Elucidation of disease-related protein networks underlying *Dyrk1A* overexpression and pro-cognitive therapies in the hippocampus

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TESI DOCTORAL UPF / ANY 2017

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DEPARTAMENT CEXS UPF – PHD PROGRAMME IN BIOMEDICINE

A la família, als meus pares

“Todo hombre puede ser, si se lo propone,
escultor de su propio cerebro”

Santiago Ramón y Cajal

AGRAÏMENTS

Molta gent ha estat essencial durant aquests últims anys, el seu suport, els seus inacabables ànims, els discursos per a ajudar-me a no rendir-me, les orelles que pacientment escoltaven els meus assaigs de conferències, la companyia des de la distància, el saber-me fer riure quan em feia tanta falta, i el treure'm al carrer quan ja el cap no em donava per més. Totes aquestes persones ja saben qui són, així que és aquí on els dic: moltes gràcies per acompanyar-me en aquest gran viatge!

En primer lloc, és imprescindible mencionar que res del que aquí segueix hagués estat possible si la Mara no m'hagués donat l'oportunitat de dur a terme aquest gran repte, ara ja fa quatre anys, la meva tesis doctoral al Centre de Regulació Genòmica (CRG). Gràcies a ella i la seva oportunitat he pogut conèixer a fons un laboratori d'investigació, endinsar-me en la neurobiologia i la biologia de sistemes, però sobretot he pogut aprendre a fer ciència. Amb tot allò que fer ciència comporta, entre elles el pensament crític i la resolució de dificultats. També gràcies a ella he descobert un món que desconeixia, el món de la divulgació científica, en el qual m'hi he endinsat i en el que espero seguir durant molt de temps. Moltes gràcies Mara.

En segon lloc, res tampoc hagués estat possible sense l'Eduard. Ell em va donar l'oportunitat de treballar a la unitat de proteòmica del CRG tot i no tenir cap experiència prèvia. Va confiar en mi sabent que no havia vist mai un espectròmetre de masses, i va ser ell, juntament amb tots els membres de la unitat, qui em va ensenyar tot el que sé sobre proteòmica. Gràcies Eduard per l'oportunitat, per la paciència demostrada i la tranquil·litat que sempre m'has trasmès, i sobretot per tenir sempre un moment per mi. Gràcies també a tota la gent de la unitat de proteòmica del CRG, especialment a la Cristina, la Eva, la Guadalupe, la Ivonne, el Roger, l'Amanda i l'Olga. Per tot el que m'han ensenyat, que és tant, però també per escoltar-me quan només he baixat a parlar, o a explicar la meva vida.

M'agradaria agrair també la seva ajuda als membres que han format part del meu comitè de tesis durant aquests quatre anys: James Sharpe, Rafael de la Torre i Susana de la Luna. Ells m'han escoltat cada any i m'han donat el seu suport, i sobretot els seus consells científics, per tirar endavant aquest projecte. Gràcies també al Jordi Garcia-Ojalvo, el qual tot i no formar part del comitè, ha aportat molt bones idees al projecte.

No puc deixar d'esmentar tota aquella gent que científicament ha tingut una contribució especial en aquest gran projecte: el servei de microscòpia del CRG i

el personal de l'estabulari del PRBB, ells han estat sempre allí quan ho he necessitat. Moltes gràcies.

Sempre he considerat que cada etapa acadèmica ha estat millor que l'anterior, i aquesta no ha estat pas menys. L'he viscut amb intensitat, hi he posat ganes, esforç i tenacitat. He donat tot el que he pogut i una miqueta més, he defallit i he tornar a començar. He plorat, però també he rigut molt, però sobretot tot ho he pogut fer perquè he treballat amb grans persones, companys que m'han recolzat quan ho he necessitat.

La primera persona que vaig conèixer, fins i tot abans de tenir un lloc al laboratori, va ser la Maria. ¿Que habría hecho yo sin ti? Nunca tendrás un compañero/a de mesa como yo y lo sabes… Nunca nadie te podrá sorprender tanto con mis movidas ni contarte tantos cotilleos. Gracias por la paciencia de vivir laboralmente a mi lado, por escucharme cuando lo he necesitado, que han sido varias veces, y por las risas que nos hemos echado. Tu y yo nos llevamos la experiencia de la FENS en Copenhagen, pasear por sitios un tanto chungos, "tirarme" de la cama y volar en un avión con ceniceros en mi estado de animo...

Marta, nosotras nos llevaremos el viaje a Varsovia. Fue allí donde de verdad empezó nuestra amistad, nuestras confidencias y nuestras risas, muchas risas. Creo que en Varsovia quedó claro que, para mí, ¡el WiFi es lo más importante! ¡Creo que nunca me he reído tanto como allí! Gracias por estar aquí siempre que lo he necesitado. Para lo bueno, pero sobre todo para lo malo. Ha sido genial trabajar contigo y también ha sido genial disfrutar de los postres de tu tía. Te deseo (a ti y a Marceau) lo mejor en París.

Júlia, ànims amb la tesis, que això només acaba de començar, així que moltíssima paciència. Gràcies per estar en els bons i els mals moments. Gràcies per les festes, pels balls, per compartir les millors cançons dels estius, amb festival inclòs! Però mai res superarà la SENC a Granada! Mai cap congres serà igual!

Linus, quatre anys han donat per moltes converses filosòfiques, i aquestes donen molt que pensar. Ets una persona única i amb les coses molt clares, no dubto que aconseguiràs tot el que et proposis. Recorda't de sortir ben aviat del pou i tirar endavant!

Ilario, porque en cuatro años he sido incapaz de aprender a programar, solo me queda darte las gracias por toda tu incansable ayuda. Disfruta de este tiempo que aun te queda por aquí, y te deseo todo lo mejor.

Marcos, tú y yo empezamos al mismo tiempo y acabamos también más o menos a la vez. Y aunque tu trabajo y el mío no se parecen en nada, ha estado genial tenerte en el laboratorio. ¡Siempre va bien tener un médico en la sala!
Silvina, ¡tú ya lo tienes! Suerte en tus nuevas metas en la vida y sobretodo, disfruta de lo que hagas. Tomas, Belén, Álvaro, Sergi, Marcin, Ruggero, Jose, Laura, Cesar, Edu, Ibo... con vosotros no he coincidido durante toda mi etapa, pero os deseo la mejor suerte del mundo en todo lo que hagáis. Ha sido un placer conoceros y poder compartir ciencia y risas.

Gràcies també a tota la gent del grup del Julio Saez-Rodríguez a Aachen. Gràcies per l'hospitalitat i l'enorme ajuda rebuda en tot moment durant el primer viatge. Inoblidable. Gràcies especialment a Aurelien, Enio, Luis, Asier i Angeliki per ser uns grans companys durant el meu segon viatge. Va ser una estada única en tots els sentits i mai oblidaré aquells tres mesos. Sempre recordaré aquells dies de tranquil·litat, d'excursions, de sushi, de compres… Alemanya em va enamorar completament. La seva gent, el seu idioma, el seu menjar, els seus carrers, el seu Nadal... No podré explicar mai la gran connexió que m'uneix a aquesta meravellosa ciutat, i les ganes que tinc de poder tornar-hi una i mil vegades més. Amb Aachen tinc un vincle massa especial, una estada que necessitava i que em va fer créixer com a persona, i em va fer veure que sóc forta, molt més forta del que em pensava.

Fora del CRG també he tingut un gran suport. El més important ha estat el de la meva família i el de la meva ex parella, els quals han aguantat tot tipus de moments. Ells han passat nervis i angoixa durant els meus viatges a congressos, i molta enyorança, neguit i por durant la meva estada a Aachen. Però sobretot han tingut molta paciència i han sabut escoltar i aconsellar-me sempre que ho he necessitat, tot i que no ha estat fàcil. Tots junts vam haver de passar moments molt durs, i van saber estar amb mi, i no em van fallar. Moments de por, d'incertesa i de moltes i moltes llàgrimes, però finalment junts ens hem vam sortir. I un temps més tard vam poder gaudir tots plegats en un viatge a Aachen que va esdevenir inoblidable, ple de la màgia del Nadal, de llegendes, de paisatges i de fred que sempre, passi el temps que passi, recordarem. I és que aquest viatge és un dels regals més grans que he rebut mai. Aquestes coses no es fan per a qualsevol, només es fan per amor del gran. Gràcies família! Gràcies especialment als meus pares que SEMPRE estan quan els necessito i que saben aconsellar com cal. Gràcies també a les meves germanes, Raquel i Lorena i als seus marits Jordi i Joan. Gràcies també als meus petits nebots Miquel, Joan i Arnau, pels vostres somriures, les paraules amb mitja llengua i els comentaris esbojarrats, i per fer un viatge tant llarg carregats d'il·lusió per veure a la tita Mireia. Us adoro i espero veure-us una mica més ara que tot està a punt d'acabar.

Gràcies al Mingo, el meu company de vida durant gairebé 10 anys i que ha estat sempre amb mi. Tu m'has vist acabar la carrera, el màster i que espero que vegis com em converteixo en doctora. Per moltes paraules que et dediqués sempre em quedaria curta. T'he estimat, t'estimo i sempre t'estimaré.

Moltes i sinceres gràcies a tots. Durant aquesta experiència no només he crescut i millorat com a científica, sinó també com a persona. Aprenent dels errors i mirant sempre cap endavant tot i les dificultats. Això és el més important que m'emporto.

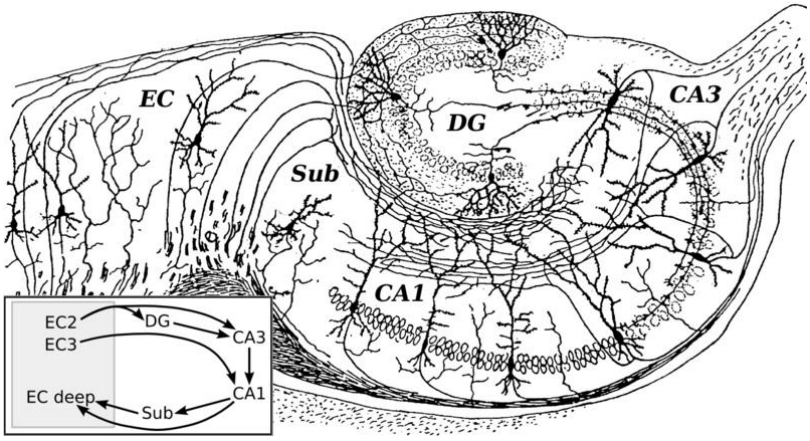
Ja sé que ens trencarem,
que ens caurà a terra el món.
Ja sé que plorarem
i que podem perdre-ho tot.

Per sempre no hi ha res
i el tren del temps no frena.
Tinc un desig només:
que tot valgui la pena.

El tren del temps
Txarango

Mireia Ortega Crespo received a FPI Severo Ochoa doctoral fellowship from the Spanish Ministry of Economy and Finance (MINECO) SVP-2013-068022 during the period 2014-2018; and a short research international stay fellowship (EEBB-I-16-11305).

The work has received financial help from Fondation Jérôme Lejeune (París, France), MINECO (SAF2013-49129-C2-1-R and SAF2016-79956-R) and Centre for Genomic Regulation Severo Ochoa excellence grant (SEV-2012-0208).



Modified drawing of the neural circuitry of the rodent hippocampus by Santiago Ramón y Cajal. Histologie du Système nerveux de l'Homme et des Vertébrés, *Paris*: A. Maloine

ABSTRACT / RESUM

ABSTRACT

Down syndrome (DS) is the most frequent genetic cause of intellectual disability. It is a multifaceted condition characterized by impairments in cognition, communication, behavior and/or motor skills resulting from abnormal brain development and function. Although DS is caused by the trisomy with more than 300 triplicated genes located on chromosome 21, there are a reduced number of dosage-sensitive candidate genes that play a critical role in the pathogenesis of the disorder.

Our group has made important contributions demonstrating that overexpression of a single gene, *DYRK1A*, is sufficient and necessary to recapitulate some of the DS phenotypes. Importantly, we could also demonstrate that the genetic, pharmacological or environmental normalization of its overdosage rescues behavioral, cognitive and neuronal phenotypes in preclinical studies with DS mouse models and in clinical trials in humans.

In this Thesis I propose that the consequences of *DYRK1A* overexpression could spread along a complex intracellular network leading to a disease-causing network. For this reason, DYRK1A kinase normalization could be a good molecular target to restore the protein network functionality in DS.

Here we have examined large-scale protein and phosphoprotein profiling to explore alterations caused by *Dyrk1A* overexpression and of pro-cognitive therapeutic strategies previously shown in the laboratory to normalize DYRK1A kinase activity and to promote cognition: i) environmental enrichment (EE), ii) and (-)-epigallocatechin-3-gallate (EGCG) and iii) the combination of both EGCG+EE. Using high-throughput tandem mass spectrometry (LC-MS/MS) in the hippocampus of TgDyrk1A mice we show that *Dyrk1A* overexpression in mice hippocampus primarily affects MAPK signaling and plasticity processes that could be related with impairments in recognition memory. Treatment with EGCG, EE, or their combination (EGCG+EE) restore some of the proteome and phosphoproteome alterations of *Dyrk1A* overexpression, possibly through the same mechanisms.

RESUM

La síndrome de Down (SD) és una de les causes mes freqüents de discapacitat intel·lectual. És una condició multifacètica caracteritzada per dèficits en cognició, comunicació, comportament i motors com a resultat d'un mal desenvolupament cerebral. Encara que la SD està causada per una trisomia que afecta a més de 300 gens del cromosoma 21, hi ha un reduït número de gens sensibles a dosis que juguen un paper crucial en la patogènesis d'aquesta síndrome.

El nostre grup de recerca ha fet contribucions importants en aquest camp i ha demostrat que la sobreexpressio d'un sol gen, *DYRK1A*, és suficient i necessària per recapitular alguns dels fenotips de la SD. També hem pogut demostrar que la normalització de la dosi de DYRK1A a nivells basals ja sigui de manera genètica, farmacològica, o a través d'una estimulació per enriquiment ambiental rescata alguns dels fenotips cognitius i neuronals en estudis preclinics amb models de ratolí però també amb humans.
En aquesta tesi, es proposa que la sobreexpressió de *DYRK1A* condueix a canvis moleculars que s'expandeix a través d'una complexa xarxa interaccions intracel·lulars. Per aquesta raó, la normalització de DYRK1A podria ser una bona diana terapèutica per restaurar la funcionalitat de la xarxa proteica alterada en la SD.

Hem examinat a gran escala el perfil proteòmic i fosfoproteòmic per tal d'explorar les alteracions causades per la sobreexpressió de *Dyrk1A*, així com les alteracions causades per les diferent estratègies que han sigut provades al laboratori que normalitzen la seva activitat quinasa: 1) enriquiment ambiental (EE), 2) (-)-epigallocatechin-3-gallate (EGCG) i 3) la combinació de EGCG+EE.
Utilitzant espectrometria de masses per explorar les alteracions moleculars en l'hipocamp de ratolins que sobreexpressen Dyrk1A (TgDyrk1A) hem trobat alteracions que afecten la cascada de senyalització de les MAP quinases aixi com en processos relacionats amb la plasticitat que podrien estar relacionats amb els dèficits de reconeixement de memòria. Els tractaments amb EGCG, EE o la seva combinació han demostrat rescatar algunes de les alteracions proteòmiques i fosfoproteomiques induïdes per la sobreexpressió de *Dyrk1A*, possiblement mitjançant mecanismes comuns.

PREFACE

PREFACE

Down syndrome (DS) is the most frequent genetic cause of intellectual disability. It is a multifaceted condition characterized by impairments in cognition, communication, behavior and/or motor skills resulting from abnormal brain development and function. Although DS is caused by the trisomy of part or the whole chromosome 21, our group has made important contributions demonstrating that overexpression of a single gene, *DYRK1A*, is sufficient and necessary to recapitulate some of the DS phenotypes. Importantly, we could also demonstrate that the genetic, pharmacological or environmental normalization of its overdosage rescues behavioral, cognitive and neuronal phenotypes in preclinical studies with DS mouse models and in clinical trials in humans.

This Thesis originates from the interest of the Cellular and Systems Neurobiology group at the Center for Genomic Regulation in further understanding the molecular mechanisms underlying the neuropathological consequences of *DYRK1A* triplication, and the beneficial effects of its normalization. A key concept behind this interest, is that a disease is rarely a consequence of an abnormality in a single gene. Instead, the disease phenotype is a reflection of the consequences of how genetic abnormalities spread along the complex intracellular network, altering the expression or the activity of gene products that carry no defects. Thus, classical reductionist molecular biology approaches might not be sufficient to capture the complexity of the *DYRK1A*-driven (disease) status and its recovery.

Consequently, I used high-throughput proteomic and phosphoproteomic techniques for large-scale protein profiling in order to gain insight into the complexity of the problem. With this approach, we explored alterations caused by *DYRK1A* overexpression and of therapeutic strategies, environmental enrichment (EE), and (-)-epigallocatechin-3-gallate (EGCG), previously shown in the lab to normalize DYRK1A kinase activity and to promote cognition. We also explored their combination (EGCG+EE) to identify possible synergistic effects.
Our model system was a transgenic mouse strain overexpressing *Dyrk1A*, and the experiments included behavioral studies and mass spectrometry analysis of *Dyrk1A* overexpressing hippocampus of young-adult mice to resolve the proteomic and phosphoproteomic profiles of the effects of DYRK1A, and the treatments EGCG, EE and EGCG+EE, that were carried out in the Proteomics Unit of the Center for Genomic Regulation. I also performed the bioinformatics analysis of the results, and finally, I linked molecular phenotype with behavioral phenotype. The whole work was performed under the direction of Mara Dierssen and Eduard Sabidó, co-director of this thesis, and director of the Proteomics Unit.

During my Thesis I have managed a plethora of frameworks and theoretical perspectives keeping in mind the challenge of using consistent cross-species

terms and concepts, one over another. Additionally, the outcomes of this Thesis have set the basis for further research lines in Dierssen´s lab involving mechanistic studies that will address the molecular effects of the treatments to other levels.

I had the opportunity to be part of several international and national scientific collaborations with renowned research groups both in the neuroscience and systems biology field. Concretely, I would like to highlight and thank Prof. Julio Saez-Rodríguez to welcome me in his research group in RWTH Aachen University. During my four-month stage in his group I learnt the basics of common bioinformatics strategies used in the analysis of proteomic and phosphoproteomic data. I had also the opportunity to integrate my mass spectrometry data into mechanistic mathematical tools. However, due to the high biological variability of our data and the structure of the tools at that moment we were unable to use them. I would like also to highlight the collaboration with Prof. Cedric Boeckx (Universitat de Barcelona) where we hypothesize that DS could offer a special window into the mechanism underlying the domestication syndrome. Finally, Dr. Jordi García-Ojalvo introduced me to the concepts and techniques for modelling molecular networks.

I had the specific support of a predoctoral fellowship Severo Ochoa from the Spanish Ministry of Economy Competitivity and my project was funded by a grant from the Jerome Lejeune Foundation. During this Doctoral Thesis, I had the opportunity to present my work in three national and four international meetings, and I have participated also in other outreach activities of the laboratory.

List of scientific publications:

1. Cristina Chiva; **Mireia Ortega**; Eduard Sabidó (2014) **"Influence of the digestion technique, protease and miscleavaged peptides in protein quantitation."** J Proteome Res 13(9):3979 - 3986. 05/09/2014

2. **Mireia Ortega**; Eduard Sabidó; Mara Dierssen; Cedric Boeck. **"What Down syndrome can tell us about self-domestication?"** (*in preparation*)

3. **Mireia Ortega**; Ilario de Toma; Eduard Sabidó; Mara Dierssen **"Mass spectrometric analysis of Dyrk1A overexpressing mice and pro-cognitive treatments reveals proteome alterations in hippocampus"** (*in preparation*)

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INTRODUCTION

“The human brain has 100 billion neurons, each neuron connected to ten thousand other neurons. Sitting on your shoulders is the most complicated object in the known universe.”

Michio Kaku

1. INTRODUCTION

1.1 Intellectual disability

The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) defines intellectual disability (ID) as characterized by significant limitations of intellectual functioning (intelligent quotient (IQ) below 70) and adaptive behavior, which includes conceptual, social, and practical skills. As neurodevelopmental anomalies their impact is not only limited to cognitive systems, but also affect neural networks involved in a broad range of behaviors. Furthermore, ID is often part of a syndrome that affects other organs and their functions. Only in Europe, there are 4.2 million individuals affected by ID (Wittchen *et al*., 2011) that have a huge impact on public health, as these are chronic disorders. ID can be caused by a variety spectrum of genetic alterations and environmental insults such as including infections, trauma and tetarogens. Still, ~60% of cases of ID do not have a known etiology (Rauch *et al*., 2006).

There is an important phenotypic variability in individuals with ID, not only in IQ levels, which can range from severe to moderate, but also compromise quality of life with other neurologic and neurobehavioral manifestations. Compared to the general population, ID individuals have a significantly rate of comorbid psychopathology of which autistic spectrum disorder (ASD) is one of the most common co-occurring disorders in individuals with ID. As much as 40 % of the ID population meets diagnostic criteria for ASD (Cervantes *et al*., 2015).

1.1.2 Genetic etiology of intellectual disability

The causative factors (genes, epigenetic and environmental) of ID are quite varied and likely interact and this has further complicated the identification of candidate genes for ID. Historically, genetic diagnosis of ID started under the microscope with the identification of cytogenetically visible abnormalities as in the case of trisomy 21, the cause of Down syndrome, and a marker chromosome X, the cause of Fragile X syndrome. The use of microarrays led to the identification of numerous pathogenic gains and losses of chromosomal segments that escaped detection by the microscope (Tysen *et al*., 2005). Recent decades have witnessed numerous advances in genetics research highlighting the importance of genetic factors as an etiology for developmental disabilities Specifically, the advances in gene sequencing technology, especially next-generation sequencing (NGS), have led to the identification of an increasing number of causative genes for intellectual disability (Figure 1). Single gene mutations, as well as copy number variants (CNVs), either duplications or deletions, have been associated with ID conditions. Additionally, hypomorphic alterations in multiple genes or excess of genetic material are also noted. There is an extensive literature with

curated lists of genes involved in ID (Gilissen *et al.*, 2014; Wrigh *et al.*, 2015, Grozeva *et al*., 2015; Lisenka *et al.*, 2015; Chiurazzi and Pirozzi, 2016).

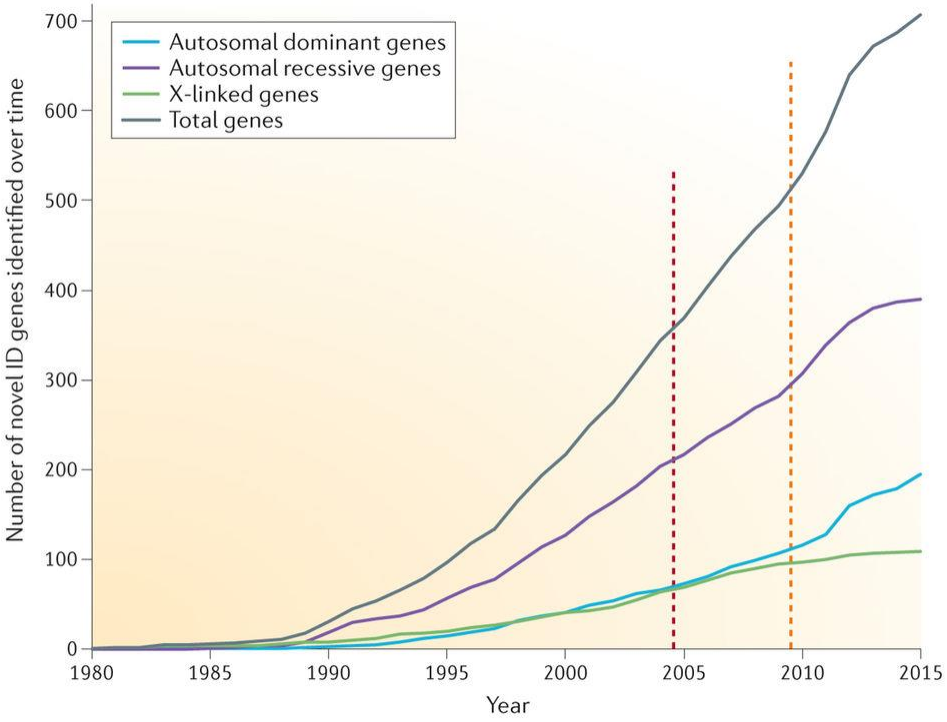


Figure 1: Increase of genes linked to isolated ID and ID-associated disorders. Graphical overview of the increase in gene discovery for isolated intellectual disability (ID) and ID-associated disorders over time, specified by the type of inheritance. Vertical dashed lines represent the introduction of genomic microarrays (red) and next-generation sequencing (NGS)-based technologies (orange) for the detection of new ID genes. From this figure it is clear that we have not reached any saturation in ID disease gene identification, except perhaps for X-linked forms of ID. (Adapted from Lisenka *et al*., 2015).

Except for some specific ID cases (e.g., Angelman syndrome, fragile X syndrome, Prader-Willi syndrome, Rett syndrome, Rubinstein-Taybi syndrome, Smith-Magenis syndrome, velocardiofacial syndrome, Williams syndrome, etc.) knowing the possible genetic etiology, does not inform about clinical features, prognosis, or possible treatments. This is because primary causative factors (monogenic causes, epigenetic and environmental factors or other as yet unidentified causative factors) do not directly result in cognitive impairment. Rather, the mutant genes or other primary causative factors directly or indirectly cause disturbances in downstream molecular pathways, which lead to altered neurodevelopment (for example, interference in cell proliferation and/or migration), which then lead to the brain abnormalities that result in cognitive and behavioral disabilities. It is thus likely that common groups of genes, proteins and metabolites or a combination of these are affected. In such scenario, the proteins encoded by ID genes can be hypothesized to play a part in one or more shared pathways (Lisenka *et al.*, 2015). Thus, more than the concrete genes or proteins

affected, it is important to identify those pathways or biological processes affected. Chiurazzi and Pirozzi used a Gene Ontology (GO) enrichment analysis to understand the functions of the proteins encoded by genes related to ID. The results showed that multiple essential metabolic pathways, especially those related to energy production in the mitochondria, were highly enriched. Also in the top GO biological processes enriched they found processes related with brain function, such as central nervous system development, neurogenesis, neuron differentiation, synapse formation and neurotransmission (Chiurazzi and Pirozzi, 2016; Rauch *et al*., 2006).

Components of common interaction networks and biological processes associated with these genes/proteins are likely critical and unique to normal cognitive and behavioral function. Among those, diverse cellular signaling pathways have also been reported to have an important role in etiology of ID. As an example, the RAS-MAPK (mitogen-activating protein kinase) pathway is associated with a particular set of intellectual disabilities, the so-called rasopathies (e.g. Noonan syndrome and Costello syndrome). Another emerging cellular signaling cascade is the RHO GTPase pathway. Guanine-nucleotide binding proteins that act as a “molecular switches” in variety of cellular functions including the morphogenesis of dendritic spines. In total, over a 20 GTPses are known, of which the effectors of RAC1, cell division cycle 42 (CDC42) and RHOA have established roles in spine formation and synapse plasticity. Also mutations in downstream effector of RHO including phosphatases and calcium/calmodulin-dependent kinase type II (CAMKII) have been reported in patients with ID. Also transcriptional and translational control, epigenetic regulation and chromatin remodeling are deregulated by mutations leading to some forms of ID such as methyl CpG binding protein 2 (MECP2) and chromodomain helicase DNA remodeling protein 8 (CHD8) (Lisenka *et al*., 2015).

1.2 Down syndrome

Down syndrome (DS), is the most prevalent cause of intellectual disability due to a genetic aneuploidy affecting 1 each 800-1000 newborn children worldwide (Roizen and Patterson, 2003), with prevalence increasing with maternal age (Hook, 1983). DS results from an extra (full or partial) copy of chromosome 21 (HSA1) and depending on the cytogenetic origin, trisomy 21 is divided into different types. In about 95% of cases, trisomy results from meiotic non-disjunction of the chromosome 21 pair. About 4% of persons with DS have 46 chromosomes, one of which is carrying the Robertsonian translocation between chromosome 21q and the long arm of one of another acrocentric chromosome (usually chromosome 14 or 22). 1% of DS cases are mosaicisms, with cell populations showing either a normal or a trisomic 21 karyotype; in this case, the phenotype may be milder than the typical trisomy 21 (Patterson, 1987; Nadal and Mila *et al*., 1996). Nevertheless, those genetic abnormalities produce an

excessive expression of tens to hundreds of genes and thus, a genetic and proteomic imbalance affecting all cell types in the body leading to a wide range of phenotypic abnormalities.

1.2.1 Neuropsychological and Neuropathological aspects of Down syndrome

The most limiting feature in DS is the congenital limitation in intellectual functioning and adaptive behaviour, affecting 100% of individuals displaying specific deficits in learning and memory, language and executive functions that lead to a moderate-severe IQ ranging from 30-70 (Chapman and Hesketh, 2000; Vicari, 2004; Liogier d'Ardhuy *et al*., 2015) affecting from early childhood, due to a central nervous system maldevelopment, to adolescence and adulthood (Brown *et al*., 1990; Carr and Carr, 1995; Vicari, 2004). Cognitive phenotypes in DS are diverse and while some aspects are present throughout lifespan, others appear at specific temporal windows. In fact, 3-month-old-infants with DS show psychomotor learning abilities similar to euploid infants (Ohr and Fagen., 1991; 1993), but it is during their early childhood between 6 month and 2 years of age that their cognitive capacities suffer a deceleration probably associated to the neurodevelopmental delays with a further decline in adolescents. Specifically, children exhibit incomplete and delayed acquisition of motor, linguistic, cognitive, and adaptive functions, compared with typically developing children of the same mental age (Hesketh and Chapman, 1998; Chapman and Hesketh, 2001; Silverman, 2007).

The presence of moderate-severe impairment in intellectual functioning can be explained with the structural and cellular alterations in brain structures involved in the processing and storage of information, especially, the neocortex and the hippocampus. Post-mortem observations have revealed reduced brain volumes (microcephaly) with small hippocampus, cerebellum and prefrontal cortex (Aylward, Li *et al*., 1999; Pinter *et al*., 2001) that appear in the first months of life. Of note, hippocampal volume continues to decrease with age in DS individuals (Teipel *et al*., 2003) and was found to be inversely correlated with the degree of cognitive impairment (Smigielska-Kuzia *et al*., 2011) pointing the hippocampus as one of the main structures underlying cognitive impairments in DS. Those impairments include deficits in their ability to create and retain new lasting memories for facts and events (declarative memory) including visuospatial and contextual information (Carlesimo *et al*., 1997; Pennington *et al*., 2003; Visu-Petra *et al*., 2007; Lavenex *et al*., 2015). Also, difficulties in the acquisition of information (learning) and the long-term storage and retrieval of information (memory) (Nadel, 2003).

It is important to mention here that in addition to neuropsychological aspects already described, individuals with DS present a higher incidence and early onset

of Alzheimer's disease (AD)-like cognitive deficits and dementia compared to general population (Ballard *et al*., 2016). Individuals with DS older than 40 years show a rapid and progressive cognitive decline resembling the cognitive profile found in sporadic AD, due to the presence of three copies of the amyloid precursor protein gene *APP*.

1.2.2. Genetic causes of Down syndrome

The prevailing hypothesis for the genetic causes underlying DS pathology is that individual phenotypes are caused by an extra copy of one or more of the ~310 genes present on Hsa21 (Ensembl release 62, including known and newly identified protein-coding and RNA genes but excluding pseudogenes; http://www.ensembl.org/Homo_sapiens/Location/Chromosome?r=21:1-48129895). Such genes are described as being dosage sensitive, and much effort is being made to identify the dosage-sensitive genes underlying each of the DS phenotypes. The hope is that identification of such genes will lead to a better understanding of the molecular mechanisms underlying the pathologies, and hence to more effective therapies.

The search for these dosage-sensitive genetic culprits has taken advantage of both human and mouse genetics. In humans, rare partial trisomies of Hsa21 have been used to narrow down regions of the chromosome that might contain dosage-sensitive genes. Early studies suggested that a limited region of Hsa21, termed the Down syndrome critical region (DSCR), might contain one or more dosage-sensitive genes that contribute to many of the DS phenotypes (McCormick et al., 1989; Rahmani et al., 1989; Korenberg et al., 1990; Sinet et al., 1994). However, further studies that included larger numbers of partial trisomy cases and more-detailed genetic mapping have shown that different regions of Hsa21 contribute to different phenotypes, arguing against a single DSCR (Delabar *et al*., 1993; Korenberg *et al*., 1994; Korbel *et al*., 2009; Lyle *et al*., 2009). Nonetheless, the data do not exclude the possibility that it contributes to the phenotype (Korbel *et al*., 2009; Lyle *et al*., 2009). Analysis of mouse models with or without three copies of the DSCR showed that trisomy of this region was necessary but not sufficient to cause a defect in the Morris water maze test (Olson *et al*., 2007). However, a separate study using the novel-object-recognition test, a different assay of learning and memory, concluded that trisomy of the DSCR was sufficient to result in cognitive defects (Belichenko *et al*., 2009). More work is needed to resolve the basis for these distinct conclusions, but the use of human partial trisomies to identify dosage-sensitive genes is limited by the rarity of partial trisomies, heterogeneity of the specific phenotype and genetic variation between individuals.

On one hand, transcriptomic studies revealed that dysregulated genes are not present solely on HSA21, but throughout the whole genome. Letourneau *et al*.,

(2014) showed the existence of chromosomal domains of gene expression dysregulation so that the overexpression of one or more HSA21 candidate gene(s) modified the chromatin environment of the nuclear compartments in trisomic cells, leading to a general perturbation of the transcriptome. Other studies that integrated DNA methylation and RNA-seq data detected 43 genes with demethylation or upregulated methylation of promoter regions in DS samples suggesting also epigenetic modifications affecting DS. In addition, 24 DS genes have transcriptional regulatory function, among which are *RUNX1, NR4A2, EGR2, EGR3* and *ID4* (Zhang *et al*., 2016).

On the other hand, many of the dysregulated genes exhibit highly specific temporal and regional expression profiles and these dysregulated genes form distinct co-expression networks associated with distinct biological categories (Olmos-Serrano *et al*., 2006).

Given that changes in gene dosage affect the expression levels of virtually all genes present in three copies, but also non-HSA21 genes, it is reasonable to assume that some of those genes will be neutral for organism fitness, whereas others will exert pathological effects when expression reaches a critical threshold above basal level. However, it is not straightforward to predict which genes will be deleterious when they are over-expressed modestly, even with knowledge of the genes' functions.

1.3 Dyrk1A: a candidate gene for Down syndrome

The identification of dosage-sensitive genes has become a major focus in DS research because it is essential for a full understanding of the molecular mechanisms underlying pathology, and might eventually lead to more effective therapy. A number of genes located in the DSCR have been proposed as candidate dosage-sensitive genes that might contribute to DS-associated brain phenotypes, including *DYRK1A, SIM2, DSCAM* and *KCNJ6*.

DYRK1A (Dual specificity Yak1 Related Kinase) is a DS candidate gene overexpressed in fetal and adult DS brain (Guimerá *et al*., 1996; Guimerá *et al*., 1999). It is located in the long arm of HSA21 (Guimerá *et al*., 1996; Song *et al*., 1996), specifically in 21q22.13, and encodes two main protein isoforms of 763 and 764 amino acids. DYRK1A is a dual-specificity protein kinase of approximately 90kDa that belongs to DYRK family as a part of CMGC group of kinases (named after the initials of some members), which includes cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), glycogen synthase kinases (GSK) and CDK-like kinases (Aranda *et al*., 2010).

DYRK1A autophosphorylates on tyrosine (Tyr) 312/321 in the activation loop for the full catalytic activity and then, only phosphorylates on serine (Ser) and

threonine (Thr) residues (Himpel *et al*., 2001; Becker and Sippl, 2011). The kinase domain is located centrally in the primary structure of the protein and contains four leucines (Leu) probably forming a Leucine-zipper domain for DNA binding. DYRK1A shares with the other DYRKs a conserved motif situated on N-terminal to the kinase domain and known as DYRK homology (DH)-box. It also contains a functional, bipartite nuclear localization signal (NLS) N-terminal to the DH-box and a second NLS between subdomains X and XI within the kinase domain. Also, Dyrk1A contains a C-terminal PEST motif involved in protein signaling degradation, and a polyhistidine tract that acts as a nuclear speckle-targeting signal (Figure 2).

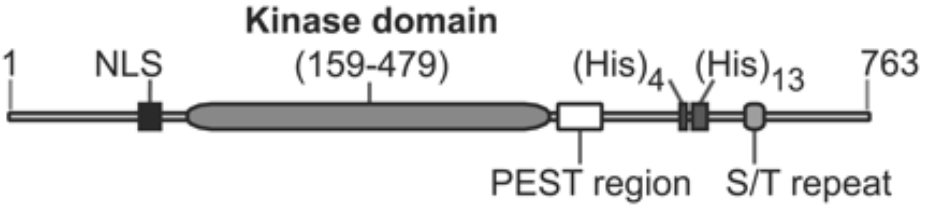


Figure 2: Schematic representation of Dyrk1A protein. NLS: nuclear targeting localization signal. Kinase domain is located centrally in the primary structure of the protein and contains four leucines (Leu) probably forming a Leucin-zipper domain for DNA binding. PEST: PEST motif involved in protein signaling degradation. His: Polyhistidine extension that acts as a nuclear speckle targeting signal. S/T: Serine and Threonine-rich region. (Adapted from Hendrik *et al*., 2004).

In the brain, DYRK1A is expressed in both the nucleus and in the cytoplasm of neurons and astrocytes (Weigel *et al*., 2004; Kida et al., 2011). Although the majority of studies on DYRK1A subcellular localization have been done in cellular lines, some works have demonstrated that, in vertebrates, DYRK1A is expressed during prenatal brain development with a specific sequence of distinct temporal and subcellular patterns starting in neural progenitor cells and finishing in neuronal dendritic tree and synapses (Hämmerle *et al*., 2008). This spatio-temporal distribution indicates a high functional versatility. Substrates of Dyrk1A are also localized in different subcellular regions (TableX)

1.3.1 DYRK1A functions in the central nervous system

DYRK1A is highly and ubiquitously expressed during vertebrate's embryonic brain development while in adult mouse brain its expression is reduced and restricted to certain regions such as cortex, hippocampus, cerebellum, deep motor nuclei, hypothalamic nuclei and olfactory bulb (Marti *et al*., 2003). This differential expression along life, suggests that DYRK1A may have different functions along life. In fact, some of the brain regions where DYRK1A is

expressed are highly affected in DS especially the cortex, cerebellum and hippocampus. The fact that DYRK1A is expressed in adult brain regions involved in cognition such as the hippocampus and cortex, phosphorylates proteins involved in synaptic plasticity and neuronal differentiation and produces cognitive alterations when overdosed in mouse models (Altafaj *et al*., 2001; Ahn *et al*., 2006), reinforces the idea that DYRK1A could participate in cognitive processes and their disturbance in DS.

1.3.2 Dyrk1A targets

The phosphorylation of multiple targets DYRK1A is implicated in diverse biological processes that are critical for cell function. DYRK1A has an important role in transcription activity and alternative splicing through diverse substrates such as SF2/ASF which controls splicing of many transcripts. The kinase has been reported to interact with several transcription factors, but it is not a general transcriptional activator. For instance, DYRK1A regulates the transcriptional activity of glioma-associated oncogene 1 (GLI1), a major effector of sonic hedgehog (SHH) signaling, which is a key pathway in the regulation of the proliferation during vertebrate nervous system development (Mao *et al.*, 2002; Ruiz and Altaba *et al*., 2002). Most of the transcription factors previously described as DYRK1A substrates are phosphorylated by DYRK11A. Remarkably, DYRK1A phosphorylates NRSF/REST chromatin remodeling complex (Canzonetta *et al*., 2008) and NOTCH receptor, contributing to cell fate definition of pluripotent embryonic stem cells and neuronal progenitors (Fernandez-Martinez *et al*., 2009). In fact, recently, results from Di Vona *et al*., confirmed the role of DYRK1A as a transcriptional regulator by acting as a CTD kinase. However, it remains to be determined which of the signaling cascades leading to gene induction is most predominantly regulated by DYRK1A or even requires DYRK1A under physiological conditions in the mammalian brain.

DYRK1A also plays a key role in structural and synaptic plasticity processes such as neurite formation, dendritic growth and synaptic vesicle trafficking through the phosphorylation of proteins such as the transcription factor cAMP responsive element binding (CREB) (Yang *et al*., 2001), cytoskeleton-related proteins, MAP1B and WASL (Scales et al., 2009, Park et al., 2012), and component of the endocytic protein complex machinery, amphiphysin, dynamin 1, endophilin 1 and synaptojanin 1 (Chen-Hwang *et al*., 2002; Hammerle *et al*., 2003; Murakami *et al*., 2006, 2009).

DYRK1A is also involved in neurodegenerative processes through its participation in the phosphorylation and alternative splicing regulation of Alzheimer disease-associated proteins such as TAU and APP. DYRK1A directly contribute to the formation of neurofibrillary tangles by the phosphorylation of TAU (Woods *et al*., 2001) and also has an indirect role through the promotion of

GSK3β activity upon TAU (Liu *et al*., 2008; Azorsa *et al*., 2010). Through the phosphotylation of the amyloid precursor protein APP (Ryoo *et al.*, 2011) and presenilin 1(Ryu *et al*, 2010), DYRK1A contributes to an increase in the proteolytic cleavage of APP and elevation of Aβ40 and Aβ42 in DS and AD. Other Dyrk1A substrates are represented in Table 1.

Table 1: List of DYRK1A substrates. Based on Duchon and Herault *et al*., 2016 and Aranda *et al*., 2010.

SUBSTRATE	SUBCELLULAR LOCALIZACTION	FUNCTION	REFERENCE
14-3-3	C	Regulatory protein	(Kim *et al*., 2004)
Amph	C	Synaptic vesicle endocytosis	(Murakami *et al*, 2006)
App	M	Apoptosis, cell adhesion, endocytosis	(Kimura *et al*., 2007; Ryoo *et al*., 2008)
Arip4	N	ATP, DNA and nucleotide-binding	(Sitz *et al*., 2004)
Srsf1	C/N	mRNA processing, splicing and transport	(Shi *et al*., 2008)
Braf	C/N	ATP, metal and nucleotide-binding	(Kelly and Rahmani., 2005)
Casp-9	C/N	Apoptosis	(laguna *et al*., 2008; Seifert *et al*., 2008)
CHC	C	Endocytosis	(Murakami *et al*., 2009)
Ccnd1	C/N	Cell cycle and division, transcription	(Chen *et al*., 2013; Najas *et al*., 2015)
Ccnl2	N	Transcription regulation	(de Graaf *et al*, 2004)
Creb	N	Differentiation, transcription regulation	(Yang *et al*., 2001)
Cry2	C/N	Biological rhythms, sensory transduction, transcript regulation	(Kurabayashi *et al*., 2010)
CTD Rnap II	N	Transcription	(Di Vona *et al*., 2015a)
Dnm1	C	Endocytosis	(Chen-Hwang *et al*., 2002)
Endophilin1	C	Endocytosis	(Murakami *et al*., 2009)
Fkhr	C/N	Apoptosis, autophagy, differentiation, transcription regulation	(Woods *et al*., 2001)
Gli1	C/N	Differentiation, transcription regulation	(Mao *et al*., 2002)
Grb2	C/N	Cell differentiation	(Abekhoukh *et al*., 2013)
Gsk3B	N/C/M	Differentiation, neurogenesis	(Skurat and Dietrich, 2004)
Glun2a	M	Transport	(Grau *et al*., 2014)
Hip1	N	Apoptosis, differentiation, endocytosis, transcription regulation	(Kang *et al*., 2005)

H3	N	Chromatin assembly	(Himpel *et al*., 2000)
HPV16E7	N	Viral oncoprotein	(Liang *et al*., 2008)
Kip1	C	Cell cycle	(Soppa *et al*., 2014)
Lin52	N	Cell cycle, transcription	(Litovchick *et al*., 2011)
Map1b	C	Axon extension, intracellular transport	(Scales *et al*., 2009)
Mek1	C/N	ATP-binding, nucleotide binding	(Kelly and Rahmani, 2005)
NCID	N	Cell signaling	(Fernandez-Martinez *et al*., 2009)
Nfat	C/N	Transcription regulation	(Arron *et al*., 2006; Gwack *et al*., 2006)
Notch	N	Angiogenesis, differentiation, transcription	(Fernandez-Martinez *et al*., 2009)
Nrsf/Rest	N	Transcription regulation	(Canzonetta *et al*., 2008)
P53	C/N	Apoptosis, cell cycle, necrosis, transcription	(Park *et al*., 2010)
Phyhip	C	Activation of mitophagy	(Bescond and Rahmani., 2005)
Park2	C/N	Autophagy, transcription regulation	(Im and Chung., 2015)
Psen1	C	Apoptosis, cell adhesion, Noch signaling pathway	(Ryu *et al*., 2010)
Ras	C	Cell proliferation	(Kelly and Rahmani, 2005)
Rcan1	C/N	Calcineurin-NFAT signaling cascade	(Song *et al*., 2013)
SF2/ASF	N	RNA splicing	(Qian *et al*., 2011)
Sept4	C	GTP-binding, nucleotide-binding	(Sitz *et al*., 2008)
Sf3b1/Sap155	C/N	mRNA processing, mRNA splicing	(de Graaf *et al.*, 2006)
Sirt1	C/N	Apoptosis differentiation, myogenesis, transcription	(Guo *et al.*, 2010)
Snca	C/N	Synaptic function	(Kim *et al.*, 2006a)
Snr1	N	Cell cycle, neurogenesis transcription regulation	(Kinstrie *et a.l*, 2006)
SPRED1/2	C	Cell signaling	(Li *et al*., 2010)
Spry2	C	Developmental protein	(Aranda *et al*., 2008)
Stat3	N	Transcription	(Matsuo *et al*., 2011)
Synj1	C	Endocytosis	(Chen *et al*., 2014)
α-Synuclein	N	Membrane trafficking	(Kim *et al*., 20069
Tra2β	N	Splicing	(de Graaf *et al*., 2004)
Tau	C	Brain development	(Ryoo *et al*., 2007)
Wasl	C/N	Cell cycle, cell division, mitosis, transcription	(Park *et al*., 2012)
Wdr68	C/N	Ubl conjugation pathway	(Morita *et al*., 2006; Miyata and Nishida *et al*., 2011)

1.4 Down syndrome mouse models

For most of the last century, the study of the molecular genetics of DS was an undertaking only in humans, focused on human postmortem material or biochemical indicators in blood. In the last 20 years, the generation of genetically modified mouse models has revolutionized the study of DS. The sequencing of human and mouse genomes at the beginning of 21st century confirmed that the long arm of HSA21 contains approximately 552 genes, being around 160 of them coding-protein genes. Among them, 157 genes are orthologous to genes located to syntetic region of three mouse chromosomes: MMU16, MMU17 and MMU10 (Hattori *et al*., 2000; Sturgeon and Gardiner, 2011) (Figure 3).

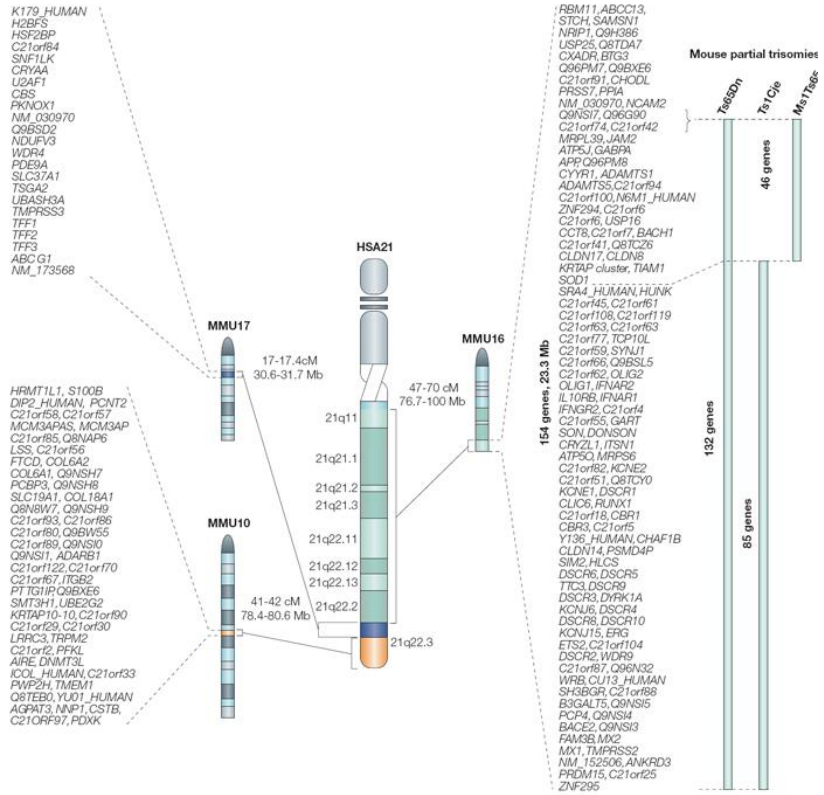


Figure 3: Regions of synteny between human chromosome 21 (Hsa21) and mouse chromosome (Mmu) 16, 17 and 10. Schematic comparative genetic maps of degree of conservation between Hsa21 and Mmu 16, 17 and 10 with three partial trisomy mouse models of human trisomy 21. (Adapted from Antonarakis *et al*., 2004).

Mice trisomic for chromosome regions syntenic to HSA21 may be more complete models of the DS phenotype, since they are trisomic for many genes triplicated in individuals with DS. On the other hand, trisomy of multiple genes makes interpretation of results more complex. Numerous trisomic or transchromosomal mice have been produced (Vacano *et al*., 2012). With the advent of chromosome engineering approaches, it is now possible to produce mice trisomic for any chromosome region. On the other hand, transgenic mice contain additional,

artificially introduced foreign genetic material, often a single gene, resulting in gain of function or overexpression of a certain protein(s). The use of transgenic mice provides thus an opportunity to study the biochemical and phenotypic implications of overexpression of individual trisomic genes in vivo (Vacano *et al*., 2012).

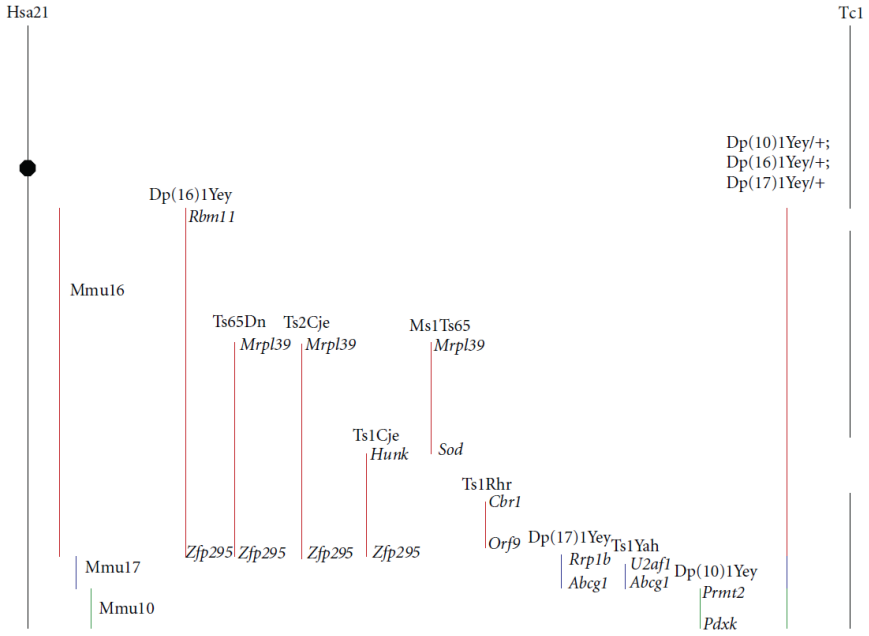


Figure 4: Schematic representation of Hsa21 and syntenic regions of Mmu16, Mmu17, and Mmu10 and the different mouse models trisomic for different set of genes orthologous of Hsa21. The flanked genes found at the boundaries of the triplicated region in each model are written in italics. (Adapted from Rueda *et al*., 2012).

1.4.1 Trisomic mouse models

The first attempt to create a mouse model of DS was to develop a trisomic model for the entire MMU16 named Ts16, trisomic for the entire MMU16 (Gropp *et al*., 1975). Although this allowed the first experimental studies, the Ts16 had poor construct validity, since it bears in trisomy many genes that are not triplicated in DS because MMU16 also present syntenies with other chromosomes such as HSA3, HSA8 and HSA16. Besides, this model lacks from several genes triplicated in HSA2. Its face validity was also limited since pups die in utero making impossible to test postnatal or adult phenotypes.

In the early 1990s, Muriel Davisson created a genetic mouse model for DS, the Ts65Dn strain. The Ts65Dn, bearing a partial trisomy of a segment of MMU16 (extending from the *MRP139* to *ZNF295* genes), is the most commonly used and widely studied mouse model of DS. Still, it contains approximately 55% of HSA21 conserved genes (Davisson *et al*., 1990; Reeves *et al*., 1995) and thus, is also questioned by its construct validity. However, extensive research has

demonstrated its face validity. Ts65Dn recapitulates most of the cognitive, and neuromorphological alterations of DS individuals. Similar to DS, Ts65Dn mice show learning and memory impairment in performing hippocampal-dependent tasks in spontaneous alternation in T-maze, contextual fear conditioning, novel object recognition and spatial memory in the Morris water Maze test (Reeves *et al*., 1995; Escorihuela *et al*., 1995, Fernandez and Garner, 2008). Young Ts65Dn also present spatial working and reference memory impairment in the radial arm maze (RAM) (Demas *et al*., 1996). The strain shows age-related cognitive decline associated to cholinergic neurodegeneration approximately at the age of 6-8 months, similar to humans.

Recently, a new strategy called CRISMERE has revealed as a powerful tool to manipulate rodent genomes in a fast and efficient manner (Birling *et al.*, 2017). This strategy opens a window to generate a better trisomic mouse model bearing all the DS genes in triplicate without extra genomic regions (Yann Herault, personal communication).

1.4.2 Transgenic mouse models

Over the last years, substantial research has revealed the biological relevance of specific HSA21 genes that play a fundamental role in the pathogenesis of DS. To study the role of these particular genes, several transgenic mouse models overexpressing a single gene have been created. Some examples are TgS100beta (Gerlai *et al*., 1993), TgSOD1 (Gahtan *et al*., 1998), TgSIM2 (Ema *et al*., 1999) and TgDyrk1A (Altafaj, Dierssen *et al*., 2001). In this Thesis, we will focus on the last model that overexpress our main target: *DYRK1A* and explained in the following sections.

Dyrk1A overexpressing strains

To study the specific contribution of the single overexpression of Dyrk1A on DS phenotypes, two transgenic mouse models have been generated. One is the mBACTgDyrk1A mouse model, in which a Bacterial Artificial Chromosome contains the *Dyrk1A* gene with its endogenous regulatory sequences. This allows the interaction of the transcription factors and thus, mimics the best endogenous *Dyrk1A* overexpression (Guedj *et al*., 2012. These mice show learning and memory impairments as well as synaptic plasticity alterations that are associated with LTP and LTD modifications (Ahn *et al*., 2006) and hyperphosphorylation of tau (Ryoo et al., 2007) among other alterations associated with DS pathology. In this Thesis, we have used a transgenic mouse model overexpressing *Dyrk1A* (TgDyrk1A) developed by the group of Cristina Fillat. This model overexpresses the cDNA of rat *Dyrk1A* under the controls of the inducible sheep metallothionein-Ia (sMT-Ia) promoter. Previous work demonstrated that the levels of Dyrk1A overexpression were similar to those observed in DS when the transgene was

not induced (Altafaj et al., 2001; Toiber *et al*., 2010), without ectopic expression in the CNS (Marti, *et al*., 2003). Behavioral analysis showed that TgDyrk1A mice present a delayed acquisition of mature locomotor activity, reduced motor coordination during neurodevelopment and maintained to some extent in the adult, impaired motor learning and altered visuospatial learning and reference memory in the adult (Altafaj et al., 2001). These behavioral alterations have suggested a dysfunction in some brain structures involved in learning and memory (cortex, hippocampus and cerebellum) and motor functions (cerebellum, striatum and motor cortex) in TgDyrk1A mice. During postnatal periods, *Dyrk1A* overexpression produces motor developmental alterations possibly contributing to DS motor phenotypes and modifies the number of cholinergic neurons suggesting that the kinase may have a role in the development of the brainstem and spinal cord motor system (Arque *et al*., 2013). Furthermore, TgDyrk1A mice also exhibit altered hippocampal LTP and LTD associated with learning and memory defects (Ahn *et al*., 2006) and reduced dendritic length and branching accompanied with fewer spines have been observed in pyramidal neurons in layer II of the secondary motor cortex (Martinez de Lagran *et al*., 2012). TgDyrk1A show an important alteration in adult neurogenesis including reduced cell proliferation rate, altered cell cycle progression and reduced cell cycle exit leading premature migration, differentiation and reduced survival of newly born cells. Not only that, but less proportion of newborn hippocampal tgDyrk1A neurons are activated upon learning, suggesting reduced integration in learning circuits. Some of these alterations are DYRK1A kinase-dependent since they were rescued upon its normalization (Pons-Epinal *et al*., 2013)

1.5 Therapeutic strategies for intellectual disabilities

Given that genetically determined neurodevelopmental disorders leading to intellectual disability as DS caused by a full or partial extra copy of a chromosome including hundreds of genes, it is generally viewed as a too complex genetic perturbation to be amenable to postnatal interventions. Thus, until relatively recently, DS was considered an “incurable” disease by most people and, what is more, it was viewed as a disorder that hindered individuals from acquiring education (Smith *et al*., 1976). However, a number of therapies of different nature have been implemented or examined in an attempt to attenuate the cognitive impairments in individuals with DS. Our group has contributed to demonstrate that despite this broad spectrum of possible causes, there are common neuropathological alterations that converge on the same molecular pathways.

Some of these pathways are related with plasticity suggesting neuroplasticity is a key mechanism contributing to cognitive impairment (Dierssen et al., 2003; Dierssen and Ramakers, 2006). It is important then, to elucidate the main responsible of the phenotype to find pharmacological therapies that target those abnormalities. Neural plasticity is the capacity of the brain to reorganize itself,

both physically and functionally, throughout the life due to the environment, behavior, thinking, emotions and experiences. These changes could include modification in: (2) strength of synaptic functions, (2) transient fluctuations in neurotransmission and (3) changes in morphology and number of spines, (4) neurogenesis, (5) synaptogenesis and (6) changes in dendritic arborization and complexity. These are functions found to be disturbed in most cases of ID, especially regarding dendritic spine alterations (also known as dendritic pathology), which are hypothesized to lead to a suboptimal number of efficient synaptic connections associated to information processing and storage, giving rise to ID. In fact, it has been proposed that neuroplasticity-targeted pharmacologic interventions that promote neuroplasticity could exert positive effects in different forms of IDs including DS (Benavides-Piccione *et al*., 2004). Interestingly many of those phenotypes are also present in transgenic mice only overexpressing DYRK1A suggesting its key role in neuroplasticity processes.

1.5.1 Non-pharmacological interventions for Down syndrome

Being identifiable at birth, DS is a suitable target for early efforts to ameliorate neuropsychological and linguistic development. The early intervention literature concerning Down syndrome has been reviewed on several occasions (Gibson and Fields, 1984; Gibson and Harris, 1988; Gunn and Berry, 1989 and Spiker, 1990). At the moment the early intervention programs that are primarily focused on infants and young individuals, aimed at providing cognitive stimulation and special education to promote children's development of skills and support them to fully participate in family, school and community life (Odom and Diamond, 1998). These programs consist on specific interventions that emphasize education and training, targeted to cognitive domains that are especially affected in individuals with DS, such as speech, language and nonverbal communication, motor and problem-solving skills, attention, learning and memory. Training strategies involve reinforcement principles and stimulus-response learning models and behavior modification in relevant aspects for self-development, peer interactions and integration in society.

Several studies have shown that early intervention programs induce beneficial effects on children with DS, including acceleration of skill acquisition, prevention of abnormal patters of functioning, promotion of better parent-child interactions and encouragement of inclusion (Bailey *et al*, 1997; Meisels and Shonkoff, 1990; Rondal *et al.*, 2011; Engevik *et al*., 2016). Additionally, cognitive and physical exercise programs improved health status and wellbeing in adults with DS (Moni and Jobling, 2001; Heller *et al.*, 2004b). However, although these improvements made a huge impact in the way individuals with DS are integrated in society, they are limited since the skills learnt through these programs are rarely generalized to everyday situations (Moni and Jobling, 2001; Mahoney *et al*., 2006; Bonnier,

2008), suggesting that intervention programs are still insufficient to mitigate cognitive impairment and provide only moderate relief in DS.

1.5.1.2 Environmental enrichment studies in rodents

From the biological perspective, multiple studies mainly in rodents but also in humans have strengthen the idea that experience is able to regulate the structure and function of different areas of brain both in young and adult individuals (Watanabe *et al*., 1992; Maguire *et al*., 2000; Bermudez *et al*., 2009; May, 2011). Along the past fifty years, the paradigm to study the effects of experience and environmental stimulation in experimental settings with rodents has been environmental enrichment (EE), which consist of housing conditions involving a complex combination of physical activity, learning experiences and social interactions (Rosenzweig and Bennett, 1996). The beneficial effects of EE on behavior and brain function have ever since been reported in a multitude of studies using rodent spatial memory, neuroanatomical, cellular and molecular assays (Greenough *et al*., 1973; Rosenzweig and Bennett, 1996). In particular, changes such as increased brain weight, neurotransmitter content, synaptic plasticity, and dendritic spine growth, as well as upregulation of neuronal signaling molecules, neurotrophin levels, and adult hippocampal neurogenesis have been associated with cognitive enhancement (for reviews Praag *et al.*, 2000; Nithianantharajah and Hannan, 2006; Baroncelli *et al*., 2010; Voss et al., 2013). It is widely accepted that EE is a cognitive enhancing intervention that promotes synaptic plasticity, adult neurogenesis, and epigenetic modifications, among other processes (Sale *et al*., 2014). These biochemical, morphological and functional changes in the brain are due to posttranslational modifications affecting protein activity or to changes in the expression of genes.

Several research groups have shown that it is possible to partially rescue DS phenotypes using nonpharmacological strategies such as postnatal handling or cognitive training by EE that ameliorate behavioral and brain alterations in the Ts65Dn mouse model of DS (Martínez-Cué *et al*., 2002; Dierssen, 2003; Begenisic *et al*., 2011; Chakrabarti *et al*., 2011; Golabek *et al*., 2011). However, despite its beneficial effects, EE is not sufficient to promote long-lasting dendritic spine remodeling in Ts65Dn mice (Dierssen, 2003) or significant developmental changes in DS children (Mahoney *et al*., 2004).

1.5.2 Pharmacological interventions for DS

So far, pharmacological interventions in DS have been mainly targeted to restore the neurotransmitter imbalance found in the disorder or the increased synaptic inhibition. Some of these drugs include acetylcholinesterase inhibitors, such as donepezil and rivastigmine (Heller *et al*., 2004a, 2010; Prasher, 2004; Spiridigliozzi *et al*., 2007; Kishnani *et al*., 2010), nicotine (Seidl *et al*., 2000),

acetyl-L-carnitine (Pueschel *et al.*, 2006) and N-methyl-D-aspartate (NMDA) receptor antagonist memantine (Hanney *et al.*, 2012). Unfortunately, results from clinical trials do not support the use of NMDA antagonists like memantine and nor GABA(A) antagonists to conclude the efficacy of approaching DS therapeutics by modulating neurotransmission systems altered in this pathology (De la Torre and Dierssen, 2012). In addition, the potential of diverse compounds, such as vitamins and mineral supplements, has been assessed to ameliorate DS symptoms. Antioxidants have been used to counteract increased oxidative stress resulting from the over activity of CuZnSOD1 and folate supplementation to normalize the folate deficiency derived from Cystathionine b-synthase (Ellis *et al*., 2008). Although some of these interventions promoted positive outcomes for some singular participants, most of them have yielded disappointment due to their limited efficacy or complete failure to provide improvement in DS cognition (reviewed in detail in de la Torre and Dierssen, 2012).

Alternatively, to the modulation of specific neurotransmission systems, therapeutic approaches have also attempted at normalizing the expression levels or function of candidate genes. One interesting example is *Dyrk1A*. Ortiz-Abalia and colleagues demonstrated for the first time that normalization of *Dyrk1A* expression in the striatum of TgDyrk1A mice through the injection of an adeno-associated virus type 2-mediated Dyrk1A inhibitor (AAVshDyrk1A), rescued motor alterations in these animals (Ortiz-Abalia *et al*., 2008). In 2013, Altafaj and colleagues also demonstrated that the normalization of Dyrk1A expression in the hippocampus of Ts65Dn mice rescued cognitive impairments in the MWM (Altafaj *et al*., 2013). Moreover, those effects were possibly dependent on its kinase activity, since specific DYRK1A kinase activity inhibitors, such as harmine, were able to rescue neuritogenesis alterations in cortical cultures from TgDyrk1A mice (Martinez de Lagran *et al*., 2012). However, the use of harmine in vivo in animal models of DS, is not really useful because of its toxicity and its inhibitory activity on monoamine oxidase-A (MAO-A) (Kim *et al*., 1997). Thus an effort was done to identify clinically suitable alternatives for DYRK1A kinase inhibition.

1.5.2.1 Epigallocatechin-3-gallate

In 2003, Bain and colleagues described the properties of 30 inhibitors, tested with 25 kinases. One of them was epigallocatechin-3-gallate (EGCG) (Bain *et al*. 2003), the most abundant catechin found in green tea, with antioxidant and neuroprotective properties that has been shown to efficiently improve cognitive phenotypes in DS individuals and mouse models (De la Torre *et al*., 2014), ameliorate synaptic plasticity impairment in vitro (Xie *et al*., 2008), and restore excitatory/inhibitory (E/I) imbalance in Ts65Dn mice potentially modulating the GABAergic pathway (Souchet *et al*., 2015). EGCG is a natural inhibitor of the kinase activity of Hsa21 candidate gene Dyrk1A with an apparent IC50 of 0.33 μM (Bain *et al*., 2003) as recently confirmed (Wang *et al*., 2012a). EGCG had

been reported to be able to cross both the blood brain barrier in conscious and freely moving rats (Lin *et al*., 2007) and the placental barrier in gestating rats (Chu *et al*., 2007). A study in mouse embryonic fibroblast immortalized NIH-3T3 cells, showed that the mechanism by which EGCG acts on DYRK1A kinase activity involves a non-competitive inhibition against ATP binding site (Adayev *et al*., 2006). Interestingly, when EGCG was given to pregnant BACTgDyrk1A females, it prevented the alterations in brain volume and cognitive deficits of their pups (Guedj *et al*. 2009).

Our group also assessed the effects of one-month oral treatment with EGCG (30 mg/Kg) on post-weaning TgDyrk1A mice and found a normalization of the excessive proliferating cells and their accelerated cell cycle exit in the granular cellular layer of the DG, a phenotype that possibly contributes to deficient spatial learning and memory in these mice (Pons-Espinal *et al*, 2013). These changes were accompanied by a normalization of hippocampal DYRK1A kinase activity levels (Pons-Espinal *et al*., 2013), suggesting a potential pharmacological role of EGCG to tackle DS altered neurodevelopment and neuronal differentiation, at least partially due to its ability to normalize DYRK1A kinase activity. So far, the above data along with other multiple studies indicate that DYRK1A exerts effects on synaptic neuroplasticity, brain and skeletal development, neuronal cell cycle and differentiation and, hippocampal and cortical excitation/inhibition balance. Thus, DYRK1A had been shown to be a good candidate gene for many DS related phenotypes, considerable interest grew around the therapeutic potential of EGCG as it provided the means to rescue DS phenotypic features with a natural and apparently safe polyphenolic compound.

However, it is rather unlikely that the benefits of EGCG are limited to the inhibition of Dyrk1A kinase activity. As pointed by Gardiner (2014), the reduction of DYRK1A kinase activity occurs in a context of elevated expression of other multiple HSA21 genes. Among those other overexpressed Hsa21 genes, some proteins are phosphorylation substrates of DYRK1A, such as APP, SYNJ1 and RCAN. Thus, if EGCG optimally and specifically decrease Dyrk1A activity in the context of elevated expression of those Hsa21-encoded substrates, additional imbalances relevant to DS phenotypic features could arise. Additionally, EGCG participates in a multiple signaling pathways that could contribute to the beneficial effects observed in Ts65Dn and other partial trisomic mice (Figure 5).

In vitro studies demonstrated that EGCG affects a wide array of signal transduction pathways including JAK/STAT, MAPK, PI3K/AKT, WNT and NOTCH (Singh *et al*., 2011).

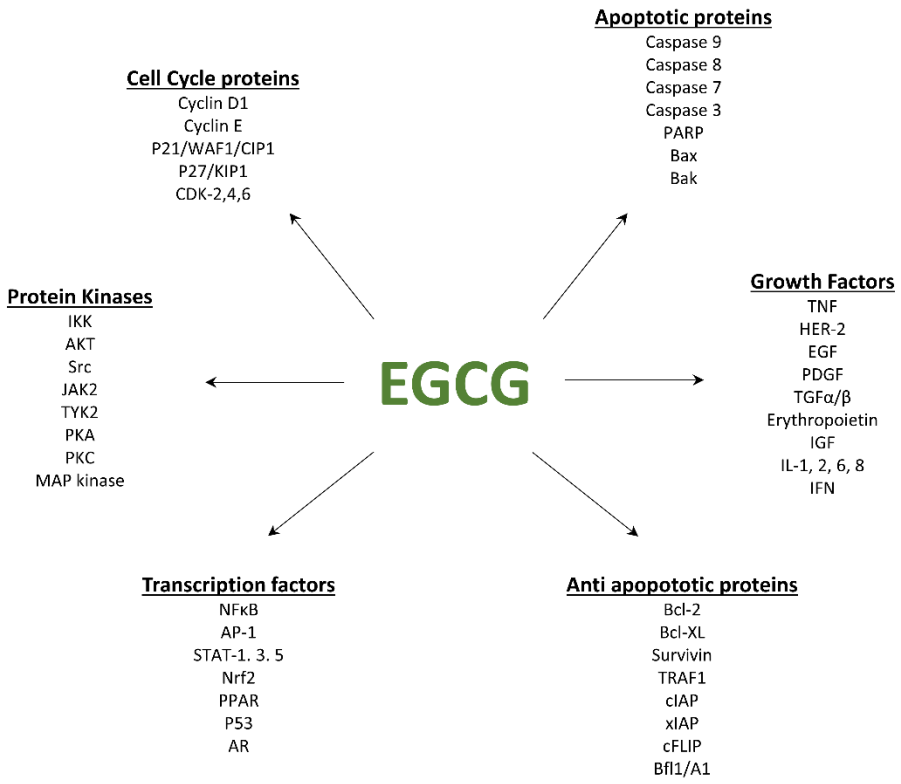


Figure 5: Different mechanisms of action of EGCG. EGCG targets include cell cycle proteins, protein kinases, transcription factor, anti-apoptotic proteins, growth factors and apoptotic proteins. (Adapted from Singh *et al*., 2011).

1.6 Addressing the molecular complexity of Down syndrome: challenges and limitations

The functional genomic exploration of the post-sequencing years of HSA21, DS is the model human phenotype for genomic gain dosage imbalances, including microduplications. This leads to a molecular complexity that possibly explains the high phenotypic variability detected in this syndrome, and the fact that drugs as EGCG, highly promiscuous in its molecular targets, are efficacious in DS.

DS is inherently complex with hundreds of over- or underexpressed genes with heterogeneous transcriptional modulation, molecular mechanisms that may be heterogeneous among individuals and may change over the lifespan in each individual. To make it even more complex, specific genes, such as *DYRK1A*, have the potential of impacting a myriad of molecular systems. However, to date, the scientific framework in DS has been largely reductionist in nature, making it extremely difficult to address the most basic questions.
Emerging technologies, including proteomic approaches now allow investigating the complexity of interactions of complex molecular networks within an individual with a unique behavioral profile and developmental history. Even so, several limitations are challenging the study of DS: i) the expected relatively small protein

level fold change caused by 1.5-fold gene overdosage that poses a technical challenge for many omic technologies, ii) the reduced availability of postmortem human tissues with well-characterized neuropsychological phenotypes, iii) the marked cell type-specific phenotype in DS that challenges the interpretation of the omic signature that is found at the tissue level.

Given that the majority of the DS phenotypes are probably related to alteration of gene expression, several studies have compared trisomic versus euploid cells or tissues at the transcriptional level (Ait Yahaya-Graison *et al*., 2007; Altug-Teber *et al*.,2007, Conti *et al*., 2007; Costa *et al*., 2011; Prandini *et al*., 2007; Sommer *et al*., 2008; Sultan *et al*., 2007). Different methods from SAGE (Serial Analysis of Gene Expression), microarrays, qPCR, or more recently, RNA sequencing have been used to assess the gene expression changes in human or mouse. In the absence of any dosage compensation mechanism, HSA21 genes should theoretically be expressed 1.5 times more in trisomic tissues than in normal tissues. However, it has been shown that the changes in copy number do not always correlate with the level of gene expression (Kahlem *et al*., 2004; Lyle *et al*., 2004). In fact, only a subset of HSA21 genes seems to be affected by the triplication. This subset is highly variable (6-62% depending on the study) and moreover, some of those genes appear to be more overexpressed than others. Those observations suggested that some mechanisms could compensate or modulate the gene dosage effect on HSA21. The same studies have shown expression changes of genes outside the HSA21, suggesting direct or indirect functional interactions between genes in HSA1 and genes in other chromosomes. Finally, the picture gets even more complex when taking into account that gene expression is tissue specific and thus trisomy would most possibly affect differentially the transcriptome in different tissues.

Even though the transcriptional deregulation is *per se* informative, a major disturbance such as an extra copy of a chromosome is expected to strongly interfere protein production and expression. Proteins are main actors in the cells and may have multiple levels of disturbance, including protein abundances and various post-translational modifications (PTMs), such as phosphorylation or glycosylation. A proper maintenance of the proteome and its PTM is essential to preserve cell functionality and the ability to respond and adapt to the changing environment. Unfortunately, the changes at the proteome level cannot be predicted from transcriptional studies, since the correlation between mRNA and protein abundances in the cell is notoriously poor as the levels of protein depend on a number of biological factors, which are difficult to identify (e.g. protein half-life and turn over) (Maier *et al*., 2009). This is especially applicable to a situation of genetic overrepresentation of a number of genes, such as DS, where a weakly correlation has also been demonstrated recently in a study from Letourneau *et al*., 2014 who detected discordances between the transcriptome analysis and the proteomic changes in fetal skin fibroblast derived from 11 DS and 11 unrelated,

sex and age-matched normal controls (Liu *et al*., 2017) even in fetal fibroblast from a pair of monozygotic twins discordant for trisomy 21. However, although the impact of trisomy 21 on the transcriptome has been relatively well studied, we still lack in depth understanding of how it affects the proteome.

1.6.1 Proteomic studies in Down syndrome

It has been suggested that the comparison of protein abundances between conditions may already reveal alterations in cellular functions. Also, relevant PTM readouts, such as phosphorylation levels will help understanding deviation in activities of signaling pathways providing important information to decode diverse activities of cellular protein networks (Park *et al*., 2015).

Proteomics studies on DS samples started in 2000 with the work by Opperman which employed two-dimensional electrophoretic (2DE) separation to analyze fetal brain samples with the aim of producing a 2D map of the most abundant proteins from fetal human brain. The comparison of DS and control subjects showed 84% homology between the two groups, and all the proteins found differentially suggested alteration of brain development (Oppermann *et al*., 2000).

From there on, the group of Lubec, who collaborated with our lab, initiated a comprehensive proteomics analysis of fetal DS brain that allowed identification and quantification of 10 protein spots with different expression in DS vs. control brain (Cheon et al., 2001). Among these, septin 6 showed decreased expression in DS that could be involved in the defective development of fetal DS brains. In 2002 the same group, using proteomics techniques, evaluated the protein expression levels of several enzymes involved in different metabolic pathways of intermediary metabolism in fetal DS and control brains. The researchers demonstrated alterations of energy metabolism pathways as indexed by increased expression of mitochondrial aconitase and mitochondrial NADP-isocitrate dehydrogenase, and suggested that brain intermediary metabolism is deranged during prenatal development of DS (Bajo et al., 2002). In Fountoulakis *et al*., 2002 employed two-dimensional (2D) electrophoresis followed by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI) to analyze human fetal DS brain. Those initial studies already revealed downregulation of several proteins, associated to the impaired brain development and deteriorated synaptic functions in fetal DS cortex. Other proteins from the neuronal structure, and dendritic spines, as septins, the synaptosomal associated protein (SNAP25) or DREBRIN, a microfilament-associated protein in the post-synaptic sites were also found to be deregulated. In 2004, the Lubec group applied 2-DE and MALDI-MS to analyze the number of differently expressed spots between fetal DS and normal brain (Shin *et al*., 2004), and identified three proteins encoded on HSA21: cystathionine β-synthase (CBS), pyridoxal kinase (PHK), and ES1 protein homolog, mitochondrial precursor, among which, only ES1 showed a significant

increase in DS. In 2006, Lubec and co-workers using mass spectrometry, successfully identified 9 proteins encoded on HSA21 with different expression levels between DS and control fetal brain: PHK, SOD1, CBR1, ES1, CBS, TCPQ, T-complex protein 1, theta subunit, cystatin B (CSTB), 6-phosphofructokinase, liver type (PFKL), and glycinamide ribonucleotide synthetase (GARS) (Shin *et al*., 2006). Interestingly CSTB inhibits cathepsin B and blocks apoptosis; indeed, mice with a gene deletion of CSTB exhibit increased apoptosis of specific neurons (Brännvall et al., 2003). Moreover, increased levels of cathepsin B with co-localization in senile plaques have been observed in brains of adult DS individuals and AD (Lemere *et al*., 1995).

More recently, reports from Lubec laboratory (Sun *et al*., 2011) aimed to complement the previous studies on the fetal DS partial proteomes or altered expression levels of individual proteins that may play a role for the abnormal development of the DS brain. The major outcome of their work was to show altered protein pathways and cascades possibly involved in the pathological mechanisms of fetal DS brain development, before morphological changes are detectable. Proteins found to be deregulated were not previously observed; however, this novel pattern of alterations is consistent with previous studies that demonstrate that aberrant expression of proteins leads to the impairment of specific functions in DS such as synaptic plasticity, brain development and energy metabolism, directly involved in DS pathology. Due to the high prevalence of Alzheimer's like disease (AD) in people with Down syndrome other proteins have been studied as dihydropyrimidinase related protein 2 (DRP-2) (Lubec *et al*., 1999) and Stathmin (STMN1), which is distributed in neurons and involved in the various transduction pathways as well as regulation of microtubule destabilization, thus playing a critical role in (AD) in patients with AD and DS finding decreased levels in frontal and temporal cortices in adult brains (Cheon *et al*., 2001).

Also PTMs have been explored in DS. Regarding protein kinases and phosphatases, critical proteins in transduction pathways, the findings of the study of Weitzdoerfer *et al*., (2015) revealed alterations in the phosphorylation of important protein kinases such as CAMKIIa, a prominent kinase in synaptic plasticity in the central nervous system, TAK1, a member of the MAPK signaling pathway and PTEN, a phosphatase involved in development of neuronal and synaptic structures.

1.6.2 Proteomic studies in mouse models

In mouse models of DS there have been several attempts to understand the proteomic signature. The Ts65Dn is the most well validated model of DS, since it displays many features relevant to DS. This model contains 50% of the genes homologous for HSA21 in three copies, exhibits craniofacial skeletal

malformation and reduced cerebellar volume and granular and Purkinje cell densities. TS65Dn mice also display learning and behavioral deficits, including impaired performance in tasks, such as the Morris water maze. However, other models have been also evaluated.

The laboratory of Gert Lubec was again pioneering in this field. In 2006 they analyzed the quantitative protein changes in WT mice and the 141G6 mouse model of DS. YAC141G6 mice were generated, by inserting yeast artificial chromosomes (YACs) containing a fragment of the human DSCR-1 region in the mouse genome. 2D proteomics analyses demonstrated significantly altered expression levels of a series of identified proteins correlate with results obtained in reports on human brain (Shin *et al*., 2006). Among others were α and β tubulin, HSP60 and 90, peptidyl-prolyl cis-trans isomerase A (Pin-1), aspartate aminotransferase (AAT), ATP synthase, laminin receptor, suggesting aberrant expression of proteins belonging to antioxidant response, chaperone system, cytoskeleton, proteostasis network and metabolic pathways implicated in neurodegeneration and cognitive decline known to occur in DS.

In 2009 Wang *et al*. (2009) performed a proteomic study on Tc1 mice embryonic stem cells. The Tc1 DS model contained a single supernumerary HSA21 and reproduces a number of DS phenotypes including heart defects, learning difficulties, and a reduced cerebellar neuron count (O'Doherty *et al*., 2005). Using ITRAQ and absolute quantification, 52 proteins were identified to be differently expressed when an extra human HSA21 was present. From those, 15 proteins were down-regulated, and 37 showed higher expression in DS cells. Among the protein identified several have direct associations with DS and the extra copy of Chr21 such as: DSCR1, DSCR3, DSCR5, TIAM1, TTC3, DYRK1A and APP. Other proteins are associated with the premature onset of AD. Indeed, alterations in CTSB, LRP2, and LRPAP1 expression levels are consistent with previous studies on Aβ formation and clearance in AD (Dierssen, 2012). Overall, this study demonstrates the correlation between expression differences in embryonic stem cells from mouse and human DS fetal tissue.

A study by Ishihara *et al*. (2009) on primary cultured astrocytes and neurons from Ts1CJe mouse model of DS demonstrated an increased level of ROS and mitochondrial dysfunction using a redox proteomics approach. The authors identified the putative target proteins modified by lipid peroxidation-derived products, that were involved in ATP generation, the neuronal cytoskeleton and antioxidant enzymes, suggesting the dysfunction of these pathways as a consequence of oxidative damage.

1.6.3 Recent advances and future of proteomics studies in Down syndrome

Even though those studies have importantly contributed to the understanding of the pathogenesis of DS, the proteomic techniques used only allowed the study of very few number of proteins, usually with a hypothesis-driven behind, and lacking of information about the whole proteome profile. In the last recent years, mass spectrometry has improved rapidly relying on tandem mass spectrometry (LC-MS/MS) as a method referred to as bottom-up proteomics (Figure 6). Briefly, bottom up (or shotgun) LC-MS/MS is currently geared toward the discovery or validation of differential protein regulation on a large scale in response to biological perturbations (e.g., normal versus disease, or control versus treated samples). MS data acquisition, quantification, and data analysis and multiple tools have been developed and reviewed extensively (Cox and Mann, 2011; Yates *et al*., 2009; Domom and Aebersold, 2010; Nesvizhskii *et al*., 2007 and Mallick and Kuster, 2010).

In fact, the use of mass spectrometry (MS) has revolutionized proteome studies, in a manner analogous to the impact of next generation sequencing on genomics and transcriptomics. MS has allowed to cataloging complete proteomes of unicellular organisms as yeast or Mycobacterium tuberculosis (Nagaraj *et al*., 2012; Picotti et al., 2013; Kelkar *et al*., 2011) and to deeply explore proteomes of higher organisms including the generation of a tissue-specific atlas of mouse protein phosphorylation and expression (Huttlin *et al*., 2010), a cell-type and brain region-resolved mouse brain proteome (Sharma et al., 2015), or draft maps of the human proteome (Wilhelm *et al*., 2014; Kim *et al*., 2014; Schwenk *et al*., 2017). Also, new *in vivo* quantitative proteomics with SILAC has allowed metabolic labelling in whole animals (Zanivan *et al*., 2012) and new protein-protein interaction methods can screen for physiologically relevant protein interactions that occur in living cells (Roux *et al*., 2013).

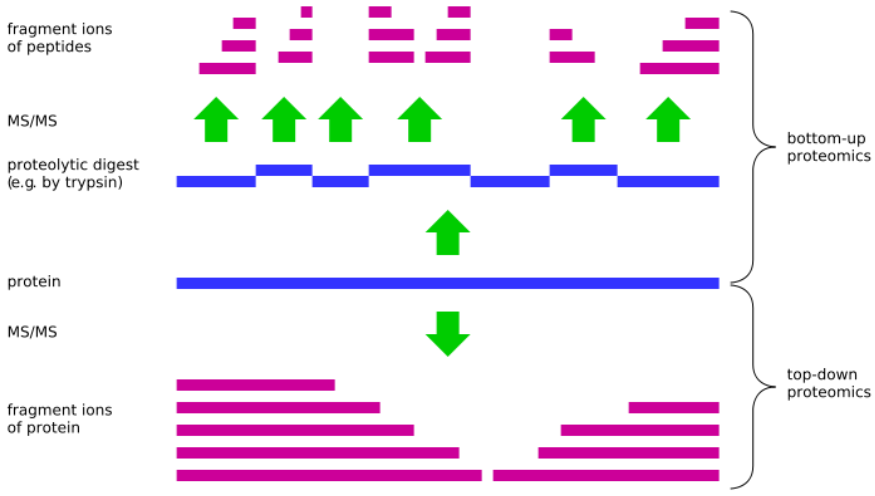


Figure 6: Schematic representation between bottom-up versus top-down proteomics. In the conventional bottom-up approach, purified proteins or complex mixtures of proteins are digested into peptides via proteolytic cleavage, separated by a chromatographic column and the peptide products analysed in mass spectrometer. In top-down proteomics, intact proteins ions are generated by electrospray mass spectrometry, introduced to the mass analyser and subjected to fragmentation.

The field of neurosciences has benefited from these recent advances in the MS field. Indeed, in DS, bottom-up MS has also been applied to study the pathology of both mouse models and human samples. Quantitative proteomic analysis by MS in amniocytes (amniotic fluid cells) of DS and euploid fetuses (Cho *et al*., 2013) found, among 4,900 proteins identified, 900 proteins potentially dysregulatedin amniocytes wit trisomy 21 in pathways including NF-κB or APP.

Fernandez *et al*. (2009) analyzed the protein composition of synapses from Ts65Dn. The authors employed synaptosomes or postsynaptic densities from the Ts65Dn cerebrum and evaluated synaptic protein profiles via two quantitative methods: Odyssey-based fluorescence Western blotting and iTRAQ technique. Results on synaptosomal fraction showed only modest changes in protein expression, including increased levels of synaptojanin and decreased levels of ERC1/CAST2/ELKS, the PSD proteins PSD-95 and CaMKIIa, as well as the a1 subunit of the GABA receptor. In PSD preparations from Ts65Dn mice the few synaptic proteins that exhibit changes included Munc13, fragile X mental retardation protein, the beta4 subunit of the voltage-dependent calcium channel, and liprin. These biochemical data are consistent with other reports showing little change in the expression of proteins from synaptosomes and PSDs isolated from the cerebri of adult Ts65Dn mice (Pollonini *et al*., 2008; Belichenko *et al*., 2009). However, shifts in the phosphorylation of a variety of synaptic proteins including pre- and post-synaptic scaffold proteins and receptors such as synapsin, piccolo, liprin, dynamin, PSD-95 or NMDA receptors were observed. Recently, protein arrays have assayed the expression levels of proteins/protein modifications in six brain regions/subcelullar fractions from Ts65Dn providing useful information about upregulated and downregulated proteins mainly in hippocampus and cortex (Ahmed *et al*., 2015).

1.7 Network biology and network pharmacology

High-throughput molecular data such as proteomic and RNA expression data have to be systematically analyzed to better understand how the different molecular entities, either genes or proteins, and their interactions determine changes in specific functions. This is driven by the fact that molecules rarely function alone, but exert their functions through stable complexes or engaging in transient interactions. This subcellular interconnectivity implies that the impact of a specific genetic abnormality is not restricted to the activity of the encoded gene

product, but can spread along the links of a network, and alter the activity of gene products that otherwise carry no defects (Barabási *et al*., 2011). Recently, network biology has emerged as an integrative and systems-level approach to aid the interpretation of data in the context of health and disease, with the limitation of the incompleteness of human and other species protein-protein interaction databases (Furlong *et al*., 2013). Networks can resolve many analytical problems in proteomics, including incomplete coverage and inconsistency.

Protein-protein interaction networks represent and analyze proteins as computable sub-units (nodes) that are linked through their interactions (edges). However, other physical interactions between molecules in those networks include protein-protein, protein-nucleic acid, and protein-metabolite. The nodes and edges form a network, or, in a more formal mathematic language, a graph. Nodes can be characterized by their role in the network as peripheral, hubs or bottlenecks. The relative importance of a node in a network is often measured by the magnitude of changes in the network structure caused by its removal. Thus, the more partners a node has in a network, the higher the probability of this node (called hub) to engaging in essential protein-protein interactions. Hence, hubs are more important than non-hubs to the maintenance of the global network structure (He and Zhang, 2006). In directed networks, the interaction between two nodes has a well-defined direction, for example the flow from a substrate to a product or the flow from a transcription factor to the gene it regulates. In undirected networks, the links do not have an assigned direction, for example a protein-protein interaction representing a mutual binding relationship. Their architecture is governed by a few simple principles that are common to most networks. Finally, the concept of modular biology, proposed by Leland Hartwell and his colleagues, states that the biological functions of the cell are carried out by multicomponent modules (Hartwell *et al*., 1999). Networks can also be modular. In a network representation, a module (or cluster) appears as a highly interconnected group of nodes reflected by a clustering coefficient. In protein-protein interaction networks these modules usually contain proteins involved in the same biological process.

In the field of DS, studies that combine high-throughput data with network analyses have mainly been undertaken by the group of Kathleen Gardiner that introduced network analysis to the study of up- and downregulation of genes. The rationale behind was that this is not only causing modest perturbation of reduced processes but can affect the whole intracellular network. They were the first to propose that assembling data from high-throughput experiments or text-mining experimental literature could be combined in networks to build next generation computational models with predictive capabilities (Ma'ayan *et al*., 2006).

Network biology theory predicts that modulating multiple nodes simultaneously is often required for modifying phenotypes (Barabasi and Oltvai, 2004) but also plays a role in drug-target identification. It has been suggested that targets of these drugs tend to have more interactions than average proteins, thus essential proteins, even it's not necessary to be an essential hub in the biological network. Over the past two decades, the concept of designing highly selective ligands or drugs that act on single targets to avoid unwanted side effects, which was the predominant paradigm in drug discovery, has been strongly questioned. The 'one-drug one-disease' model, related to the 'one-gene one-disease' idea, is a recipe for inevitable failure in modern pharmaceutical development, often with multiple side effects and inefficacy. This is due to the fact that those models do not consider the interactions of the complex drug-target network environment and the discovery of compensatory signalling pathways However, a new paradigm called network pharmacology is emerging revealing a far more complex picture of drug action. In fact, in the recent years, it has been appreciated that many effective drugs in different therapeutic areas act on multiple rather than single targets (Roth *et al*., 2004), a phenomenon known as poly-pharmacology or "promiscuous drugs" (Paolini *et al*., 2006), less toxic drugs targeting multiple protein families are considered. In fact, network pharmacology is an approach to drug design that encompasses systems biology, network analysis, connectivity, redundancy and pleiotropy. Network pharmacology re-introduces the old idea that understanding the biological and kinetic profile of the drug is more important than individual validation of the targets or combinations of targets (Hopkins, 2008).

HYPOTHESIS AND OBJECTIVES

“What used to be called prejudice is now called a null hypothesis”
A. W. F. Edwards

2. HYPOTHESIS AND OBJECTIVES

In the last years, it has become clear that the scale-free nature of many biological networks results in systems resilient against random deletion of any one node. This fact also includes molecular networks, in which the main players are the physical and functional interactions among the biomolecules of the cell. This inherent robustness has profound implications for disease and drug discovery; instead of searching for the 'disease-causing' genes, network biology suggests that the strategy is to identify the changes that occur in the disease-causing network after a perturbation. Since Down syndrome (DS) is caused by a trisomy of the chromosome 21, hundreds of triplicated genes could contribute to the phenotype. Thus, it would be even more challenging to identify bona fide disease-causing genes. However, pioneering studies in our lab indicate that the dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A), a dose-sensitive gene in HSA21 is necessary and sufficient to recapitulate some of the DS cognitive deficits and that restoration of DYRK1A overdosage can rescue behavioral, cognitive and neuronal phenotypes in preclinical studies with DS mouse models and clinical trials in humans.

2.1 Hypothesis

Here we propose that *DYRK1A* is a disease-causing gene for cognitive and neuronal phenotypes of DS. Given the multiple substrates, interactors, subcellular location, cell functions etc. described for the encoded protein, the consequences of *DYRK1A* overexpression could spread along a complex intracellular network leading to a disease-causing network. Thus we also propose DYRK1A as a good molecular target to restore the network functionality in DS.

2.2 Objectives

The main objective of this Thesis is to elucidate the biological pathways and the structure of the disease-causing proteome and phosphoproteome network and its putative rewiring upon pro-cognitive therapies using DS mouse models.

Specific objectives:

1. Evaluate the impact of *in vivo Dyrk1A* overexpression on hippocampal learning and memory in *Dyrk1A* overexpressing mice (TgDyrk1A).

2. Quantify the proteomic and phosphoproteomic alterations that result from *in vivo Dyrk1A* overexpression in the hippocampus of TgDyrk1A mice.

3. Identify the biological pathways and structure of the disease-causing network in the hippocampus of TgDyrk1A mice.

4. Quantify the hippocampal proteomic and phosphoproteomic alterations upon pro-cognitive treatments (epigallocatechin-3-gallate, environmental enrichment and its combination) in the hippocampus of TgDyrk1A mice.

5. Elucidate the treatment-driven rewiring disease-causing network in the hippocampus of TgDyrk1A mice.

MATERIALS AND METHODS

“To consult the bioinformatician after an experiment is finished is often merely to ask him to conduct a post mortem examination. He can perhaps say what the experiment died of.“

Ronald Fischer

3. MATERIALS AND METHODS

In this Thesis we used TgDyrk1A mice as biological model for behavioral analysis and high-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) to elucidate the biological pathways and the structure of the disease-causing networks and their rewiring upon pro-cognitive therapies in the hippocampus of TgDyrk1A mice.

3.1 Animal model and genotyping

All the experiments were performed using 2-month-male transgenic mice overexpressing *Dyrk1A* (TgDyrk1A) generated by Dr. Cristina Fillat (Altafaj *et al*., 2001). The transgene was constructed using the rat full-length *Dyrk1A* cDNA that shares high sequence homology with the corresponding mouse cDNA (Song *et al*., 1996), under the control of the heterologous sheep metallothionein-Ia (sMT-Ia) promoter, which is inducible by $ZnSO_4$. In this thesis the transgene was not induced because we aimed at moderate levels of *Dyrk1A* overexpression more similar to those observed in individuals with DS (Toiber Azkona *et al.*, 2010). Mice used in this thesis were obtained by crossing transgenic males with C57BL6/SJL female. For genotyping, genomic DNA obtained from the mice tail was amplified. Briefly, tail biopsies were digested during 30 min at 98º C in 300 µl of 50 mM NaOH. Digestion was stopped adding 30 µl of Tris-Hcl 1 mM (pH = 8) and with a centrifuge of 6 min at maximum speed. In vitro DNA amplification was performed by polymerase chain reaction (PCR) technique using the oligonucleotides DyrkF (5'-GTCCAAACTCATCAATCTATC-3') and DyrkR (5'-CTTGAGCACAGCACTGTTG-3'). PCR conditions were as follows: 96 ºC for 3 min, 32 cycles at 96 ºC for 30 s, 54 ºC for 30 s and 74 ºC for 40 s, finally 74º C for 10 min.

Except for the environmental enrichment treatments (see below), mice were reared in standard cages (20 x 12 x 12 cm (length x width x height), Plexiglas cage) in groups of 2-3 animals and maintained under a 12-hour light-dark cycle (8:00h to 22:00h) in controlled environmental conditions of humidity (60%) and temperature (22 ± 1ºC) with *ad libitum* access to food and water. All procedures were approved by the local ethical committee (Comité Ético de Experimentación Animal del PRBB (CEEA-PRBB); MDS 0035P2), and met the guidelines of the local (law 32/2007) and European regulations (EU directive e no. 86/609, EU decree 2001-486) and the Standards for Use of Laboratory Animals no. A5388-01 (NIH). The CRG is authorized to work with genetically modified organisms (A/ES/05/I-13 and A/ES/05/14).

3.2 Treatment with epigallocatechin-3-gallate, environmental enrichment and the combination of both

At the age of two months, TgDyrk1A and wild type mice were randomly assigned to the control and treated groups that were administered with a) epigallocatechin-3-gallate (EGCG) and b) reared under non-enriched (NE) or enriched environment (EE) conditions. We also tested c) the combination of both EGCG and EE. The experiments were repeated for a *n* of 147 animals (non-treated: 38 (18 TgDyrk1A and 20 WT) EGCG: 39 (18 TgDyrk1A and 21 WT) EE: 36 (16 TgDyrk1A and 19 WT) EGCG-EE: 34 (17 TgDyrk1A and 17 WT). In each experiment, animals of all the conditions were included to avoid batch effects (Figure 7).

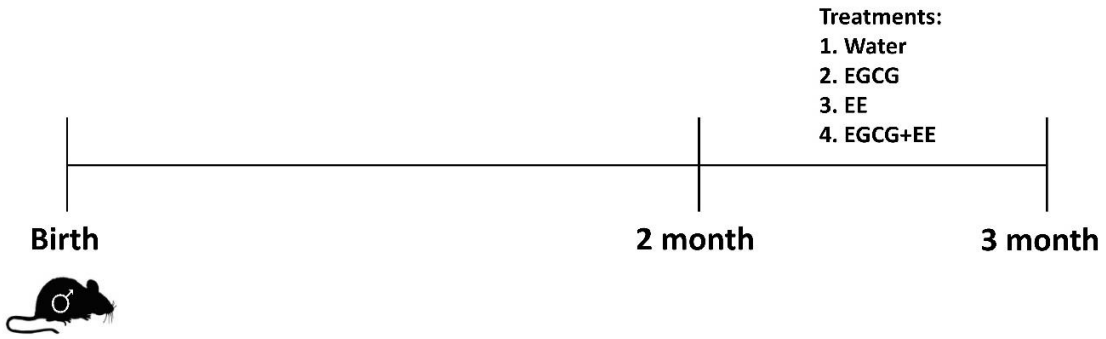


Figure 7: Scheme of treatment schedule. Representation of the experimental schedule used to analyze the effect of one month of treatment of EGCG, EE and EGCG+EE in 2-month wild type and TgDyrk1A male mice.

EGCG solution was freshly prepared every 2-3 days from a green tea leaf extract (Mega Green Tea Extract, Decaffeinated, Life Extension®, USA; EGCG content of 326.25 mg per capsule). EGCG was dissolved in drinking water at 0.33 mg/ml corresponding to average dose of 42 mg/Kg per day.

Mice under NE condition were reared in conventional cages in groups of 2-3 animals. The EE consisted in a spacious cage (55 x 80 x 50 cm, length x width x height; Plexiglas cage) with toys, small houses, tunnels and platforms of different shapes, sizes, colors and textures (Figure 8). Wheels were not introduced in the cages to avoid the effect of physical exercise on adult neurogenesis. The arrangement was changed every 3 days to keep novelty conditions. We also stimulated social interactions by housing 6-8 mice per cage. To avoid territorial aggressiveness (Haemisch and Gartner, 1997), we reared the animals in the same cage just after the weaning period before they reached sexual maturity.

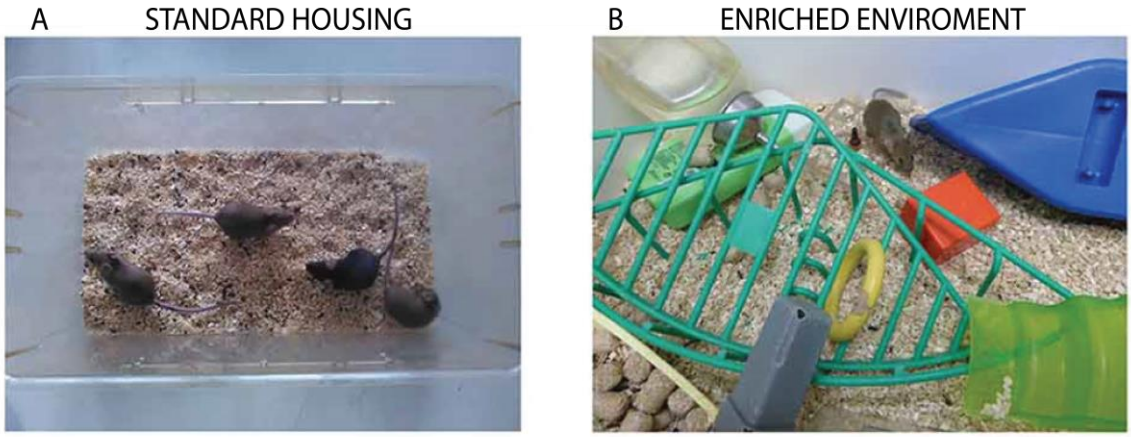


Figure 8: Housing conditions in the different experimental groups. (A) Mice reared in a standard cage (B) The enriched environment (EE) has an area of 55 cm x 80 cm and includes cages, igloos, plastic tubing, wooden logs, and other plastic enrichment toys.

3.3 Behavioral analysis: novel object recognition (NOR)

After one-month treatment, at 3 months of age, mice were tested on a hippocampal-dependent task, the novel object recognition test. This test would allow to proofing that changes on the proteome/phosphoproteome were reflecting the effective rescue of cognitive deficits. Mice were placed into a maze that consisted of three adjacent arms made of black methacrylate (each arm 30 x 5 x 6 cm, length x width x height) delineating a Y shape and surrounded by curtains. The task was performed under non-aversive low lighting conditions (50 lux). An overhead camera connected to the video-tracking software (SMART, Panlab) was used to monitor the animal's behavior. To eliminate odor cues, the arena and the objects were thoroughly cleaned with 10% odorless soap and dried. The location of the objects in the familiarization and test session was counterbalanced between animals. Sniffing time was used as the measure of exploration and was registered manually by an experimenter who was blind to genotype and treatment.

The test consisted of three different sessions: i) habituation, ii) familiarization and iii) test. The first day during the habituation session, mice were allowed to explore the arena of the Y-maze during 10 min. On the second day, during the familiarization, the animals had to explore during 10 min two identical objects placed at the end of the arms of the Y-maze. One hour after, in the test session, mice were allowed to explore for 5 min the same arena, but one of the familiar objects was changed for a new one (Figure 9).

The exploration time for the familiar (TF) and the new object (TN) during the test phase was recorded. Memory was operationally defined by the discrimination index (DI) calculated by the time spent investigating the novel minus the time spent investigating the familiar one in the testing period and divided by the total exploration time as described in the following equation:

$$Discrimination\ Index\ (DI)(\%) = \frac{[(\mathrm{TN} - \mathrm{TF})]}{\text{Total Exploration Time}} x\ 100$$

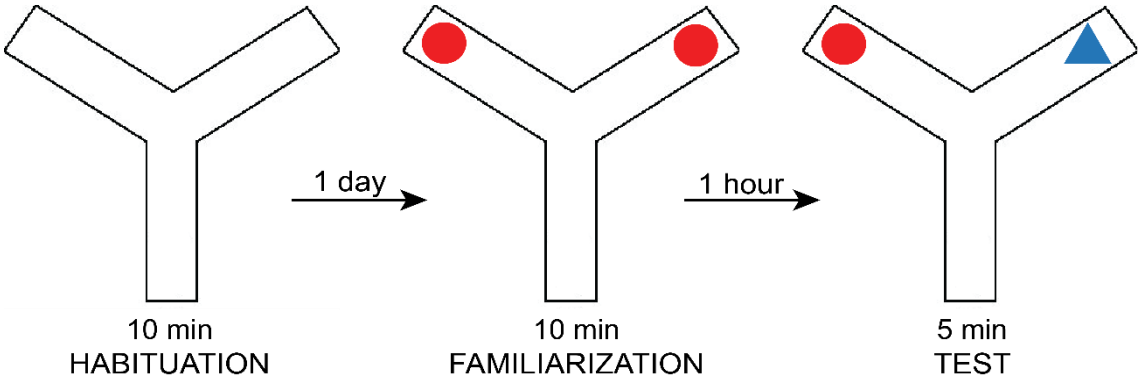


Figure 9: Novel object recognition paradigm. Schematic representation of the experimental outline used in the NOR to test recognition memory. The three phases of the NOR paradigm are represented: habituation, familiarization and test.

Behavioral statistical analysis

Significant differences between experimental groups were assessed using a one-way ANOVA taking into consideration the interaction between the genotype and the treatment effect, the ANOVA model was implemented using the *lm* function in the R package *stats*. Four contrasts (TgDyrk1A vs WT; TgDyrk1A vs TgDyrk1A+EGCG; TgDyrk1A vs TgDyrk1A+EE and TgDyrk1A vs TgDyrk1A+EGCG+EE) were assessed with the model. The computation of simultaneous confidence intervals and adjusted p-values in order to guarantee a family-wise error rate of 0.05 was based on the multivariate t distribution of the vector of test statistics using the function *glht* from the *multcomp* R package; for technical details, see Hothorn *et al*., (2008)

Observed differences in discrimination index and the time of exploration in the different session were considered statistically significant at adj. p-value<0.05. However, a p-value higher than 0.05 but lower than 0.07 was considered to indicate a strong statistical tendency due to the small sample size.

3.4 Mass-spectrometry based proteomics

Proteome and phosphoproteome analysis of the hippocampus (Figure 10) were performed with five mice per experimental condition which were selected based on the distribution of the discrimination index (DI) with DI closer to the average of the population distribution.

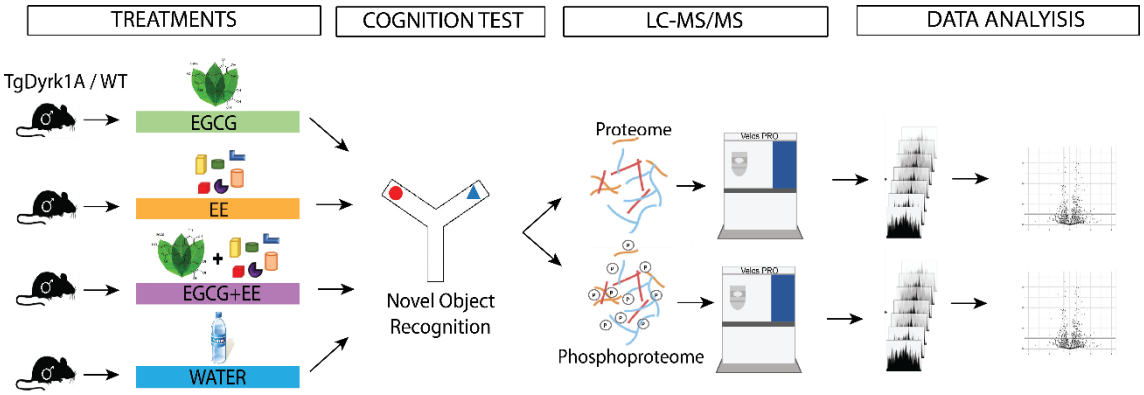


Figure 10: Scheme of treatment and proteomics schedule. 2-month wild type and TgDyrk1A male mice received water, EGCG, EE or EGCG+EE for a month. After the treatment mice were tested for novel object recognition (NOR). After the test, the dissected hippocampus was analysed using a label-free based mass-spectrometry based approach in an Orbitrap Velos Pro. Finally, bioinformatics analysis was performed.

3.4.1 Influence of the digestion protocols on peptide and protein identification and quantitation

Prior to shotgun LC-MS/MS, different digestion techniques were tested with *E.coli* samples to find the best digestion techniques for our experiments: i) in-solution with trypsin (TINSOL), ii) in solution with endopeptidase Lys-C and trypsin (LTINSOL), iii) in solution with Lys-C (LINSOL), iv) in solution with chymotrypsin (CINSOL), v) FASP with trypsin (TFASP) and vi) FASP with Lys-C and trypsin (LTFASP) (Figure 11A) (for more details, see Chiva C, Ortega M, Sabidó E (2014) " Influence of the digestion technique, protease, and missed cleavage peptides in protein quantitation." J Proteome Research 13(9):3979-3986)

Results revealed that digestion protocols in which tryptic protease specificity was used led to the highest number of identified peptides and proteins (Figure 11B). Among them, digestion protocols combining Lys-C and trypsin in a filter-aided approach (*LTFASP*) gave ~10% more identified protein groups than standard tryptic digestions but in contrast, *TINSOL* rendered the highest number of identified peptides. This fact is due to the presence of a higher number of peptides with missed cleavages in TINSOL that generated redundant information at the peptide level and thus, has a little impact on the final number of identified proteins. Indeed, the filter-aided digestions as well as digestions that included Lys-C resulted in a high percentage of peptides without missed cleavages (Figure 11C). In the case of *CINSOL*, only ~ 60% of protein groups observed with tryptic specificity. Moreover, assuming cleavage after Tyr, Phe and Trp as normal cleavage for Chymotypsin, this protocol resulted in a high percentage of peptides with one, two or three missed cleavages, evidencing low digestion efficiency.

To evaluate in-solution and FASP (filter-aided sample preparation) reproducibility on accuracy, specificity, and sensitivity of the estimation of protein abundances five complex protein mixtures with known amounts of spiked-in proteins were prepared and digested with the digestion protocols detailed above to evaluate accuracy and precision of protein quantification as well as the impact of including peptides with missed cleavages.

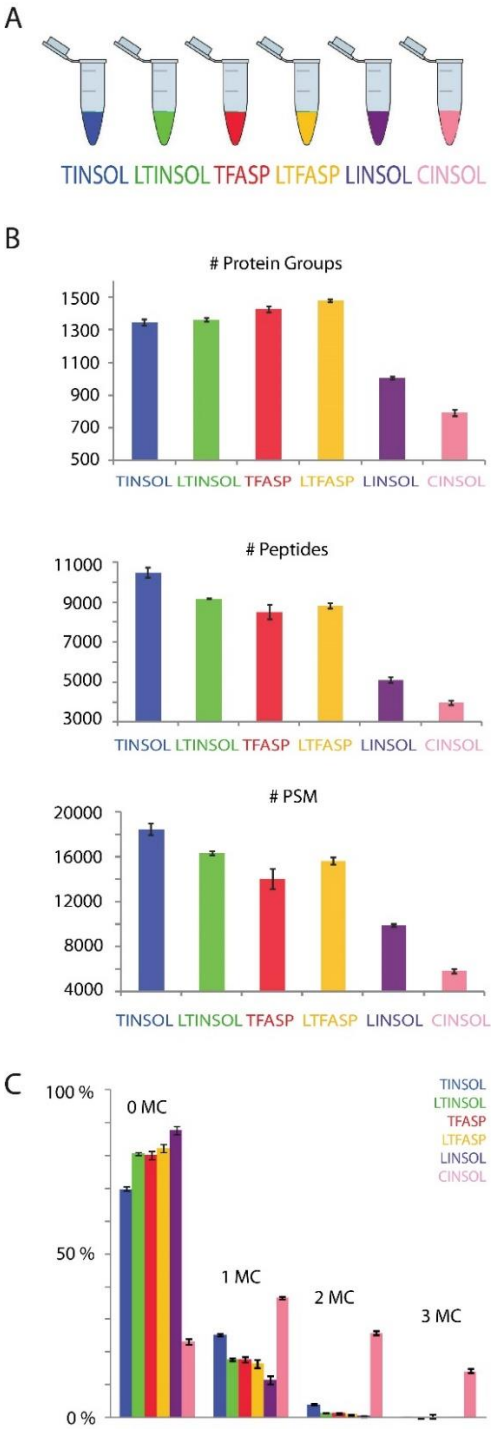


Figure 11: Influence of digestion protocols in peptide and protein identification (A) Representation of the six digestion protocols used in the study. TISNSOL: in-solution with trypsin; LTINSOL: in solution with endopeptidase Lys-C and trypsin; TFASP: FASP with trypsin; LTFASP: FASP with Lys-C and trypsin; LINSOL: in solution with Lys-C and CINSOL: in solution with

chymotrypsin. (B) Number of proteins groups, peptides and peptide-spectrum matches (PSMs) identified in the *E. Coli* sample spiked with controlled proteins under different digestion protocols used. (C) Percentage of peptides bearing zero, one, three or three missed cleavages as a result of the different digestion protocols tested.

Protein relative quantification was achieved by extracting peptide areas, followed by a statistical analysis using MSstats (Choi *et al.*, 2014) of only unique peptides regardless of the number of missed cleavages or using only the ones with no missed cleavages. In both cases, the precision and accuracy of relatively protein quantification was assessed using the mean squared error (MSE), which sums the differences between the estimated protein fold change and their true values. Precision and accuracy were slightly better when using all peptides in cases such as LINSOL and LTFASP and the observed results were consistent regardless of the fold-change analyzed (Figure 12). Similarly, the obtained values for sensitivity and specificity were not significantly different between the analyses with only peptides without missed cleavages and the analyses in which peptides with missed cleavages were also included (data not shown).

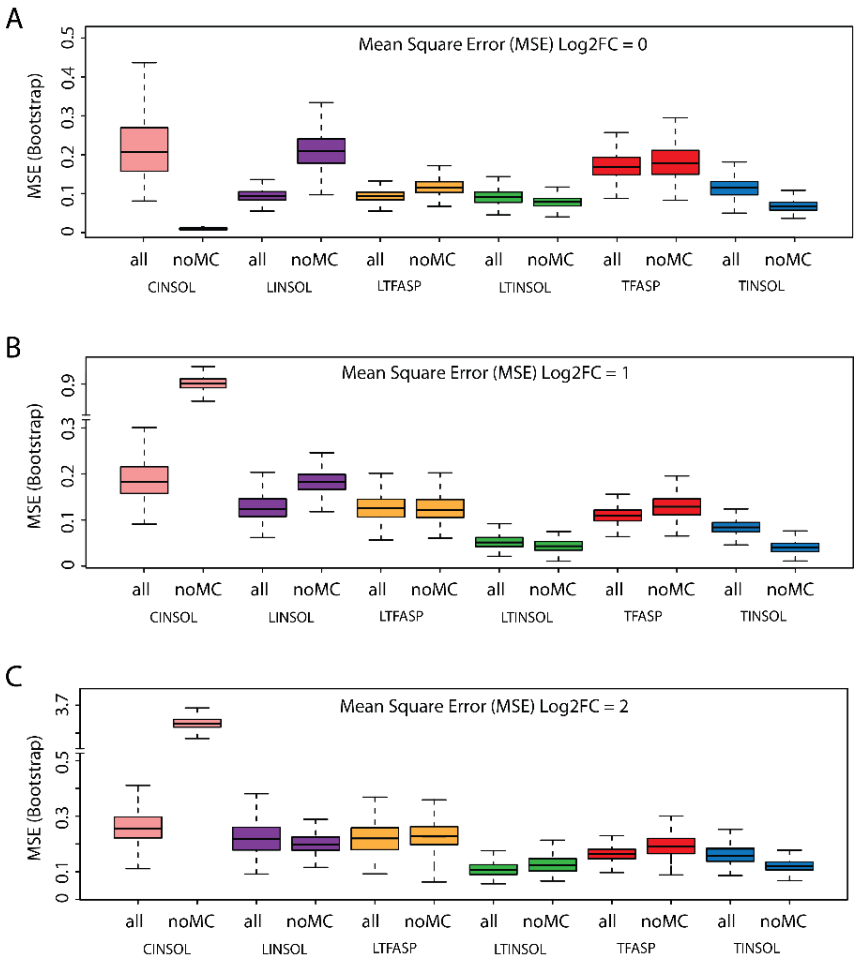


Figure 12: Precision and accuracy of relative protein quantitation assessed by the use of the mean-squared error (MSE) for protein fold-change 1 (log2FC = 0) (A), protein fold-change 2 (log2FC = 1) (B), and protein fold-change 4 (log2FC = 2) (C) when including all peptides (all) or only peptides without any missed cleavages (no MC). The mean-squared error (MSE) was calculated with the formula MSE = (1/n)Σi N(xi − μ)2, which sums the differences between the

estimated protein fold-changes and their true values, and its variability was estimated by data resampling (bootstrap method, n = 1000).

Overall, the results showed that LTINSOL was the digestion technique that showed the minimum error (MSE) and thus a suitable digestion technique for our experiments with TgDyrk1A mice. Moreover, our results also confirmed that using these digestion procedures, the inclusion of peptides with missed cleavages does not introduce a higher degree of variability, and no significant differences in precision, accuracy, specificity, and sensibility in protein relative quantification were found compared with the use of fully tryptic peptides. Thus, peptides with missed cleavages can be introduced as proteotrypic peptides in quantitative proteomic experiments without major concerns.

3.4.2 TgDyrk1A sample preparation for MS analysis

Immediately after the novel object recognition test, mice were sacrificed, and the dissected hippocampus was kept at -80 ºC before so that all samples were processed at the same time. The hippocampi, were homogenated with a RIPA-modified buffer (50mM tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate with the addition of 5 mM b-glycerophosphate, 10 mM sodium fluoride, 10 mM sodium orthovanadate, and one Complete Protease Inhibitor Cocktail tablet Roche, 04 693 159 001) per 10 ml of solution).

Samples were sonicated using a diagenode BIORUPTOR for 5 min with 30 on/off cycles, always maintaining the samples on ice to avoid heating. Samples were centrifuged for 10 min at 10.000 rpm at 4 ºC and the supernatants were collected. Proteins from the supernatant were precipitated overnight at −20 °C by adding a volume of ice-cold acetone in six-fold excess. The acetone-precipitated proteins were solubilized in denaturation buffer (6 M urea + 200 mM ammonium bicarbonate in water). Final protein content was quantified using the BCA assay (Pierce). Proteins were reduced with dithiothreitol (DTT, 10 mM, 37 °C, 60 min), and alkylated with iodoacetamide (IAM, 20 mM, 25 °C, 30 min). In the sequential Lys-C/ trypsin (Wako, 129-02541/Promega, V-5111) digestion protocol, samples were diluted with 200 mM ammonium bicarbonate up to 2 M urea, digested overnight with Lys-C at 37 °C, and then diluted two-fold again and digested overnight with trypsin at 37 °C. Peptides were desalted using a C18 membrane packed on a MicroSpin C18 300A silica pipet tip (The Nest Group Inc, SEMSS18V.), evaporated to dryness using a speedvac, and dissolved in 30 µL of 0.1% formic acid in water.

Titanium dioxide (TiO_2) phosphopeptide enrichment

Phosphopeptides were enriched using titansphere chromatography as described previously in PRIME-XS Protocol TiO_2 phosphopeptide enrichment (http://www.primexs.eu/downloads/func-startdown/103/). Briefly, tryptic peptides were desalted and completely evaporated to dryness, and then they were dissolved with 100 µl of Loading Buffer [80% ACN (vol/vol) and 6% TFA (vol/vol)] at ~1 µg/µl concentration of peptides. Samples were passed through a constricted TiO2 loaded spin tip (see webpage for complete details of TiO_2 packing), previously equilibrated with Loading Buffer, applying 2x50 µl and using a centrifuge at ~50 g in order to achieve a complete binding. TiO_2 spin tip was washed once with 50 µl of Loading Buffer and once with 50 µl of Washing Buffer [50% ACN (vol/vol) and 0.1% TFA (vol/vol)]. Finally, phosphopeptides were eluted from the TiO_2 spin tip with 30 µl of Elution Buffer (85% NH_3-H_2O, pH 11.0) into a tube that contains 30 µl of 20% formic acid. Second elution was made in the same tube with 3 µl Elution Buffer 2 [(80% ACN (vol/vol) and 2% formic acid (vol/vol)]. The eluted phosphopeptides could be evaporated to dryness and dissolved with 0.1% formic acid in water for being analyzed by MS (Figure 13).

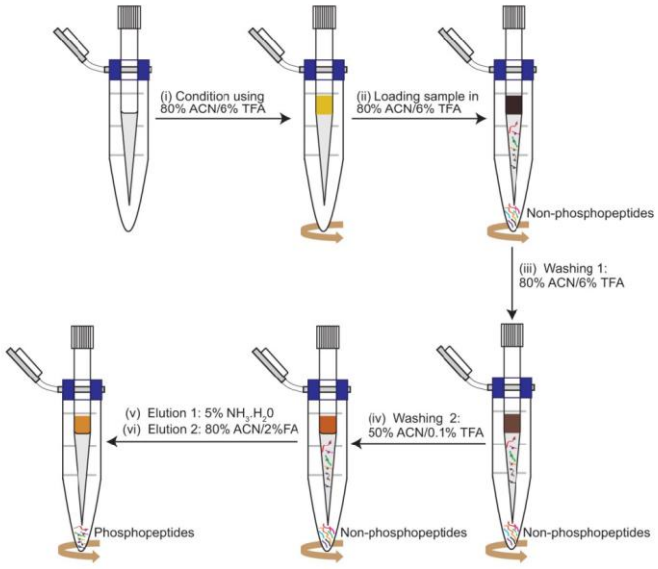


Figure 13: Graphical outline of the steps performed during the enrichment process using TiO_2. (Adapted from Zhou *et al.*, 2013)

3.4.3 Liquid chromatography-tandem mass spectrometry

For each sample, 1µg of tryptic peptides from digested hippocampal tissue and 100µg of phosphoenriched peptides from the same tissue were injected in a LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific) coupled to a nano-LC (EASY-nLC, Proxeon). Nano-LC was equipped with a reversed-phase chromatography column of 25 cm with an inner diameter of 75 µm, packed with 3 µm C18 particles (Nikkyo Technos, NTCC-360/75-3-25L), and a Nano Trap column Acclaim PepMap100 100 µm x 2 cm C18, 5 µm, 100A (Thermo, 164199). Chromatographic gradients started at 93% of buffer A and 7% of buffer B with a flow rate of 250 nL/min during 5min and linearly changed to 65% buffer A and 35% buffer B after 240 min. After each analysis, the column was washed for 16 min with 90% buffer A and 10% buffer B (Buffer A: 0.1% formic acid in water. Buffer B: 0.1% formic acid in acetonitrile).

The mass spectrometer was operated in positive ionization mode with the nanospray voltage set at 2.2 kV and the source temperature at 250°C. Ultramark 1621 for the FT mass analyzer was used for external calibration prior the analyses. The background polysiloxane ion signal at m/z 445.1200 was used as lock mass. The instrument was operated in data-dependent acquisition (DDA) mode with 1 microscan at resolution of 60,000 at 400m/z and survey scans were recorded over a mass range of m/z 350−2,000 with detection in the Orbitrap mass analyzer. Auto gain control (AGC) was set to 10^6, dynamic exclusion was set at 60 s, and the charge-state filter disqualifying singly charged peptides for fragmentation was activated. Following each survey scan, the top 20 most intense ions with multiple charged ions above a threshold ion count of 5,000 were selected for fragmentation at normalized collision energy of 35%. Fragment ion spectra produced via collision-induced dissociation (CID) and collision-induced dissociation MultiStage activation (CID MSA) for proteome and phosphoproteome respectively were acquired in the linear ion trap, AGC was set to $5 \cdot 10^4$ and isolation window of 2.0 m/z, activation time of 0.1 ms, and maximum injection time of 100 ms were used.

3.4.4 Mass spectrometry data analysis

Aquired mass spectra were processed using the MaxQuant computational platform (Cox *et al*., 2008) version 1.5.2.8. The MS2 spectra were searched by using the Andromeda search engine (Cox *et al*., 2011) against the Uniprot sequence database for *Mus musculus* (17,263 forward entries; version from July 2015). The search included cysteine carbamidomethylation as a fixed modification, and N-terminal protein acetylation and methionine oxidation as variable modifications. In the case of phosphoproteome analyses, phosphorylation on Ser, Thr and Tyr was also added as variable modification. FDR was set to 1% at the peptide and protein level, and protein identification

required at least one unique or razor peptide per protein group. More parameters details are summarized in table 2.

Table 2: Summary of parameters used for peptide identification and quantification by MaxQuant. For more details: http://www.coxdocs.org/doku.php?id=maxquant:manual:beginner

Parameters	Description	Choice
Variable modifications	The variable modification(s) used during the identification of peptides.	Oxidation (M), Acetyl (Protein N-term) For phosphoproteome: Phospho(STY)
Fixed modifications	The fixed modification(s) used during the identification of peptides.	Carbamidomethyl (C)
Multiplicity	The number of labels used: 1 for label-free and 2 for SILAC.	1
Label-free quantification	Apply the algorithm for label-free quantification LFQ.	LFQ
Enzyme	The protease used to digest the protein sample.	Trypsin/P
Include contaminants	When checked protein sequences from a contaminant list are automatically added to the list of proteins for the in-silico digestion.	
Max. missed cleavages	The maximum allowed number of missed cleavages.	3
Instrument type	Type of instrument the data was generated on.	Orbitrap
First search peptide tolerance	Mass tolerance that is applied to the peptide masses during the first round Andromeda search which is used for the mass and time dependent recalibration of the peptide masses. For “normal” orbitrap data without severe calibration problems 20 ppm are sufficient.	20 ppm
Main search peptide tolerance	The peptide mass tolerance used during mass search. This refers to mass deviations after the time and mass dependent peptide mass calibration has been performed.	7 ppm
Individual peptide mass tolerance	If checked peptides will be filtered according to individual peptide mass tolerances.	Checked
Min. peptide length	Peptides shorter than this value will not be reported nor considered during protein identification and quantification. Short peptides are usually not unique in the protein database and therefore not statistically informative.	6
Mas. Peptide mass (Da)	Peptides that are heavier than this mass will be discarded in the Andromeda search.	4600
Min. peptide length for unspecific search	During unspecific searches peptides that have fewer amino acids than this value will be discarded.	8
Max. peptide length for unspecific search	During unspecific searches peptides that have more amino acids than this value will be discarded.	25
PSM FDR	Desired false discovery rate at the peptide spectrum match level.	0.01 (1%)

Protein FDR	Desired false discovery rate at the protein level.	0.01 (1%)
Min. peptides	The minimum number of total peptides a protein group should have to be considered as identified and reported.	1
Min. razor + unique peptides	The minimum of razor + unique peptides a protein group should have to be considered as identified and reported.	1
Min. unique peptides	The minimum number of unique peptides a protein group should have to be considered as identified and reported.	0
Min. score for unmodified peptides	Minimum Andromeda score for accepting an MS/MS identification for unmodified peptides. The recommended value is 0, corresponding to no additional filtering in addition to imposing the desired FDR.	0
Min. score for modified peptides	Minimum Andromeda score for accepting and MS/MS identification for modified peptides.	40
Min. delta score for unmodified peptides	Minimum Andromeda delta score for accepting an MS/MS identification for unmodified peptides. The recommended value is 0, corresponding to no additional filtering in addition to imposing the desired FDR.	0
Min. delta score for modified peptides	Minimum Andromeda delta score for accepting an MS/MS identification for modified peptides. The recommended value is 6, corresponding to imposing the desired FDR on PSMs.	6
Match between runs	Identification are transferred to non-sequenced or non-identified MS features in other LC-MS runs.	Checked
Peptides for quantification	Specify how the protein ratios will be calculated.	Unique + razor

The MaxQuant algorithm was used to retrieve accurate extracted ion currents (XICs) per each peptide feature for quantification purposes. Areas under the curve for each peptide were calculated and later areas used to estimate protein intensities during the statistical analysis. Statistical analysis was performed using a statistical software package in R MSstats (Choi *et al.*, 2014) version 2.6.0 with the main parameters specified in table 3. MSstats is a reference statistical software for proteomics statistical inference that uses the list of identified peptides with their corresponding peak areas to identify the list of differentially abundant proteins relying on a flexible family of linear mixed models. In some of the experimental groups, one of the five biological replicates was excluded from the analysis, when the number of peptides identified was lower (at least 1,000 peptides) from the average of peptides identified in the whole experimental set thus, one replicate was excluded from the TG_EGCG+EE and WT_EE groups in the proteome analysis, and one replicate was excluded from the TG_EGCG group in the phosphoproteome analysis. To ensure high confidence in our quantitative data, only peptides observed at least in three of the five biological replicates (or at least in two when we remained only with four biological replicates), were used, and no imputation of missing values was performed.

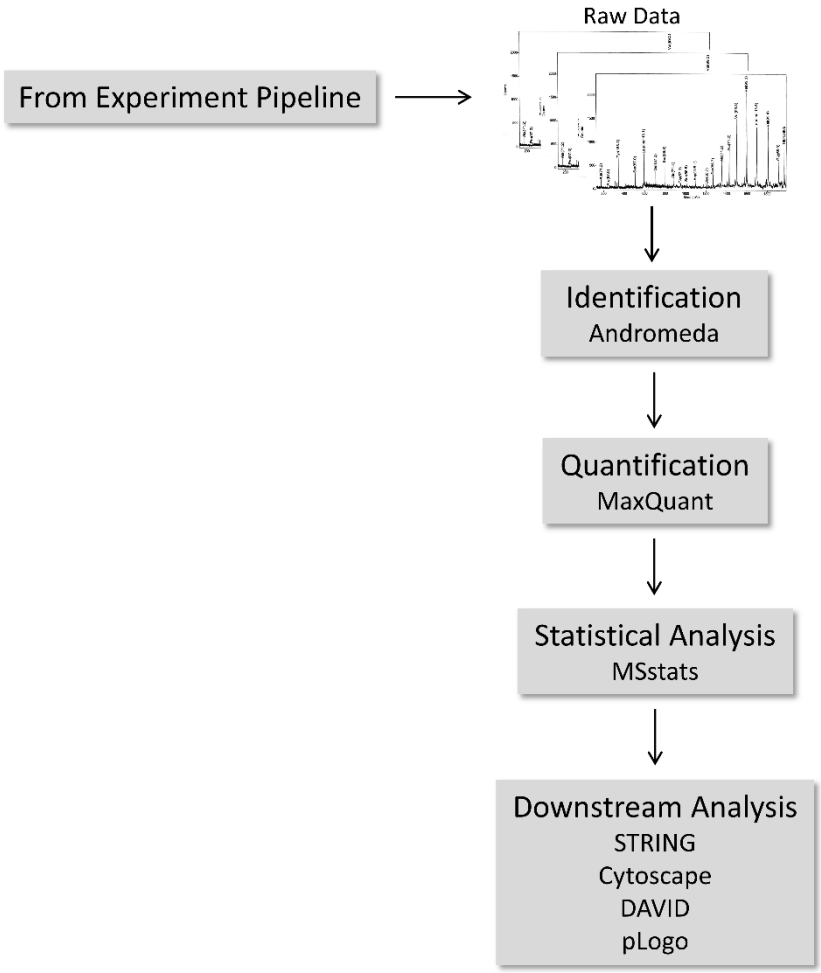


Figure 14: Bioinformatics pipeline for large-scale proteomics in which the involvement of various bioinformatics tasks in processing and interpreting proteomics data. Raw mass spectra data collected from an experimental pipeline are subjected to proteomics identification, quantification and statistical packages. Protein identifications are acquired based on the comparison between raw data and protein sequence databases using Andromeda. The results are quantified by using MaxQuant with a relative quantification and finally MSstats package performed statistical analysis of the proteins comparing all the experimental groups. Finally, downstream analysis including protein-protein interactions and network-based analysis are performed using different public tools. (Adapted from Wastling *et al*., 2012)

Table 3: Summary of parameters used statistical analysis by MSstats. For more details: http://master.bioconductor.org/packages/3.1/bioc/manuals/MSstats/man/MSstats.pdf

Function/parameters	Description	Choice
DataProcess	**Data pre-processing and quality control of MS runs of raw data for model fitting and group comparison.**	
raw	Name of the raw (input) data set.	
logTrans	Logarithm transformation with base 2(default) or 10.	Default
normalization	Remove systematic bias between MS runs. Three different normalizations supported: 'constant'(default) represents constant normalization based on reference	False (performed by median)

	signals. 'quantile' represents quantile normalization based on reference signals. 'globalStandards' represents normalization with global standards proteins. FALSE represents no normalization is performed.	
nameStandards	Vector of global standard protein names. Only for normalization with global standard proteins	NULL
betweenRunInterferenceScore	Interference is detected by a between-run-interference score. TRUE means the scores are generated automatically and stored in a .csv file. FALSE (default) means no scores are generated.	default
groupComparison	**Tests for significant changes in protein abundance across conditions based on a family of linear mixed-effects models in LC-MS**	
contrast.matrix	Comparison between conditions of interest.	Comparison
labelled	Choice of labeling technique. TRUE(default) represents the label-based study. FALSE represents label-free study.	False
data	Name of the (processed) data set.	QuanData
featureVar	Logical variable for whether the model should account for heterogeneous variation among intensities from different features. Default is FALSE, which assume equal variance among intensities from features.	True
interference	Choice of interference data. TRUE(default) means data contain interference transitions and need additional model interaction to address the interference. FALSE means data contain no interference transitions and no need additional model interaction to address the interference.	False
scopeOfTechReplication	Choice of scope of biological replication. "restricted" represents restricted scope of biological replication by specifying subject term as fixed effect in the model. "expanded" (default) represents expanded scope of biological replication by specifying subject term as random effect in the model.	Restricted
scopeOfTechReplication	Choice of scope of technical MS run replication. "restricted" represents restricted scope of technical MS run replication by specifying run term as fixed effect in the model. "expanded" (default) represents expanded scope of technical MS run replication by specifying run term as random effect in the model.	Restricted
missing.action	Specifies the action to take in presence of extreme missing values; must be one of 'nointeraction','impute', or 'remove'. Default is 'nointeraction'.	nointeraction

3.5 Bioinformatics analysis

3.5.1 Differentially expressed proteins and phosphopeptides

Downstream bioinformatics analysis was performed on proteins and phosphopeptides that showed a significant change in abundance having an adj. p-value lower than 0.05 and a $\log_2$ Fold Change (log2FC) greater than 0.3 or lower than -0.3. For the phosphoproteomic analysis, only phosphorylation sites with a localization probability of 0.5 or higher were considered. Those proteins or phosphopeptides uniquely present in one condition of the ones compared were added to the lists of differentially abundant proteins and phosphopeptides. A

peptide was defined as "*absent*" when it was detected in less than 3 out of 5 biological replicates for a given condition (or less than 2 out of 4 biological replicates). Since blood contamination is a common problem in sample collection from dissected tissues, proteins belonging to the Gene Ontology cell component "*blood microparticle*" were filtered out from all datasets before proceeding with downstream analyses.

3.5.2 Defining "*rescued*" proteins/phosphopeptides

The term "*Rescued*" proteins/phosphopeptides used in the results was defined as those proteins/phosphopeptides that showed a significant change in abundance in TgDyrk1A mice compared to wild type mice but their abundance was significantly restored to wild type values upon treatment. Therefore, we defined as restored proteins those proteins/phosphopeptides considered also significant with the thresholds defined in 3.5.1 but with an opposite sign of the log_2FC.

3.5.3 Motif and logo analysis tool

Two different tools were used for motif analysis and logo generation: Peptidextender (version 0.2.2 alpha) (http://schwartzlab.uconn.edu/pepextend/) and with Probability Logo generator (pLogo; version 0.9.0) (O'Shea *et al*., 2013; https://plogo.uconn.edu/). Phosphorylated peptides significantly changing in abundance in TgDyrk1A vs. WT contrast were divided into phosphopeptides with an increased or a decreased abundance in TgDyrk1A compared to wild type. The Peptidextender was used to align the differentially phosphosites/residues and extend the proteolytic peptide to 15 amino acids so that the phosphosite remained in a central position. Finally, residues aligned in Peptidextender were introduced into pLogo as a foreground to extract overrepresented patterns using all the quantified peptides as a background and removing possible duplicate sequences. In pLogo phosphopeptides were scaled proportional to their logo-odds bionomial probabilities under the background of all quantified phosphosites in that specific contrast. Thus, the larger a residue/position, the more statistically significant was. By default, residues were colored according to their physicochemical properties.

3.5.4 Gene Ontology enrichment analysis

Gene Ontology (GO) enrichment analysis was performed using the functional annotation tool (Dennis *et al.*, 2003; https://david.ncifcrf.gov/). For the enrichment analysis, *Mus musculus* database implemented in david.ncifcrf.gov was used. Proteins being analyzed (e.g significant proteins in a given comparison, rescued proteins, etc) were considered foreground while all quantified proteins per each comparison were used as a background universe. If no significant results were found, all proteins from *Mus musculus* database implemented in David bioinformatic database was used.

3.5.5 Network building and visualization

Information from mouse protein-protein associations were extracted from the STRING database (version 10) (Szklarcyk *et al*., 2015; https://string-db.org/) with a minimum required interaction of score of 0.4 (medium confidence). To avoid predicted interactions, only interactions assigned as experimentally were used as an interaction source. Therefore, only physical and functional interactions were used for further studies. The protein network visualization was performed using Cytoscape version 3.2.1 (Cline *et al.*, 2007) taking into account the combined score of the interaction computed by STRING database. Briefly, the combined score computes the probability of the interaction of each pair of proteins taking into account the individual sub-scores of the origin of the interaction.

Cluster analysis

The plugin ClusterONE (version 1.0) (Nepusz et al., 2014) implemented in Cytoscape was used to discover densely connected regions in protein-protein interaction networks. A subgraph representing a protein complex should satisfy two simple structural properties: it should contain many reliable interactions between its subunits, and it should be well-separated from the rest of the network. Developers formalized these two properties in a quality measure called cohesiveness and developed an algorithm that detects possibly overlapping protein complexes from weighted networks.

Default values of the basic parameters (described in table 4) were maintained with exception of the weight of the edges in which the combined score provided by STRING databased was used to increase the confidence in the internal algorithms of the plugin. Finally, only significant clusters with significant p-values lower than 0.05 were colored in the network. The biological processes associated to these subclusters were identified using David bioinformatics resource.

Table 4: Summary of parameters used by ClusterONE. For more details: http://www.cs.rhul.ac.uk/home/tamas/assets/files/cl1/cl1-cytoscape-0.1.html

Basic Parameters	Description	Choice
Minimum size	Minimum size of clusters: whenever Cluster ONE finds a cluster smaller than the minimum size, the cluster will be discarded immediately.	3
Minimum density	Minimum density of clusters. The density of a cluster is the total sum of edge weights within the cluster, divided by the number of theoretically possible edges within the cluster. Whenever Cluster ONE finds a cluster that has a smaller density than the value given here, the cluster will be discarded immediately.	Auto
Edge weigh	A numeric edge attribute to be used for the edge weights	Combined score

3.5.6 Transcription factor prediction analysis.

Transcription factor enrichment analyses were performed using the iRegulon plugin (version 1.3) (Janky *et al*., 2014) implemented in Cytoscape. The iRegulon plugin allows to identifying transcription factors using motif discovery in a sets of proteins. Proteins changing in abundance were analyzed in two different set of networks depending on the sign of the fold change in logarithmic scale. Thus, one network corresponding to proteins with a log2FC>0 (increased abundance) and another one with proteins with a log2FC<0 (decreased abundance). Default values of the plugin were maintained except for the database that was changed to *Mus musculus* (Figure 15). Normalized Enrichment Score (NES) was used to select the most confident predicted transcription factors. Thus, only NES values higher than 4 were considered. This score corresponded to a FDR estimate.

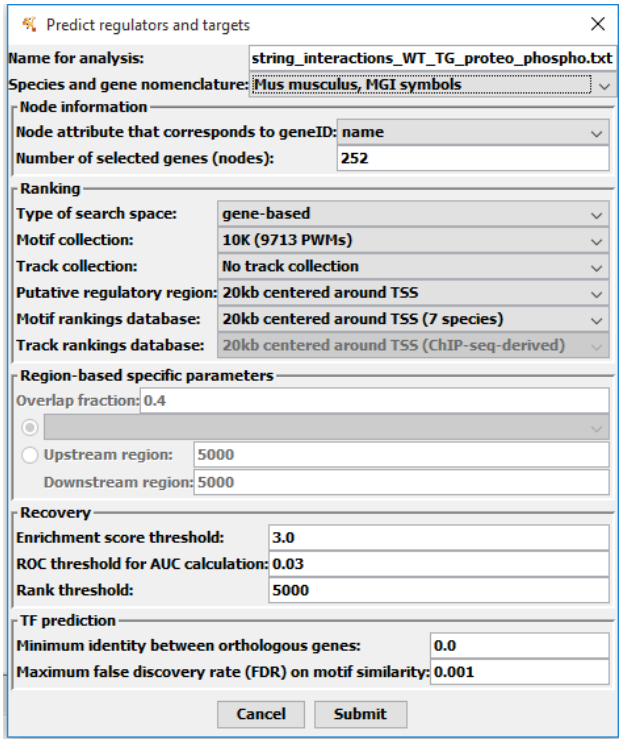


Figure 15: Summary of parameters used by iRegulon. For more details: http://iregulon.aertslab.org/manual_input.html

3.5.7 Expansion of proteome and phosphoproteome

One of the common problems in mass spectrometry is that it cannot identify all components of the proteome and phosphoproteome creating a certain bias. Therefore, lists of differentially abundant and phosphopeptides were expanded with their primary interactors so that if not detectable by mass spectrometry, changes could be set into context. For the expansion, a list of bona fide physical

interactors mainly coming from two-hybrid experiments with high IMEX index (Orchard *et al*., 2012) was used.

3.5.8 Enrichment of DYRK1A targets

DYRK1A targets were taken from the mammalian verified targets present in Aranda *et al*., 2010 and Duchon A. *et al*.,2016. The significance of the overlaps between DYRK1A targets and the differentially abundant proteins, or between DYRK1A targets and the proteins with differentially abundant phosphopeptides, was assessed using a Fisher Exact Test checking whether the number of DYRK1A targets was enriched compared to random appearance taking into account the quantified proteins and phosphoproteins in each comparison.

The enrichment was assessed both for the detected protein and the sum of detected + primary interactors (see section 3.5.7).

3.5.9 Intellectual disability and autism related proteins

Two list of proteins involved in intellectual disabilities and autism were generated from the literature.

- A list of 1,152 genes described to be involved in intellectual disabilities was manually compiled from the genes described in Gilissen *et al.*, 2014; Wrigh *et al.*, 2015, Grozeva *et al*., 2015; Lisenka *et al.*, 2015; Chiurazzi and Pirozzi, 2016. Only genes with a homologous gene in mouse and being reviewed by UniProtKB/Swiss-Prot database were used.

- A list of 60 genes from the highest ranking candidate autism risk genes ranked according by SFARI Gene (https://www.sfari.org/resource/sfari-gene/) were used.
  Given the high number of autism related genes and that this was not the main aim of this thesis, only the most bona fide genes were used according to the scoring process of SFARI Genes. The scoring criteria were based on 7 categories depending on the study as described in: https://gene.sfari.org/about-gene-scoring/criteria/.

3.5.10 Principal component analysis and correlation with behavior

Principal component analysis (PCA) of the NOR test was performed using as input for each mouse the behavioral variables measured in the test: percentage of time spent with the familiar object (% familiar); percentage of time spent with the novel object (% novel); the distance travelled through the maze (distance); and spontaneous alternation (SA) that is the measure of the sequence of arm entries and total amount of arm entries scored as a percentage.

The obtained PC1 values were correlated with proteomics experiments per each animal used in mass spectrometry analysis. Based on the distribution of rho values (Spearmans), a cut off +/-0.4 +/-0.4 (i.e. rho > 0.4 or rho < 0.4) was applied obtaining two lists of proteins were obtained: i) a list of protein whose expression levels were highly correlating (or anti-correlating) with PC1 and ii) a list of proteins whose phosphopeptides levels were highly correlating (or anti-correlating) with PC1.

A Principal Component 1 value was obtained for each animal as a result of the combination of our behavioral variables that explained most of the variance. The PC1 values (Spearmans's rho) corresponding to the animals used in the proteomic experiments with the expression and phosphorylation levels of each protein.

The list of proteins and phosphopeptides correlating with NOR attributes (PC1) were compared to the lists of significant proteins and phosphopeptides founs in each of the comparison studied. The significance of the overlaps was assessed using a Fisher Exact Test and the results were summarized by a heatmap in which the color reflected the p-value of the test.

3.6 Validation of protein candidates using western blot

Protein extracts were fractionated by SDS-PAGE (Thermo, NP0336) and transferred to a nitrocellulose membrane (Thermo, IB301001) using the Original iBlot® Gel Transfer Device from Thermo. After incubation with 5% non-fat milk (Thermo, 70166) in TBS-Tween 0.1% (TBST) for 60 min, the membranes were washed one with TBST and incubated with the primary antibody at 4º overnight in a 1% non-fat milk with TBST. Membranes were washed three times with for 10 min with TBST and incubated with secondary antibody (anti-mouse or anti-rabbit) for one hour. Membranes were again washed three times with TBST and developed with the ECL system (Amersham Biosciences, RPN2232) according to the manufacturer's protocol.

After chemiluminescence detection, if stripping steps were needed, membranes were washed 2 times with TBST for 5 min and incubated with stripping solution for 30 min at 50º C [2-mercaptoetanol 100 mM, SDS 2%, Tris-HCl 62.5mM (pH = 6.8), PBS 1x]. After the incubation, membranes were washed 2 more times with TBST for 10 min and blocked again with 5% non-fat milk.

Table 5: Antibodies used for Western Blot.

Antibody	Dilution	Specie	Company
pERK1/2 p44/42$_{(Thr202/Tyr204)}$	1/1000	Rabbit	Cell Signaling
ERK1/2	1/1000	Rabbit	Sigma
pMEK1/2$_{(Ser717/721)}$	1/350	Rabbit	Cell Signaling
MEK1/2	1/1000	Rabbit	Cell Signaling
HRAS	1/1000	Rabbit	Proteintech
GAPDH	1/4000	Mouse	Chemicon

Western blot statistical analysis

Significant differences between experimental groups were evaluated using a one-way ANOVA with repeated measures that took into consideration the interaction between the genotype and the treatment effect. Technical replication (3 per each western blot) was also considered in our model (since experiments were repeated "n" times) using the *nlme* R package with the lme function. The computation of simultaneous confidence intervals and adjusted p-values in order to guarantee a family-wise error rate of 0.05 was based on the multivariate t distribution of the vector of test statistics using the function *glht* from the *multcomp* R package; for technical details, see Hothorn *et al*., 2008.

Observed differences in discrimination index as well as time of exploration in the different session were considered statistically significant at adj. p-value<0.05. However, a p-value higher than 0.05 but lower than 0.07 was considered to indicate a strong statistical tendency due to the small sample size.

RESULTS

"…the constantly evolving gravimetric balance has been a faithful servant of the laboratory chemist and has played a major role in developing the analytical methods that are the foundation of contemporary chemical science. Perhaps the ultimate stage in the evolution of that balance is represented by the modern mass spectrometer. It is able to determine with high precision the masses of individual atoms and molecules by transforming them into ions and measuring the response of their trajectories in vacuo to various combinations of electric and magnetic fields."

John B. Fenn 2002 Nobel Laureate in Chemistry

4. RESULTS

4.1 Effect of *Dyrk1A* overexpression, drug and environmental enrichment on hippocampal dpendent learning and memory in TgDyrk1A mice

We evaluated the impact of *Dyrk1A* overexpression on hippocampal learning and memory and whether the treatment with epigallocathechin-3-gallate (EGCG), a pharmacologically DYRK1A kinase inhibitor, and an enriched environment (EE) improved the cognitive deficit observed in young-adult TgDyrk1A mice using a novel object recognition test (NOR; Antunes and Biala, 2012; Leger *et al*., 2013).

4.1.1 Novel object recognition in untreated TgDyrk1A mice and upon epigallocatechin-3-gallate, environmental enrichment and their combination

In the familiarization session, in which mice explore two identical objects, total time of exploration was similar in both genotypes (Figure 16A) indicating a similar exploratory activity. This was also the case in the test session, in which no differences were observed in total exploration time (Figure 16B). However, TgDyrk1A mice showed significantly impaired discrimination index compared to wild type mice (Figure 16C) (one-way ANOVA **p<0.01).

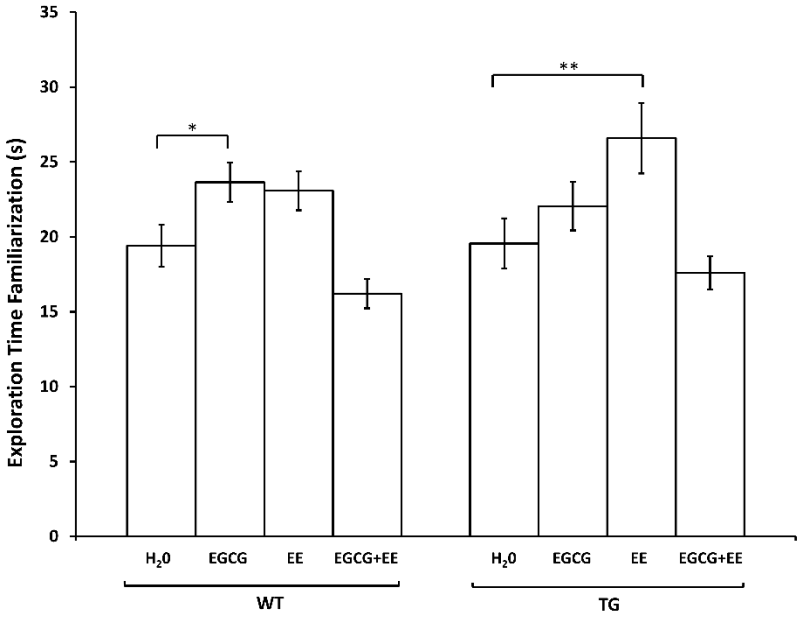
We then evaluated whether one-month of treatment with EGCG (42 mg/kg/day) improved object recognition impairments in TgDyrk1A mice. During the familiarization phase, EGCG produced a significant increase in exploration time in wild type (Figure XA) (WT vs. WT EGCG one-way ANOVA test *p<0.05) but not in TgDyrk1A mice. EGCG significantly improved the object recognition in TgDyrk1A (Figure XC, one-way ANOVA **p<0.01) but did not change the NOR performance in wild type mice.

One month of exposure to an enriched environment (EE) produced a significant increase in the total exploration time in the familiarization phase (one-way ANOVA p<0.05) and in the test phase (one-way ANOVA p<0.05) in TgDyrk1A mice (Figure 16A and 16B) but not in wild type mice. Regarding object recognition, the EE treatment slightly, though not significantly, improved discrimination index in TgDyrk1A (Figure 16C, one-way ANOVA *p<0.05), and showed no effects in wild type mice.

Finally, when evaluating the effects of combining of both treatments (EGCG+EE) during one month we did not observe significant changes in total exploration time during the familiarization or the test phase neither in wild type nor in TgDyrk1A mice, with respect to untreated mice. However, the combined treatment slightly,

though not significantly, improved recognition memory in TgDyrk1A mice (Figure 16C, one-way ANOVA; p = 0.055).

A



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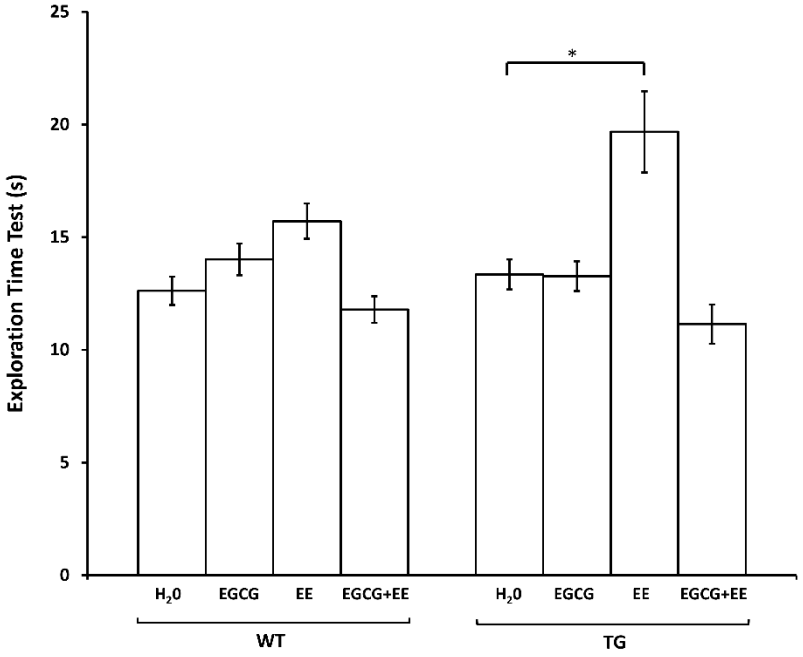

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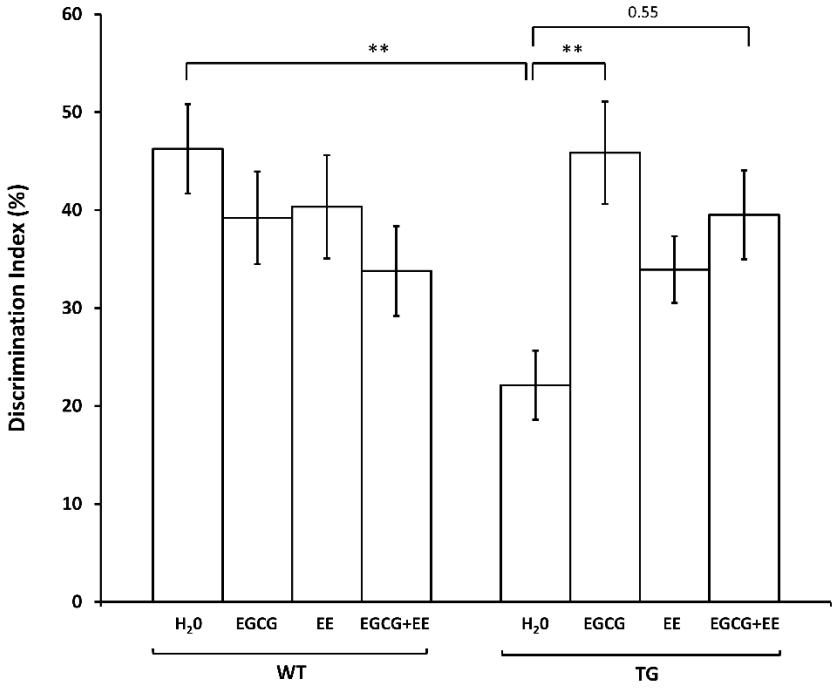


Figure 16: Novel object recognition test (NOR). (A) Time of exploration (in seconds) during the familiarization session. (B) Time of exploration during the test session (in seconds). (C) Discrimination index during the test session (%). Data are represented as mean ± SEM. One-way ANOVA with adjusted p-values based on the multivariate t distribution of the vector of test statistics; for technical details, see Hothorn *et al*., (2008). WT (wild type) n = 20; TG (TgDyrk1A) n = 18; TG EGCG n = 18; TG EE n = 16; TG EGCG+EE n = 17; WT EGCG n = 21; WT EE n = 19 and WT EGCG+EE n = 17. **p<0.01 * p<0.05

Using PCA we found that spatial learning parameters (% time exploring the familiar object and % time exploring the novel object) contributed similarly to PC1 and explained a large proportion of the variance among groups (60.64%). High values of PC1 corresponded to increased novel object recognition. Interestingly, TgDyrk1A mice and WT but also TgDyrk1A mice treated with EGCG were nicely separated along this dimension, with untreated transgenic mice showing the lowest values. On the other hand, PC2 explained 24.77% of the between-group variance and was mainly composed of distance and spontaneous alternation (Figure 17).

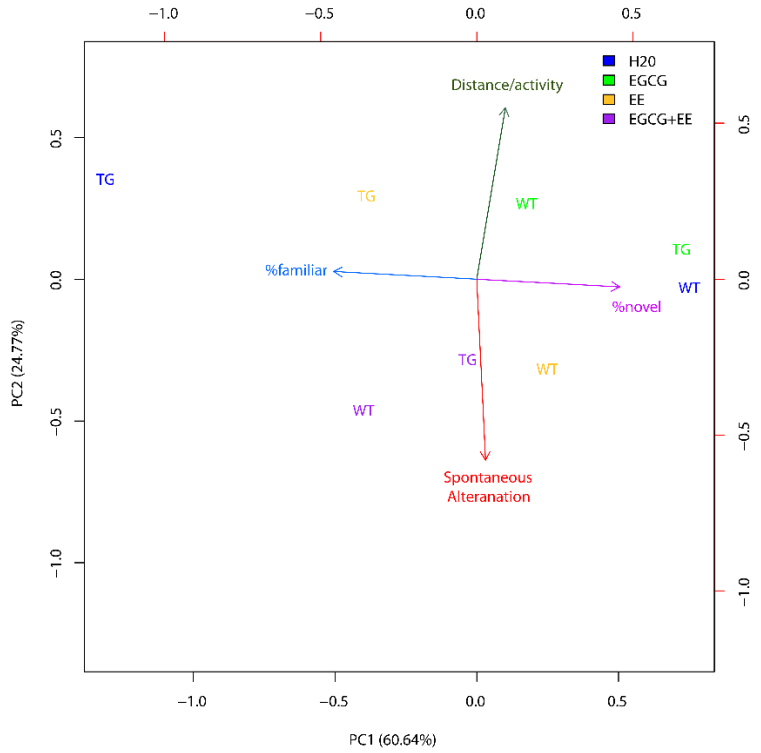


Figure 17: Principal component analysis: effects of EGCG, EE and EGCG+EE in a novel object recognition task. Principal component analysis of treatments effect on recognition memory giving as input for each mouse the behavioral variables measured in the test: % familiar, % novel, distance and Spontaneous Alternation (SA). Uppercase words represent the mice used for LC-MS/MS analysis. For visualization purposes, we plotted the centroid for each group of mice (the mean of the PC1 and PC2 values for mice belonging to the same group).

4.2 Hippocampal proteomic and phosphoproteomic investigation of the effect of *Dyrk1A* overexpression and it kinase activity normalization in TgDyrk1A mice

Our goal was to quantify the proteome and phosphoproteome alterations of *in vivo Dyrk1A* overexpression and its kinase activity normalization (Pons-Espinal et al., 2013) with EGCG and EE in the hippocampus of TgDyrk1A mice using liquid chromatography tandem-mass spectrometry (LC-MS/MS) and establish the biological pathways and the structure of the disease-causing network. Thus, with such information of the proteome and phosphoproteome it was possible to identify the alterations in the proteome and phosphoproteome and infer into functional differences among the different cellular states.

4.2.1. Selection of samples for mass spectrometry analysis

After evaluating the effect of *Dyrk1A* overexpression and the impact of the treatments, we performed LC-MS/MS on hippocampal samples of the same mice. Five mice per group tested in the NOR, were selected (n = 40 mice analyzed). To avoid outliers but also possible biases introduced by choosing animals with

extreme phenotypes, we selected the five prototype mice with a discrimination index close to the median of the intra-group distribution of values obtained in the NOR (Figure 18). This probably limited the changes observed among experimental conditions, but ensured the robustness of the analysis, since the phenotypes are representative of the average population.

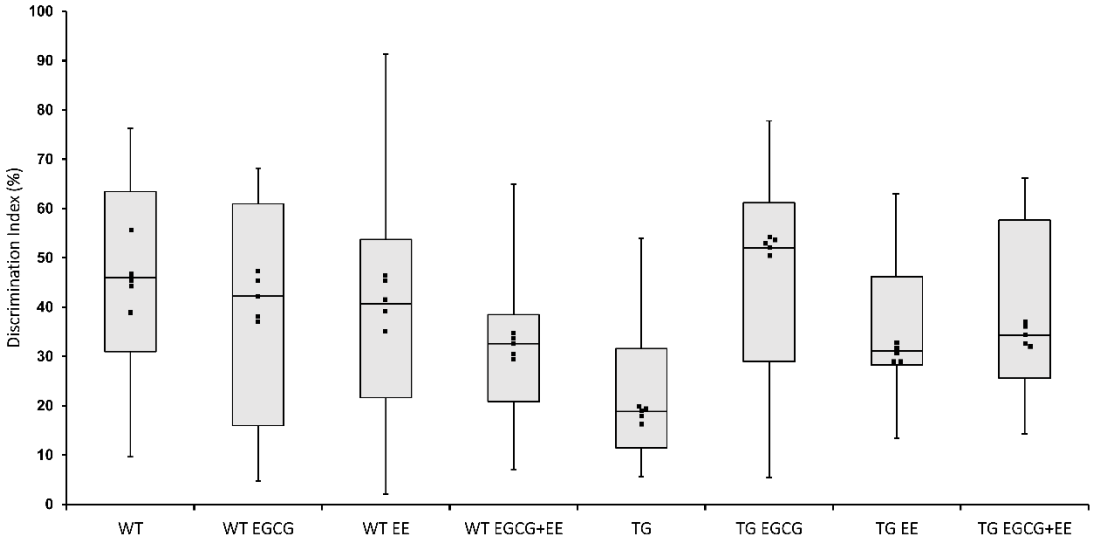


Figure 18: Box plot of the Discrimination Index (DI) in the novel object recognition test. Box plot showing the DI distribution of mice tested in the novel object recognition in the eight experimental conditions. Black squares correspond to mice with DI closer to the media and thus, the ones chosen for LC-MS/MS studies.

4.2.2 Quantitative proteomics and phosphoproteomics

Detecting changes in the protein or phosphopeptide abundance in response to a disease compared to a non-diseased state in biological samples is one of the goals of this Thesis. Quantitative information can be obtained by using several high-throughput methods of which LC-MS/MS is the most commonly used technology for monitoring changes in proteome profiles.

Proteome and phosphoproteome mass spectrometry results

A total of 40 hippocampal samples from 5 mice per experimental group were prepared for LC-MS/MS analysis. Obtained spectra were interpreted using the Andromeda, the peptide database search engine integrated in MaxQuant, resulting in the identification of 22,018 unique peptides corresponding to 3,001 proteins and 3,678 unique phosphopeptides corresponding to 1,134 proteins.

Peptide and phosphopeptide areas were extracted using the label-free algorithm implemented in MaxQuant (Cox and Mann 2008), and compared among biological replicates (intra-group) and across all experimental groups (inter-

group) to further determine the technical quality. High reproducibility was observed across biological replicates both in proteome and phosphoproteome by Pearson's correlation (rho>0.9), indicating good quality of sample preparation and mass spectrometer stability. Also a high reproducibility was observed (rho>0.9) across all experimental groups both in proteome and phosphoproteome when considering the average of biological replicates (Figure 19).

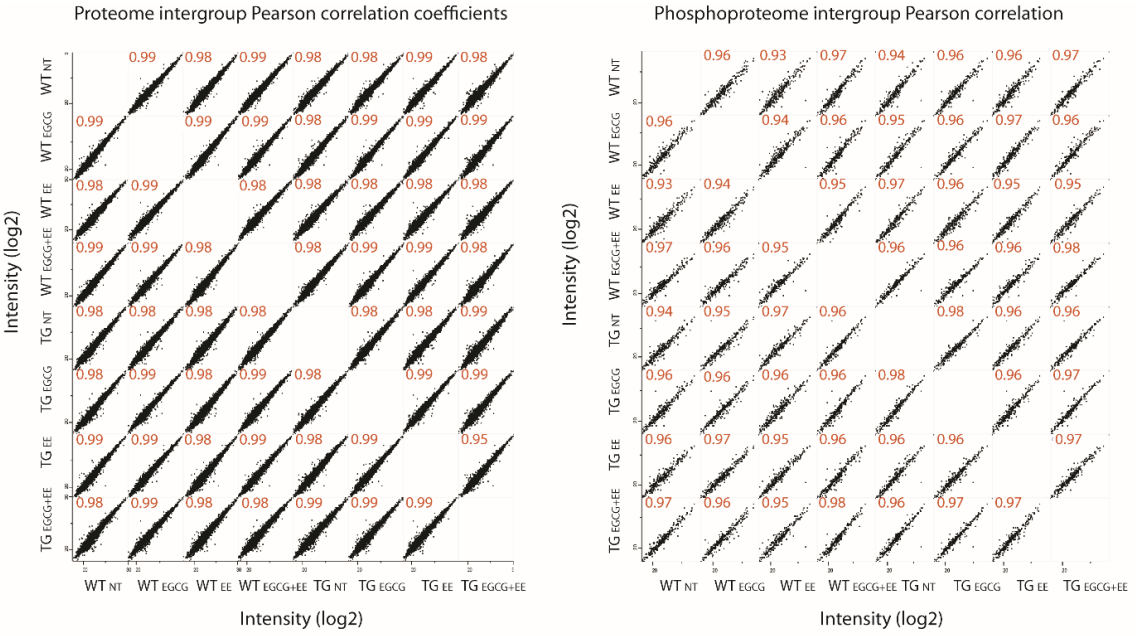


Figure 19: Reproducibility of the LC-MS/MS workflow. Density plots depicting the median log2 transformed peptide intensities for each experimental group. Proteome (left), phosphoproteome (right) Pearson correlation coefficients are represented in orange for each pairwise correlation. WT (wild-type), TG (TgDyrk1A), (NT) not treated.

Proteome and phosphoproteome label-free quantification

Label-free differential quantitative analysis of the proteins and phosphorylated peptides was performed using MSstats (Choi *et al.*, 2014). To avoid the introduction of artificial values and ensure high confidence in our statistical analysis there was no imputation of missing values. Overall, more than 2,600 proteins and 700 phosphopeptides were quantified. Criteria to define significant proteins and phosphopeptides are detailed in section 3.5.1 of materials and methods. Briefly, proteins or phosphopeptides were considered differentially expressed if having a p-value lower than 0.05 and a $\log_2$ (Fold Change) greater than 0.3 or lower than -0.3. Moreover, for the phosphoproteomic analysis, only phosphorylation sites with a localization probability of 0.5 or higher were considered. Also, proteins and phosphopeptides, present only in one of the conditions were added. A summary of the quantification results of the seven comparisons of interest is shown in table 6.

Table 6: Summary of the quantification results of the LC-MS/MS analysis. Peptides or phosphopeptides uniquely present in only one condition (present/absent proteins and phosphopeptides) are included. WT (wild-type), TG (TgDyrk1A), (NT) not treated.

	PROTEOME		PHOSPHOPROTEOME		
CONTRAST	**Proteins quantified**	**Significant proteins**	**Phosphopeptides quantified**	**Significant phosphopeptides**	**Significant proteins**
TG_{NT} – WT_{NT}	2685	98	1248	196	169
WT_{NT} - WT_{EGCG}	2695	115	1323	221	179
WT_{NT} - WT_{EE}	2758	124	1330	309	245
WT_{NT} - $WT_{EGCG+EE}$	2690	110	1349	364	290
TG_{NT} - TG_{EGCG}	2650	98	1372	349	291
TG_{NT} - TG_{EE}	2680	113	1288	186	161
TG_{NT} - $TG_{EGCG+EE}$	2756	204	1204	182	162

4.2.3 Analysis of proteins and phosphopeptides altered in abundance in TgDyrk1A mice and in response to treatments

The dynamics of protein abundance and activity that drive biological processes is regulated at different levels, including transcription, translation, post-translational modification and degradation.

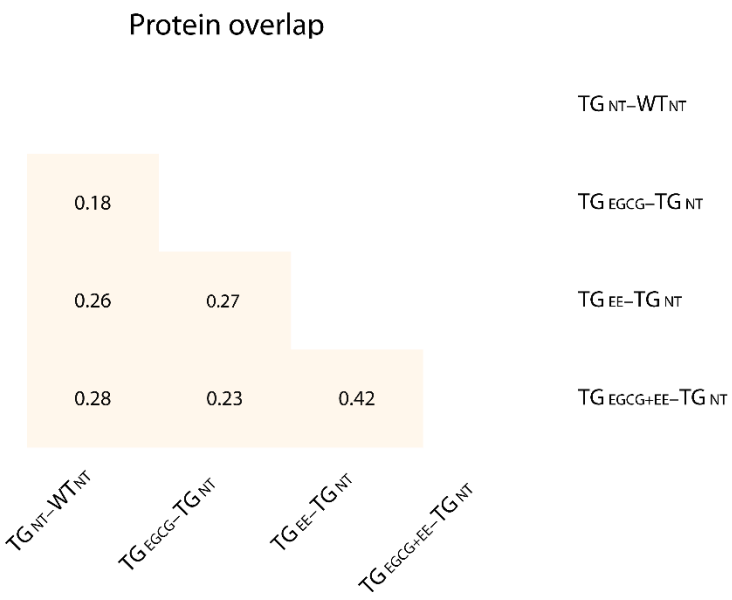
To determine to what extent proteins and phosphopeptides that changed in abundance after label-free quantitative analysis were similar across the different comparisons, we analyzed the overlap of proteins and phosphorylated peptides using a Fisher's exact test.
The test revealed that proteins showing a significant change in abundance in every contrast, significantly overlapped among comparisons (p-value Fisher's test < 0.05) (Figure 20A). This indicates that EGCG, EE and their combination (EGCG+EE) modulated many proteins perturbed by *Dyrk1A* overexpression. From the 98 proteins showing significant changes in abundance in TgDyrk1A compared to wild type, 44 overlapped with those changing abundance in TgDyrk1A mice treated with EGCG, EE or EGCG+EE. Only one third of these 44 proteins were enriched in the GO term transport category that includes mainly ion channels, vesicle-mediated transport and solute carriers (Figure 20D).

Similarly, phosphorylated peptides with significant changes in abundance overlapped across all the comparisons (p-value Fisher's test < 0.05) (Figure 20B). In this case, from the 169 proteins with phosphopeptides with significant change in abundance in TgDyrk1A compared to wild type, 127 overlapped with those showing significant changes in abundance in TgDyrk1A mice treated with EGCG, EE or their combination (EGCG+EE) (Figure 20E). More than half of those overlapping proteins are enriched in GO terms related to plasticity processes

such as axonogenesis, long-term synaptic potentiation and cytoskeleton processes.

Finally, to determine to what extent proteins that changed in abundance were also phosphorylated the same test was applied comparing proteome and phosphoproteome. In this case, proteins changing their abundances in the proteome showed minimal overlap (less than 10%, e.g., no more than 5 proteins) with proteins that exhibit changes in their phosphorylation levels in the phosphoproteome (Figure 20C). This indicates that proteins that significantly change in abundance due to *Dyrk1A* overexpression or upon treatments are different from those changing their phosphorylation level. These results suggest that *Dyrk1A* overexpression acts differentially on the proteome and phosphoproteome.

A



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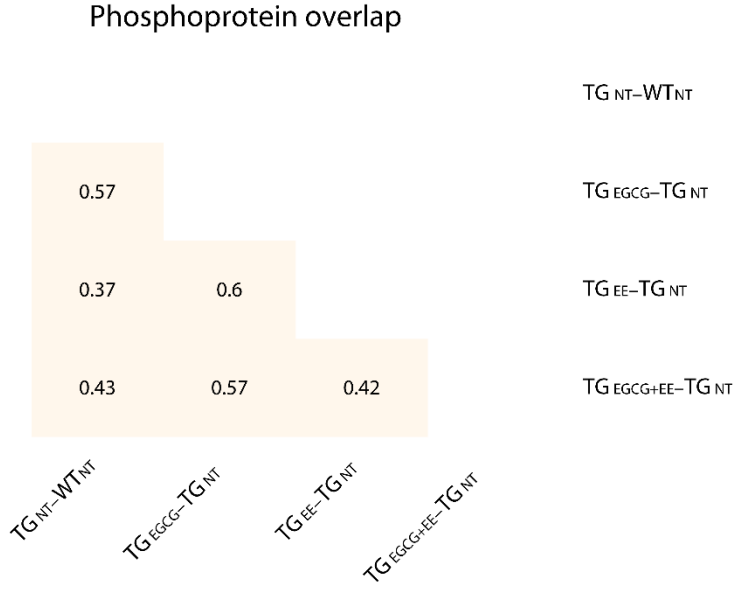
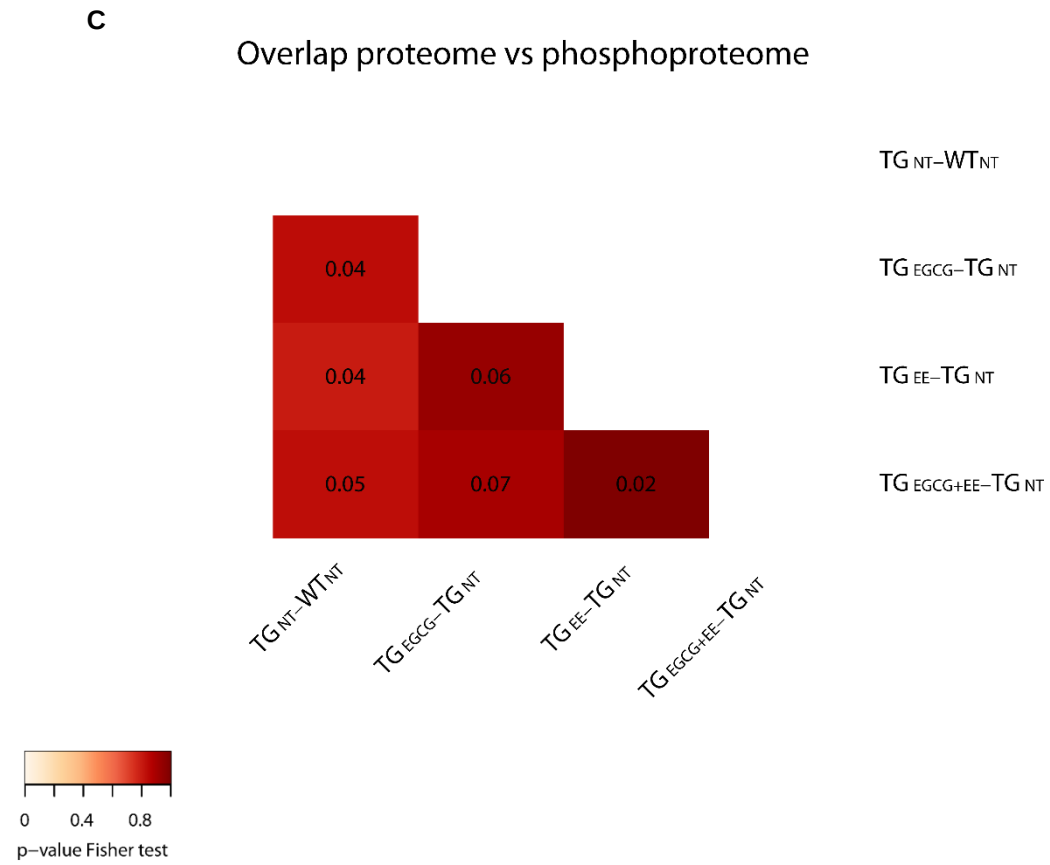




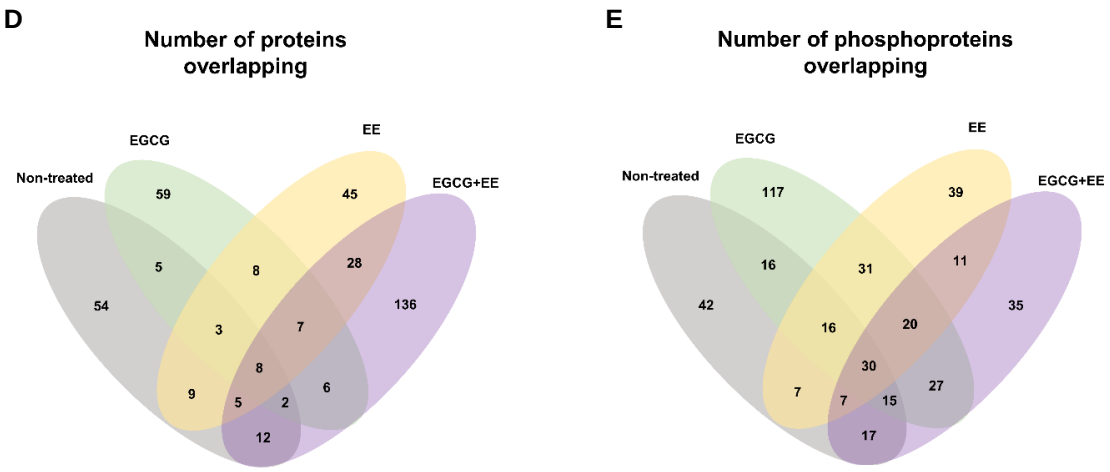


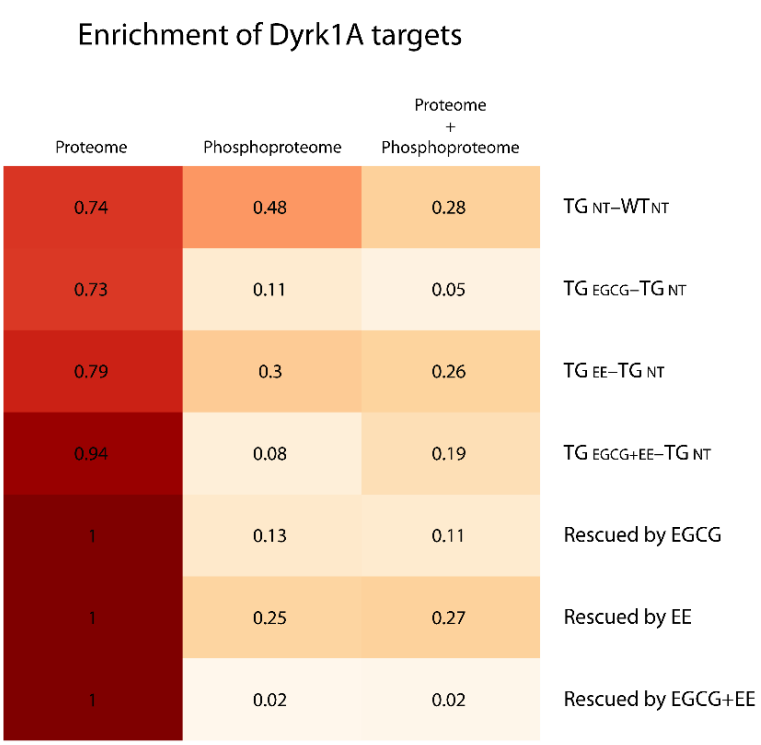
Figure 20: Proteome and phosphoproteome overlap. Heatmaps representing (A) the overlap of the differentially expressed proteins, (B) the overlap of the differentially phosphorylated peptides, (C) the overlap between differentially expressed proteins versus differentially

phosphorylated ones, in all the contrasts studied. Colour-code corresponds to –log10 of the p-value from the Fisher Test and numbers in the heatmaps correspond to overlap coefficient. (D) Venn diagram representing the overlap of the proteins differentially expressed by *Dyrk1A* overexpression and by the three treatments (E) Venn diagram representing the overlap of phosphoproteins differentially expressed by *Dyrk1A* overexpression by the three treatments.

Enrichment of protein interactors and/or substrates (phosphorylation targets) for mammalian DYRK1A

For all the comparisons, the Fisher's exact test revealed an enrichment of DYRK1A targets in the phosphoproteome compared to the proteome (Figure 21A). When analyzing the proteome and phosphoproteome together, we found that TG vs. TG EGCG had the strongest effect, as shown by the significant enrichment of DYRK1A targets (more DYRK1A targets; detailed in table 7) in the TG_{NT} - TG_{EGCG} contrast (Figure 21A). It should be noted, though, that the sites phosphorylated in the DYRK1A targets did not correspond to those described as Dyrk1A phosphorylation sites.

A



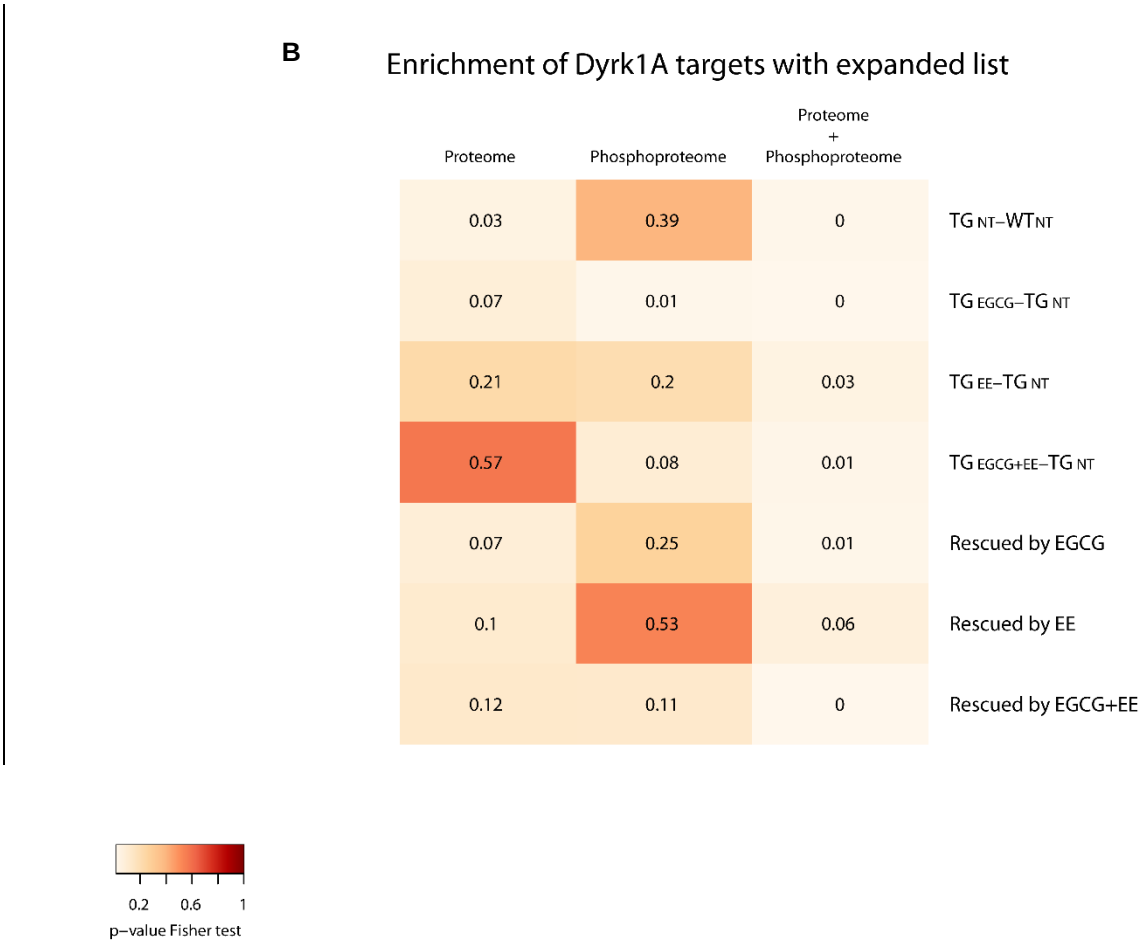


Figure 21: Enrichment for Dyrk1A targets. (A) Heatmap depicting the enrichment of DYRK1A targets in all the contrasts (right label) in the proteome, phosophoproteome and both; (B) Heatmap depicting the enrichment of Dyrk1A targets described among all the contrast with the expanded list of differentially abundant proteins or differentially abundant phosphorylated peptides in which we included those proteins not differentially expressed but that were present or absent and their main interactors. Colour code corresponds to –log10 of the p-value from the Fisher Test and numbers in the heatmap correspond to the exact p-value.

One of the limitations of our analysis is that mass spectrometry cannot identify the whole proteome and phosphoproteome and it has a certain bias towards the most abundant components. Given that proteins are organized in linked networks so one can assume that altered proteins might also alter the function, activity and/or localization of their interaction partners (Oliver, 2000). Therefore, we expanded our list to include proteins and phosphorylated peptides the first-degree interactors (for details see Materials and Methods, section 3.5.7). Proteins and phosphorylated peptides that were present and absent in the different conditions were also considered in the analysis.

The enrichment analysis of the expanded list showed a significantly higher enrichment of DYRK1A targets compared to the non-expanded list, suggesting that more DYRK1A targets could be affected (Figure 21B and table 7).

Table 7: DYRK1A substrates detected by MS. The table shows the DYRK1A substrates detected with mass-spectrometry (MS) from the list of proteins and phosphopeptides changing in abundance as well as the expanded list (including first-degree interactors)

	DYRK1A TARGETS			
	Proteome (from MS)	**Proteome first interactors**	**Phosphoproteome (from MS)**	**Phosphoproteome first interactors**
$TG_{NT} - WT_{NT}$	Hras	Dnm1 Psen1 Braf Sirt1 Grb2	Synj1 Gys1 Map1b Srsf2	14-3-3 p53 Sirt1 Polr2a
$TG_{EGCG} - TG_{NT}$	Spred1	Dnm1 Psen1 Stat3 Grb2	Synj1 Grin2a Gys1 Map1b Sept4 Srsf2 Eif2b5	14-3-3 Creb1 Dnm1 Gsk3β Mek1 p53 Psem1 Sirt1 Polr2a Grin2a Prkkn Mapk1
$TG_{EE} - TG_{NT}$	Spred1	App Dnm1 Psen1	Synj1 Map1b Sept4	Dnm1 Gsk3β Psen1 Stat3 Srsf2
$TG_{EGCG+EE} - TG_{NT}$	Cdkn1b	Nfat1 Psen1 Ccnd1	Synj1 Map1b Sept4 Srsf2 Gsk3β	14-3-3 Dnm1 Mek1 Psen1 Stat3 Polr2a

4.3. Genotype-dependent proteome and phosphoproteoeme changes

After this first general analysis of the data obtained by LC-MS/MS, we focused on the effect of *Dyrk1A* overexpression on the hippocampal phosphoproteome and proteome in TgDyrk1A mice.

4.3.1 Proteomic signature of *Dyrk1A* overexpression in the hippocampus of TgDyrk1A mice

The comparison of genotypes (WT vs TG) revealed 61 proteins that changed significantly their abundance (Figure 22) and 37 that were present in one genotype and absent in the other. From those, only one protein was a DYRK1A substrate, HRAS, a signal transduction protein, but if we include also first neighbors of significant proteins in the expanded list, we detect 5 more none of those was found in the phosphoproteome analysis: i) serine-threonine-protein kinase B-raf (BRAF), involved in transduction of mitogenic signals from the cell membrane to the nucleus with a possible role in postsynaptic responses of hippocampal neurons; ii) growth factor receptor-bound protein 2 (GRB2), an adapter protein in the Ras signaling pathway; iii) dynamin 1 (Dnm1), a microtubule-associated force-producing protein involved in producing microtubule bundles; iv) presenilin-1 (PSEN1), the catalytic subunit of γ-secretase complex that catalyzes the cleavage of proteins such Notch receptors and APP and v) NAD-dependent protein deacetylase sirtuin-1 (SIRT1), deacetylase that links transcriptional regulation directly to intracellular energetics and participates in the coordination of several separated cellular functions such as cell cycle, response to DNA damage, metabolism, apoptosis and autophagy.

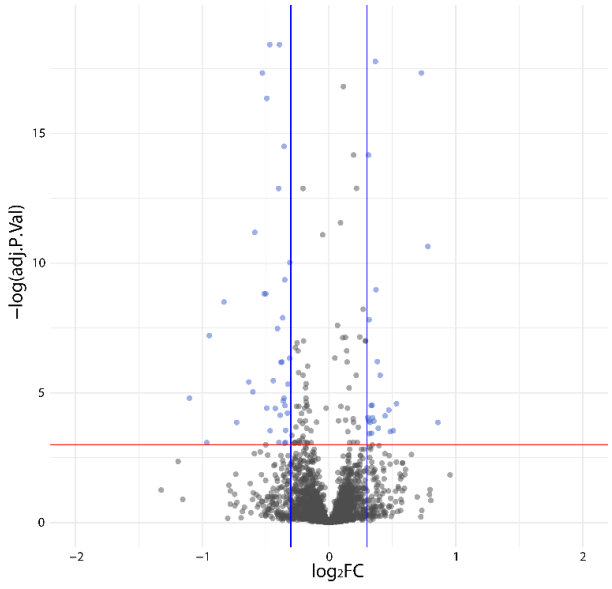


Figure 22: Proteome quantification. Volcano plot of quantified proteins in the WT vs. TgDyrk1A comparison. X-axis reports the log2 of the fold change, on the y-axis –log10 the adjusted p-value. Each dot represents a protein. Significant proteins are printed in blue. The red line shows the adjusted-p-value <0.05. Blue lines the threshold used for the Log_2(Fold Change 0.3/-0.3). Proteins present and absent in the WT-TgDyrk1A contrast are not represented in the volcano plot.

Some proteins are selectively expressed in TgDyrk1A hippocampus

We found 20 proteins present only in TgDyrk1A hippocampus and 17 proteins present only in WT hippocampus, and thus, absent in TgDyrk1A (see table 8) reinforcing the idea of DYRK1A affecting transcription in this brain region. Among proteins uniquely found in TgDyrk1A, three were proteins involved in MAPK signaling regulation: Caveolin1 (CAV1), regulator complex protein LAMTOR1 (LAMTOR1) and Protein Kinase C delta type (PRKCδ). Another interesting observation was the Craniofacial development protein 1 (CFDP1), that was absent in TgDyrk1A hippocampus. These results correlate with the transcript expression values reported for these gene products at the Allen Brain Atlas (http://mouse.brain-map.org). Proteins only expressed in TgDyrk1A showed low transcript in wild type mice in the Allen Brain Atlas.

Table 8: Present and absent proteins in TgDyrk1A hippocampus

PRESENT IN TGDYRK1A	ABSENT IN TGDYRK1A
Oxsm	Homer3
Lyrm4	Tmed2
Cmp	Npc2
Cav1	Esyt2
Snw1	E2f8
Ptprn2	Aoc3
Etv6	Sel1l
Tkfc	Stxbp3
Akr1e2	Arhgap39
Naga	Commd4
Lamtor1	Taco1
Pla2g12a	Dock10
Prkcd	Bsdc1
Pts	Usp8
Get4	Arvcf
Vps51	Cfdp1
Tubgcp3	Dnajb12
	Anp32b
	Nanp
	Dhdh

Dyrk1A as a transcription factor regulator

Even though, DYRK1A is mainly expected to induce changes in phosphorylation, we detected a number of changes in protein abundance. We thus reasoned that, for these changes to occur, DYRK1A should modulate the activity of transcription factors or proteins regulating transcription factors. We used iRegulon (Janky *et al*., 2014) implemented in Cytoscape to analyze the transcriptional factors regulating the set of proteins that change in abundance due to *Dyrk1A* overexpression (for details see Materials and Methods, section 3.5.6). With this purpose, we built a protein-protein interaction (PPI) network for upregulated and downregulated proteins in TgDyrk1A with data retrieved from mouse interactome collected from the publically available STRING database (Szklarczyk *et al*., 2015) (figureX). The analysis revealed ten transcription factors that could explain the protein abundance changes observed in the TgDyrk1A. Four of them explained the up-regulation or presence of the proteins more expressed in TgDyrk1A and six explained the proteins down-regulated or absent in TgDyrk1A (Table 9).

Table 9: Transcription factors predicted by iRegulon. The "targets" column indicates the number of proteins predicted as targets of the transcription factors. The NES (normalized enrichment score) column indicates the score of the enrichment prediction. Only transcription factors with a NES higher than 4 where considered.

UPREGULATED IN TGDYR1A			**DOWNREGULATED IN TGDYR1A**		
Transcription factor	**Targets**	**NES**	**Transcription factor**	**Targets**	**NES**
Srf	10	5.1	Msx1	17	5.1
Cebpa	8	4.7	Nkx2-2	13	4.9
Arntl	9	4.5	Ddx43	10	4.7
Nr2c2	10	4.0	Tcf3	10	4.6
			Smc3	20	4.6
			Foxo1	12	4.1

We then performed a nearest neighbor analysis of the predicted transcription factors and using DYRK1A as seed protein. First neighbors of the transcription factors did not reveal any relationship with DYRK1A except for FOXO1 that has been described as DYRK1A substrate (Woods *et al*., 2001). In fact, FOXO1 explained the down-regulation of twelve of the proteins that reduced their abundance or were absent in TgDyrk1A (Figure 23).

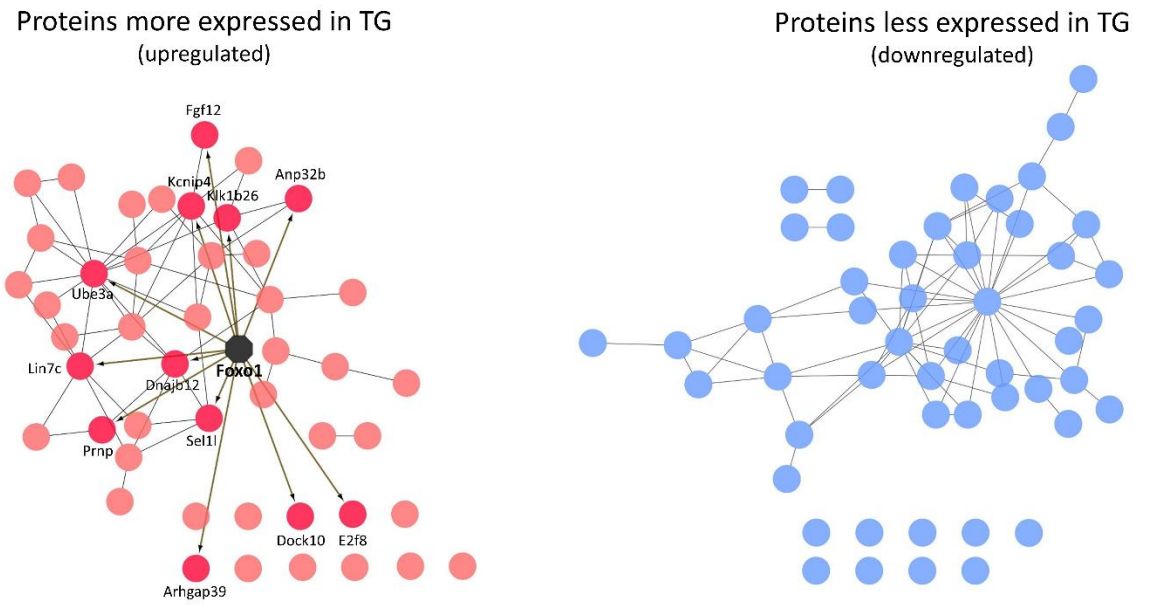


Figure 23: Transcription factor prediction. Protein-protein interaction network representation of proteins upregulated (red, left) and downregulated (blue, right). Only FOKO1, for having a direct relation with DYRK1A, is represented in the network of proteins less abundant in TgDyrk1A hippocampus. Edges represent physical, functional or predicted interactions and each node correspond to one protein. Arrows represent regulatory function of FOXO1 and dark red correspond to the nodes that can be regulated by FOXO1 activity. Dark pink nodes correspond to the targets predicted for FOXO1.

4.3.2 Phosphoproteomic signature of *Dyrk1A* overexpression in the hippocampus of TgDyrk1A mice

When comparing the hippocampus of TgDyrk1A mice to wild type mice, phosphoproteomic analysis revealed 196 differentially abundant phosphopeptides including presence/absence (Figure 24). Significant phosphopeptides corresponded to a total of 169 proteins.

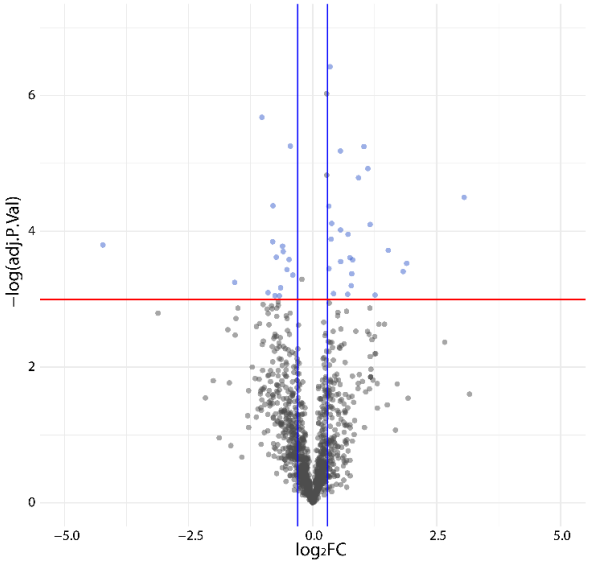


Figure 24: Phosphoproteome quantification. Volcano plot of quantified phosphopeptides in the WT vs. TgDyrk1A comparison. X-axis reports the log2 of the fold change, on the y-axis –log10 the adjusted p-value. Each dot represents a protein. Significant proteins or phosphopeptides are printed in blue. The red line shows the adjusted-p-value <0.05. Blue lines the threshold used for the Log_2(Fold Change 0.3/-0.3). Absent and present proteins in the WT-TgDyrk1A contrast are not represented in the volcano plot.

Among the significantly phosphorylated proteins there were four DYRK1A targets: i) synaptojanin1 (SYNJ1), a phosphatase proposed to participate in the endocytosis of synaptic vesicles and actin function; ii) microtubule-associated protein 1B (MAP1B), involved in the cytoskeletal changes that accompany neurite extension acting through tubulin and required for synaptic maturation; iii) glycogen synthase 1 (GYS1), involved in the pathway glycogen biosynthesis; and finally iv) serine/arginine-rich splicing factor 2 (SRSF2), necessary for the splicing of pre-mRNA (Table 7).

When considering the expanded list of first neighbors of significant phosphoproteins in the analysis, we found four more DYRK1A targets: i) 14-3-3, an adapter protein implicated in the regulation of multiple signaling pathways including AKT and MAP kinase signaling; ii) cellular tumor antigen p53 (p53), involved in cell cycle regulation; iii) sirtuin 1 (SIRT1), a deacetylase known to regulate epigenetic gene silencing and iv) DNA-directed RNA polymerase II (POLR2A), that catalyze the transcription of DNA into RNA.

DYRK1A consensus motif analysis

Even though there is not a unique consensus motif for DYRK1A phosphorylation, we considered the RPX(S/T)P motif as a proxy of DYRK1A-dependent phosphorylation (Himpel *et al*., 2001). When analyzing the WT vs. TgDyrk1A contrast, we found a significant enrichment of proteins containing this motif

(Figure 25A), suggesting that *Dyrk1A* overexpression could have led to their increased phosphorylation.

When we aligned the phopshopeptides with significantly increased abundance or exclusively present in TgDyrk1A for motif enrichment phosphorylation sites (figure XB), 83 out of 100 phosphorylation sites, were phosphorylated in a serine residue, of which 35 had a proline in position +1. This proline residue immediately after a serine or a threonine is highly conserved in proline-directed kinases, which include MAP kinases, cyclin-dependent kinases but also members of the DYRK family.

Also from those 35 phosphorylation sites we found 11 with a proline in position -2. The resulting most represented motif, PXSP, fits with published consensus motifs for DYRK1A (RPX(S/T)P) but also for MAP kinases (Figure 25B). Finally, two phosphorylated peptides exactly matched the consensus motif of DYRK1A (RPX(S/T)P). Those correspond to SGIP1, a protein that mediates clathrin endocytosis, and SHANK3, a postsynaptic density protein with a role in dendritic spine and synapse formation and described to be involved in intellectual disabilities and autism.

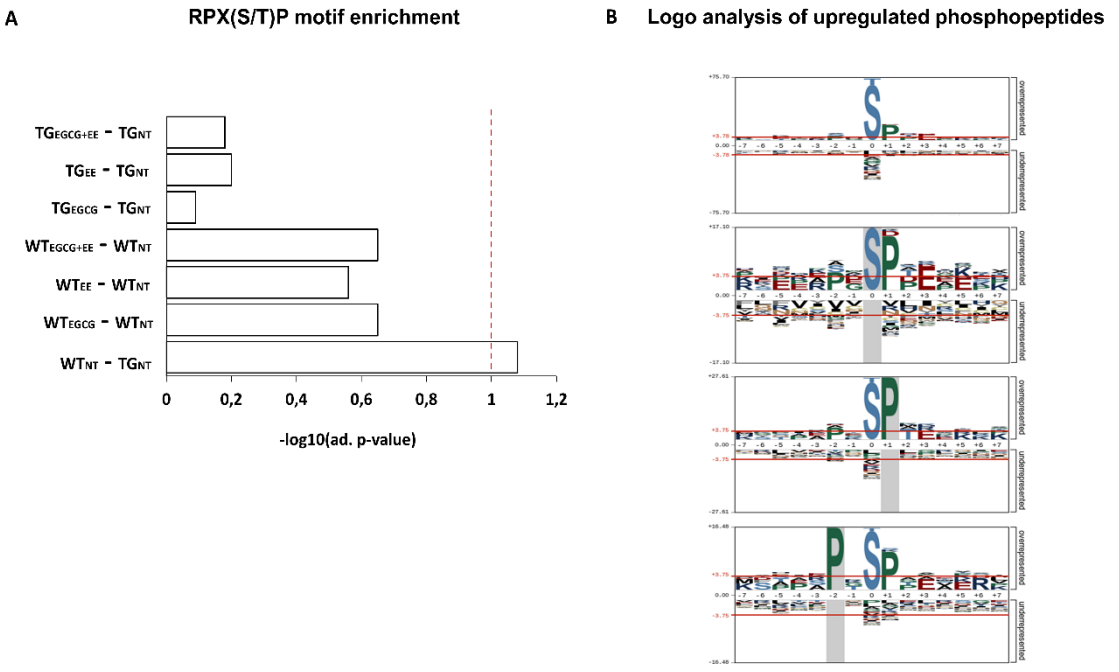


Figure 25: DYRK1A consensus motif analysis. (A) Barplot showing the degree of enrichment (-log10 of the p-value of the Fisher test) of our analyzed comparisons in protein containing a RPX(S/T) motif. Significance was defined to X>1 (C) Sequence logo of the aligned phosphopeptides which are upregulated or exclusively present in TG_{NT}.

4.3.3 Effect of proteomic and phosphoproteomic alterations on cellular functions

Both changes in protein abundance and changes in phosphorylation can have an impact on cell function. Thus, we searched for cellular functions of both the significant proteins from the proteome and the proteins with at least one significant phosphopeptide.

Proteomic and phosphoproteomic network

To better understand the changes caused by *Dyrk1A* overexpression in the hippocampus of TgDyrk1A mice, we built PPI network with data retrieved from mouse interactome collected from the publicaly available STRING database. It has been suggested that if two proteins interact, then they are likely to have related functions, a phenomenon known as “guilt-by-association” (Oliver, 2000). Therefore, a PPI map will provide insight into the DYRK1A dependent disturbances in cell biology.

Proteins that significantly changed in abundance were represented as circles, phosphoproteins as rectangles and proteins modified in both protein abundance and phosphorylation abundance as triangles (the later accounting for only four nodes). Physical and functional interactions between proteins were represented as edges. This network representation of proteins and phosphoproteins altered by *Dyrk1A* overexpression allowed the study of protein interactions as well as the analysis of functional modules and the identification of highly interconnected proteins or hubs.

From the 252 nodes in the network, 135 have at least one connection with another node (Figure 26A). When analyzing the degree distribution of the nodes in the network, we found a power-law degree distribution (Figure 26C) indicating a scale-free network with a total average node degree of 3.1 (each node has 3.1 direct partners in the network). Note that there were no differences in average node degree between proteins and phosphoproteins.

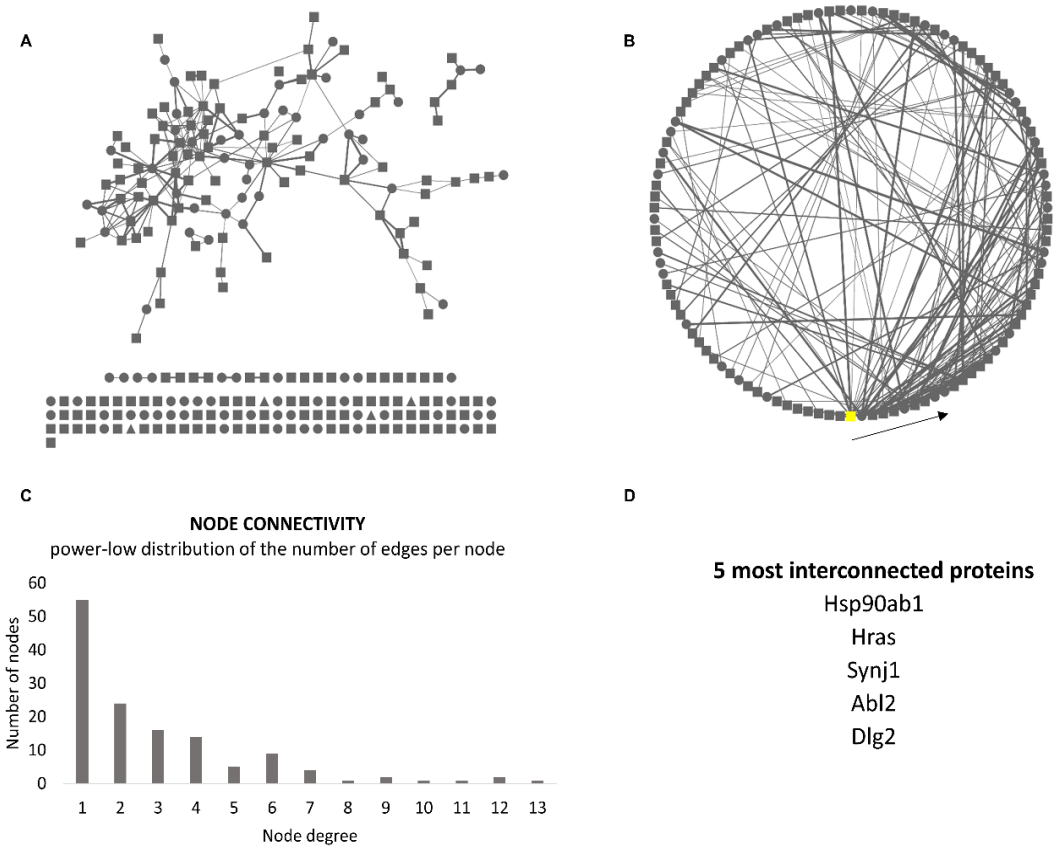


Figure 26: Interaction network of differentially expressed and phosphorylated proteins in TgDyrk1A hippocampus (genotype network). (A) Protein-protein interaction network representation of proteins differentially expressed (circle), differentially phosphorylated (rectangle) and both differentially expressed and differentially phosphorylated (triangle). Edges represent physical and/or functional of the nodes with the thickness depending on the strength of the interaction (combined score in String database). (B) Degree sorted circle representation of the network. Nodes are sorted from the most interconnected node or hub (red node) to less connected nodes (right following the arrow). (C) Node degree distribution showing the number of edges per node. (D) List of the most interconnected proteins in the network.

Highly interconnected proteins or hubs in the genotype network

In scale-free networks, the majority of nodes (proteins) have only a few connections to other nodes, whereas some nodes (hubs) are highly connected to many other nodes in the network. A degree sorted circle representation of the network (Figure 26B) helped us to better visualized the hubs in the network being the most interconnected one in red at the bottom of the circle and decreasing its connectivity following the direction of the arrow. The 5 most interconnected proteins (with more than 10 connections) that were altered by *Dyrk1A* overexpression in mice hippocampus are listed in a table (Figure 26D).

These were i) HSP90AB1, a molecular chaperone; ii) GTPase Hras (HRAS), a GTPase protein with a critical role in MAPK signaling; iii) synaptojanin-1 (SYNJ1), a phosphatase with a role in the rearrangement of actin filaments; iv) Abelson tyrosine-protein kinase 2 (ABL2), a tyrosine kinase with key roles in cell growth, survival and cytoskeleton remodeling and in brain, regulating neurotransmission phosphorylating proteins at the synapse and v) disk large homolog 2 (DLG2), interactor on NMDA receptor and involved in the synaptic stability at cholinergic synapses and part of the postsynaptic protein scaffold of excitatory synapses.

Functional modules in the genotype network

A functional module is defined as a group of proteins which are related by one or more genetic or cellular interactions, e.g. co-regulation, co-expression or pertaining to a protein complex, a metabolic or signaling pathway or a cellular aggregate (e.g. chaperone, ribosome, protein transport facilitator, etc.) (Tornow *et al*., 2006). An important property of a module is that its members have more interactions among themselves than with members of other modules, which is reflected in the network topology. Thus, we used the plugin ClusterONE (Nepusz et al., 2012) implemented of Cytoscape (Cline *et al*., 2007) to discover those more densely connected regions of the network and DAVID Bioinformatics Database (Dennis *et al*., 2003) to elucidate which cell functions are more represented and thus, more affected by Dyrk1A overdosage. We identified six significant functional modules: vesicle-mediated transport, cell adhesion, regulation of protein phosphorylation (MAPK activity), protein folding, RNA processing and synaptic vesicle exocytosis (Figure 27). At least three of them, synaptic vesicle exocytosis, cell adhesion and regulation of protein phosphorylation (MAPK activity) are directly related with synaptic plasticity.

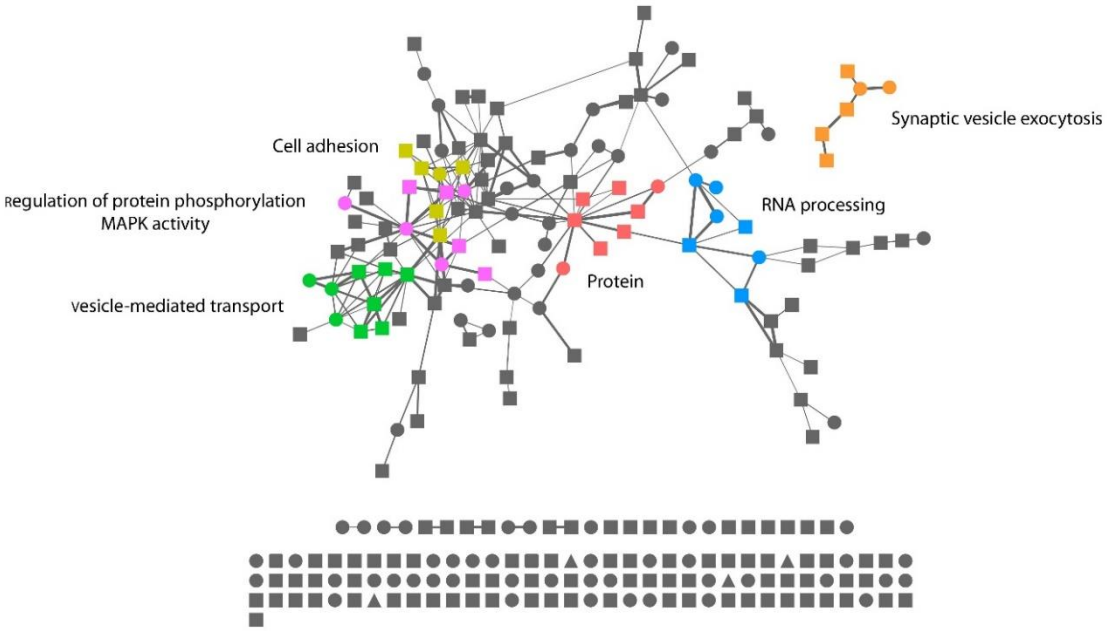


Figure 27: Clustering of the interaction network of differentially expressed and phosphorylated proteins in TgDyrk1A hippocampus. Protein-protein interaction network representation of proteins differentially expressed (circle), differentially phosphorylated (rectangle) and both differentially expressed and differentially phosphorylated (triangle). Edges represent physical and/or functional of the nodes with the thickness depending on the strength of the interaction (combined score in String database). Significant clusters from ClusterONE are represented with different colors.

Gene ontology enrichment analysis of proteins and phosphoproteins deregulated by *Dyrk1A* overexpression

To gain further insight into alterations caused by *Dyrk1A* overexpression in the hippocampus of TgDyrk1A mice, in this case we also took into consideration the non-connected proteins. To this end, cluster analysis using Gene Ontology (GO) biological processes and Gene Ontology (GO) cellular components was performed using the DAVID bioinformatics database. All the proteins and phosphoproteins quantified in the TgDyrk1A vs. WT comparison were used as a background universe.

Biological Process

GO enrichment analysis revealed 23 gene ontology terms corresponding to the biological processes significantly enriched among the proteins changing the abundance (Figure 28) which, in general terms, were related to neuronal plasticity and signal transduction. There were also processes affecting signaling cascades such us regulation of MAP kinase activity, regulation of ERK1 and ERK2 cascade and G-protein coupled receptor protein signaling. However, among the significant ones (dark blue bars), there were processes related with neuronal plasticity such as axonogenesis, neurotransmitter secretion, regulation of long-term synaptic

potentiation, behavior and microtubule cytoskeleton organization but also protein localization and regulation of protein binding.

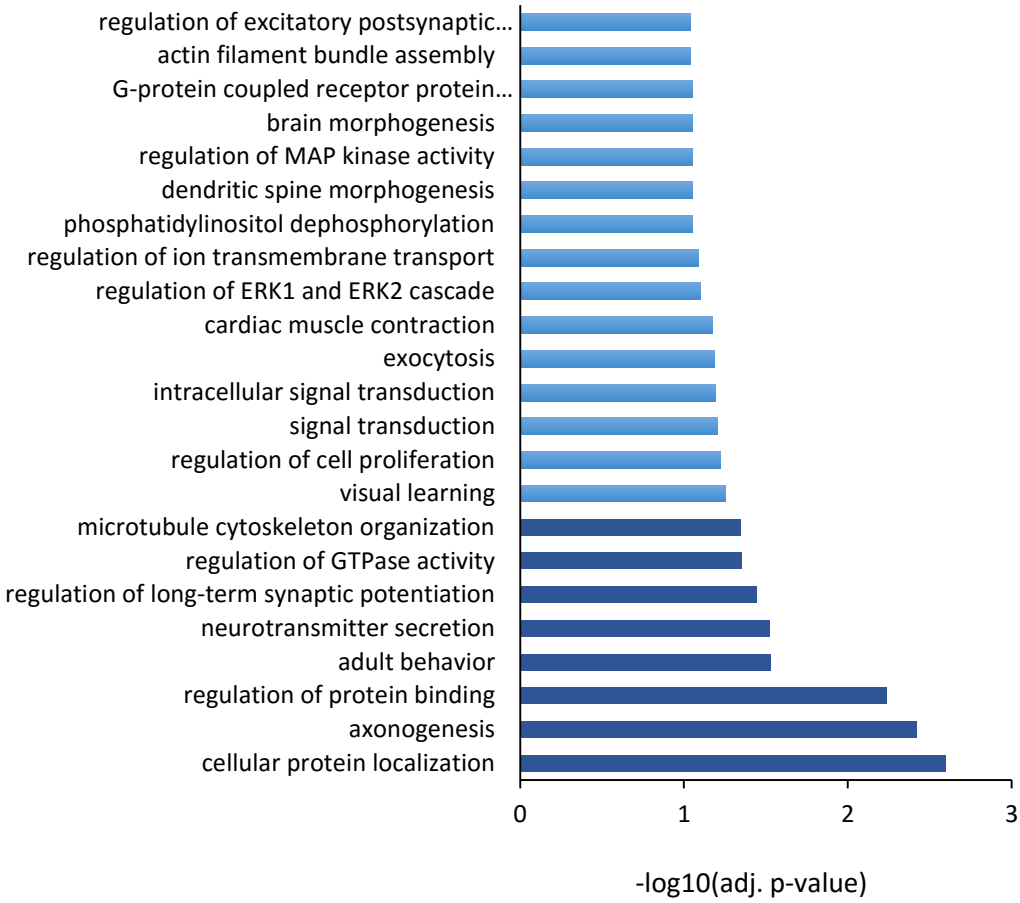


Figure 28: Enriched biological processes in proteins and phosphoproteins altered in TgDyrk1A hippocampus. Biological processes found to be enriched among the set of proteins and phosphoproteins significantly altered in TgDyrk1A hippocampus. Dark blue indicates the significant enriched ones with an adjusted (Benjamini) p-value < 0.05.

Results of GO enrichment analysis of biological processes of alterations caused by *Dyrk1A* overexpression revealed mainly signal transduction and neuroplasticity processes. To understand if these two main categories were clearly separated at the proteome and phosphoproeome levels, the same analysis was repeated but now separating proteins differentially abundant and significant phosphoproteins. Results are shown in table 10 with only the significant GO terms enriched with an adjusted p-value <0.05. Signal transduction and specific signaling cascades such as MAPK and ERK cascades, were present in both altered proteome and phosphoproteome but neuroplasticity GO terms were exclusive of the phosphoproteome.

Table 10: Biological processes from differentially expressed proteins and proteins with differentially expressed phosphoproteins by separate. Only GO terms are in the table ordered from smallest to larger adjusted (Benjamini) p-value < 0.05

BIOLOGICAL PROCESSES PROTEOME	BIOLOGICAL PROCESSES PHOSPHOPROTEOME
Regulation of protein ubiquitination *cellular senescence* *transport* *protein transport* *regulation of GTPase activity* *regulation of MAPK cascade* *cholesterol homeostasis* *regulation of cation channel activity* *vesicle-mediated transport* *nuclear envelope reassembly*	axonogenesis cellular protein localization endocytosis regulation of long-term synaptic potentiation exocytosis visual learning regulation of ERK1 and ERK2 cascade regulation of protein binding actin filament bundle assembly nervous system development regulation of synaptic transmission, GABAergic transport synapse assembly cardiac muscle contraction regulation of GTPase activity long-term synaptic potentiation cell adhesion microtubule cytoskeleton organization neuromuscular process controlling balance small GTPase mediated signal transduction neuron projection morphogenesis brain morphogenesis learning regulation of exocytosis protein targeting to plasma membrane intracellular signal transduction regulation of GTPase activity regulation of excitatory postsynaptic potential regulation of endocytosis neurotransmitter secretion neurofilament bundle assembly postsynaptic density assembly regulation of cell migration regulation of neuron projection development regulation of extrinsic apoptotic signaling pathway in absence of ligand actin cytoskeleton organization regulation of G-protein coupled receptor protein signaling pathway vesicle-mediated transport phosphatidylinositol-mediated signaling platelet-derived growth factor receptor signaling pathway adult behavior regulation of I-kappaB kinase/NF-kappaB signaling intermediate filament bundle assembly

Cellular Component

Cellular component enrichment analysis revealed mainly important cell components for the information transmission among neuronal networks as dendrites, synapse, presynaptic and postsynaptic sites, but also axon and neuronal projection (Figure 29).

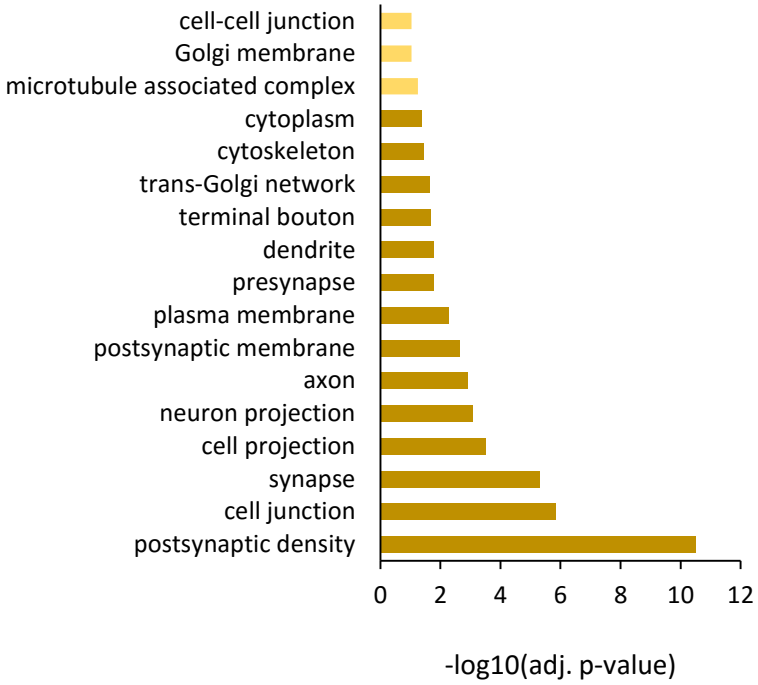


Figure 29: Enriched cellular components in proteins and phosphoproteins altered in TgDyrk1A hippocampus. Cellular components found to be enriched among the set of proteins and phosphoproteins significantly altered in TgDyrk1A hippocampus. Dark orange indicates the significant enriched ones with an adjusted (Benjamini) p-value < 0.05.

MAPK, ERK and phosphatidylinositol cascade

Among the three signaling cascades found in the GO enrichment analysis, a total of 27 proteins involved in MAPK, ERK and phosphatidylinositol cascades were identified (Table 11). Its noteworthy to remind that MAP kinase was also enriched as functional module in the genotype network.

Table 11: Proteins involved in singling cascades. Proteins altered by *Dyrk1A* overexpression that belong to MAPK, ERK and phosphatidylinositol signalling cascades

Proteins involved in signalling cascades (MAPK, ERK and Phosphatidylinositol)	
Pten	Ralbp1
Slc9a3r1	Rabl6
Dab2ip	Arhgef
Dmd	G3bp2
Rgs14	Rab3a
Dock10	Ppp1r1b
Agap2	Dgkg
Scg2	Pi4kb
Mink1	Irs2
Rgs14	Hras
Arfgef2	Prkcd
Sh3bp5l	Gpr37l1
Stk32c	
Synj1	Fgf12

Validation of signaling cascades perturbation

Due to the crosstalk between signaling cascades, with the current data it was difficult to elucidate the contribution of each of the proteins to the aforementioned pathways. However, as i) both MAPK signaling and phosphatidylinositol signaling cascade have a direct or indirect effect in the regulation of ERK (Mendoza *et al*., 2011) and ii) HRAS was upregulated in TgDyrk1A hippocampus, the activation of ERK1/2 was analyzed using phospho-specific antibodies p-ERK1/2 (Thr202/Tyr204) by Western Blot from total hippocampus protein extract from the same animals used in LC-MS/MS. The measure of pERK1/2 showed a trend to increase its phosphorylated form in the hippocampus of TgDyrk1A compared to wild type mice and a decrease with the treatments, especially with EE and the combined treatment EGCG+EE even without reaching significant levels (Figure 30).

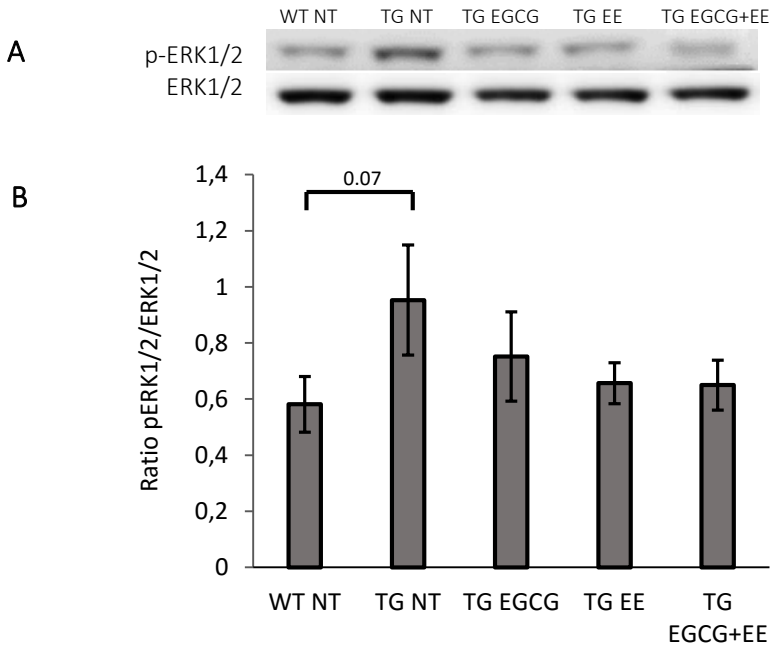


Figure 30: Effect of *Dyrk1A* overexpression and treatments on ERK activation in the hippocampus of TgDyrk1A mice. (A) Slot blots showing ERK activation (p-ERK p44/p42) in the hippocampus of wild type mice (WT), TgDyrk1A mice (TG) and TgDyrk1A mice treated with EGCG, EE and combined treatment EGCG+EE. Proteins were subjected to immunoblot analysis using antibodies specific to p-ERK p44/p42. After stripping, the membranes were incubated with anti-ERK total form antibody for control. (B) Relative protein expression was determined by normalization intensity of images from p-ERK p44/p42 with that of ERK total form of the same blot. The blots represent the three independent experiments with *n*=5 per each experimental condition. Data are expressed as mean ± SEM and statistical analysis was done with one-way ANOVA repeated measures.

The activation of the immediate kinase downstream of HRAS, MEK1/2, was also measured using phospho-specific antibodies pMEK1/2 (Ser217/221) by Western Blot from total hippocampus protein extract from the same animals used in LC-MS/MS. However, no significant changes were found in the phosphorylation of MEK in the hippocampus of TgDyrk1A compared with wild type mice but neither with any of the treatments (Figure 31).

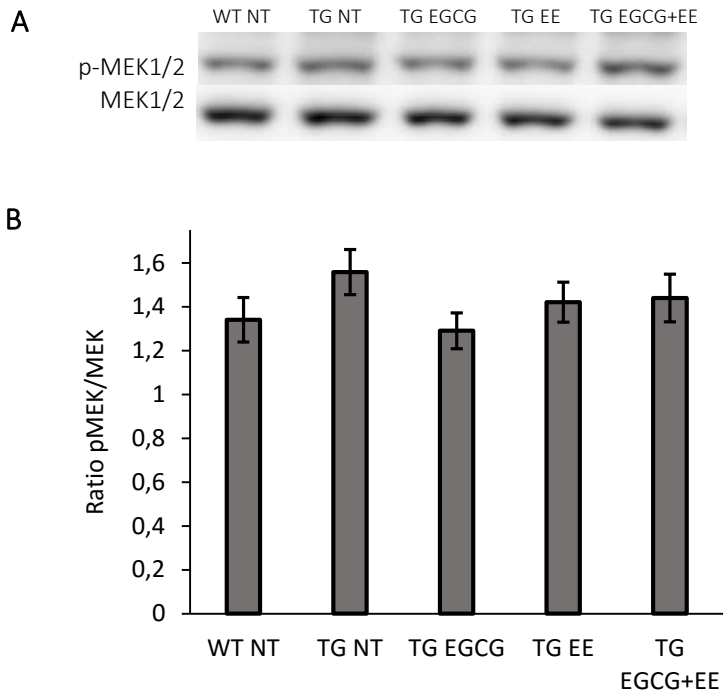


Figure 31: Effect of *Dyrk1A* overexpression and treatments on MEK1/2 activation in the hippocampus of TgDyrk1A mice. (A) Slot blots showing MEK1/2 activation (p-MEK1/2) in the hippocampus of wild type mice (WT), TgDyrk1A mice (TG) and TgDyrk1A mice treated with EGCG, EE and combined treatment EGCG+EE. Proteins were subjected to immunoblot analysis using antibodies specific to p-MEK1/2. After stripping, the membranes were incubated with anti-MEK1/2 total form antibody for control. (B) Relative protein expression was determined by normalization intensity of images from p-MEK1/2 with that of MEK1/2 total form of the same blot. The blots represent the three independent experiments with *n*=5 per each experimental condition. Data are expressed as mean ± SEM and statistical analysis was done with one-way ANOVA repeated measures.

The results suggest that the small increased of abundance of HRAS found in TgDyrk1A hippocampus by LC-MS/MS (log_2 (Fold Change = 0.37; ratio 1.3 respect to wild type mice), and not detected by Western blot (Figure 32), was probably not the responsible of the increased activation of ERK1/2 suggesting that other proteins could be involved.

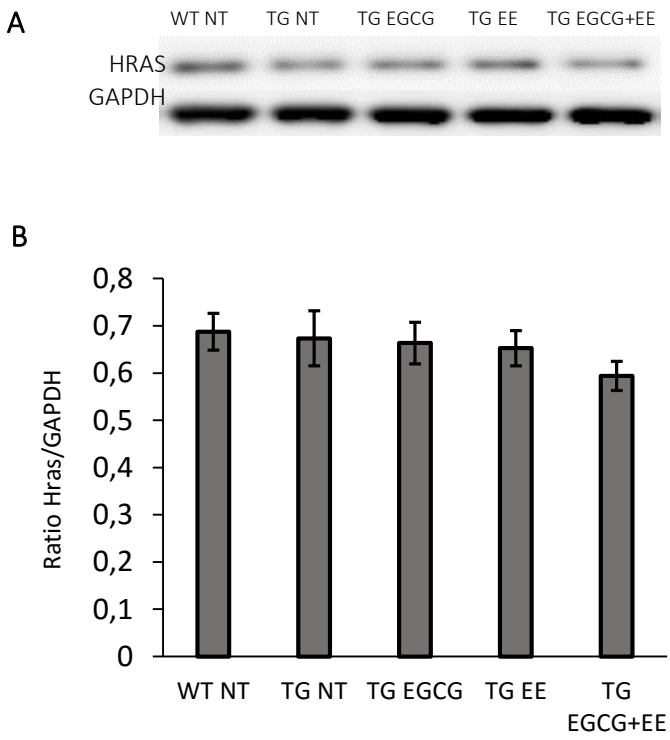


Figure 32: Effect of *Dyrk1A* overexpression and treatments on levels of HRAS in the hippocampus of TgDyrk1A mice. (A) Slot blots showing HRAS levels in the hippocampus of wild type mice (WT), TgDyrk1A mice (TG) and TgDyrk1A mice treated with EGCG, EE and combined treatment EGCG+EE. Proteins were subjected to immunoblot analysis using antibodies specific to HRAS. After stripping, the membranes were incubated with anti GAPDH for the control. (B) Relative protein expression was determined by normalization intensity of images from HRAS with that of GAPDH of the same blot. The blots represent the three independent experiments with *n*=5 per each experimental condition. Data are expressed as mean ± SEM and statistical analysis was done with one-way ANOVA repeated measures.

Intellectual disability proteins

As people with Down syndrome have a mild to moderate intellectual disability (ID), we investigated if *Dyrk1A* overexpression could affect proteins involved in intellectual disability. From the 1152 genes described for ID (for details see Materials and Methods, section 3.5.9), 30 were present among the proteins and phosphoproteins altered in TgDyrk1A hippocampus and mapped in the genotype network (Figure 33). The total average node degree of these 30 intellectual disability-described proteins was 4.2 (total average node degree of the genotype network was 3.1) suggesting that proteins described to be involved in ID are not highly interconnected proteins than the rest of the proteins in the network.

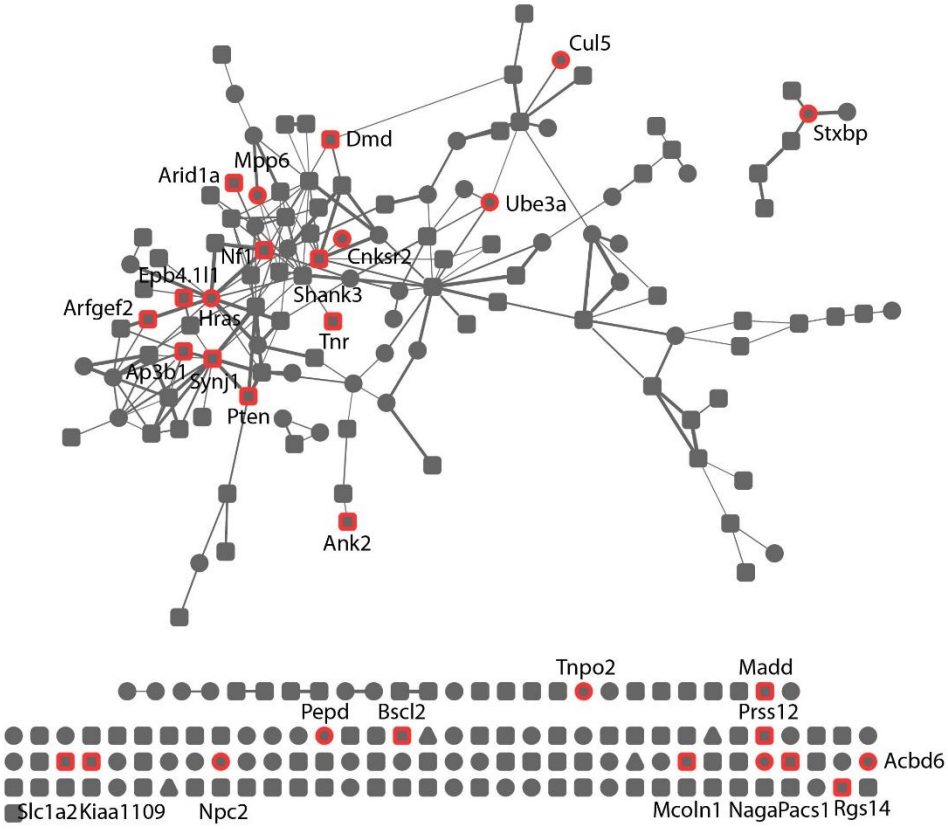


Figure 33: Overlap of differentially expressed and phosphorylated proteins in TgDyrk1A hippocampus and proteins related with intellectual disability. Protein-protein interaction network representation of proteins differentially expressed (circle), differentially phosphorylated (rectangle) and both differentially expressed and differentially phosphorylated (triangle). Edges represent physical and/or functional of the nodes with the thickness depending on the strength of the interaction (combined score in String database). Red border indicates proteins described to be involved in intellectual disabilities.

Autism-related proteins

The prevalence of autism spectrum disorder (ASD) in children with DS (ages 2-11) is significantly higher than in the typical population (DiGuiseppi *et al*., 2010). Genes associated with ASD have been listed in different databases such as AutismKB, AutDB or SFARIgene and among them, several genes from Hsa21, have also been listed suggesting their role in ASD (Table 12). Given that *Dyrk1A*, also present in the list, have been associated with autism (Rachubinski *et al*., 2017), we investigated if *Dyrk1A* overexpression could lead to changes in proteins involved in autism.

Table 12: Hsa21 genes with a potential role in DS+ASD. (Adapted from Rachubinski *et al*., 2017)

Hsa21 genes with potential roles in DS+ASD
DYRK1A
APP
DSCAM
ITSN1
TMPRSS2
BRWD1
WDR4
CBS
DIP2A
ITGB2
SUMO3
PTTG1IP
CSTB

The list of the 60 most confident proteins to be involved in autism described by SFARI Gene (https://www.sfari.org/resource/sfari-gene/; for details see Materials and Methods, section 3.5.9) was used to check if the overexpression of *Dyrk1A* could lead changes to them. From the 60 proteins, 25 overlapped with intellectual disability genes. Among the proteins differentially abundant or with differentially abundant phosphopeptides, four proteins were described to be involved both in intellectual disabilities and autism and mapped in the genotype network (Figure 34): i) PTEN, a phosphatase involved in different signaling cascades including PI3K-AKT; ii) SHANK3, a postsynaptic density protein with a role in dendritic spine and synapse formation; iii) ARID1A, involved in transcriptional activation and repression of selected genes by chromatin remodeling and iv) ANK2, a protein that attached integral proteins to cytoskeletal elements and required for regulation of ion exchangers. One protein, BCKDK, a mitochondrial dehydrogenase, is described to be involved only in autism.

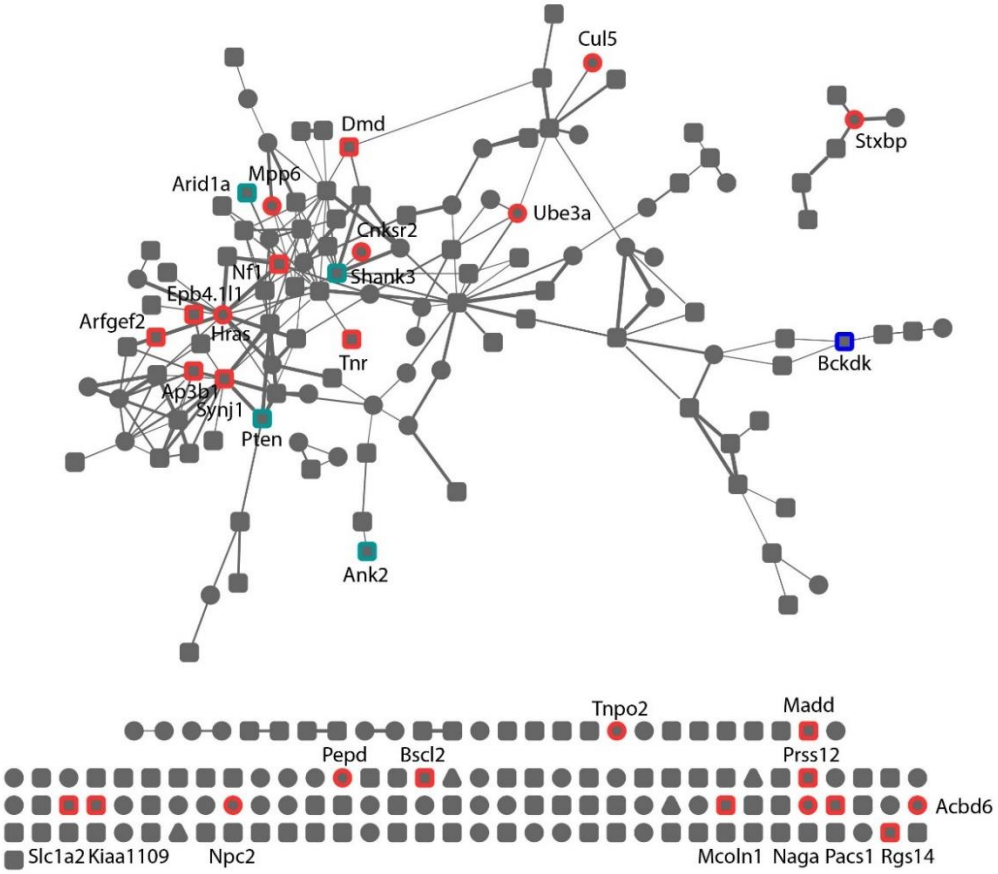


Figure 34: Overlap of differentially expressed and phosphorylated proteins in TgDyrk1A hippocampus and proteins related with intellectual disability and autism. Protein-protein interaction network representation of proteins differentially expressed (circle), differentially phosphorylated (rectangle) and both differentially expressed and differentially phosphorylated (triangle). Edges represent physical and/or functional of the nodes with the thickness depending on the strength of the interaction (combined score in String database). Red border indicates proteins correspond to intellectual disabilities, green borders correspond to both intellectual disabilities and autisms genes and blue border correspond only to autism.

4.4 Treatment-dependent proteome and phosphoproteome changes

After the proteome and phosphoproteome characterization of the hippocampus of TgDyrk1A mice, we analyzed the effect epigallocatechin-3gallate (EGCG), environmental enrichment (EE) and the combination of both (EGCG+EE) on the hippocampal proteome and phosphoproteome of TgDyrk1A mice. Both treatments decrease DYRK1A kinase activity in the hippocampus of TgDyrk1A mice and improve cognitive deficits in Down syndrome mouse models. The number of proteins and phosphopeptides with significant differences in abundance summarized in table 6 and plotted in figure 35.

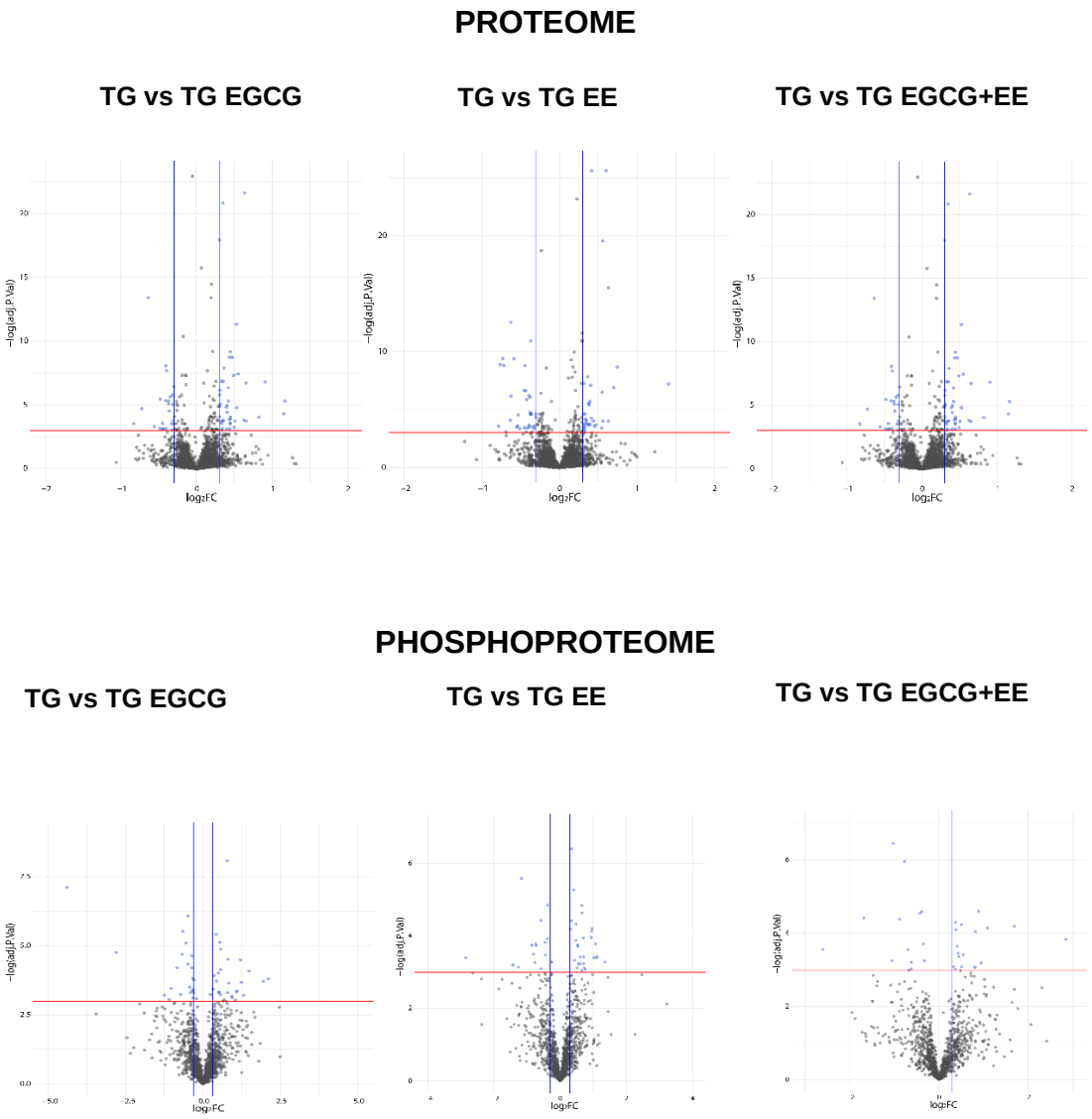


Figure 35: Proteome and phosphoproteome quantification. Volcano plot of quantified proteins and phosphoproteins in the treatments comparison. X-axis reports the log2 of the fold change, on the y-axis –log10 the adjusted p-value. Each dot represents a protein or phosphopeptide. Significant proteins or phosphopeptides are printed in blue. The red line shows the adjusted-p-value <0.05. Blue lines the threshold used for the Log2fold change (0.3/-0.3). Absent and present proteins in the comparisons are not represented in the volcano plot.

TgDyrk1A proteome alterations are partially restored by EGCG, EE and EGCG+EE

We first focused on what we called “rescued” proteins/phosphoproteins, e.g. those proteins or phosphopeptides up- or downregulated by *Dyrk1A* overexpression and whose altered abundance was reverted by the treatments (Table X) (for details see Materials and Methods, section 3.5.2). EGCG was the treatment rescueing more proteins in the hippocampus of TgDyrk1A,

nevertheless, it was also the treatment that caused more proteomic and phosphoproteomic changes.

Table 13: Number of rescued proteins and phosphoproteins. Right column shows the percentage of rescued proteins joining proteome and phosphoproteome.

CONTRAST FOR PROTEOMICS	PROTEOME	PHOSPHOPROTEOME	% OF RESCUED
TG_{NT} – WT_{NT}	98	169	-
RESCUED EGCG	17	73	34
RESCUED EE	24	52	28
RESCUED EGCG+EE	29	52	30

From the total of rescued proteins, 10 proteins and 19 phosphoproteins were rescued by all the three treatments (Figure 36A/B). The fact of observing overlapping proteins suggests that EGCG, EE and EGCG+EE shared common mechanistic pathways.

Among the common phosphoproteins we found rescued phosphosites from DYRK1A targets, concretely synaptojanin-1 (SYINJ1), a phosphatase with a role in the rearrangement of actin filaments and microtubule-associated protein 1B (MAP1B), another protein with a role in cytoskeletal changes that accompany neurite extension and required for synaptic maturation. Also, GYS1, glycogen synthase, was rescued only by EGCG and SRSF2, a splicing factor, was rescued only by EGCG+EE as described in table 14.

Table 14: DYRK1A targets rescued by the treatments.

	DYRK1A TARGETS	
CONTRAST FOR PROTEOMICS	**Proteome**	**Phosphoproteome**
RESCUED EGCG	-	Synj1 Gys1 Map1b
RESCUED EE	-	Synj1 Map1b
RESCUED EGCG+EE	-	Synj1 Map1b Srsf2

Four “intellectual disability” proteins were rescued by all the treatments at the phosphoproteome level: i) AT-rich interactive domain-containing protein 1A (ARID1), involved in transcriptional activation and repression of selected genes by chromatin remodeling; ii) ankyrin-2 (ANK2), a protein that attached integral proteins to cytoskeletal elements and required for regulation of ion exchangers; iii) synaptojanin-1 (SYNK1), a phosphatase with a role in the rearrangement of actin filaments and iv) seipin (BSCL2), a regulator of lipid catabolism and energy homeostasis.

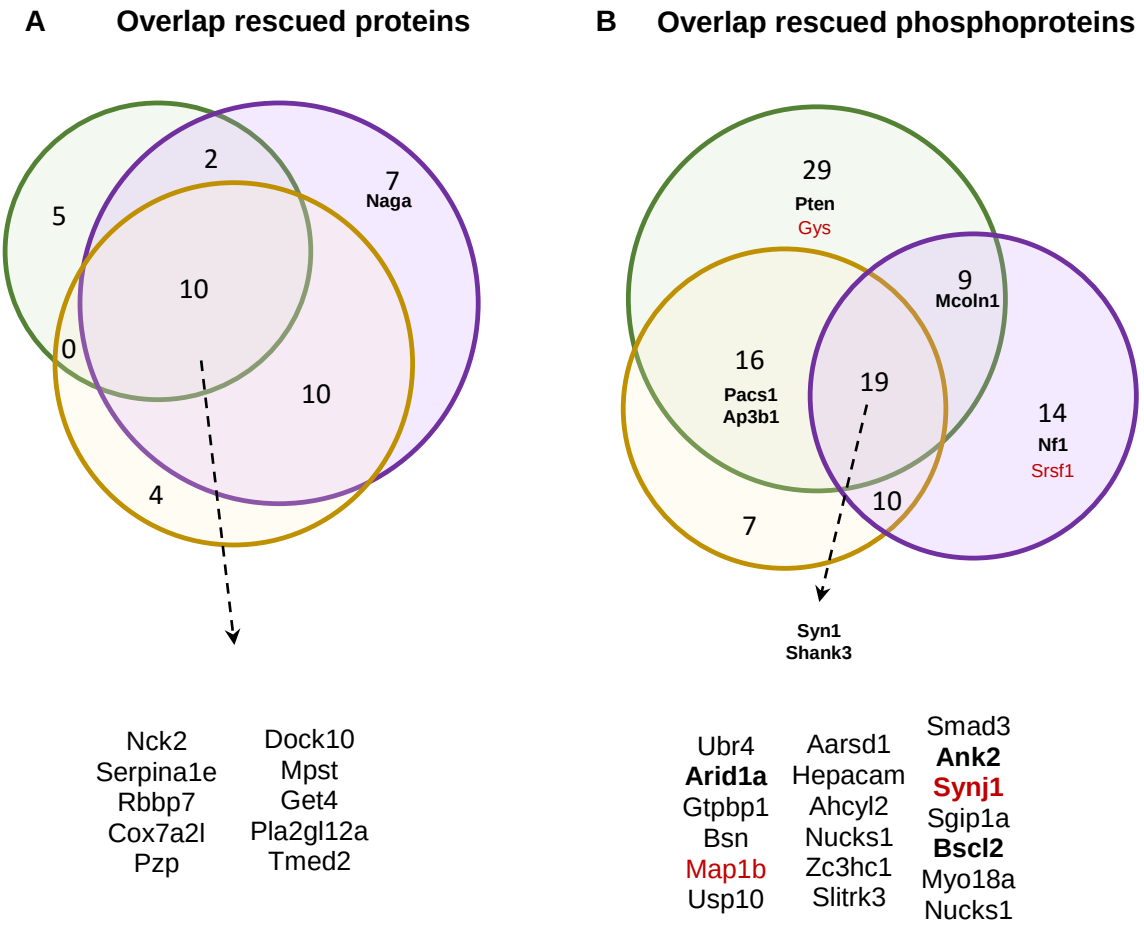


Figure 36: Overlap of rescued proteins and phosphoproteins. (A) Venn diagram of rescued proteins. (B) Venn diagram of phospopeptide proteins (proteins that have at least one phosphopeptide). Colors indicate the treatment: EGCG (green), EE (yellow) and EGCG+EE (purple). Bold proteins correspond to intellectual disability described genes. Red proteins correspond to DYRK1A targets.

Protein-protein interaction sub-network of the rescued proteins and phosphoproteins

We mapped the rescued proteins on the previously built genotype protein-protein interaction network representing the proteins and phosphoproteins altered in the hippocampus of TgDyrk1A mice. The average node degree of the rescued proteins was 2.9, similar to the average node degree of the networks (3.1). Thus, data suggested that the treatment do not only rescue specifically highly connected proteins as can be observed in figure 37. However, all treatments rescued one of the first five hubs described to be affected by *Dyrk1A* overexpression, SYNJ1, and EGCG and the combined treatment of EGCG+EE rescued also one of this five hubs, ABL2.

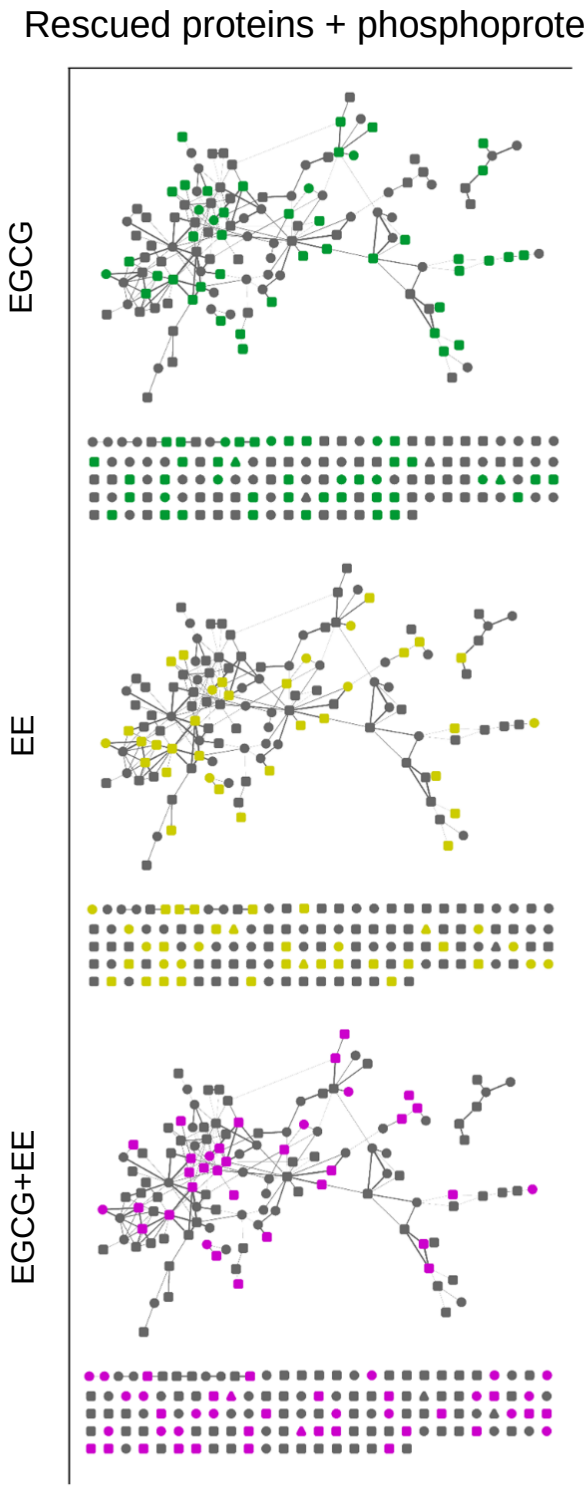


Figure 37: Overlap of rescued proteins and phosphoproteins, network view. Protein-protein interaction network representation of proteins differentially expressed (circle), differentially phosphorylated (rectangle) and both differentially expressed and differentially phosphorylated (triangle). Edges represent physical and/or functional of the nodes with the thickness depending on the strength of the interaction (combined score in String database). Green nodes indicate proteins corresponding to rescued proteins by EGCG, yellow nodes indicate proteins corresponding to rescued proteins by EE, purple nodes indicate proteins rescued by EGCG+EE.

Gene ontology enrichment analysis of proteins and phosphoproteins rescued by epigallocatechin-3-gallate, environmental enrichment and its combination

To get further insight into rescued proteins cluster analysis using Gene Ontology (GO) biological processes was performed using the DAVID bioinformatics database. All the proteins and phosphoproteins quantified in each comparison were used as a background.

Biological Process

All treatments rescued proteins related with cellular protein localization. EE rescued proteins involved in axonogenesis and the combined treatment rescued proteins involved in MAPK cascade. It necessary to mention that we found some biological processes related to cardiac functions, mainly ion channels. Those proteins, SCNB2, ANK2, ARP1A3, PTEN, GJA1, were checked manually to understand their function and why were described under cardiac terms. It was found that those proteins were directly or indirectly involved in the ion transmission and intercellular electrical and chemical transmission and thus, have the same role in neurons (Figure 38).

Figure 38: Enriched biological processes in proteins and phosphoproteins rescued by the treatments. Biological processes found to be enriched among the set of proteins and phosphoproteins rescued by EGCG (green), EE (yellow) and EGCG+EE (purple) Dark colors indicates the significant enriched ones with adjusted (Benjamini) p-value < 0.05.

Even MAPK cascade was only enriched in the combined treatment, it is worth mentioning that the three treatments had an effect rescuing proteins involved the already mentioned signaling cascades (table X). Concretely, 8, 7 and 6 proteins involved in MAPK, ERK and phosphatidylinositol signaling cascades were rescued by EGCG, EE and the combined treatment EGCG+EE respectively (Table 15). Concretely, DOCK10 and SYNJ1 were two proteins rescued by all the treatments (Figure 39).

Table 15: Rescued proteins by the treatemnts related to MAPK, ERK and phosphatidylinositol signaling cascades.

Rescued by EGCG	Rescued by EE	Rescued by EGCG+EE
Pten Dock10 Ralbp1 Arhgef G3bp2 Stk32c Synj1 Pi4kb	Dock10 Ralbp1 Rab3a Scg2 Stk32c Synj1 Pi4kb	Dock10 Rabl6 Arhgef Dgkg Scg2 Synj

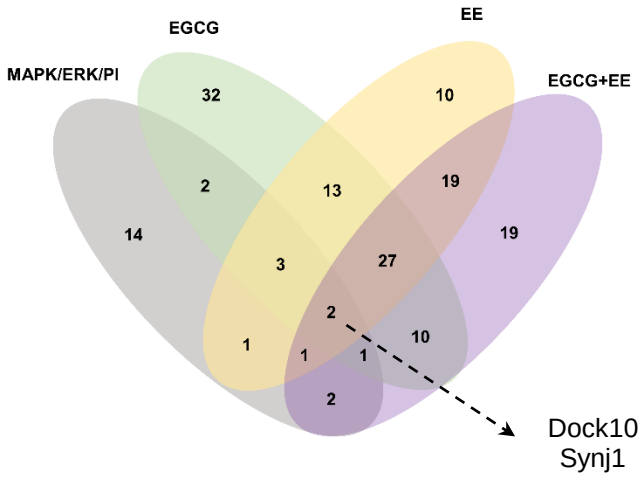


Figure 39: Rescued proteins related with MAPK, ERK and phosphatidylinositol signaling cascades. Overlap for rescued proteins belonging to MAPK, ERK and phosphatidylinositol signalling cascades with Dock10 and Synj1 as the two proteins rescued by all the treatments.

Gene ontology enrichment analysis of proteins and phosphoproteins altered but not rescued by epigallocatechin-3-gallate, environmental enrichment and its combination in the hippocampus of TgDyrk1A mice

Due to the multiple targets of the treatments there were also proteins and phosphoproteins altered by the treatments and that do not rescue any of the ones altered by *Dyrk1A* overexpression.

Even these set of proteins were not the priority of this thesis, a GO enrichment analysis was performed to elucidate which biological processes were affected by

the treatments but that not include the restoring of *Dyrk1A* overexpressing alterations.

Biological Process (EGCG)

22 biological processes enriched were enriched. There were processes affecting mainly neuro plasticity processes and no concrete signaling pathway appeared. Among eight the significant ones (dark blue) there were processes related with plasticity such as cytoskeleton and microtubule processes, cell adhesion and axonogenesis but also regulation of apoptotic process and intracellular signal transduction (Figure 40).

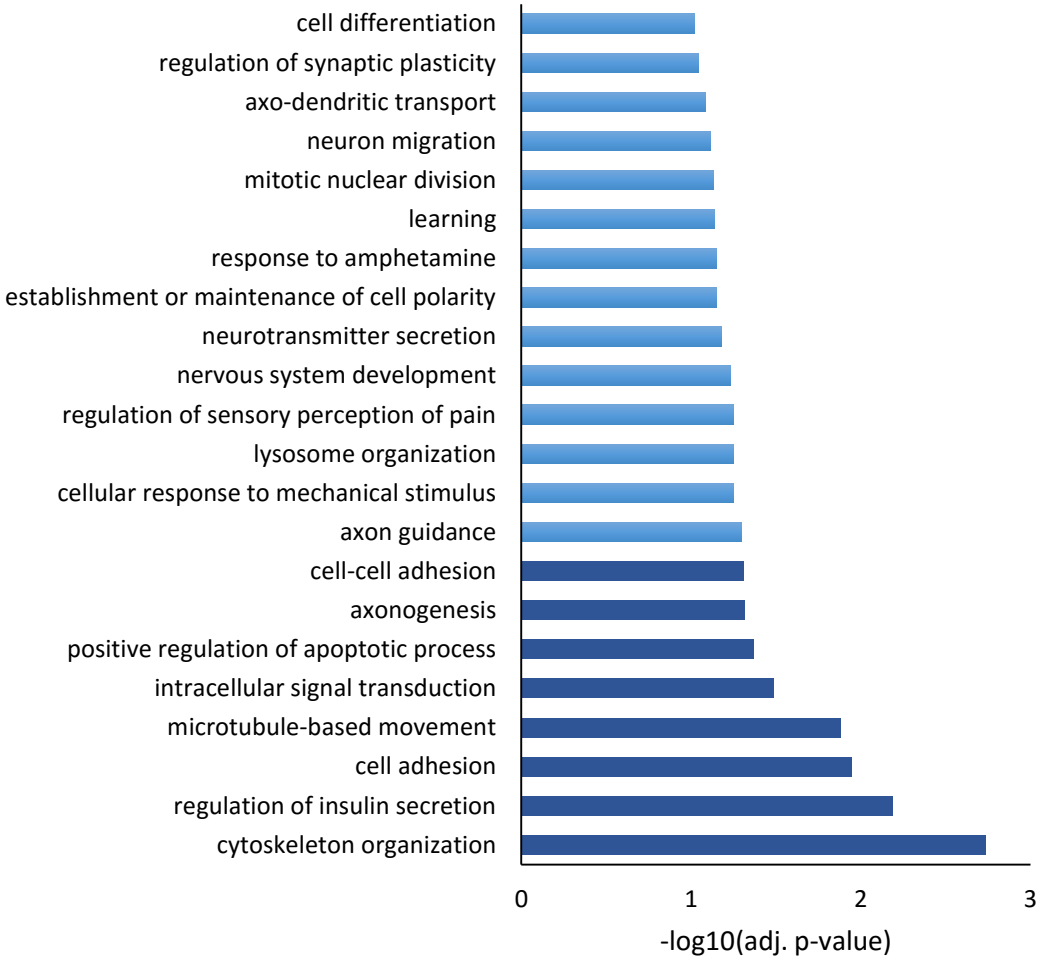


Figure 40: Enriched biological processes in proteins and phosphoproteins altered by EGCG and that not rescue alterations caused by *Dyrk1A* overexpression. Biological processes found to be enriched among the set of proteins and phosphoproteins significantly altered by EGCG in TgDyrk1A hippocampus but without the ones that rescued proteins and phosphoproteins altered by *Dyrk1A* overexpression. Dark blue indicates the significant enriched ones with an adjusted (Benjamini) p-value < 0.05.

Biological Process (EE)

10 biological processes enriched were enriched but only two were significant: positive regulation of neurogenesis and regulation of axon extension. In the other processes, apart from finding again plasticity processes such as cytoskeleton and microtubule processes, cell differentiation or brain development there was regulation of transcription (DNA templated) (Figure 41).

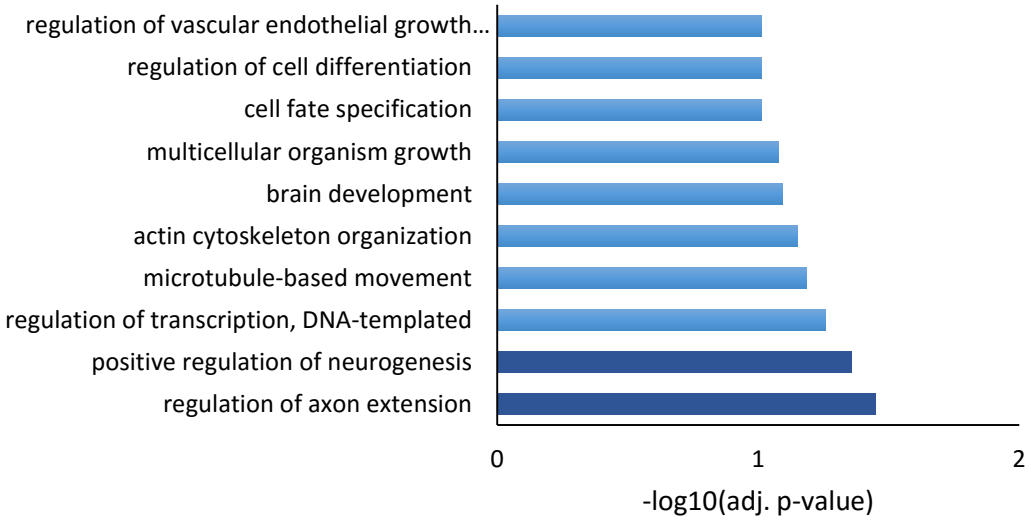


Figure 41: Enriched biological processes in proteins and phosphoproteins altered by EE and that not rescue alterations caused by *Dyrk1A* overexpression. Biological processes found to be enriched among the set of proteins and phosphoproteins significantly altered by EE in TgDyrk1A hippocampus but without the ones that rescued proteins and phosphoproteins altered by *Dyrk1A* overexpression. Dark blue indicates the significant enriched ones with an adjusted (Benjamini) p-value < 0.05.

Biological Process (EGCG+EE)

15 biological processes enriched were enriched. There were processes that also affect neuroplasticity such as cytoskeleton, differentiation or axon guidance but the difference from the other treatments is that there were processes related with transcription and transport and signal transduction. Among the nine the significant ones (dark blue) there were processes related microtubule cytoskeleton organization, cell differentiation and growth but also regulation of GTPase activity transport and regulation of transcription (Figure 42).

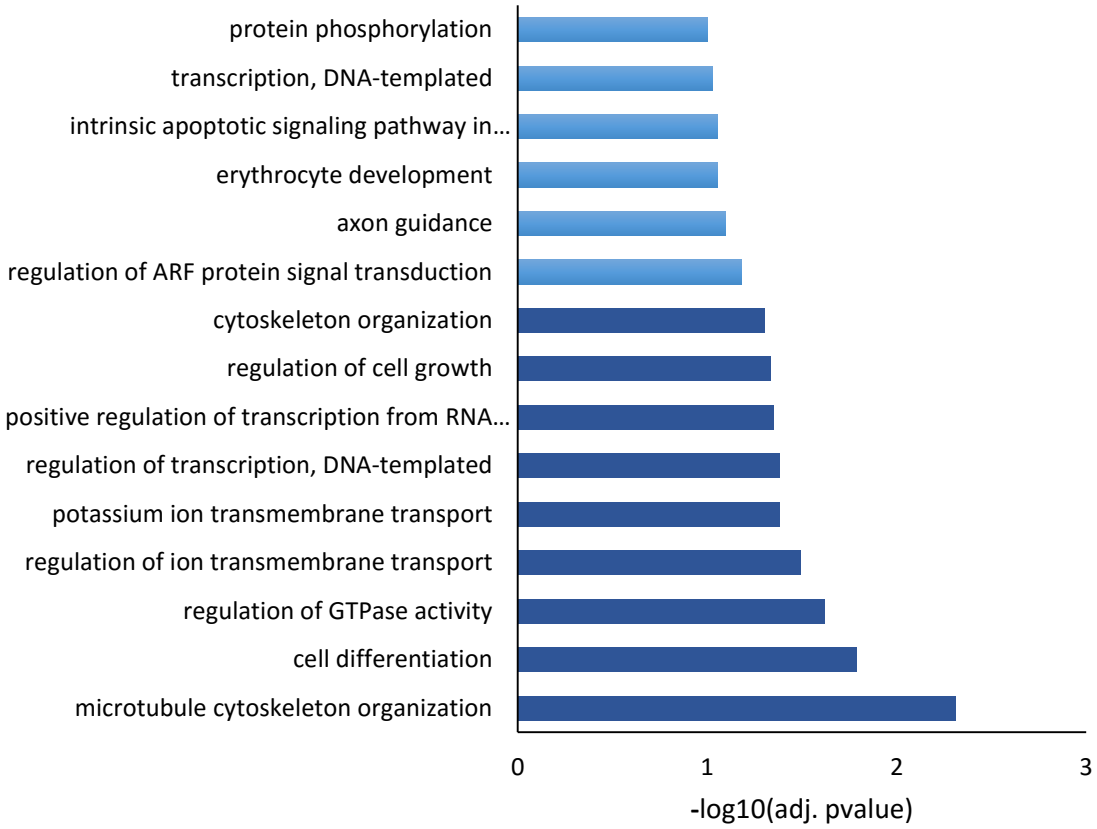


Figure 42: Enriched biological processes in proteins and phosphoproteins altered by EGCG+EE and that not rescue alterations caused by *Dyrk1A* overexpression. Biological processes found to be enriched among the set of proteins and phosphoproteins significantly altered by EGCG+EE in TgDyrk1A hippocampus but without the ones that rescued proteins and phosphoproteins altered by *Dyrk1A* overexpression. Dark blue indicates the significant enriched ones with an adjusted (Benjamini) p-value < 0.05.

4.5 Correlation of proteome and phosphoproteome changes with recognition memory in TgDyrk1A

While most of the results manifest the result of changes in protein/phosphopeptide abundance, we also wanted to correlate those changes with parameters measured during the novel object recognition (NOR) test.

Proteome and phosphoproteome correlation with behavioral variables measured in the novel object recognition test

PC1 values corresponding to the animals used in the proteomic experiments were correlated (Spearman's rho) with the expression and phosphorylation levels of each protein according to the thresholds specified in section 3.5.10 of materials and methods. 94 proteins were found to correlate or anticorrelate with PC1 values and 251 phosphoproteins were found to correlate or anticorrelate with PC1. However, only the top10 list of protein are represented in table 16. (Annex for a complete list).

Taula 16: Top10 correlating and anticorrelating proteins and phosphoproteins with PC1 values.

TOP10			
Correlating proteins	**Anticorrelating proteins**	**Correlating phosphoproteins**	**Anticorrelating phosphoproteins**
Rps13	Erlin2	Ccnjl	Ap3b1
Micu3	Ncstn	Bzw1	Gm996
Dmwd	Maob	Psmd2	Dmxl2
Trim32	UPF0687	Raly	Ttll12
RPL3	Ndufs4	Map1a	Hdgf
Osbpl6	Sh4gl3	Atp1a3	Plec
Fus	Ndufb4	If2bp1	Kcnma1
Rtn4	Atp6v1g2	Psd3	Hacd3
Glrx5	Slc6a11	Slc1a2	Slc4a3
Epb41l	Atp5o	Eif4b	Pclo

To get further insight into the biological processes of proteins and phosphoproteins correlating and anticorrelating with PC1 a GO enrichment analysis was performed. Results are displayed in a list and not a bar plot due to the high number of biological processes enriched (Table 17). To clear groups can be observed in the proteins (anti)correlating in the proteome in contrast with the ones with the phosphoproteomes. Biological processes enriched in the proteome correspond mainly to mitochondrial, energy and metabolic processes while in the phosphoproteome mainly processes related with synaptic plasticity are found such as synaptic vesicle transport and signaling but also other processes contributing to neurplasticitity such as differentiation, axon development or morphogenesis.

Taula 17: Biological processes enriched for (anti)correlating proteins and phosphoproteins. Significant enriched ones with an adjusted (Benjamini) p-value < 0.01.

Biological Processes	
(Anti)correlating proteome	**(Anti)correlating Phosphoproteome**
electron transport chain	cellular component organization
mitochondrion organization	cellular component organization or biogenesis
NADH dehydrogenase complex assembly mitochondrial respiratory chain complex I assembly	cellular process
	biological regulation

mitochondrial respiratory chain complex I biogenesis	localization
respiratory electron transport chain	regulation of biological quality
ATP synthesis coupled electron transport	regulation of cellular component organization
oxidative phosphorylation	neuron projection development
cellular respiration	establishment of localization
mitochondrial respiratory chain complex assembly	transport
mitochondrial ATP synthesis coupled electron transport	generation of neurons
organelle organization	neuron differentiation
cellular component organization	neurogenesis
energy derivation by oxidation of organic compounds	organelle organization
cellular macromolecular complex assembly	regulation of biological process
cellular component organization or biogenesis	cellular localization
ATP metabolic process	nervous system development
generation of precursor metabolites and energy	neuron development
purine ribonucleoside triphosphate metabolic process	axon development
purine ribonucleoside monophosphate metabolic process	regulation of transport
purine nucleoside monophosphate metabolic process	intracellular transport
ribonucleoside monophosphate metabolic process	establishment of localization in cell
ribonucleoside triphosphate metabolic process	regulation of localization
purine nucleoside triphosphate metabolic process	regulation of cellular process
nucleoside monophosphate metabolic process	cellular component assembly
nucleoside triphosphate metabolic process	cytoskeleton organization
transport	synaptic vesicle transport
localization	establishment of synaptic vesicle localization
establishment of localization	vesicle-mediated transport in synapse
	synaptic vesicle localization
	plasma membrane bounded cell projection organization
	cell part morphogenesis
	protein complex subunit organization
	macromolecular complex subunit organization
	cellular component biogenesis
	neuron projection morphogenesis
	cell projection organization
	plasma membrane bounded cell projection morphogenesis
	establishment of organelle localization
	cell projection morphogenesis
	axonogenesis
	negative regulation of cellular component organization
	organelle localization

	protein complex assembly protein complex biogenesis macromolecule localization establishment of vesicle localization macromolecular complex assembly protein localization chemical synaptic transmission anterograde trans-synaptic signaling cell morphogenesis involved in neuron differentiation system development trans-synaptic signaling vesicle localization synaptic signaling synaptic vesicle cycle cell development organic substance transport regulation of plasma membrane bounded cell projection organization synaptic vesicle recycling regulation of cell projection organization regulation of vesicle-mediated transport cell morphogenesis positive regulation of biological process protein transport nitrogen compound transport multicellular organism development positive regulation of cellular process establishment of protein localization cell morphogenesis involved in differentiation peptide transport cellular developmental process microtubule-based process regulation of cytoskeleton organization regulation of organelle organization cell differentiation amide transport regulation of neuron differentiation cellular component morphogenesis regulation of neuron projection development regulation of protein complex disassembly vesicle-mediated transport regulation of protein depolymerization developmental process regulation of cellular component size regulation of cellular component biogenesis anatomical structure development

Overlap among (anti)correlating and significant proteins and phosphosproteins

Correlating and anticorrelating proteins and phosphopeptides were compared to the lists of significant proteins and phosphopeptides using a Fisher Exact Test and the results were summarized by a heatmap, in which the color reflected the p-value of the test. The test results in the heatmap revealed that in the most of the cases, changes in the phosphoproteome correlate with the result of the NOR test except for EE treatment. EGCG-dependent changes in the proteome in TgDyrk1A were the ones that correlated more with the novel object recognition test (Figure 43).

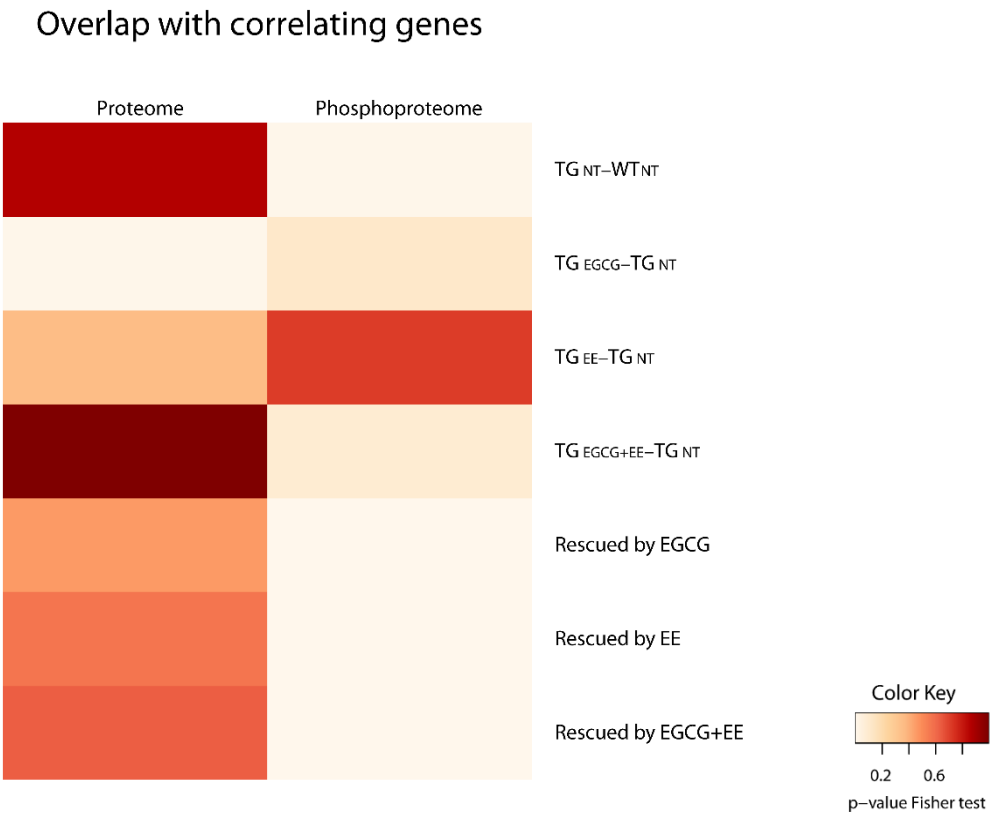


Figure 43: Overlap significant proteins and phosphoproteins with the correlating ones. Heatmap representing the significant overlap of proteins and phosphoproteins that correlates with novel object recognition test. The color-code correspond to -log10 of the p-value from the Fisher Test.

DISCUSSION

“When you make the finding yourself, even if you’re the last person on Earth to see the light, you’ll never forget it.”

Carl Sagan

5. DISCUSSION

DS is the first cause of intellectual disability and the model human phenotype for genomic gain dosage imbalances. To date, the scientific framework in DS has been largely reductionist in nature. In this Thesis we applied state-of-the-art proteomics technologies, to investigate the complexity of interactions of the molecular networks in a mouse model that overexpresses one of the main candidate genes for DS located in the DSCR: *DYRK1A.* The decision of using this model was driven by the fact that its overexpression is sufficient and necessary to produce the DS cognitive and neural phenotypes and that the only treatment reported to be effective to improve cognition in DS patients is a DYRK1A kinase inhibitor, EGCG, as previously demonstrated by our laboratory (De la Torre *et al*., 2016).

We report here a set of quantitative proteomics and phosphoproteomics analyses of the hippocampus of TgDyrk1A mice. Our results show that single overexpression of *Dyrk1A* causes important changes in the hippocampal proteomic and phosphoproteomic profiles, and that most of these changes are related to neuroplasticity and intellectual disabilities.
Molecular high-throughput proteomics analyses of the hippocampus of TgDyrk1A mice revealed that pro-cognitive treatments (EGCG, EE and EGCG+EE) restore 30% of the proteomic and phosphoproteomic alterations caused by *Dyrk1A* overexpression.

These results highlight the role of DYRK1A as a hub for neural plasticity molecular networks in the hippocampus, and indicate that EE and EGCG can partially rescue the deficits derived from its overexpression through shared mechanisms of action.

5.1 Dyrk1A overexpression produces deficits in recognition memory in TgDyrk1A mice rescued by pro-cognitive treatments

Our main goal in this Thesis was to profile the proteome and phsophoproteome in the hippocampus and to establish possible correlations between the molecular phenotype and the behavioral changes. The hippocampus was selected as our target region given its fundamental role in learning and memory and because it has been reported previously to strongly affected in trisomic mouse models and DS individuals.

As a first step towards this goal, we evaluated the hippocampal function using a novel object recognition (NOR) test. This test evaluates recognition memory that is highly dependent on the hippocampus (Clark *et al*., 2000; Broadbent *et al*., 2004; Broadbent *et al*., 2009). However, contrary to other hippocampal learning tasks, The NOR test is time-constrained, and does not require appetitive or

aversive stimuli, being thus ideal to assess the performance of mice and treatment efficiency.

We here compared the performance of TgDyrk1A mice and wild type mice in untreated conditions and in a separate group after one-month treatment with pro-cognitive therapies, to then perform our proteomic and phsophoproteomic analysis in single mice tested in the NOR.

5.1.1 Dyrk1A overexpression produces deficits in recognition memory in TgDyrk1A

We found no differences in the exploration time in TgDyrk1A during the training session in the NOR test. This is in line with previous studies in the laboratory (data not published) and humans (MacTurk *et al*., 1985) that showed no differences in the amount of exploration. However, in the test session, TgDyrk1A mice were not able to recognize a novel object indicating a significant failure in recognition memory. Importantly, similar cognitive impairments were also reported in Dyrk1A heterozygous mice (Dyrk1A +/-) (Arque *et al*., 2008), indicating that correct dose of *Dyrk1A* is necessary to efficiently perform this hippocampal dependent task. Also, and as mentioned before, altered performance on tasks of object discrimination have been demonstrated in DS trisomic mice (Fernandez and Garner, 2008) and humans (Nelson *et al*., 2005), confirming that the single overexpression of *Dyrk1A* is sufficient to produce the phenotype.

The fact that TgDyrk1A showed impaired novelty recognition confirms previous results and suggests that overexpression of *Dyrk1A* is sufficient to induce learning defects in hippocampal dependent tasks, and may contribute to the hippocampal-dependent learning and memory impairments observed in DS.

5.1.2 Pro-cognitive therapies rescued deficits in recognition memory in TgDyrk1A mice

Mice were under pro-cognitive treatments for one-month before the NOR test. We used EGCG, the major catechin in green tea leaves, that has been identified as one of the most specific inhibitors of DYRK1A kinase activity (Bain *et al*., 2003, Wang *et al*., 2012a). Several studies have demonstrated a positive impact of EGCG on brain and behavior (for a review see Stagni *et al*., 2017). Previous studies in our laboratory had demonstrated that one-month oral administration of EGCG normalizes DYRK1A kinase activity in the hippocampus of TgDyrk1A female mice without affecting it in wild types (Pons-Espinal *et al*., 2013). This provided us the ideal model to perform the proteomic studies that are the main aim of my work.

In our experiments, EGCG rescued recognition memory deficits in TgDyrk1A, as previously described in trisomic and transgenic mice and in humans (Pons-Espinal *et al.*, 2013 De la Torre *et al*, 2013). As shown in previous studies, this rescue was not due to differences in total exploration time.

Conversely, in our experiments, EGCG did not impair the performance of wild type mice, opposite as shown by previous results of Pons-Espinal *et al.* (2013) and De la Torre *et al.* (2013). It is possible that the use of a Y maze instead of an open field for the experiments may influence mice performance, since the open field is a more aversive and stressful environment than Y maze (Carter and Shieh, 2015).

Our results reinforce the idea that treatment with EGCG can reverse the cognitive impairment in conditions of single D*yrk1A* overdose and allowed us to establish a specific correlation of behavioural improvement and proteome changes.

Besides the pharmacological treatments, we also aimed at exploring the effects of an environmental therapy. This was motivated by the fact that DYRK1A is involved in activity dependent plasticity and that in humans, early intervention programs are the only effective non-pharmacological therapy to improve cognitive impairments in intellectual disability disorders, and specifically in DS individuals (Mahoney *et al*., 2006; Bonnier 2008). Moreover, EE also decreases hippocampal DYRK1A activity (Pons-Espinal *et al*, 2013).

In trisomic DS mouse models, a number of studies have reported that postnatal environmental enrichment (EE) was able to recover hippocampal-dependent learning and memory deficits (Martinez-Cue *et al*., 2002; Dierssen *et al*., 2003; Chakrabarti, *et al*., 2011; Kida *et al*., 2013), and also in TgDyrk1A, EE improves hippocampal learning and memory as measured in the Morris water maze (Pons-Espinal *et al.*, 2013). In wild-types, a myriad of reports provided evidence that this improvement occurs through the modulation of neuronal structural plasticity mechanisms involving dendritic structure, synaptic plasticity and adult neurogenesis (Kempermann *et al*., 1997; Rampon *et al*., 2000; Nithianantharajah and Hannan; 2006). Thus we would expect that the possible beneficial effects of this treatment would be the consequence of an underlying neural plasticity enhancement.

In our experiments, EE increased the time exploring objects in TgDyrk1A, but did not significantly improve object discrimination in TgDyrk1A nor in WT mice. Given that most studies in the literature have shown that rearing mice under EE improves recognition memory (Mesa-Gresa *et al*., 2013), our results may be interpreted as a failure of our EE conditions. However, it has to be noted that, while the EE effects on other learning paradigms are more reproducible, a

number of studies did not find any effect on the NOR (see Viola *et al*., 2010 as an example). The main reasons for these discrepancies are related to the mouse strains, but also the duration of the EE treatment. We had previously used the same strain with positive results of one month of EE in the Morris water maze test (Pons-Espinal *et al*., 2013), thus discarding a strain effect or treatment duration biases in our case. Another important factor is also the gender of the selected animals, because EE can lead to increased levels of stress when performed with males (Haemisch and Gartner, 1997). This was reported in Ts65Dn males, which showed increased levels of stress hormones upon EE, causing even a deleterious effect on hippocampal learning while positive effects were observed in hippocampal-dependent tasks in females (Martinez-Cue *et al*., 2005, Martinez-Cue *et al*., 2002). To avoid this problem, we reared together mice of the same experimental group before sexual maturity. In fact, we did not notice any abnormal, aggressive or dominant behavior before or during the enrichment experiments. However, although this possibility cannot be completely discarded, the significant increase of exploratory behavior may be an important contributor.

Finally, the combination of cognitive training and EGCG (EE and EGCG) has been used in previous clinical trials in humans, showing significant cognitive and brain remodeling effects in human individuals with DS (De la Torre *et al.*, 2017). Also in Ts65Dn mice, Catuara-Solarz *et al*., 2015 and Catuara-Solarz *et al*., 2016 showed a better performance in the Morris water maze when combining both EE and EGCG treatments. In our experiments, however, we only found a trend to rescue recognition memory impairments in TgDyrk1A when combining both therapies. Although we cannot discard a specific batch effect in our cohort, it may be argued that the NOR would be less sensitive to the EE and thus to the combined treatment or that the possible stressor effects of EE could explain the reduced rescuing effects of the combined treatment.

Both in the EE and EE+EGCG groups, the high intra-group variability revealed by the DI distribution may also be contributing to the lack of significance. This could be due to the intrinsic characteristic of the NOR, which does not require long periods of learning, external motivation, reward or punishment. In turn, this makes it a particularly attractive test (Antunes and Biala, 2012; Leger *et al*., 2013) for proteomic studies, since it reduces possible confounding effects.

5.2 Proteome alterations in TgDyrk1A hippocampus

To understand the molecular mechanisms of Dyrk1A-dependent phenotypes and the effects of our pro-cognitive treatments, we used multi-dimensional proteomic analyses to acquire multiple types of protein data including changes in protein abundances and post-translational modifications (PTMs), such as phosphorylation.

From all the mice studied with the NOR test, we selected those closer to the average DI value for the proteomics and phosphoproteomics characterization, and thus extreme phenotypes that would have probably result in more significant differences, but would have certainly been less reflective of the real changes in a general population.

Being a single-gene transgenic mouse, our initial thought was that TgDyrk1A would only overexpress the DYRK1A kinase, in the hippocampus (Pons-Espinal *et al*., 2013), and might not have important differences in protein abundances. Instead, when we compared the proteome of TgDyrk1A hippocampus with wild type, the MS analysis resulted in the quantification of 2,685 proteins. Among those, 98 proteins exhibited changes in abundance. It is of note that we did not detect DYRK1A, most likely due to sensitivity limitations, given that the kinase is not an abundant protein in the cell (also according to estimation by Protein Abundance Database (PaxDB; https://pax-db.org/).

Of the 98 proteins that changed in abundance, 37 proteins were only present in one genotype. It is well-known that among the DYRK1A targets there are a number of proteins directly or indirectly involved in transcription and translation. In fact, from the 43 DYRK1A targets described in a recent review (Duchon and Herault, 2016), ~40% (17) were transcription factors or proteins involved in transcription regulation. For example, Di Vona *et al*. (2015) suggested that DYRK1A could act as a RNA polymerase II carboxy terminal domain (CTD) kinase, and activate transcription when recruited to regulatory regions of the chromatin, preferentially to proximal promoter regions. To unravel the contribution of DYRK1A to transcription events, we performed a transcription factor prediction analysis using i) significant proteins more abundant in TgDyrk1A (upregulated) or only present in TgDyrk1A and ii) significant proteins less abundant in TgDyrk1A (downregulated) or absent in TgDyrk1A. Results revealed a total of 10 transcription factors (4 for upregulated and 6 for downregulated proteins) that could explain changes in abundance of most of the significant proteins.

However, only one of them had a described relationship with DYRK1A, FKHR or FOXO1 (Woods *et al*., 2001). FOXO1 is a transcription factor implicated in apoptosis and cell cycle regulation (Lu *et al*., 2011). FOXO1 activity is regulated through acetylation, phosphorylation and ubiquitination, but phosphorylation plays a key role (Wang *et al*., 2014). Nuclear export of FOXO1 can occur through direct phosphorylation by protein kinase B (AKT) and serum/glucocorticoid inducible kinase (SGK1) on three sites T24, S256 and S319. Also, it has been shown that FOXO1 loses its interaction with DNA when phosphorylated by AKT/SGK1 at S256 (Wang *et al*., 2014) and cyclin-dependent kinase 2 (CDK2) at S249 (Hedrick *et al*., 2012).

Of interest for our study, the phosphorylation of FOXO1 by DYRK1A in S329 can prevent its activity as a transcription factor recruiting the protein in the cytosol (Woods *et al*., 2001) (Figure 44). This would agree with the fact that FOXO1 was explaining downregulated proteins in our analysis.

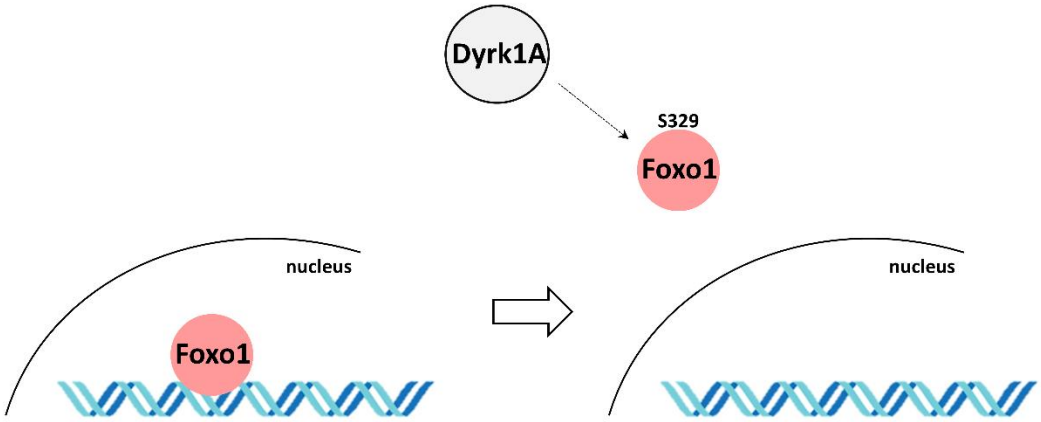


Figure 44: Proposed mechanism for DYRK1A and FOXO1. Phosphorylation of DYRK1A to S329 retain FOXO1 in the cytosol preventing its transcription factor activity (Woods *et al*., 2001).

We thus suggest that the phosphorylation of FOXO1 by DYRK1A overexpression could prevent 12 proteins to be transcribed in TgDyrk1A. However, as the over activation of AKT has also been demonstrated (Abekhoukh *et al*., 2013) in the mBACtgDyrk1A (another model of overexpression of Dyrk1A), we cannot firmly conclude that DYRK1A is the only protein affecting the inhibition of FOXO1 activity. Thus, further validation of the phosphorylation of S329 is to be done by Western blot.

We did not find any direct or indirect relationship with Dyrk1A of the other nine transcription factors predicted in our analysis, suggesting that other downstream mechanisms of DYRK1A could be involved including DYRK1A-regulated epigenetic mechanisms.

In fact, DYRK1A is able to regulate several proteins involved in epigenetic mechanisms. DYRK1A promotes both histone deacetylation by phosphorylating SIRT1 (Guo *et al*., 2010) and histone acetylation by phosphorylating CREB transcription factor (Yang *et al*., 2001), it also interferes with chromatin remodeling by binding nBAF and reducing levels of the NRSF/REST neuron-restrictive silencing factor (Lepagnol-Bestel *et al*., 2009) (for a complete review see De Toma *et al*., 2016), it phosphorylates in vitro H3 at Thr45 (Himpel *et al*., 2000), and it was recently reported that DYRK1A acts as a transcriptional

regulator by phosphorylating H3T45, resulting in increased H3Ac and antagonizing heterochromatin protein 1 binding (Jang *et al*., 2014).

From all those candidates, we found SIRT1 in our list of expanded proteins in the TgDyrk1A vs. WT comparison (Table 7). Activation of SIRT1 could cause deacetylation of chromatin, and have an inhibitory effect on transcription activity. Interestingly, we also found SIRT1 as a possible DYRK1A target altered in our list of expanded proteins in the TgDyrk1A vs. TgDyrk1A EGCG comparison.

5.3 Phosphoproteome alterations in TgDyrk1A hippocampus

Changes in the phosphoproteome due to overexpression of *Dyrk1A*, a kinase with more than 40 described direct substrates, were highly expected. Even more when kinase activity over other kinases can spread along the links of the complex intracellular network introducing a myriad of changes into the cell phosphoproteome. The MS analysis of the phosphoproteome resulted in the quantification of 1,248 phosphopeptides, with 196 significant phosphopeptides corresponding to 169 phosphoproteins. Noteworthy, no significant overlap was found between the proteins with significant changes in abundance and phosphoproteins with significant phosphopeptides in abundance indicating that *Dyrk1A* overexpression acts differentially on the proteome and phosphoproteome.

5.3.1 DYRK1A affects proline-directed kinases

The main activity of a kinase is to catalyze the transfer of γ-phosphate from ATP (or GTP) to its protein substrate known as a phosphorylation event. This phosphorylation regulates protein function by inducing conformational changes or by disruption and creation of protein-protein interaction surfaces but also by disrupting the surfaces for protein-ligand interaction without inducing any conformational changes (Cheng *et al*., 2011).

Thus, changes in the phosphoproteome due to overexpression of *Dyrk1A*, a kinase with more than 40 described direct substrates, were highly expected. Even more when kinase activity over other kinases can spread along the links of the complex intracellular network introducing a myriad of changes into the cell phosphoproteome.

A motif analysis was performed to identify the most overrepresented amino acid motif in phosphopeptides upregulated in TgDyrk1A. The results revealed that the amino acids overrepresented were serine (S) or threonine (T) followed by a proline (P) forming the SP or TP motif. A proline residue immediately preceding a serine or threonine is a highly conserved motif in proline-directed kinases, which include members of the CMGK kinase group including mitogen-activated protein

kinases (MAPKs), cyclin dependent kinases (CDK), glycogen synthase kinase (GSK) but also members of the DYRK family. Thus, our results suggested a possible role of DYRK1A in modifying the activity of proline-directed kinases. This is interesting given that several studies have suggested that sustained activation of proline-directed kinases may be a general phenomenon in neurodegenerative diseases as proline-directed kinases are implicated in the hyperphosphorylation of the Tau protein and in the regulation of neurofilament phosphorylation associated with Alzheimer's disease (Pelech, 1995) and neurodegenerative diseases (Holmgren *et al*., 2012; Rudrabhatla, 2014). In fact, *DYRK1A* has also been implicated in neurodegenerative diseases, not only in DS. but also in taupathies, β-amiloidosis or α-synucleinopathies (For a complete review see Wegiel *et al*., 2011)

5.3.2 Phosphoproteome analysis reveal two new possible targets of DYRK1A

Among the SP and ST motifs overrepresented, 11 phosphorylation sites with the overrepresented PXSP motif were found. This motif almost fitted with the first and most studied DYRK1A consensus motif (RPX(S/T)P) published in Himpel *et al*. (2001) and is the favorite motif for MAPKs and CDKs (Garai *et al*., 2010; Daub *et al*., 2008). However, we identified two phosphorylated peptides that matched exactly with the consensus motif of DYRK1A (RPX(S/T)P). The first belonged to SGIP1, a protein that mediates clathrin endocytosis and is required for the endocytosis of the transferrin receptor. It is well-known that DYRK1A phosphorylates multiple proteins engaged in regulated endocytosis in neurons: dynamin1, amphiphysin 1, endophilin and synaptojanin1 (for details see table 1). Moreover, a study demonstrated that overexpression of *Dyrk1A* causes defects in clathrin-mediated endocytosis in fibroblasts as well as in neurons of *Dyrk1a* overexpressing mice by reducing the co-localization between dynamin and clathrin at the plasma membrane producing delays in vesicle internalization and thus synaptic malfunctions (Kim *et al*., 2010).

Another study identified DYRK1A substrates associated with brain clathrin coated vesicles (CCV) suggesting that DYRK1A may regulate multiple steps of the CCV cycling processes (Murkami *et al*., 2012). Our finding, together with the increased levels in abundance of transferrin receptor that we found in TgDyrk1A hippocampus suggest that DYRK1A and SGIP1 could have a close relation in the regulation of endocytosis. In fact, transferrin receptor (TFR) is an important iron transporter regulating iron homeostasis and has long been used as a marker for clathrin mediated endocytosis, and recently it has been shown to function as a novel regulator to control AMPA trafficking efficiency and synaptic plasticity (Liu *et al*., 2016).

The second phosphopeptide we found with the exact consensus motif belonged to SHANK3, a postsynaptic density (PSD) protein with a role in spine morphogenesis and synaptic plasticity. It acts as scaffold protein to bind NLGN-NRXN and NMDAR at the PSD and has been involved in intellectual disabilities and autism. Given that a pool of endogenous DYRK1A has been localized in the synapses, and also in the postsynaptic zones (Alvarez *et al*., 2003; Martí *et al*., 2003; Arque *et al*., 2013), it is plausible that phosphorylation of SHANK3 could occur through DYRK1A kinase. Of course, these hypotheses have to be validated with classical biochemical methods such as *in vitro* kinase assays in which the purified kinase, DYRK1A, is incubated with the substrates, SGIP1 and SHANK3, in the presence of ATP. The phosphorylated substrates can then be assessed by various measurements including colorimetric, radioactive, chemiluminescence, and fluorometric detection (Johnson and Hunter, 2015)

5.4 Proteome and phosphoproteome network analysis

We here analyzed the combined alterations of proteome and phosphoproteome due to *Dyrk1A* overexpression in the hippocampus to better understand the perturbations in cell functionality. To this aim we took advantage of network analysis methods. Large-scale data, in our case proteins, can be represented as networks given that they interact with each other forming a complex network of intracellular connections. Contrary to the original belief that one protein had a single function, proteins have different functions and cellular roles depending on their immediate environment. Thus, the study of molecular networks helps us to predict abnormalities in biological processed and assume “guilt by association” of other proteins not identified or quantified in the experiments (Oliver, 2000).

In our study, 135 differentially abundant proteins and phosphoproteins had a protein-protein interaction (PPI) in a network representation. Given the power-law distribution of the nodes degree (average of 3.1), the topology of the network corresponded to scale-free. Scale-free networks are characterized for having a heavy-tailed degree distribution of the number of links connecting to a node which means a small number of highly connected proteins known as hubs and many poorly connected nodes (Barabasi and Albert, 1999).

In our dataset, the 5 most interconnected proteins (with more than 10 connections) corresponded to HSP90AB1, HRAS, SYNJ1, ABL2 and DLG2. Genome wide studies (He *et al*., 2006) have shown that deletion of a hub protein/gene is more likely to be lethal than deletion of a non-hub protein, a phenomenon known as the centrality rule (He and Zhang, 2006) simply because the hub has more interactions. This would suggest that these proteins may be important in the pathogenesis of DS. However, no relation with DS has been previously for HSP90AB1 or DLG2. Instead, HRAS, an important protein in signal transduction, is the main player in rasopathies (Rauen, 2013), suggesting that its

alteration can cause alterations in important cellular functions, some of which related to intellectual disability. Also, SYNJ1 is encoded by an HSA1 gene and overexpressed in DS brains but in AD. Interestingly, it has an important role in synaptic plasticity and neuronal migration and synaptogenesis (Arai *et al*., 2002; Martin *et al*., 2014).

On the other hand, ABL2 has been associated with acute lymphoblastic leukemia (ALL) (Moorman *et al*., 2016) interesting given that children with DS have an increased risk of ALL (Zwaan *et al*., 2008).

Noteworthy, 30 proteins described to be involved in intellectual disabilities overlap with the proteins in the network but do not coincide with the most interconnected nodes. This suggests that single overexpression of *Dyrk1A* is sufficient to cause massive alterations in proteins associated to intellectual disability pathogenesis.

5.4.1 *Dyrk1A* overexpression might affect MAPK signaling independent of HRAS and produces changes into phosphorylation of plasticity-related proteins

Of the 135 connected proteins in our network, some were clustered in the same cellular functions, as revealed by cluster analysis. Synaptic vesicle exocytosis, regulation of MAPK activity, Cell adhesion, vesicle-mediated transport, protein folding, and RNA processing, appeared as major clusters in our network. Those processes have extensively been described to be affected by DYRK1A overexpression (Table 1).

A GO enrichment analysis of both connected and non-connected nodes revealed 23 gene ontology terms corresponding to mainly two main biological processes: i) neuronal plasticity and ii) signal transduction. Further GO enrichment analysis with only significant proteins and only phosphoproteins revealed that GO terms referring to signal transduction especially to MAPK activity, regulation of ERK1/2 and phosphatidylinositol were shared across proteome and phosphoproteome. Interestingly, GO terms related to neural plasticity were unique to the phosphoproteome, suggesting that DYRK1A and its downstream effectors exert their effects on plasticity through the phosphorylation of targets involved in the regulation of cytoskeleton and neurofilaments, long-term synaptic potentiation and synapse assembly, among others. This is especially important in the hippocampus, given the neuronal dendritic abnormalities and synaptic plasticity alterations (Kaufmann and Moser, 2000; Benavides-Piccione *et al*., 2004; Martinez de Lagran *et al*., 2012) found in pyramidal neurons on DS individuals (Ferrer and Gullota, 1990) and the anatomo-functional alterations detected in previous studies in the Ts65Dn mouse (Dierssen, 2012). This thesis suggests that phosphoproteome alterations caused by *Dyrk1A* overexpression underlie

biological processes related to neuroplasticity leading to learning and memory deficits, revealing new possible candidates for intellectual disability.

Our results identified signaling cascades similar to those found by Ahmed *et al*. (2012) that found extensive abnormalities in players of MTOR, MAP kinase, AKT and NMDA pathways in Ts65Dn mice. Our analyses suggest that *Dyrk1A* overexpression could be the main responsible of the abnormalities in MAPK/ERK signaling. In addition, to GO enrichment analysis, we have validated by Western Blot a trend to significantly increase the activation levels of ERK1/2 in TgDyrk1A suggesting a possible alteration of this signaling cascade in accordance to previous work in *Dyrk1A* transgenic mice (Abekhoukh *et al*, 2013). However, even being ERK1/2 activity increased, and contrary to Abekhoukh, we could not detect changes in MEK1/2 activation, suggesting that mechanism not directly downstream of HRAS may be influencing the activation of ERK1/2. We found that 90% of the proteins involved in MAPK, ERK and phosphatidylinositol signaling had phosphosites changes. In the case of PTEN, the phosphosite we found has been described to inhibit its phosphatase activity and to be decreased in TgDyrk1A mice. However, in most cases, we lack functional information about the phosphosites making it difficult to predict which protein could be the responsible of ERK1/2. One candidate could be PKC which involvement in the signaling pathway leading to MAP kinase activation was previously suggested (Castagna *et al*., 1982; Blumber *et al*., 1988; Niedel *et al*., 1983). In fact, at least two studies demonstrate that PKCδ is sufficient for the activation of MEK1/2 and ERK1/2 (Ueda *et al*., 1996; Kuriyama M *et al*., 2004). In our results PKCδ was only present in TgDyrk1A and thus it could be speculated that, if activated, it could be responsible of the increase of ERK1/2 activation in an HRAS-independent manner. Also, we found DOCK10 absent in TgDyrk1A. DOCK10 is a guanine nucleotide-exchange factor (GEF) that activates CDC42 and RAC1 and is essential for dendritic spine morphogenesis in hippocampal neurons (Jaudon *et al*., 2015). This could lead to inhibition of RAC1 or CDC42 and thus to ERK activation via PI(3)K, AKT, RAF, and MEK, but not RAS (Rul *et al*., 2002). At this stage, we can only suggest that there is an activation of ERK in TgDyrk1A hippocampus that seems to be independent of HRAS upregulation and that two proteins, PKCδ and DOCK10, could have a role in its activation through mechanisms that are still not clear (Figure 45).

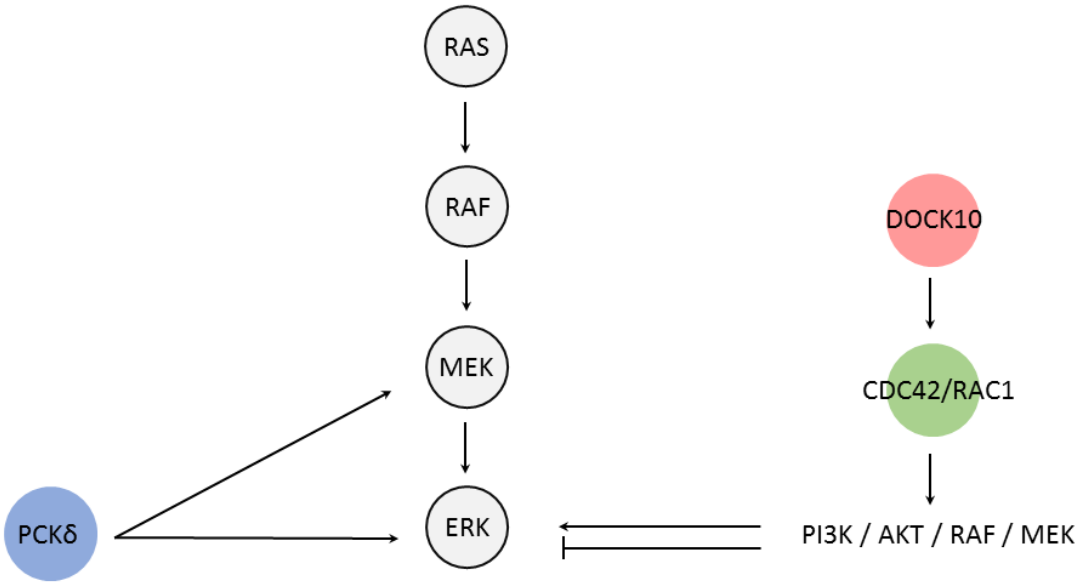


Figure 45: Possible mechanisms that can explain ERK1/2 activation.

5.5 Pro-cognitive treatments-dependent changes in proteome and phosphoproteome in TgDyrk1A hippocampus

A second objective of this thesis was to elucidate the treatment-driven molecular rewiring produced by pro-cognitive treatments in *Dyrk1A* overexpressing mice. Thus, we focused our interest in "rescued" proteins/phosphoproteins, e.g. those proteins or phosphopeptides up- or downregulated by *Dyrk1A* overexpression and whose altered abundance was reverted by the treatments.

5.5.1 Pro-cognitive treatments have an effect on Dyrk1A alterations possible sharing common mechanisms

One-month of treatment with EGCG, EE and EGCG+EE rescued not only proteins but also phosphopeptides. Surprisingly, a similar percentage of rescued proteins and phosphoproteins was found across the three different treatments. Concretely, an average of 30% of the protein/phosphopeptide alterations caused by *Dyrk1A* overexpression in TgDyrk1A were rescued and the overlap between the rescued proteins and phosphoproteins suggested some common mechanisms of action among treatments.

Proteins and phosphoproteins rescued by the treatments correspond both to connected and no-connected proteins in the genotype network suggesting a broad spectrum of targets, and not only to those highly connected. Noteworthy, all treatments rescued the phosphosites affected in SYNJ1 DYRK1A target described as intellectual disability protein. As reported above, SYNJ1 was a hub

in the genotype network with 13 interactors and its alteration with *Dyrk1A* overexpression, and recovery with the treatments could have a huge impact on the network deserving special attention for future studies. Interestingly, all the treatments also rescued some of the proteins and/or phosphoproteins involved in MAPK, ERK and phosphatidylinositol signaling cascades, including SYNJ1 but also DOCK10. The increased levels of DOCK10 upon the treatments could be related with the trend to decrease ERK activation that we detected.

It is also interesting that all the treatments rescued proteins and phosphoproteins involved in intellectual disabilities, four of which, ARID1A, ANK2, SYNJ1 and BSCL2, were common in the three treatments. This would suggest that the use of the treatments could be beneficial also for other disorders such as Fragile X, Alzheimer's disease or Parkinson disease (Andrade *et al*., 2012; Mandel *et al*., 2012).

Finally, among the multiple protein/phosphoprotein alterations driven by the treatments, 70% of the "*Dyrk1A* overexpression-related protein alteration" were not rescued. A GO enrichment analysis on these proteins showed EGCG was mainly related with plasticity processes while EE and EGCG+EE were related with regulation of neurogenesis and transcription processes. Interestingly, in our lab, Pons-Espinal *et al*., 2013 described rescuing of adult hippocampal neurogenesis by environmental enrichment in TgDyrk1A. In summary, our results suggest that the treatments do not only revert protein and phosphoprotein alterations caused by *Dyrk1A* overexpression but also boost plasticity in TgDyrk1A hippocampus.

CONCLUSIONS

6. CONCLUSIONS

1. We confirmed that Dyrk1A overexpression impairs hippocampal-dependent recognition memory in TgDyrk1A mice relevant for Down syndrome. Similar to the human situation we found a high phenotypic variability with some individuals having better recognition memory and responding better to the treatments.

2. Hippocampal-dependent recognition memory impairments in TgDyrk1A are reversed by treatment with epigallocatechin-3-gallate (EGCG) and slightly improved by the combination of environmental enrichment and epigallocatechin-3-gallate (EGCG+EE).

3. *Dyrk1A* overexpression causes independent changes in the hippocampal proteome and phosphoproteome suggesting the existence of both DYRK1A kinase dependent and DYRK1A transcription-related mechanisms.

4. The proteome and phosphoproteome alterations involve signaling pathways, especially MAPK. However, only the phosphoproteome changes involved neuroplasticity, suggesting DYRK1A kinase activity as a main player in plasticity processes.

5. Transcription factor analysis of up and downregulated proteins in TgDyrk1A revealed FOXO1 as a DYRK1A-related mechanism to explain transcriptional inhibition of 12 proteins. Other transcription-related mechanisms could involve DYRK1A-related epigenetic mechanism, possibly through SIRT1.

6. Motif analysis of upregulated phosphopeptides in TgDyrk1A analysis revealed overrepresentation of proline-directed kinases and two new DYRK1A targets, SGIP1 and SHANK3 involved in clathrin-mediated endocytosis with a role in spine morphogenesis and synaptic plasticity.

7. Dyrk1A overexpression produces changes in MAPK signaling, and other proteins affecting neuroplasticity, such as ERK1/2. PKCδ and DOCK10, and possibly SYNJ1, could be two potential candidates to explain its activation.

8. Network analyses revealed 5 proteins highly interconnected (with more than 10 interactions): HSP90AB1, HRAS, SYNJ1, DLG2 and ABL2, considered hubs in TgDyrk1A hippocampal network.

9. Pro-cognitive treatments (EGCG, EE and EGCG+EE) rescued genotype-dependent proteome and phosphoproteome alterations including plasticity-related and signaling proteins.

10. Correlation of proteome and phosphoproteome changes with recognition memory in TgDyrk1A mice revealed that only the changes in the phosphoproteome correlate with recognition memory, except for the EE treatmen

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7. BIBLIOGRAPHY

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ANNEX

ANNEX I: ABREVIATIONS

ACN	Acetonitrile
AD	Alzheimer's disease
AGC	Auto gain control
APP	Amyloid precursor protein
ASD	Autism spectrum disorder
BCA	Bicinchoninic acid
cm	Centimeter
CID	Collision-induced dissociation
CNS	Central nervous system
DDA	Data dependent aquisition
DI	Discrimination index
DS	Down syndrome
DTT	Dithiothreitol
DYRK1A	Dual specificity Yak1-Related Kinase
EDTA	Ethylenediaminetetraacetic acid
EE	Environmental enrichment
EGCG	Epigallocatechin-3-gallate
ESI	Electrospray ionization
FA	Formic acid
FDR	False discovery rate
FT	Fourier transform
GABA	Gamma aminobutyric acid
h	Hour
HCD	Higher energy c-trap dissociation
HCl	Hidrogen chloride
HSA21	Homo sapiens autosome 21
IAM	2-iodoacetamide
ID	Intellectual disability
iTRAQ	Isobaric tags for relative and absolute quantitation
LC-MS/MS	Liquid chromatography tandem-mass spectrometry
LTD	Long-term depression
LTP	Long-term potentiation
M	Molar
min	Minute
mg	Milligram
ml	Milliliter
mM	Millimolar
mm	Millimeter
MMU10	Mus musculus chromosome 10
MMU16	Mus musculus chromosome 16
MS	Mass-spectrometry
ms	Milliseconds
MWM	Morris water maze
n	Number
NE	Non enriched
NOR	Novel object recognition

P	Proline
PBS	Phosphate buffered saline
PBS-T	Saline phosphate buffer with triton X-100
PCA	Principal aomponent analysis
PCR	Polymerase chain reaction
PD	Parkinson’s disease
PTM	Post translational modification
Kg	Kilograme
R	Arginine
RT	Room temperature
s	Second
S	Serine
SILAC	Stable amino acid labeling in cell culture
SEM	Standard error of the mean
T	Threonine
TF	Time familiar
TFA	Trifluoroacetic acid
TG	Transgenic mice overexpressing Dyrk1A
TgDyrk1A	Transgenic mice overexpressing Dyrk1A
TN	Time novel
Ts65Dn	Trisomic mouse model for Down syndrome
WT	Wild type
µl	Microliter
µm	Micrometer
µM	Micromolar

ANNEX II: PUBLICATIONS AND CONGRESSES

Original papers:

4. Cristina Chiva; **Mireia Ortega**; Eduard Sabidó (2014) **Influence of the digestion technique, protease and miscleavaged peptides in protein) quantitation**. J Proteome Res 13(9):3979 - 3986. 05/09/2014

5. **Mireia Ortega**; Eduard Sabidó; Mara Dierssen; Cedric Boeck. **What Down syndrome can tell us about self-domestication?** (*in preparation*)

6. **Mireia Ortega**; Ilario de Toma; Eduard Sabidó; Mara Dierssen **"Mass spectrometric analysis of Dyrk1A overexpressing mice and pro-cognitive treatments reveals proteome alterations in hippocampus"** (*in preparation*)

Scientific international meetings

- **Mireia Ortega**, Eduard Sabidó, Mara Dierssen. Impact of gene overdosage in the hippocampus and cerebellum proteome and phosphoproteome. Proteomic Forum 2017, Potsdam – April 2-5, 2017 (oral communication).
- **Mireia Ortega**. Dyrk1A conference. Dyrk1A, related kinases & human disease, Saint-Malo – March 28-April 1st, 2017 (assistant).
- **Mireia Ortega**, Ilario de Toma, Silvina Catuara, Eduard Sabidó, Mara Dierssen Impact of gene overdosage on signaling networks in down syndrome mouse models, a proteomic approach. International Conference on Systems Biology (ICSB), Barcelona – September 16-20, 2016 (poster presentation).
- **Mireia Ortega**, Eduard Sabidó, Mara Dierssen. Effect of DYRK1A overexpression on signaling networks: a system biology approach. 10th Forum of Neuroscience (FENS), Copenhagen – July 2-6, 2016 (poster presentation).

Scientific national meetings

- **Mireia Ortega**, Ilario de Toma, Eduard Sabidó, Mara Dierssen. Impact of gene overdosage in the hippocampus and cerebellum proteome and phosphoproteome. VI Congreso Nacional de la Sociedad Española de Proteomica (SEProt), Cádiz – November 15-18, 2016 (oral communication).
- **Mireia Ortega**, Eduard Sabidó, Mara Dierssen. Effect of DYRK1A overexpression on signaling networks: a system biology approach. XVI Congreso Nacional Sociedad Española de Neurociencia (SENC), Granada – September 23-25, 2015 (poster presentation).
- **Mireia Ortega**, Jordi García Ojalvo, Eduard Sabidó, Mara Dierssen. Effect of DYRK1A overexpression on signaling networks: a system biology approach. Barcelona Computational, cognitive and Systems Neuroscience (BARCSYN), Barcelona – 18 and 19 of June, 2015 (poster presentation).

ANNEX III: PUBLICATIONS

Chiva C, Ortega M, Sabidó E. Influence of the digestion technique, protease, and missed cleavage peptides in protein quantitation. J Proteome Res. 2014 Sep 5;13(9):3979–86. DOI: 10.1021/pr500294d