The results confirmed as previously shown, that all the mouse strains except the Ts65Dn,
294 remembered where the platform was located after the learning sessions. Interestingly, the



295

296 **Figure 1. Standardized behavioural profiling of DS mouse models.**

297 Y-maze spontaneous alternation. Arm visited (A upper panel) and alternation rate (A lower
 298 panel) are presented as box plots with the median and quartiles (upper and lower 90%
 299 confidence interval are indicated by a grey box). Only the Tg(*Dyrk1a*) mice were showed

300 hyperactivity in this test with increased arms entries compared to the wild type ($p=0,017$).
301 Alternation rate in Dp1Yey ($p=0,002$), Ts65Dn ($p<0,001$), Dp1Rhr ($P=0,012$), Dp5/Dp1Rhr
302 ($p=0,018$) and Tg(*Dyrk1a*) ($P=0,010$) mice was significantly lower than respective wild-type
303 mice. Exploratory activity in a novel environment. Distance travelled (B upper panel) and % of
304 distance travelled in peripheral zone recorded in the Open field arena (B lower panel). The
305 total distance travelled was significantly higher in Ts65Dn ($p=0,022$), Tg(*Dyrk1a*) ($p=0,008$)
306 and Dp5Yah/Tg(*Dyrk1a*) ($p>0,001$). Moreover, the % of distance in the peripheral zone was
307 increased in Ts65Dn ($p>0,001$) mice compared to wild type mice. Novel Object Recognition
308 with 24 hour (C upper panel) or 1 hour retention time (C lower panel). The results are presented
309 as % of sniffing time (as box plots with the median and quartiles) for the novel object (NO).
310 For 24 hours time laps, one sample t test vs 50% (hazard) showed that Dp1Yey ($p=0,837$),
311 Dp3Yah ($P=0,173$), Ts65Dn ($p=0,432$), Dp1Rhr ($p=0,492$), Tg(*Dyrk1a*) ($p=0,144$) and
312 Dp5Yah/Tg(*Dyrk1a*) ($P=0,488$) failed to recognize the new object. The Dp5Yah genomic
313 fragment restored the capacity of the Dp1Rhr in the Dp5Yah/Dp1Rhr mice ($p=0,0157$). For 1
314 hour retention time, all the mice were able to discriminate the NO except for the Tg(*Dyrk1a*)
315 ($p=0,011$ preference for FO). Probe test session in Morris Water Maze (D). The results are
316 presented as % of time in the target quadrant. All the mice have spent more time in the target
317 quadrant versus non target excepted for the Ts65Dn mice ($p=0,398$) (* $p<0.05$, ** $p<0.01$,
318 *** $p<0.001$).

319

320 Ts65Dn model, and to a lower extent the Tg(*Dyrk1a*) one, presented marked thigmotaxic
321 behaviour, spending higher percentage of time in the peripheral zone as compared to controls
322 (S3 FigC). Finally, to check the visual ability of mice, we performed a visual training version
323 of the MWM during which the platform position is indicated by a flag. All mice were able to
324 find the visible platform without any significant difference with controls except for the
325 Tg(*Dyrk1a*) that presented a small delay in session 2 (S3 FigA).

326 We then evaluated non-spatial recognition memory using the novel object recognition
327 (NOR) paradigm with the retention time of 24h. The percentage of sniffing time for the novel
328 object was analysed and compared to 50% (hazard). This analysis showed that Dp1Yey,
329 Dp3Yah, Ts65Dn, Dp1Rhr and Tg(*Dyrk1a*) were not able to discriminate the familiar and the
330 novel objects unlike the Dp5Yah and more surprisingly the Dp5/Dp1 (Fig 1C). to further
331 characterize the effect/lack of effect of Dp5Yah mutation on novel object recognition, the
332 Dp5Yah mouse line was crossed with the Tg(*Dyrk1a*) and compared to new sets of wild-type,
333 Dp5Yah and Tg(*Dyrk1a*) mice. Interestingly, we found that the Dp5Yah/Tg(*Dyrk1a*) and as
334 expected the Tg(*Dyrk1a*) displayed altered novel object discrimination while the Dp5Yah spent

335 more time exploring the novel object than the familiar one. We also assessed Dp1Yey, Dp3Yah,
336 Ts65Dn and Tg(*Dyrk1a*) short term memory performing the NOR with 1 hour delay between
337 acquisition and retention and only the Tg(*Dyrk1a*) mice showed a deficit.

338 All the trisomic lines were also tested for associative memory in the Pavlovian fear
339 conditioning test. All the groups showed higher percentage of freezing during the 2 min post-
340 shock compared to the habituation session, indicating that all groups developed fear responses
341 during the training session (S4 Fig). When animals were re-exposed 24 h later to the same
342 context, the level of freezing in all groups was increased compared to the habituation (PRE2
343 and PRE4). However, freezing time for Ts65Dn mice was lower compared to the respective
344 control littermates. When we assessed cued fear conditioning in a new context, all the mice
345 presented an increased time of immobility with a strong difference between pre-cue and cue
346 periods (S4 Fig). In addition, Dp1Yey and slightly Ts65Dn showed lower freezing during
347 presentation of the cues as compared to wildtype counterparts. These data suggest altered
348 emotional associative memory Ts65Dn.

349

350 **Dissecting the contribution of Mmu16 sub-regions to the DS-related brain morphological** 351 **phenotypes in mouse models**

352 DS models have been reported to show brain morphological alterations of specific
353 regions (35). Thus, we wondered if we could detect changes in the brain morphology using
354 MRI on these different partial trisomic mice models. Data were first analyzed using a volume
355 approach and a brain region atlas. We confirmed that the brain of Tg(*Dyrk1a*) mice was larger
356 ($p < 0,001$) (35) and the brain of Dp1Yey mice was smaller than the respective wildtypes. Then,
357 we analyzed different brain regions/structures taking into consideration/correction the whole
358 brain volume. Even with this correction, the Tg(*Dyrk1a*) mice were the most affected in terms
359 of the brain structures volume, and on the contrary, the Dp1Rhr mice did not show any
360 significant variation compared to the wt mice. Several regions, such as the basal forebrain

361 septum, central gray matter, the rest of midbrain, and superior colliculi were significantly larger
362 in Tg(*Dyrk1a*), Ts65Dn and Dp1Yey DS models. Moreover, the cerebellum, hypothalamus,
363 inferior colliculi and caudate putamen were significantly different in Dp1Yey and Tg(*Dyrk1a*)
364 compare to the control group (S2 table, S5 Fig) and a few additional areas were altered
365 specifically in certain models (Amygdala, Globus pallidus, Hippocampus, Neo Cortex, and
366 Thalamus for Tg(*Dyrk1a*); External capsule, Fimbria, and Ventricles for Dp1Yey). Altogether
367 this brain morphometric analysis showed a greater similarity between Dp1Yey and *Dyrk1a*
368 overexpression transgenic models with intermediate overlapp with the Ts65Dn mouse model.

369

370 **Dissecting the contribution of Mmu16 subregions to the DS-related transcriptome profiles** 371 **in mouse models**

372 Various studies have shown the consequences of trisomy on gene expression (61-70).
373 Here we took the opportunity to dissect the alteration of gene expression and functional
374 pathways in various DS trisomic models carrying different duplications of the Mmu16. We
375 analyzed the Ts65Dn, Dp1Yey, Dp3Yah, Dp5/Dp1, Dp5Yah, Dp1Rhr, and we included the
376 trisomic model for *Dyrk1a* alone, Tg(*Dyrk1a*). Considering the hippocampal formation as a hub
377 structure involved in learning and memory, we performed gene expression analysis in the adult
378 hippocampus comparing the DS models with their own littermate controls using a unique
379 pipeline for all the models. For each DS model, we defined the expressed genes (noted as EGs)
380 as the genes whose expression level was detected, the differentially expressed genes (noted as
381 DEGs) as the genes whose expression level was found to be significantly altered in the trisomic
382 model compared to the controls littermates, and then the trisomic expressed genes (TEGs) as
383 the DEGs that are included inside the duplicated chromosomal regions for each model (S3 table,
384 Table 1).

Mouse lines	Dp1yey	Dp3Yah	Ts65Dn (Mmu16)	Dp5/Dp1	Dp5Yah	Dp1Rhr	TgDyrk1A	Ts65Dn (Mmu17)
Nb of probes detected by the array								35556
Nb of annotated probes detected (without control probes)								27359
Number of trisomic genes detected by the array	155	21	130	127	87	40	1	40
Differential expressed genes (DEGs)	711	826	1074	922	736	1306	850	
Differential expressed trisomic genes (DETGs)	66	13	64	54	39	18	1	28
% of **DETGs	43%	62%	49%	43%	45%	45%	100%	70%
% of compensated trisomic genes detected	57%	38%	51%	57%	55%	55%	0%	
Number of GAGE KEGG and GOs (CC,BP,MF) terms disregulated in the trisomic model FDR<0.1	244	67	12	111	318	225	231	
Number of GAGE KEGG and GOs (CC,BP,MF) terms upregulated in the trisomic model FDR<0.1	207	60	3	33	4	132	222	
Number of GAGE KEGG and GOs (CC,BP,MF) terms downregulated in the trisomic model FDR<0.1	37	7	9	78	314	93	9	
Number of GAGE KEGG and GOs (CC,BP,MF) terms in the trisomic model unique to each mice line FDR<0.1	80	1	2	30	195	107	64	

*DEGs/DETs: Differential expressed genes/ Differential expressed

**DETGs/DETTs: Differential expressed trisomic genes/

385

386

387 **Table 1.** Differential expression analysis results of the seven models analysed. DEGs are
 388 Differential Expressed Genes and TEGs for Differential Expressed trisomic genes. * Analysis
 389 done with Mmu17 and 16 trisomic genes. GO are Go functional terms involved in cellular
 390 compartment (CC), molecular function (MF) and biological processes (BP).
 391

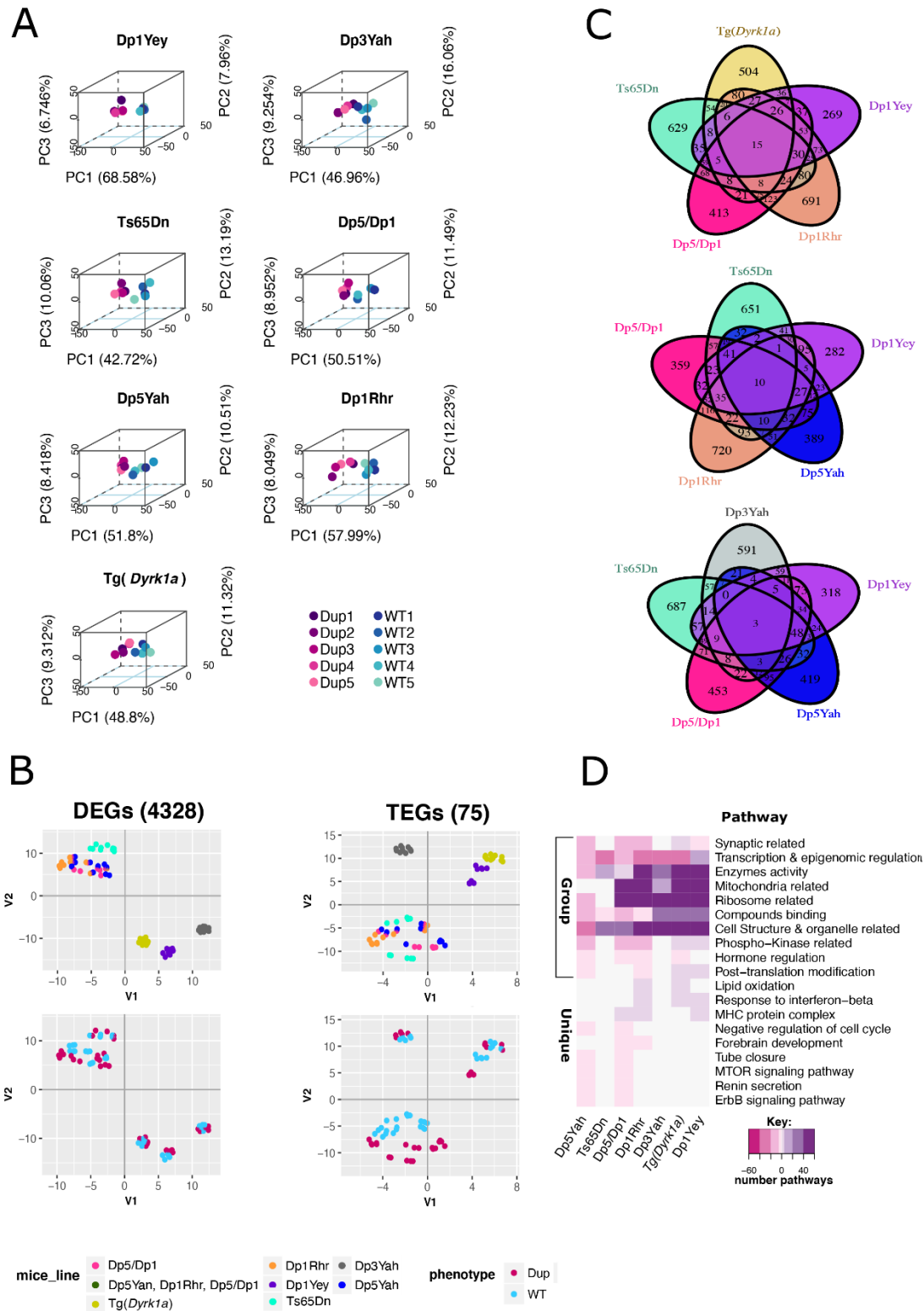
392 Although most of the genes in 3 copies were overexpressed in the relevant mouse model-
 393 derived hippocampi with a ratio around 1.5 (S6 Fig), from 38% to 57% of the trisomic genes
 394 showed a dosage compensation (Table 1, S3 table). While this compensation is expected, we
 395 noticed that most of the compensated genes behave similarly in the different trisomic context,
 396 even if the experiments were done independently. As such the genes from *Cldn17* to *Krtap11-*
 397 *1*, including the keratin cluster were not overexpressed when trisomic in any model (Dp1Yey,
 398 Ts65Dn, Dp5/Dp1 or Dp5Yah). This could be due to the fact that this region seems to be under
 399 strong regulatory constrains as on the borders two REST sites plus a LaminB1 peak
 400 encompassing this region were found (UCSC browser), while *Btg3* (*BTG Anti-Proliferation*
 401 *Factor 3*) and *C21orf91* (*Chromosome 21 open reading frame 91*, also known
 402 as *D16Erd472e*), or *Mrpl39* (*Mitochondrial Ribosomal Protein L39*), *Jam2* (*Junctional*

403 *Adhesion Molecule 2*), *Atp5J* (*ATP synthase peripheral stalk subunit F6*), *Gabpa* (*GA binding*
404 *protein transcription factor subunit alpha*) and *App* (*amyloid beta precursor protein*) are
405 overexpressed in various DS models. We also found that the genes located on the trisomic
406 segment not homologous to Hsa21 on the Mmu17 in Ts65Dn hippocampi, were overexpressed
407 (S7 Fig). This was also observed in the Ts65Dn heart in a previous study (16). Looking in detail
408 into the homologous Hsa21 region in Mmu17, we saw two main genetic effects due to the
409 overdosage of the Mmu16 region homologous to Hsa21. Noticeably, *Cbs*, coding for the
410 Cystathionine beta-synthase, another driver gene for DS cognitive phenotypes (71), was found
411 down-regulated in all the models, except Dp3Yah and Tg(*Dyrk1a*); suggesting a direct control
412 of the *Cbs* transcription by at least two loci, one located in the Dp5Yah, and another one, not
413 due to *Dyrk1a* overdosage, in the Dp1Rhr trisomic region. Similarly, under-expression of the
414 *glucagon like peptide 1 receptor (Glp1r)* was observed in Dp1Yey, Ts65Dn, Dp5/Dp1 and
415 Dp5Yah. Oppositely, this gene was overexpressed in Dp1Rhr and not affected in Tg(*Dyrk1a*).

416 Here too, *Dyrk1a* dosage was not involved, but at least two loci controlling *Glp1r*
417 expression with opposite and epistatic effects should be found respectively in the Dp5Yah and
418 Dp1Rhr genetic intervals. Thus a complex genetic interaction took place between different loci
419 and controlling subsequent gene expression.

420

421 The analysis of DEGs in each model separately highlighted the capabilities to separate
422 the trisomic individuals from the wild-type littermates (Fig 2A) by principal component
423 analysis (PCA), t-SNE (Fig 2B) or OPLS techniques (See supplementary information). A
424 genome-wide misregulation was found independently of the model, as DEGs were spread in all
425 the chromosomes (S3-S4 tables, S8 Fig), as shown previously (67), although with a stronger
426 impact of the Dp1Rhr duplication on the number of total DEGs detected. The most
427 overexpressed genes in terms of log₂ fold change (log₂FC) of expression and significance in
428 various genetic conditions were visualised using Volcano plots (S9 Fig, S4 table). For example,



429

430 **Figure 2: The differential expression analysis discriminates trisomic from disomic**
 431 **hippocampi and identifies common dysregulated genes and pathways.** (A) 3D-PCA on the
 432 DEGs for each sample allows to separate trisomic (Dp) from disomic (wt) adult hippocampi.
 433 (B) Left column: two dimensional Principal Component Analysis (2D-PCA) on the 5599
 434 transcripts of the 4328 DEGs over all the samples identified using $f_{cros} 0.025 < \alpha < 0.975$. Right
 435 column: 2D-PCA on the 75 trisomic genes with a measured expression in all the models. (C)
 436 Venn Diagrams showing the overlap in gene expression between the different mouse lines
 437 represented in different colours. (D) Heatmap representation of the number and regulation sense

438 of the pathways shared at least by two mice lines identified using the genome expression for
439 each mice line by GAGE R package and filtered by q-value cut off < 0.1 . Grouped in the
440 categories showed on the ordinate: synaptic related, synaptic related: representation of the
441 pathways involved in Myelin sheath and SNARE complex formation, synaptic related: all the
442 synaptic related pathways excluding myelin sheath and SNARE complex formation,
443 Transcription & epigenomics regulation, Enzymes activity, Ribosome related, Mitochondria
444 related, Cell Structure & organelle related, Phospho-kinase related... The color key breaks
445 represents the number of pathways within the categories 60,40,20,5,0.5. The minus or pink
446 color represents down regulated pathways, the white color represents no pathway found in the
447 category and the purple or positive numbers stands for up regulated pathways respectively.
448

449 the *listerin E3 ubiquitin protein ligase 1 (Ltn1)* gene, coding for a major component of the
450 ribosome quality control and causing neurodegeneration in mice (72), was found overexpressed
451 in Dp1Yey, Ts65dn, Dp5/Dp1, and Dp5Yah or *Ifnar2*, coding for the Interferon Alpha and Beta
452 Receptor Subunit 2, is overexpressed as expected in models that carry three copies of this gene
453 (Dp1Yey, Ts65Dn, Dp5/Dp1 and Dp5Yah). Instead, a more controlled gene like the *neuronal*
454 *acetylcholine receptor subunit alpha-3 (Chrna3)*, is found upregulated only in Dp1Rhr and
455 Dp1/Dp5, certainly due to the overexpression of one gene from the *Cbr1- Fam3b* region but
456 not *Dyrk1a*. Nevertheless, when we performed the intersection between the list of DEGs from
457 the different models, we only found a few genes in common (Fig 2C, S4 table). We decided to
458 combine the analysis of all the lines together using PCA and t-SNE and revealed a strong
459 clustering of models that share the same number of trisomic genes (Fig 2B). t-SNE analysis,
460 based on 4328 DEGs, adding all DEGs detected in each mouse model together, showed
461 different contribution of the various DS models to the transcriptome variation (Fig 2B, left
462 panel) with 2 distinct groups: one encompassing four overlapping trisomies: Ts65Dn, Dp5/Dp1,
463 Dp5, Dp1Rh and three isolated models: Dp1Yey, Dp3Yah and Tg(*Dyrk1a*) that are closer
464 together although Dp3Yah is clearly farthest from the other two. Similar distinct groups were
465 seen when analysing the TEGs (Fig 2B, right panel) and overall, the trisomic and the wild-type
466 individuals in each mouse line were nicely separated. As expected, the expression level of the
467 TEGs and the DEGs in the different trisomic conditions were strongly correlated (S10 Fig).
468 Interestingly, the 4328 DEGs showed a level of mis-regulation strongly correlated between

469 Dp1Yey and Dp3Yah (33%), Dp5/Dp1 (50%), Dp1Rhr (40%) and Tg(*Dyrk1a*) 42%. Of the 75
470 TEGs, the correlation was quite strong between Dp1Yey and Tg(*Dyrk1a*) with 28%. Thus, the
471 correlation in gene deregulation showed that *Dyrk1a* overdosage is a key driver of
472 transcriptome deregulation in the Dp1Yey and Dp1Rhr models. Unexpectedly, the correlation
473 of DEGs mis-expression level was lower between Ts65Dn and Dp1Yey (29%) or Dp1/Dp5
474 (28%). On the opposite, a high number of TEGs are mis-regulated in the same way between
475 Ts65Dn and Dp1Yey (49%) or Dp1/Dp5 (52%; S10 Fig) suggesting that the other region found
476 in 3 copies in the Ts65Dn over Mmu17 must be affecting the general DEGs landscape. We
477 confirmed by qPCR the mRNA overexpression of first, *Dyrk1a* and *Sod1* genes in the DS
478 models where they are trisomic; second, of *Synaptojanin2* and *T lymphoma invasion and*
479 *metastasis inducing gene 2* that are located on the Mmu17 centromeric region in the Ts65Dn
480 and third, of *Cholinergic Receptor Nicotinic Alpha 1 Subunit (Chrna1)* a gene misregulated in
481 Dp1Yey, Dp5/Dp1, Dp1Rhr and Ts65Dn models (S15 fig). As expected *Cbs*, expression was
482 down regulated in all the models except Tg(*Dyrk1a*) and Dp3Yah. We also detected alterations
483 of the expression of immediate early-response genes *Arc*, *FosB*, *Fos* and *Npas4* that are
484 important for cognition.

485

486 **Differential functional analysis unravel a few common altered pathways in DS models**

487 To go further, we performed a differential functional analysis and found 12 to 318 mis-
488 regulated pathways in the DS models (table 1, S5 table). Interestingly, the regulation of
489 pathways is trisomic region-dependent, as the Dp5Yah (99%) region produced an overall
490 downregulation whereas the Dp3Yah (89.5 %) and Dp1Rhr (56 %) regions together with the
491 full Hsa21 syntenic model Dp1Yey (84.8 %), an upregulation. To facilitate the understanding,
492 we clustered the broad functional dysregulation into 8 major functionality groups or meta-
493 pathways. We found ribosomal components and mitochondrial processes pathways altered in
494 all models with a high number of intermodel shared genes (S12 Fig). Others meta-pathways

495 like cell structure and organelles, transcription and epigenetic regulation, interferon and
496 synaptic pathways were more affected in some models than in the others (S13 and S14 Fig). As
497 such, we observed strong and connected effects in the control of transcription and epigenetic
498 regulation, enzyme activity and cell structure and cellular organelles involved in membrane and
499 protein processing (endoplasmic reticulum, Golgi body, lysosome, peroxisome,...; Fig 2D) in
500 the Dp1Yey, Dp5/Dp1, Dp1Rhr, and Tg(*Dyrk1a*) models, whereas the myelinization and 10
501 SNARE components, such as the *Synaptosome Associated Protein gene 25 and 23* (*Snap25* and
502 *Snap23*), were specifically dis-regulated in the Dp1Yey Dp5Yah and Tg(*Dyrk1a*) models.

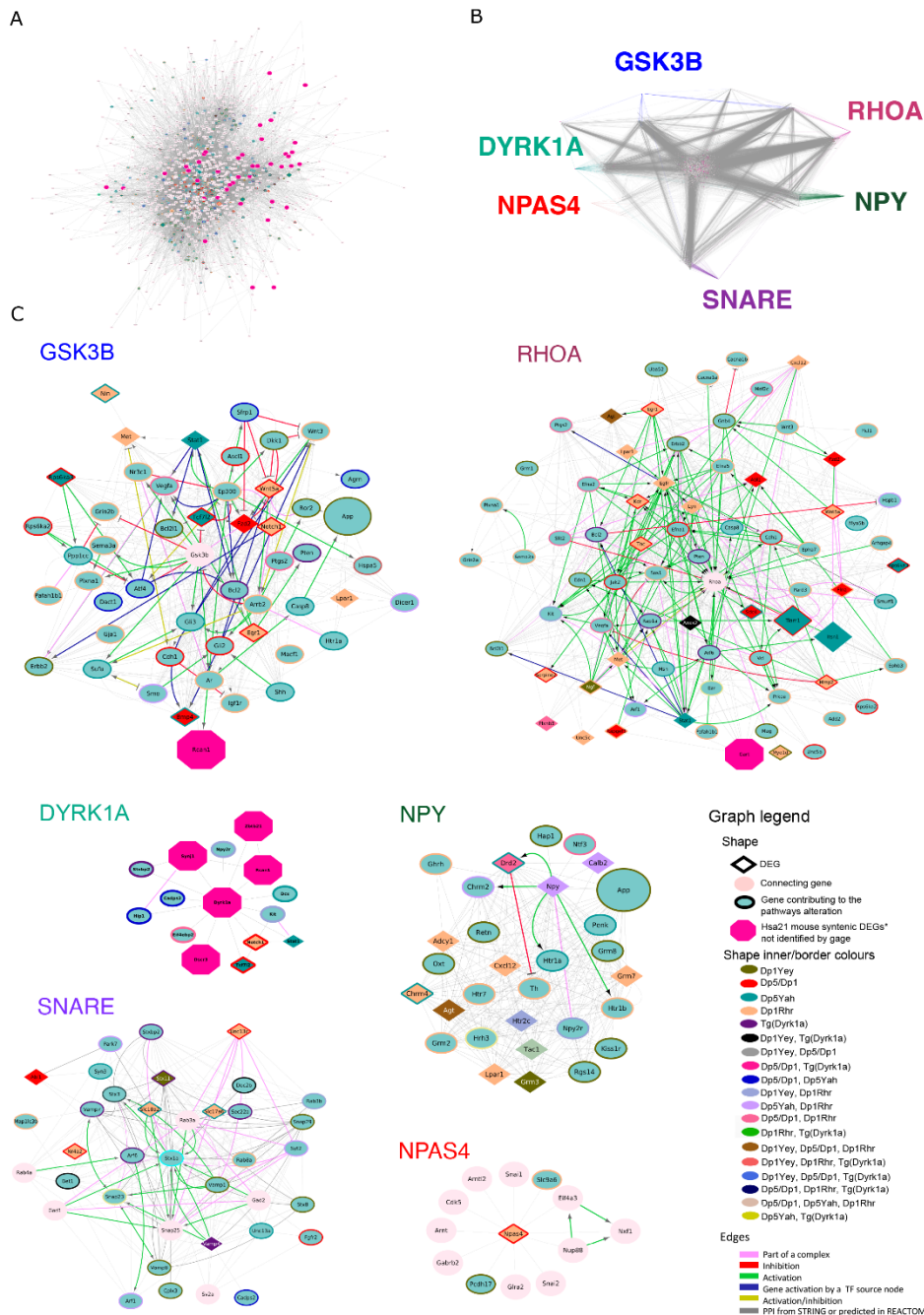
503
504 Interestingly, we saw a high number of shared genes between these pathways and the models
505 giving rise to a high intermodel pathway connectivity. To assess the gene connectivity & cross
506 talk, we build a protein-protein interaction (PPI) network of genes involved in synaptic function
507 (noted MinPPINet) and furthermore we added regulatory PPIs to build the final RegPPINet.
508 Then, we analysed the betweenness centrality to identify hubs, keys for maintaining the network
509 communication flow that are most susceptible to targeted drugs attacks and the main signalling
510 cascades affected. The relevance of the connecting nodes unknown to play a role in brain
511 dysfunction, was further predicted by the machine learning algorithm Quack¹¹⁸ and we found
512 several evidences linking 449 (22 with a high confidence score) DEGs to GO/KEGG pathways
513 involved in brain dysfunction, not previously known (S5 table). In fact, several evidences make
514 us consider that those genes should be included in the pathways:gene associations. Inside the
515 evidences we can point that these DEGs were found dis-regulated in hippocampus, were
516 included in the network built by STRING⁷³ as connecting nodes (not seeds) to produce a fully
517 connected DS network, and their relevance to brain dysfunction was also predicted by Quack,
518 in some cases, with a high score.

519 Looking into the DS synaptic MinPPINet (Fig 3A), first we analysed the DS network
520 topography and betweenness connectivity and found hubs and genes more central for the

521 network information flow. As expected from a PPI biological network, after computing 100000
522 random networks with a similar degree, the likelihood of observing such connectivity in the DS
523 network was more than one can expect by chance (P-value < 2e-16) and it showed a small world
524 effect and scale-free topology. Using a network decomposition approach (see supplementary
525 information), we highlighted 6 major subnetworks or biological cascades that strongly
526 centralized 6 different proteins: DYRK1A, GSK3B, NPY, RHOA, SNARE and NPAS4
527 proteins (Fig 3B-C). Here, ten genes from the SNARE complex were dysregulated, from those,
528 we validated the dysregulated expression of *Snap25* and *Snap23* by qRT-PCR (Fig 4F).

529 The network analysis showed that DYRK1A controls 42,3 % of the network nodes and
530 69,4 % of the network seeds, (DEGs known to be involved in brain synaptic pathways) via 2nd
531 level interactors. Hence, DYRK1A could control the DS synaptic network via PPI and
532 regulatory interactions. Furthermore, the biological cascades centred on GSK3B, DYRK1A and
533 RHOA are highly interconnected (S15 fig) and in fact several interactors of RHOA are
534 connected and could somehow modulate a higher percentage (75 and 68.5%) of the nodes of
535 the network and synaptic seeds. Interestingly, *RhoA* was not found altered in the differential
536 expression analysis, instead it was a node introduced in the network to have a fully connected
537 synaptic PPI network. We checked whether the RHOA pathway was altered in the Dp1Yey (Fig
538 4A). We found no changes in the expression of RHOA, confirming the transcriptomics
539 analyses, but a significant decrease of the phosphorylation of the c (P-MLC) in the Dp1Yey
540 hippocampi compared to control. Thus, the RHOA pathways appeared to be down-regulated in
541 the Dp1Yey DS mouse models.

542



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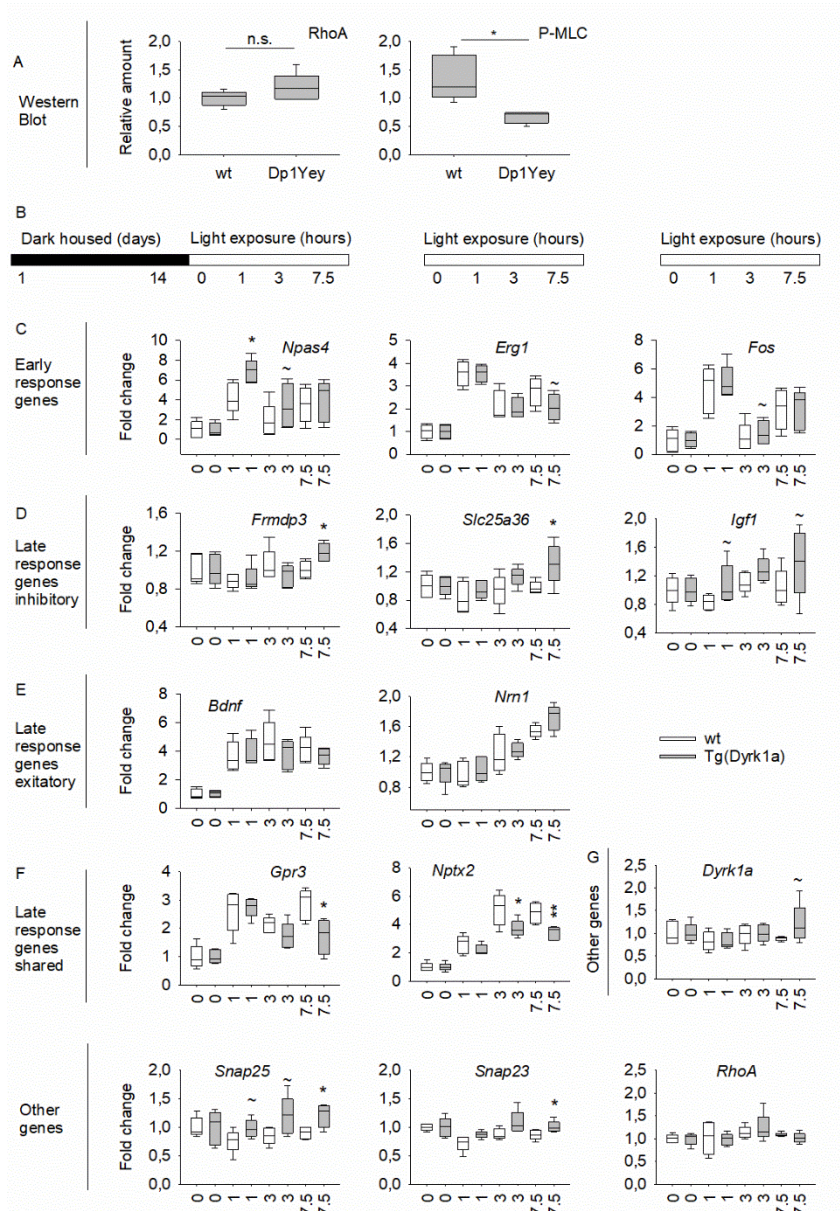
545 **Figure 3: Protein-protein interaction networks involving DEGs linked to the synaptic**
 546 **function.** (A) STRING04 MinPPINet of genes involved in synaptic function visualized using
 547 the edge weighed spring embedded layout by betweenness index in Cytoscape. The network was
 548 built by querying STRING and selecting the PPIs with a medium confidence score (CS=0.4)
 549 coming from all sources of evidence. The shapes of the nodes represent the following
 550 information: Shapes: i) Pallid pink ellipses: represent connecting proteins added to assure the
 551 full connectivity of the network; ii) pink octagons, represent HSA21 syntenic genes in mouse
 552 not identified as contributing to the meta-pathway dysregulation by GAGE; iii) green inner
 553 coloured ellipses, genes identified by GAGE after q-val <0.1 cut off to be contributing even
 554 slightly, to any pathway of those found dysregulated inside the meta-pathway. If the size is
 555 similar to the octagons, they are also HSA21 syntenic genes in mouse. Additionally, the border
 556 colour represents the mouse model multi group where those genes are found altered in; iv)
 557 diamonds, genes identified by GAGE after q-val <0.1 cut off and also by FCROS as DEGs.

558 (B) Network Structure Decomposition of the STRING04 MinPPINet. Highlighting in different
559 colors the interactions of GSK3B, NPY, SNARE proteins, DYRK1A and RHOA respectively.
560 In the case of NPAS4, the interactions coloured correspond up to the first level interactions.
561 (C) The six RegPPINets were extracted from the selection of each fo the following proteins and
562 their 2nd interactors from STRING04 MinPPINet: RHOA, DYRK1A, GSK3B, NPY, SNARE
563 proteins and NPAS4. Then, those were further annotated with regulatory information using
564 REACTOME (See Supplementary information). The shapes of the nodes represent the
565 following information: Shapes: i) Pallid pink ellipses: represent connecting proteins added to
566 assure the full connectivity of the network; ii) pink octagons, represent HSA21 syntenic genes
567 in mouse not identified as contributing to the meta-pathway dysregulation by GAGE; iii) green
568 inner coloured ellipses, genes identified by GAGE after q-val <0.1 cut off to be contributing
569 even slightly, to any pathway of those found dysregulated inside the meta-pathway. If the size
570 is similar to the octagons, they are also HSA21 syntenic genes in mouse. Additionally, the
571 border colour represents the mouse model multi group where those genes are found altered in;
572 iv) diamonds, genes identified by GAGE after q-val <0.1 cut off and also by FCROS as DEGs.
573 The edges colored represent the type of interaction annotated by following the PathPPI
574 classification (Tang *et al.* 2015), and ReactomeFIViz annotations as follows i) The GERel edges
575 indicating expression were colored in blue and repression in yellow. ii) PPrel edges indicating
576 activation were coloured in green, inhibition in red. Iii) Interactions between proteins known to
577 be part of complexes in violet. Iv) Predicted interactions were represented in grey including the
578 PPI interactions identified by STRING DB (Szklarczyk *et al.* 2017) after merging both
579 networks.
580

581 **Npas4 pathway is up-regulated as a consequence of *Dyrk1a* overexpression**

582 In our transcriptomics and network analysis, we found that *Npas4* was down-regulated
583 in Tg(*Dyrk1a*), Dp1Rhr and Ts65Dn models and this gene level of expression is highly variable
584 in the models (S11 Fig). *Npas4* belongs to the class of immediate early genes with *Arc*, *Fosb*
585 and *Fos*. Light deprivation and then exposure is known to drive robust gene expression, with
586 shared early-response genes and distinct sets of late-response genes in excitatory and inhibitory
587 (E/I) neurons (73). *Npas4* was recently identified as a key in E/I balance in the visual cortex
588 after light deprivation (74).

589 To confirm the impact of *Npas4* down regulation in Tg(*Dyrk1a*) mice, we performed qRT-PCR
590 experiments to determine the specific early and late response genes altered in the visual cortex
591 after light deprivation at 3 time points (1, 3 and 7.5 hours) after de novo light exposure (Fig
592 4B). The results showed that *Npas4* was clearly induced after light deprivation following 1 hour
593 of light stimulation. Expression data revealed also a strong overexpression of *Npas4* following
594 1 hour of light stimulation in Tg(*Dyrk1a*) mice (Fig 4C). On the contrary, the expression of late



595

596 **Figure 4: Evaluating *Npas4* and *RhoA* pathways in DS models** (A) RHOA pathway was
 597 altered in the Dp1Yey. Western blot analysis was revealed no changes in the expression of
 598 RHOA but a significant decrease of the phosphorylation of the Myosin light chain (P-MLC) in
 599 the Dp1Yey hippocampi compared to control. (B) Mice were housed in total darkness for 14
 600 days and then were subsequently exposed to light for 0, 1, 3 or 7.5 h. Relative expression levels
 601 were determined, and fold change were calculated for each condition. Genotypes differences in
 602 fold change were assessed by T Test. (C) Only the fold change for early response genes *Npas4*
 603 was up-regulated in Tg(*Dyrk1a*) mice compared to wt at 1 hours of light induction. (D) The late
 604 responses genes specific to inhibitory neurons *Frm dp3*, *Slc25a36* and *Igf1* were up-regulated
 605 after 7.5 hours of light induction. (E) The fold change of late responses genes specific to
 606 excitatory neurons *Bdnf* and *Nr n1* were unchanged. (F) The fold change of late response genes
 607 shared by excitatory and inhibitory neurons *Gpr3* and *Nptx2* were downregulated after 3 and/or
 608 7.5 hours of light induction. (G) *Dyrk1a* and *RhoA* showed a similar fold change along the
 609 different condition whereas *Snap25* and *Snap23* presented an increased enrichment for the 7.5
 610 hours condition. Data are presented as box plots with the median and quartiles.
 611

612 response genes specific for inhibitory neurons (*Frmdp3*, *Slc25a36* and *Igf1*, Fig 4D) and late
613 response genes (*Grp3* and *Nptx2*, Fig 4F) were altered after 7.5 hours of light stimulation in
614 Tg(*Dyrk1a*). Interestingly, late response genes specific to excitatory neurons (*Bdnf* and *Nrn1*,
615 Fig 4E) were not affected. The *Snap25*, *Snap23* candidate genes found in our analysis showed
616 an altered expression after 7.5hours of light stimulation while *Dyrk1a* and *RhoA* levels were
617 not affected (Fig 4F). These results indicate that inhibitory pathways controlled by *Npas4* were
618 affected by *Dyrk1a* overexpression. Furthermore, the network analyses highlighted NPAS4 as
619 a potential modulator of the synaptic dysfunction as via well connected interactors, NPAS4
620 could affect the main altered biological cascades plus the GABA and NMDA receptors involved
621 in the modulation of the excitatory / inhibitory balance of the brain (73).

622

623 **Discussion**

624 In this study, we explored the in vivo DS mouse model library focusing on the region
625 homologous to Hsa21 found on Mmu16, to decode the DS genotype-phenotype relationships
626 and further underlie genetic interactions between different regions. For this, we assessed 6
627 partial DS trisomic models, a transgenic model overexpressing one copy of *Dyrk1a*, plus some
628 additional combination of models, using a standardized behavioural pipeline focused on
629 hippocampus-dependent memory processes.

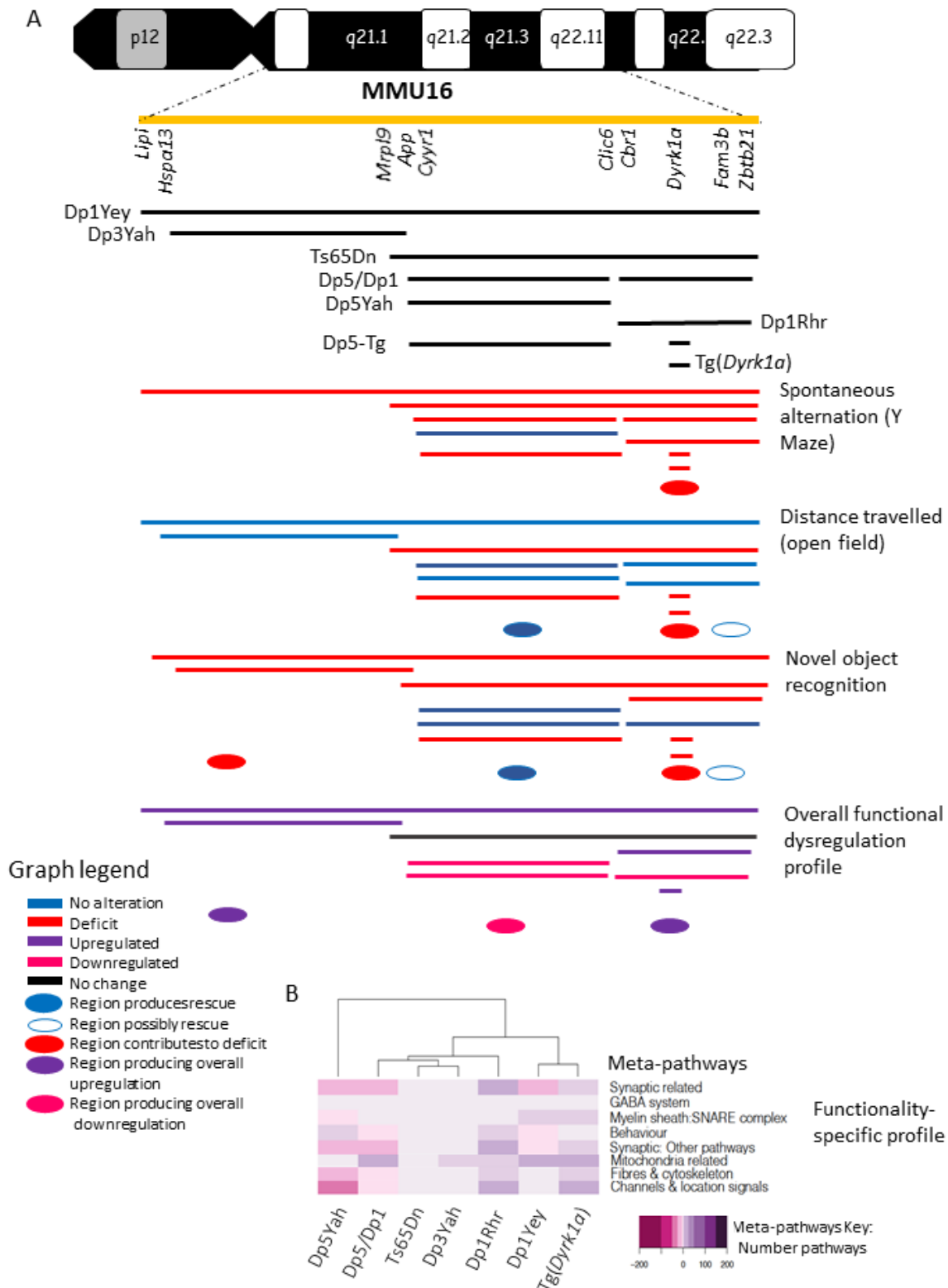
630 In this unique parallel comparison, we found that the spontaneous alternation in Y-maze,
631 was altered in most of the models (except the Dp5Yah alone; Fig 5). The minimal common
632 genetic part of these lines was the overexpression of *Dyrk1a* and the result observed for the
633 transgenic Tg(*Dyrk1a*). Altogether, our results previously obtained, support DYRK1A as a
634 main driver of this DS cognitive phenotype (25). wSimilarly, DYRK1A overdosage is a major
635 cause for the increased exploration in the open field in Ts65Dn, Dp5/Tg(*Dyrk1a*) and
636 Tg(*Dyrk1a*), but not in other models. While the Dp1Rhr Ds model was not affected, one can
637 hypothesized that another loci interfere with *Dyrk1a* overdosage in this model. The situation

638 should be even more complex with more additional genetic interaction. No phenotype is
639 observed for the distance travelled in the Dp1Yey, Dp5/Dp1, Dp5 or Dp1 models while the
640 Ts65Dn, the Dp5-Tg(*Dyrk1a*) and the Tg(*Dyrk1a*) models showed an increase in this variable.
641 Thus the overexpression of *Dyrk1a* is able on its own, or combined with Dp5Yah, to induce the
642 increase in distance travelled while some loci which are not trisomic only in the Dp5/Dp1 model
643 are able to suppress this effect, that can be reinduced by other trisomic loci specific to the
644 Ts65Dn (see below). Altogether our results suggest that many different genetic influence (at
645 least 3 for the distance travelled) acts on different behavioural variables in DS models

646 Similar analysis of the novel object recognition test with 2 distinct retention time highlight
647 several loci. Indeed the phenotype of novel object recognition test, with 1h of retention time,
648 only pointed to *Dyrk1a* overexpression, with at least one suppressing loci in the Dp1Rhr
649 trisomic region. The novel object recognition with 24h of retention unravelled deficiency in
650 most of the models, except in the Dp5Yah and Dp5/Dp1, suggesting that there are at least two
651 causative loci: one located in the Dp3Yah region and *Dyrk1a*; and presumably two modifier
652 interacting loci: one located in the Dp5Yah and another in the Dp1Rhr regions (Fig 5).
653 Altogether our results suggested that depending on the variable observed in the behavioural
654 test, many genetic interaction takes place to build the behavioural phenotyping outcome in DS
655 mouse models with several loci, spread along the Mmu16, including *Dyrk1a*. Altogether these
656 loci interacted with different degrees of complexity to induce similar behavioural phenotype
657 outcomes.

658 As expected, we found deficits in the Ts65Dn mice similar to the ones previously
659 published in the Y-maze, the open field, NOR, MWM and contextual fear conditioning (52, 75)
660 tests. Strikingly, thigmotaxis and time in the target quadrant in the probe test of the MWM
661 were two variables only modified in the Ts65Dn trisomic model while the Dp1Yey, that carry
662 a duplication of the complete syntenic Mmu16 region, was less affected as described before
663 (76, 77). Remarkably, the Dp5Yah mice, with a duplication from *Cyrr1* to *Clic6*, displayed no

664



665

666 **Figure 5: Genotype correlation associated to behaviour phenotype in partial trisomic DS**
 667 **model.** Here we highlight the duplicated region carried on each model with the corresponding
 668 syntenic region in the human chromosome 21 together with the main behavioral and
 669 transcriptomics results pointing to the existence of region specific phenotypes and functional

670 alterations. The black lines represents the duplicated syntenic regions to human chromosome
671 21 on each model (represented in the yellow line). The blue lines represents the behavioral
672 results where no alteration was found, instead the red lines identified the tests with deficits. Over
673 the transcriptomics meta-pathways functional profile summary picture, in purple is highlighted
674 upregulation whereas in pink downregulation. The intensity of the color stands for the number
675 of pathways included on each meta-pathway from the total number of pathways found altered
676 on each model.

677
678 deficits on its own in all the tests done and showed the lower number of DEGs in the
679 hippocampi. This might indicate that the *Cyrr1-Clic6* region was not sufficient to induce by
680 itself cognitive defects. However, this syntenic region includes the genes *Sod1*, *Olig1*, *Olig2*,
681 *Rcan1* and *Synj1*, proposed before as inducing early DS cognitive phenotype (78-81).
682 Nevertheless, no defects were found in the Dp5/Dp1 mice contrary to the Ts65Dn in the open
683 field or less severe in the NOR test (see below). This indicates the existence of a key modulator
684 in the *Cyrr1-Clic6* region. The major behavioural alterations found in the Ts65Dn should result
685 from the influence of different factors: first the genes homologous to Hsa21; then the presence
686 of the freely segregating mini-chromosome (82), and also the trisomy of about 60 Mmu17
687 centromeric genes, non homologous to Hsa21(16), and overexpressed in the hippocampus of
688 Ts65Dn mice. Especially the overdosage of *Tiam2* and *Synj2*, located on the Mmu17
689 centromeric region, could exacerbate the effect of the overexpression of their respective
690 paralogs *Tiam1* and *Synj1* (81, 83). Strikingly, transcriptomic analysis showed a different global
691 disruption of the genome expression in the Ts65Dn hippocampi, compared to the other trisomic
692 models with segmental duplications. This is emphasized by the low correlation of DEGs and
693 deregulated pathways between Ts65Dn, Dp1Yey and Dp5/Dp1. Overall, we can hypothesize
694 that there is a suppression effect in the Dp1Yey model compared to the Ts65Dn induced
695 phenotypes, due either to genes overexpressed and located upstream of *Mrpl39*, or to genes
696 located on the non-homologous region in the Ts65Dn minichromosome, or to the freely
697 segregating minichromosome.

698 Similar to cognition, brain morphology was affected differently in some DS models. For
699 example, the Dp1Rhr rather showed an increase of size in many brain region compared to the

700 other DS models, where a decrease of size is observed like in Dp1Yey, Ts65Dn and
701 Tg(*Dyrk1a*). Changes were found in several regions including the basal forebrain septum, a
702 predominant source of cortical cholinergic input with an early substantial loss of basal forebrain
703 cholinergic neurons (BFCN); as a constant feature of Alzheimer's disease and other deficits in
704 spatial learning and memory (84). Individuals with DS exhibit progressive degeneration of
705 BFCN (85). Similarly, significant loss of BFCN is present in the Ts65Dn (for review see (86))
706 and moreover, our results seem to indicate a default in BFCN in Dp1Yey and Tg(*Dyrk1a*) too.
707 Several findings suggest that overexpression of amyloid protein precursor (APP) play a major
708 role and could be strictly necessary for BFCN cell loss (87), thus, future investigation in
709 Tg(*Dyrk1a*) should reveal if BFCN loss is present even without APP overexpression. Besides,
710 only the Ts65Dn present an enlargement on the ventricles, which was previously associated
711 with a decrease of cortical neurogenesis in the brains of Ts1Cje and Ts2Cje mouse models (33).

712 The comparative genome wide expression profiling in the mouse hippocampus revealed
713 that not a single gene or a single region can recapitulate the whole dysregulation. The overall
714 effect results from a complex interplay of a few trisomic overexpressed genes and other genes
715 spread along the genome, shown by the majority of DEGs not being Hsa21 genes (S3 table).
716 Additionally, we identified 34 trisomic DEGs (TEGs) with regulatory activity (Transcription
717 factors, chromatin modellers...) as *Mir99a*, *Usp16*, *Erg* or *Rcan1* that may be involved in the
718 models' changed regulatory landscape. Indeed, *RCAN1* and *USP16* were found upregulated in
719 all human brain datasets (cerebrum and cerebellum) and *USP16* was also found as DEG
720 upregulated in heart and adult fibroblasts while *MIR99A* was found upregulated in adult
721 fibroblast (62). Nevertheless, the DEGs expression was strongly correlated and conserved in
722 Mmu16 based DS models. This is similar to the behavioural results obtained where related
723 phenotypes were found in models carrying correlated partial duplications. Unexpectedly,
724 Dp1Yey DEGs correlation is closer to Tg(*Dyrk1a*) than to Ts65Dn (42% against 25%) and
725 there is a negative correlation between Dp3Yah & Dp5Yah (22%) and Tg(*Dyrk1a*) & Dp5Yah

726 (13%) pointing out to a different gene dysregulation on these models and to the existence of
727 epistasis with some regulatory trisomic genes countering the effect of genes in other trisomic
728 regions. Carrying an in depth functional annotation analysis, we could not find a unique
729 common pathway altered in all models. Nevertheless, due to the high redundancy in both GO
730 and KEGG terms and the genes associated with each term, grouping the pathways shared at
731 least by two models (materials & methods) in 8 functional groups unravelled 8 major meta-
732 pathways with ribosome and mitochondrial function, transcription & epigenomics regulation,
733 and the synapse function categories highly affected. We also found a strong upregulation of
734 genes involved in the InterferonB pathway (S13 FigB) as some interferon receptors are found
735 upregulated in Mmu16 DS models. As such *Ifnar2* and *Ill10rb* were found upregulated in all the
736 mice lines (except Tg(*Dyrk1a*)) pointing to a potentially critical role in the interferon pathway
737 dysregulation. The same was observed with other genes as *Irgn1*, *Ifit1*, *Ifit2* or *Ndufa13*. This
738 upregulation of the interferonB pathways was previously reported in the Ts1Cje mouse model
739 (88, 89) and linked to a possible increase of activity of the Jak-Stat signaling pathway as
740 recorded here by the up regulation of *Stat1*.

741 Expression of genes involved in long-term synaptic potentiation (LTP) and synaptic
742 plasticity were decreased in Dp1Yey, Dp5Yah, Dp1Rhr, Dp5/Dp1 models respectively,
743 corroborating previous reports in different DS mouse models and in vitro studies. The only
744 upregulated pathways were the myelin sheath and SNARE; both found in Dp1Yey and
745 Tg(*Dyrk1A*) models. Interestingly, models carrying the Dp1Rhr region duplication showed a
746 dysregulation mainly in synapse transmission, plasticity and LTP, while models carrying the
747 Dp5Yah region duplication showed a dysregulation associated with genes involved in stemness
748 and differentiation. Together, the models with both Dp5Yah & Dp1Rhr duplicated regions,
749 were involved in post-synapse modulation and transmission. Moreover, there is a high number
750 of shared DEGs between these pathways and the models giving rise to a high intermodel inter-
751 pathway connectivity with genes strongly connected into major subnetwork biological cascades

752 centralized over 6 different cascades controlled by DYRK1A, GSK3B, NPY, SNARE complex,
753 RHOA and NPAS4 that play a crucial role in the brain function. DYRK1A is a well recognized
754 driver of DS phenotypes and the target of several therapeutic approaches (25, 51). DYRK1A
755 was also shown to have interaction with GSK3B and NPY in DS models (25, 90-94). SNARE
756 complex protein were affected and it is interesting to see that they were also found modified in
757 a DS model for the region homologous to Hsa21 on Mmu17 (71). RHOA is a member of the
758 RHO GTPase involved in several intellectual disabilities that affects dendritic structure in adult
759 neurons (95-98), a phenotype also described in some DS models (99-101) or linked to
760 DYRK1A (102, 103).

761 Organizing the network to follow the betweenness centrality index values, RHOA,
762 DYRK1A, GSK3B, and their interactors were more closely knitted together and populated the
763 central part of the network while SNARE, NPAS4 and NPY with their first- and second-layer
764 interactors were more in the periphery of the network. This strong interconnectivity has a
765 double interesting effect: it makes the full network really sensible to targeted attack against
766 these proteins and, at the same time, the network would be robust against the attacks if the
767 attack does not target several proteins simultaneously; for example during a drug trial. Thus,
768 studying further closely connected altered genes and understanding the interactions could
769 provide novel insights into the possible molecular mechanism explaining why so many
770 compounds, including DYRK1A specific kinase inhibitors, can restore learning and memory in
771 DS models (51, 104-106). Additionally, these nodes show a high number of connections.
772 Indeed, organising the network using the betweenness index, these nodes and their interactors
773 occupy the centre of the network showing the extreme importance of these nodes for the
774 stability of the network in terms of network theory. Moreover, similar to the observation in DS
775 patients, where the affectation/severity of the gene dysfunction vary for one patient, we
776 proposed here that different DS mouse models show different signalling cascades that were
777 affected with several members strongly interconnected and affecting brain dysfunction leading

778 to similar behavioural phenotypes; may be one possible explanation of the developmental
779 instability hypothesis, which postulates that the non specific triplication of a relative small
780 number of genes causes genetic imbalance with wide impact on global gene expression. This
781 hypothesis should be taken into account when therapeutic assay are planned. The result in one
782 partial trisomic mouse model should be replicated in more genetically complex models to test
783 potential genetic influential factor (43, 51, 71). This is probably the limit of the model, even if
784 the mechanisms of behaviour and memory are common between mice and human, the
785 complexity of the system is lower. Conducting the same studies in more complex animal
786 models, carrying all the trisomic genes homologous to Has21, probably would definitely permit
787 a better deciphering of genes impacting on cognitive behaviour.

788

789 **Conclusion**

790 Taking advantage of DS mouse models, we investigated behavior and cognition, brain
791 morphology and hippocampal gene expression in a standard and controlled way. We unraveled
792 how multiple genetic interactions between different regions of the chromosome 21 contribute
793 in altering the outcome of the behavioural, morphological and molecular/pathways phenotypes.
794 Nonetheless we found that overlapping DS models show convergence in the biological
795 cascades altered, observed via building protein-protein interaction and regulatory networks,
796 and centred on 6 main hubs: DYRK1A, GSK3 β , NPY, SNARE, RHOA and NPAS4. Four of
797 them were already described to be altered in some DS models, and we validated two additional
798 ones, RHOA and NPAS4. As such we have built a novel vision of the existing altered gene-
799 gene crosstalk and molecular mechanisms, with 6 specific highly interconnected hubs in DS
800 models, that should become central to advance in our understanding of DS neurobiology and
801 therapy development.

802

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812

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Supporting information

S1 Fig. Down syndrome mouse models analysed in the study. In the upper part of the plot the human chromosome 21 is represented, in yellow we highlighted the Hsa21 syntenic region found in mouse from *Lipi* to *Zbtb21* (known as *Zfp295* previously). The eight models analysed on this study Dp1Yey, Dp3Yah, Ts65Dn, Dp5/Dp1, Dp5Yah, Dp1Rhr, Tg(*Dyrk1a*), Dp5yah crossed with Tg(*Dyrk1a*) (noted as Dp5-Tg)) trisomic chromosomal regions were drawn in comparison with the Hsa21 region.

S2 Fig. Quantile-Quantile plots to assess Normal distribution. The quantile-quantile plots (Q-Q plots) and Shapiro-Wilk test for the following behavioural datasets: Y-maze, NOR and OF were analysed to study how well our data could be modelled following a normal distribution. Only the distance travelled in OF did not reach the statistical significance level in the Shapiro-Wilk test.

S3 Figure. DS mouse models and Morris water maze. The MWM results are presented as distance travelled to reach the platform (A), speed (B) and % of distance travelled in the peripheral zone (C, Mean \pm SEM). Ts65Dn mice were drastically impaired with an increase in distance travelled to reach the platform and in the distance travelled along the peripheral zone. Less drastically Tg(*Dyrk1a*) mice showed an increased distance travelled to escape the pool and in the peripheral zone (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

S4 Fig. DS mouse models and context and cue fear conditioning. The fear conditioning results are presented as inactivity periods in seconds (Mean \pm SEM). Only Ts65Dn mice present an impairment in context fear conditioning (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

S5 Fig. Comparison of the Z-score calculated from the MRI volume of different brain structures from the DS models. The structures affected in Tg(*Dyrk1a*) were also affected in Dp1Yey and Ts65Dn, whereas the Dp1Rhr mice. (Dp1Yey $n = 6$ wt and 6 Tg ; Ts65Dn $n = 5$ wt and 6 Tg ; Tg(*Dyrk1a*) $n = 8$ wt and 5 Tg ; Dp1Rhr $n = 7$ wt and 7 Tg).

S6 Fig. Fold change expression levels of the genes analysed by the microarrays homologous to the Hsa21 mouse chromosome 16 (mm16). The genes are displayed following the order of their genomic start site coordinates. The duplicated areas for each mouse model appear shaded in the following colours, purple, grey, aquamarine, red, blue and golden for Dp1Yey, Dp3Yah, Ts65Dn, Dp1Rhr, Dp5Yah and Tg(*Dyrk1a*) respectively.

S7 Fig. Fold change expression levels of genes analyzed by the microarrays. (A) Fold change expression levels of the genes analysed by the microarrays part of the centromeric region of the mouse chromosome 17. The centromeric region of the mouse chromosome 17, is defined by the UCSC table browser as the region between the coordinates chr17:110000-3000000. The genes are displayed following the order of their start site genomic coordinates. The region duplicated in the Ts65Dn model non homologous to the hsa21, appears shaded in aquamarine. (B) Fold change expression levels of the genes homologous to the Hsa21 in mouse

chromosome 10 (mm10) and chromosome 17 (mm17) analysed by the microarrays. The genes are displayed following the order of their start site genomic coordinates.

S8 Fig. Number of deregulated probes per chromosomes per models. Trisomic mice have similar patterns with a pic of deregulated genes located on mouse chromosome 16. Tg Dyrk1a at contrary, present a global pattern of deregulated probes.

S9 Fig. Overview of the top 7 dysregulated genes (higher significance and fold change difference) for each mouse model over the global transformed expression profiles illustrated over volcano plots. The x axis and y axis represent the log₂ fold change of the genes expression and the log transformed FDR values respectively. The genes whose fcros FDR value <0.05 are considered significant. These genes are represented in purple or pink and labelled as “Significant”. Instead the genes whose abs (FC)> 1.4 are represented in green or pink and labelled “FC”. Finally, the genes that don’t comply these conditions are considered not significant.

S10 Fig. Gene expression correlation analyses. (A) Correlation of the 4328 differentially expressed genes (DEGs) expression levels on the different models. B) Correlation of the 75 differentially expressed trisomic genes (TEGs) expression levels on the different models. Each row represents a model pairwise correlation with the rest of the models. The density and histograms distribution of the gene expression values for each model is found in the medial diagonal (Black for DEGs, and pink for TEGs). The LOESS smoothed fit is represented by a red line. The plots were created in R using the pairs.panel function from Psych R CRAN package (107).

S11 Fig. Weighted networks representing the connectivity of the pathways included in the Cell structure & organelles, and the Transcription & epigenomic regulation meta-pathways. (A) Representation of the strength connecting the pathways included on the Cell Structure & organelle meta-pathway. (B) Representation of the strength connecting the pathways included on the Transcription & epigenomics regulation meta-pathway. The pathways incorporated in each meta-pathway were identified in the differential functional analysis (DFA) by GAGE after imposing a q-val <0.1 cut off. The connectivity between the pathways, (the edges weight or strength connecting the pathways) is represented by the number of altered genes identified by GAGE shared by each two pathways and is calculated based on the number of common genes within the pathways shared inter and intra mouse model (by each mouse model multi group defined) also grouped by regulation sense using the following formula* and represented by the thickness of the edges connecting the nodes (pathways) of the network. Each edges colour depends on the mouse models where those genes were identified (mouse model multi groups). Formula* = number of genes shared by each pathway on each model on each sense of regulation * factorRes with factorRes= 0.6.

S12 Fig. Weighted networks representing the connectivity of the pathways included in the Ribosome and Mitochondrial meta-pathways. (A) Representation of the strength connecting the pathways included on the Ribosome meta-pathway. (B) Representation of the strength connecting the pathways included on the Mitochondria meta-pathway. The pathways incorporated in each meta-pathway were identified in the differential functional analysis (DFA) by GAGE after imposing a q-val <0.1 cut off. The connectivity between the pathways, (the

edges weight or strength connecting the pathways) is represented by the number of altered genes identified by GAGE shared by each two pathways and is calculated based on the number of common genes within the pathways shared inter and intra mouse model (by each mouse model multi group defined) also grouped by regulation sense using the following formula* and represented by the thickness of the edges connecting the nodes (pathways) of the network. Each edges colour depends on the mouse models where those genes were identified (mouse model multi groups). Formula* = number of genes shared by each pathway on each model on each sense of regulation * factorRes with factorRes= 0.6

S13 Fig. Weighted network representing the connectivity of the pathways included in the Synaptic meta-group and the network representation of the genes linked to Interferon Beta (IFN-B) ungrouped pathway. (A) Representation of the strength connecting the pathways included on the Synaptic meta-pathway. The pathways incorporated in each meta-pathway were identified in the differential functional analysis (DFA) by GAGE after imposing a q-val <0.1 cut off. The connectivity between the pathways, (the edges weight or strength connecting the pathways) is represented by the number of altered genes identified by GAGE shared by each two pathways and is calculated based on the number of common genes within the pathways shared inter and intra mouse model (by each mouse model multi group defined) also grouped by regulation sense using the following formula* and represented by the thickness of the edges connecting the nodes (pathways) of the network. Each edges colour depends on the mouse models where those genes were identified (mouse model multi groups). Formula* = number of genes shared by each pathway on each model on each sense of regulation * factorRes, factorRes= 0.6. (B) Genes linked to Interferon Beta (IFN-B) ungrouped pathway, found altered in the different mouse model multi groups by GAGE. The genes are represented by the ellipse shapes instead the mouse model multi groups by lilac cuadrangles. If the genes were found up regulated then a red border color was used around the ellipses. The genes also identified by FCROS as differentially expressed (DEG) were identified by a red colored inner ellipse.

S14 Fig. Protein-protein interaction and regulatory gene connectivity networks (RegPPINets) of genes involved in the synaptic meta-pathway highlighting biological cascades connectivity. The two sub-networks (A and B) were extracted from the STRING04 MinPPINet. This network was built querying STRING and selecting the PPIs with a medium confidence score (CS), CS=0.4 from all sources of evidence, and was further annotated with regulatory information using REACTOME (See Supplementary information). The shapes of the nodes represent the following information: Shapes: i) Pallid pink ellipses: represent connecting proteins added to assure the full connectivity of the network; ii) pink octagons, represent HSA21 syntenic genes in mouse not identified as contributing to the meta-pathway dysregulation by GAGE; iii) green inner coloured ellipses, genes identified by GAGE after q-val <0.1 cut off to be contributing even slightly, to any pathway of those found dysregulated inside the meta-pathway. If the size is similar to the octagons, they are also HSA21 syntenic genes in mouse. Additionally, the border colour represents the mouse model multi group where those genes are found altered in; iv) diamonds, genes identified by GAGE after q-val <0.1 cut off and also by FCROS as DEGs. (A) RegPPINet Sub-network extracted from the selection of RHOA 2nd interactors from STRING04 MinPPINet, highlighting RHOA 1st Interactors and DYRK1A and GSK3B interactors annotated with the regulatory information from REACTOME. (B) RegPPINet Sub-network extracted from the selection of NPAS4 2nd interactors from STRING04 MinPPINet. Inside of the ellipsoidal line shapes we highlighted NPAS4 interactors involved in the regulation of GABA receptors (cyan), of NMDA receptors (orange), SNARE complex (purple), RHOA (pink), GSK3B (Blue), both RHOA and GSK3B (black) and the genes linked to mitochondrial, ribosomal and cytoskeleton activity (yellow).

S15 Fig. Expression level of genes of interest in the different DS models. Different panels to show the expression level of genes of interest located or not in the region homologous to Hsa21. Data are presented as boxplot plots of the ratio of trisomic on disomic with the median and quartiles (Statistical analysis was done comparing wt and mutant value with a student t test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

S16 Fig. Detection of RHOA and the phosphorylated form of Myosin Light Chain (P-MLC) by Western blot in Dp1Yey hippocampal lysates and their control (wt) littermates. (A) Western blot of RHOA showed no changes in RHOA protein levels in the Dp1Yey line compared to their wt littermates (wt (n=5) and Dp1Yey (n=5)). (B) Western blot of P-MLC revealed a statistically significant decrease (* $p < 0.05$) in the amount of this protein in Dp1Yey mice (wt (n=5) and Dp1Yey (n=5)) (Student's t-test: wt vs. Dp1Yey $t(8) = 2,392$; $p = 0,0437$).

S1 Table: Summary of behavioural results including statistical assessment.

S2 Table. Volumes of each brain structure (in mm³) normalized by total brain volume derived from MRI region-based analysis.

S3 Table. Summary of the DEA and DFA results for each trisomic DS model. We included the number of trisomic genes with compensated or partially compensated expression levels, approximately half of the trisomic genes are compensated and the overall identity of DEGs are different between the models.

S4 Table. Fold-change of the gene expression in trisomic (Tg) compared to the controls for each mouse model dataset. The genes included on those lists were used to produce the S6 and S7 figs as follows: i) genes included inside the duplicated genomic regions of chr 16, ii) genes included inside the Ts65Dn chr17 duplicated region iii) genes of the centromeric region of chr17.

S5 Table. Pathways identified by GAGE as dysregulated including the genes contributing to each altered pathway per each mouse model multi group combination and including the name of the meta-pathway assigned.