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PhD THESIS

"Neuronal cell intrinsic defects in a mouse model of Down syndrome: the contribution of TTC3 gene"

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"Omnia tempus habent, et suis spatiis transeunt universa sub caelo [...]

Tempus plantandi et tempus evellendi quod plantatum est [...]"

(Ecclesiaste, Cap III, I – XV)

"Do I contradict myself? Very well then I contradict myself.

I am large, I contain multitudes"

(Walt Whitman, "Leaves of Grass", Song of Myself)

"There is GRANDEUR in this view of life"

(Charles Darwin, "On the origin of Species")

"Things change. They always do, it's one of the things of nature.

Most people are afraid of change,

but if you look at it as something you can always count on, then it can be a comfort."

("The Bridges of Madison County")

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(1) Abstract

Down Syndrome (DS) is the most common genetic disorder associated with intellectual disability (ID). DS is characterized by several brain alterations, including a simplified dendritic structure and a dendritic spine maturation impairment.

In this work we explored the contribution of cell intrinsic alterations to the abnormal cortical phenotypes of DS brains. To tackle this issue, we took advantage of a well established model of the disease, the Ts65Dn mouse ("Ts"). We cultured cortical neurons obtained from newborn pups and evaluated their ability to differentiate in *ex vivo* conditions.

Our data indicate that neuronal polarity and dendritogenesis are unaffected in *in vitro* cultures, while dendritic spines are both reduced in number and morphologically immature, evidencing an intrinsic cell defect.

We then moved to the identification of the possible molecular mechanisms underlying this defect and we focused our attention on TTC3, a negative regulator of neuritogenesis (Berto et al., 2007; Berto, et al., 2014), located on chromosome 21 and overexpressed in DS and Ts brains. Indications obtained by modulating TTC3 levels in Ts primary cortical neurons *in vitro* suggested a role for this gene in Ts abnormal dendritic spine development.

Together, these data indicate that defective Ts brain functionality can be attributed not only to abnormal circuitry or to unbalanced cellular composition, but also to specific defects within neurons, caused by the abnormal expression of chromosome 21 genes, such as TTC3.

(2) State of the art

2.1 Introduction

Down syndrome (DS) is a genetic disorder caused by the triplication of chromosome 21. Individuals with Down syndrome are characterized by a broad variety of symptoms affecting different organs and tissues; while most of these features significantly vary within Down syndrome population, intellectual disability (ID) is present in almost the hundred percent of patients, making DS the most frequent cause of ID. According to the *American Association of Intellectual and Developmental Disability,* ID is a condition characterized by significant limitations both in intellectual functioning (such as reasoning, learning and problem solving) and in adaptive behavior, which covers a range of everyday social and practical skills. ID can be caused by illnesses (e.g. meningitis, whooping cough or measles), injuries (e.g. head injury, extreme malnutrition, and toxic substances exposure), problems during childbirth (e.g. oxygen deprivation), during pregnancy (e.g. alcohol or drug use, malnutrition, certain infections, or preeclampsia) or by genetic conditions, such as in the case of Down syndrome.

2.2 Down Syndrome: an overview of the historical background

First empirical evidences of DS can be traced back to 5000 B.C: a Neolithic clay idol found in West Thessalony, in Greece, shows some peculiar features that can be attributable to DS (Diamandopoulos et al., 1997). Noticeably, a Mexican *Tolteca* culture small terra-cotta head of an infant, dated 500 A.C., presents striking features of DS: *" short palpebral fissures, oblique eyes, midface hypoplasia and open mouth with macroglossia*". Even though DS is clearly a pathology affecting humankind since time immemorial, no medical description of it has been performed before the revolutionary works of Dr. John Langdon Down. In 1862, Dr. Down first described in medical literature a distinct type of intellectual disability in his paper entitled "*Observations on an Ethnic Classification of Idiots*". In fact, in that period individuals with

cognitive impairment were often referred to as *idiots* and *imbeciles* and rarely differential diagnoses were performed (Starbuck 2011).

In his pioneering work, Dr. Down carefully described most of the phenotypical features that are representative of the pathology:

"The hair is [...] straight and scanty. The face is flat and broad, and destitute of prominence. The cheeks are roundish, and extended laterally. The eyes are obliquely placed, and the internal canthi more than normally distant from one another. The palpebral fissure is very narrow. The forehead is wrinkled transversely from the constant assistance which the levatores palpebrarum derive from the occipito-frontalis muscle in the opening of the eyes. The lips are large and thick with transverse fissures. The tongue is long, thick, and is much roughened. The nose is small. The skin has a slight dirty yellowish tinge, and is deficient in elasticity, giving the appearance of being too large for the body".

The genetic cause of DS was finally described by a French pediatrician and geneticist, Dr. Jérôme Lejeune in 1959. Working in Dr. Turpin's lab, Dr. Jérôme Lejeune together with Marthe Gauthier observed that DS fibroblasts carried one supernumerary chromosome 21 (HSA21), demonstrating for the first time that DS is a genetic disorder (Lejuene, Gauthier, & Turpin, 1959).

2.3 The triplication of Chromosome 21

According to World Health Organization (WHO), DS has an incidence of 1/1000 live births. The frequency dramatically increases with maternal age. For example, the likelihood that a woman under 30 years of age who becomes pregnant will have a baby with Down syndrome is less than 1/1000, but the chance of having a baby with Down syndrome increases to 1/400 for women who become pregnant at age of 35 years (Hook & Lindsjo, 1978) .

The American National Institute of Health (NIH) indicates that in most cases (approximately in 92% of the times), DS is caused by a meiotic *non-disjunction* event, in which a gamete (most of the times, the egg) carries an extra HSA21. After fertilization, the resulting zygote maintains the supernumerary chromosome. In another 2-4% of cases, referred to as mosaic trisomy 21, the extra HSA21 is present only in some cells (mosaicism) and the severity of the disorder is correlated with the amount of cells carrying the triplication. Moreover, there is also a small fraction of individuals that possess a normal number of chromosomes (46), but carry a supernumerary HSA21 fused to another chromosome, due to a Roberstonian translocation.

Finally, some patients carry the triplication of only a fragment of HSA21, rather than of the entire chromosome: this scenario is referred as to partial trisomy. One of the main goals of geneticists was to identify the correlation between the phenotypes of partially trisomic patients and the genes triplicated in every single case. These works led to the identification of a small region of long arm of HSA21, the "Down Syndrome Critical Region" (DSCR) (Delabar et al., 1993), that alone is sufficient to recapitulate the most prominent features of the disease. Afterwards, as extensively reviewed in Lyle et al., other regions different form the canonical DSCR have been identified in partially trisomic individuals, rising the idea that different regions of HSA21 are susceptible to rearrangements. For example, a subset of 4 patients analyzed in the mentioned paper shows DS clinical manifestation, but does not carry the triplication of the canonical DSCR. In the same work, in a cohort of 30 patients, 19 regions have been identified as triplicated: some of these were overlapping the DSCR, while others mapped to other regions within Chr21, rising the hypothesis of the presence of more than one single Critical Region.

Trisomy of HSA21 is not the only syndrome caused by triplication of a chromosome (for example triplication of HSA18 causes Edwards syndrome), but the trisomy of other chromosomes are mostly observed in aborted embryos and fetuses, indicating that the gene

dosage imbalance caused by trisomy is mostly lethal. One of the possible explanations for the viability of DS individuals, as well as the overall not so severe symptomatology that characterize them, is that HSA21 is the smallest and the poorest chromosome in terms of functional DNA elements per megabase (Antonarakis, 2017). On HSA21 we can in fact identify: 240 protein coding genes, 144 pseudogenes, 144 long non coding RNAs, 25 miRNAs, 55 antisense and 72 non annotated DNA elements (Antonarakis, 2017).

However, differently from what we could expect, data obtained from RNA-seq indicated that alteration in gene expression occurs not only on genes located on HSA21, but also on genes located on all the others chromosomes, implying that miRNA, lnRNA and transcription factors encoded by HSA21 genes act in trans to modulate overall gene expression (Olmos-Serrano et al., 2016), leading to the alteration of a large cohort of biological processes. For example, ApoE expression is consistently increased in samples obtained from DS individuals, although the gene is located on HSA19 (Lockstone et al., 2007).

2.4 Neurodevelopmental and neuroanatomical alterations of DS brain

Various anomalies of the respiratory, cardiovascular, sensory (organs), gastrointestinal, hematological, immune, endocrine, musculoskeletal, renal and genitourinary systems are congenital and progressive characteristics of DS (Arumugam et al., 2016). In addition, cognitive impairment, psychiatric disorders and an increased risk of Alzheimer's disease are also key features of DS. There is broad variability in both the occurrence (*penetrance*) and severity (*expressivity*) of these phenotypes across DS population. However, all individuals with DS share some degree of intellectual disability (ID) with mean intelligence quotients (IQs) around 50 (Chapman & Hesketh, 2000) (68% of population IQ is located between 85 and 114 (www.disabled-world.com).

Consistently with the high degree of ID, the most remarkable alterations in DS individuals occur at *neuroanatomical* and *neurological* levels.

Brains of adult DS individuals are consistently smaller (>20% reduction) as compared to healthy subjects, also upon correction for the reduced body size, which is also characteristic of the syndrome (Kemper, 1991). Magnetic Resonance Imaging (MRI) data revealed that brain volume reduction appears early during pregnancy, already in 4/5 months old fetuses (Guihard-Costa et al., 2006) (Winter et al., 2000). However, not all brain regions are similarly affected. Decreased size of the frontal lobe, brainstem and cerebellum have been reported in autoptic brain samples of DS children (Blackwood & Corsellis, 1972). MRI studies also indicated a decrease of the hippocampus and temporal lobes volume in children and young individuals with DS (Jernigan et al., 1993) (Kates et al., 2002). Neuroradiological and neuropathological findings in brains of adult DS highlighted a reduced volume of several areas, including hippocampus, entorhinal, frontal, prefrontal and temporal cortices, amygdala, cerebellum, brain stem nuclei and mammillary bodies of the hypothalamus (Aylward et al., 1999) (Kesslak et al., 1994) (Pine et al., 1997). One of the possible explanations of reduced brain volume already at a gestational level is decreased generation of neurons, which could be followed by atrophy and degeneration after birth and throughout life. Accordingly, the number of neurons is reduced in the hippocampus, parahippocampal gyrus and neocortex of DS fetuses (Guidi et al., 2008) (Larsen et al., 2008) and in the cortex of DS children (K. E. Wisniewski, 1990), that overall ends up in a decreased number of neuronal cells.

A "*diminished complement of neurons*" (Davidoff, 1928) has been reported in different brain districts (cortical layer III, precentral cortex and granule cells in temporal, parietal, and occipital lobes (Becker et al, 1991)). Interestingly, not only the number of neurons is altered, but also their distribution is affected: by 40 weeks' of gestation, neuronal layers in the visual cortex in DS fetuses are poorly defined respect to healthy matched controls. The same

phenomenon is still present at 4 months of age (Becker et al., 1991), indicating a developmental defect that is maintained after birth. It has been proposed that the reduction of neuronal number may be caused by the alteration of the timing of the mitotic cell cycle and the rate of cell proliferation during development (Mittwoch, 1972). Accordingly, it has been demonstrated that in DS the extra copy of HSA21 affects cell cycle progression of neuronal precursors during brain prenatal development. Neurogenic cell proliferation is impaired in DS fetuses at 17–21 weeks of gestation, as demonstrated by the reduced number of dividing cells in the dentate gyrus of hippocampus (DG; -65%) and ventricular germinal zone (VGZ; -32%) (Contestabile et al., 2007). Data about the genes expressed during cell cycle indicated that the G2 phase is prolonged in DS, possibly accounting for the reduced proliferation rate found during neurogenesis (Contestabile et al., 2007) (Chakrabarti et al., 2011). Subsequent studies further demonstrated that the number of differentiated neurons is also reduced in DS developing brain (Guidi et al., 2008). Analyses performed on neuronal precursors isolated from DS fetal brains and cultured as neurospheres, indicated that a reduced number of neurons can be obtained upon differentiation towards a more mature cell type (Bahn et al., 2002) (Esposito et al., 2008). Neurological alterations in DS are not only restricted to neurogenesis, but affect also differentiation and maturation. Indeed, defects in dendrites (the main receptive structure of the neurons) and dendritic spines (the main post synaptic outposts) are clearly observable. Dendritic branching is usually analyzed by Sholl analysis (Sholl, 1953), that consists in drawing concentric circles with increasing radius around the cell body. The analysis is performed by counting the number of intersections between dendrites and the drawn circles. The more intersections, the more branched the neuron is. Analyses performed in infants with DS (<6 months of age) indicated a higher number of intersections particularly evident in layer III cells. Later on, already after 6 months of age, the situation is reversed, showing a reduced dendritic arborization in DS individuals respect to

healthy age matched controls (Becker et al., 1991). These results indicate that in Down syndrome there is a dramatic cessation of the neuronal growth soon after birth, with dendritic shortening and atrophy.

Dendritic spines alterations have been described in several studies at different developmental ages: 8, 9, 19 months of age (Marin-Padilla, 1976) (Purpura, 1975) and in individuals from 3yrs to 23yrs (Suetsugu & Mehraein, 1980). In particular, the latter work, demonstrated a reduction in the number of spines on apical dendrites. Additionally, it has been described that not only dendritic spines are reduced in number, but they also show a different morphology: in visual cortex (specifically in layer III and V) some spines appeared shorter, while other were longer and thinner than controls. Moreover, no differences were detected until 40 weeks of gestation, but by 4 months of age, the number of spines was significantly decreased (Becker et al., 1991). Similar results were obtained in pre-term infants versus 4 months neonates: no differences in number or morphology were detected before delivery, while pyramidal neurons of 4 months old individuals with DS (especially in the upper part of the cortex) showed a decreased number of spines and a subgroup of them showed an unusual length as well (Takashima, Becker, Armstrong, & Chan, 1981). Similar results have been obtained with induced pluripotent stem cells (iPS): iPS obtained from patients' fibroblasts were differentiated into dorsal forebrain neurons. The iPS derived neurons showed significant synaptic deficits, such as reduced synaptic activity (Park et al., 2008). Another interesting work published in 2014 described iPS derived from fetal fibroblasts of monozygotic twins discordant for trisomy 21: trisomic iPS showed dramatic differences in proliferation, neurogenesis and neuronal differentiation (Hibaoui et al., 2014).

However, it is still unknown whether these defects (alteration of dendrites and spines) are primary (genetic) or secondary (reduced response to extrinsic cues, cortical micro environment, altered cellular composition) (Takashima et al., 1981).

In addition to neurodevelopmental alterations, early dementia occurrence and premature onset of Alzheimer's disease (AD) are common features of DS individuals. In fact, by the age of 30, virtually all individuals are affected by AD (Wisniewski et al, 1994). By the age of 12, DS patients start to accumulate Aβ lesions in diffuse plaques (Lemere et al., 1996): this time window is called "pre-amyloid". The neuropathological manifestations of AD in DS have been attributed, at least in part, to triplication and overexpression of the gene for amyloid precursor protein (APP) located on chromosome 21 (Rumble et al., 1989).

In conclusion, most of neurological abnormalities that characterize DS brain depend on developmental defects that are maintained after birth and are further exacerbated by the onset of neurodegeneration induced, for example, by Alzheimer's disease.

2.5 Mimicking the disease: the generation of DS mouse models

As with the study of most human diseases, mouse has played a prominent role among model systems. DS, however, is more complicated to model adequately in mouse because it is a contiguous gene syndrome that spans the entire *35 Mb of the long arm of HSA21. The long arm of HSA21 contains approximately 552 genes (Sturgeon & Gardiner, 2011). The orthologs of these genes are situated in mouse on the *28 Mb of the telomeric region of Mus Musculus (Mmu) chromosome 16, on an internal *1.5 Mb segment of Mmu17 and on an internal *3 Mb segment of Mmu10 (Davisson et al., 2001).

Many different experimental designs have been used to tackle the issue of the triplication of hundreds of genes (Vacano et al, 2012); however we can classify the approaches mainly in two categories: *chromosomal trisomic mice* and *transgenic mice*. The former includes most of the mouse models available in the laboratories today, such as Ts16 (trisomic for essentially all of Mmu16: generally dies during fetal development or very shortly after birth), Ts1Cje (trisomic for a short region of Mmu16, but disomic for SOD1 and APP), Ts1Rhr (trisomic for

the mouse equivalent of the hypothetical human DSCR), Ts1Yah (trisomic for the HSA21 syntenic region on Mmu17 between Abcg1 and U2af1), Dp(10)1Yey/+, Dp(16)1Yey/+ and Dp(17)1Yey/+ (trisomic for the Mmu10, Mmu16 and Mmu17 region syntenic to HSA21 respectively), Ts65Dn (extra-translocation of a chromosome composed of the distal fragment of Mmu16 attached to the centromeric region of Mmmu17) and Ts2Cje (similar to Ts65Dn, but the extrachromosome is attached to Mmu12 by Robertsonian translocation). Regarding the transgenic mice category, many genes located on HSA21 have been engineered to be overexpressed in mouse model, with the aim of phenocopying particular features of the disease and to identify the involvement of genes mainly responsible for specific symptoms. For example, mice have been developed overexpressing APP, SOD1, DSCR1, DYRK1A etc.

Mice knocked out for single Hsa21 genes were also made available and were further crossed with DS mice in order to restore the normal number of alleles as in (Chakrabarti et al., 2010), in which they crossed Ts65Dn mouse with $Olig1^{+/-}$/ $Olig2^{+/-}$ mice, thus restoring 2 copies of the gene.

Among all available models, the best characterized and studied is Ts65Dn (details, breeding and Jackson laboratories identifiers in **Supplementary Fig.1**). The mouse model was developed in 1990 by Muriel Davisson (Davisson, Schmidt, & Akeson, 1990) by irradiation of the testes of male mice, followed by a screening of the offspring for chromosomal rearrangements involving Mmu16.

Ts65Dn mice have many features that recapitulate those described in people with DS: these include anatomical features such as small size of some brain regions (hippocampus and cerebellum) and abnormal skull shape (Hill et al., 2009). Other similarities are: congenital heart defects (Williams et al., 2008), myeloproliferative disorders (Kirsammer et al., 2008), decreased bone density (Blazek et al., 2011) and altered incidence to certain cancers

(Wegelius, 1992). Moreover, Ts65Dn mice show deficits in learning, memory and behavior and early signs of neurodegeneration (Holtzman et al., 1996) (Reeves et al., 1995).

Additionally, Ts65Dn exhibit specific defects at the level of cortical pyramidal cells. Indeed, pyramidal neurons of layer III are smaller, less branched (with a reduced total dendritic length) and show a reduced number of spines, if compared to controls (Dierssen et al., 2003). No alterations of dendritic width have been revealed (Belichenko et al., 2004). Presynaptic terminals show increased area in Ts65Dn neurons both in hippocampus and in neocortex, as detected by immunoreactivity to synaptophysin staining (Belichenko et al., 2004). Electron microscopy data further corroborated those findings and revealed that not only presynaptic boutons are larger, but also the synaptic cleft is enlarged and post synaptic spines are bigger (Belichenko et al., 2004). Anomalies in the shape of spines have been observed as well, as they exhibit short necks and increased head size (Belichenko et al., 2004) .

Overall, given the molecular, behavioral and anatomical similarities between Ts65Dn and human Down syndrome, it is conceivable to use this mouse model to clarify the pathological mechanisms of the human disease.

2.6 A Down Syndrome Critical Region gene: TTC3

In order to explain how the triplication of HSA21 may lead to Down syndrome, two hypothesis have been proposed and discussed in the last (almost) twenty years: the *"dosage imbalance hypothesis"* (Antonarakis et al., 2001) and the *"amplified developmental instability hypothesis"* (Moldrich, 2007). The former postulates that the DS phenotypes are a direct result of the increased levels of genes located on HSA21, while the latter hypothesizes that the increased expression of HSA21 genes has a broad impact on non HSA21 genes as well, perturbing the expression of whole genome. Both hypothesis are actually valid: in fact general perturbation of the genome has been demonstrated (Olmos-Serrano et al., 2016) and at the

same time, mouse models with specific deletion of HSA21 genes, crossed with DS mice, ameliorated some phenotypes (e.g. APP, DSCR1 and OLIG1/2), thus highlighting the contribution of single genes to the disease. Gene Ontology (GO) analysis revealed that HSA21 genes function in 636 different Biological Processes, possess 304 different Molecular Functions and are associated with 163 Cellular Components (some terms overlap). Of particular interest is the significant enrichment in genes implicated in cytoskeletal regulation and remodeling (e.g. "cytoskeleton" is enriched with a p-value of $4{,}64 \times 10^{-16}$), suggesting a strong involvement of the cytoskeleton in the broad number of phenotypes that characterize DS. Among the possible candidate genes in this context , the tetratricopeptide repeat domain 3, TTC3 (also known as DCRR1; RNF105; TPRDIII. Gene ID 7267) is of special note due to its location within the DSCR, a small region of HSA21 found triplicated in only a fraction of patients showing typical features of DS (Delabar et al., 1993). Cloned for the first time in middle '90s (Tsukahara et al, 1996) (Eki et al., 1997), TTC3 is located on HSA21 (July 9th NCBI annotation boundaries [37073184..37203118]) and is composed of 52 exons.

Different protein domains are found on TTC3 aminoacidic sequence: four TPR domains at N-terminus, a potential coiled coil region (CC) and a Citron binding region (CBR) in the central part and a E3 ubiquitin ligase ring finger domain at the C-terminus (Tsukahara et al., 1996) (Berto et al., 2007) (**FIG.4 A**), which allows to include it in the E3 ubiquitin ligase family (Suizu et al., 2009).

Despite the well described E3 ligase domain, the only substrate of the ubiquitin ligase activity so far reported is the phosphorylated form of AKT (Suizu et al., 2009). In fact, low levels of TTC3 were found in human colon cancer cells with high proliferative potential (expressing high levels of AKT), while low AKT expressing cells, showed high TTC3 expression, suggesting that AKT signaling in this type of cancer is regulated by TTC3 post translational control of AKT via ubiquitin mediated degradation. (Dey-Guha et al., 2015). Moreover, previous data

obtained from my thesis laboratory indicated that TTC3 inhibits differentiation of rat pheochromocitoma cells (PC12) and primary mouse hippocampal cells (Berto et al., 2007) (Berto et al, 2014). This activity is elicited by the activation by TTC3 of the RAS homolog gene family member A, the small GTPase RhoA (Berto et al, 2014) through Citron N (CitN) protein. The former is implicated in several cellular mechanism, such as cell division, transcription and gene regulation: in particular, RhoA signaling pathway is involved in the formation of stress fibers and in actin cytoskeleton contractility (Spiering and Hodgson, 2011). On the other side, CitN is a gene implicated in the maintenance of Golgi compactness and in dendritic spine formation (Camera et al., 2003 and 2008). Increased levels of TTC3 in mouse hippocampal neurons induce a CitN dependent RhoA activation that ends in abnormal actin polymerization and a final block of differentiation (Berto et al., 2014). Treatment of primary hippocampal neurons with actin depolymerizing compounds or with a RhoA inhibitor as the Clostridium botulinum ADP ribosyltransferase C3 (C3) induce the rescue of the block of differentiation elicited by TTC3. On the contrary, RNA interference of TTC3 in hippocampal neurons induces an increase of the length of the axon after 3 days in culture. This effect is counteracted by the use of actin polymerizing drugs, such as Jasplakinolide (Berto et al., 2014). TTC3 also plays a role in Golgi compactness: in fact, overexpression of TTC3 in hippocampal neurons induces the fragmentation of Golgi cisternae, effect that is partially rescued by the actin depolymerizing drug cytochalasin B (Berto et al., 2014). Whether it is the abnormal actin polymerization or the fragmentation of Golgi system at the very beginning of neuronal differentiation the cause of the block of neuronal polarity induced by high TTC3 levels is still a matter of debate. Finally, a rare variant of TTC3 has been found in a cohort of patients characterized by a late onset Alzheimer's disease (Kohli et al., 2016).

Real time analysis revealed that TTC3 messenger RNA is upregulated in DS from the very early development of human embryo, both at 3 and 5 days post fertilization (pf) (Shaeffer et

al, 2017), making this gene particularly suitable for trisomy 21 screening of blastocysts before In Vitro Fertilization (IVF) procedures (where legally accepted). Expression profile studies performed on mouse model of the syndrome and on brain tissues derived from individuals both with DS and other non DS disorders associated with intellectual disability revealed that TTC3 is one of the most consistently upregulated genes (Vilardell et al., 2011). In a recent work (Guedj et al., 2016), TTC3 appeared as one of the two only genes (together with the deubiquitinase gene USP16) transcriptionally modulated in a large number of samples, both derived from humans and from mice (human fetal amniocytes, induced pluripotent stem cells (iPSCs), iPSCs-derived neurons, post-mortem human fetal brain tissue and embryonic forebrains from three DS mouse models of DS, Ts1Cje, Ts65Dn and Dp16), further corroborating the idea that TTC3 may play a functional role in the disease.

(3) Aim of the work

The aim of this work is to investigate whether the neurological phenotypes observed in Ts65Dn mouse model can be attributed to the direct effects of dosage imbalance in neuronal cells or to secondary phenomena, such as reduced response to extrinsic cues, cortical microenvironment or altered cellular composition. In fact, as described in the introductory section of this work, many alterations characterize the cortex of both DS patients and mouse models, however, at least in part in some developmental windows, neurons appear to have relatively normal intrinsic ability to generate neurites and to connect properly. It is then of great importance to understand which phenotypes are primary or secondary, in order to design suitable therapeutic approaches directed to one defect or another, thus implementing, counteracting or preventing the cognitive decline.

To discriminate between primary and secondary phenotypes, we took advantage of primary cortical neurons obtained from the Ts65Dn mouse model. The use of this experimental model allowed us to study *in vitro* all the steps of differentiation that occur *in vivo* during cortical development. Moreover, stemming from the data obtained in the first part of this work, we decided to shed light on the possible molecular mechanisms at the basis of the defects that we have observed. We have then investigated the role of a HSA21 gene, the tetratricopeptide domain protein 3 (TTC3). Specifically we studied its contribution to the dendritic spine defects that we detected in our neuronal cultures.

(4) Results

4.1 Neuronal polarity is not altered in Ts65Dn mouse cortical neurons

In order to investigate Ts65Dn cortical neurons ability to differentiate in *ex vivo* conditions, we prepared neuronal cultures from post natal 0 (P0) mice from both trisomic and euploid litters (see further details in material and methods). After 72 hours from plating, we evaluated the percentage of cells in stage 2 (multipolar) and in stage 3 (polarized cell) (Banker & Goslin, 1988) and the number of primary neurites emerging directly from the cell soma.

We stained filamentous actin and alfa-tubulin to see the structure of the cell and the organization of the cytoskeleton within the neuron itself.

Interestingly, we did not see any significant changes nor in the distribution of cells between the two stages (**Fig.1 A-C**) or in the number of primary neurites emerging directy from the cell soma (**Fig.1 D**).

Moreover, we measured the axonal length and also in this case we were not able to appreciate any differences between the two genotypes (**Fig.1 E).**

To further strengthen our results, we have evaluated the same parameters in cultures obtained from another mouse model, the Ts2Cje (comparable to Ts65Dn: in this case, the extra chromosome is fused to Mmu12 due to a Robertsonian translocation).

Also with this genetic background, stage progression, axon specification, number of sphere primary neurites and axonal length were indistinguishable between euploid and trisomic mice (**Fig.1 F-G-H**).

Together, these data indicate that neuronal polarity and the efficiency of neurite sprouting, including axons is not altered in *ex vivo* conditions, suggesting that, intrinsically, Ts65Dn neuronal cells have no major defects in their cell-autonomous initial differentiation program.

Additionally, in a fraction of cultures obtained from Ts65Dn model, we noticed an abnormal enlargement and of the growth cone (GC) (**Fig.1 B**), characterized by a roundish shape and by

the presence of looping microtubules in the innermost part of the structure (instead of parallel bundles emerging from the axon), suggestive of a paused GC. It is noteworthy that increased area of the GC has been described also in hippocampal neurons from Ts65Dn as well as in human cortical neurons derived from DS post mortem tissues (Sosa et al., 2014).

4.2 Dendritic arborization is unaffected in Ts65Dn cortical neurons

Neuronal polarity establishment is followed by the rapid growth of the minor neurites into dendrites. *In vitro*, this step takes place around seven days after plating (Takano et al., 2015). Previous work (Dierssen et al., 2003) has reported that Ts65Dn layer III pyramidal neurons show a simplified branching pattern and a shorter dendritic length *in vivo.*

We then evaluated the differentiation potential of Ts65Dn cortical neurons *in vitro,* culturing the cells up to seven days and analyzed their ability to generate dendrites.

In order to clearly delineate the dendritic arbor, neurons were transfected with a GFP-empty vector at DIV 5. Sholl analysis (Sholl, 1953) was performed on GFP positive cells. At this developmental stage we did not reveal any significant change in the number of intersections up to a radius of 60 μm (**Fig.2 A-C**). The overall number of intersections and the total dendritic length inside the Sholl' area were unchanged as well (**Fig.2 D-E**). We next asked whether the defects observed *in vivo* may be due not to the inability of the neurons to generate the dendrites, but rather to maintain them.

In fact, given that dendrites display a first dynamic phase of branching instability (with progression and retraction of protrusions) occurring around at DIV 7-9, followed by a stabilization of the dendritic arbor occurring between DIV 10-15 (Baj et al, 2014), we wondered whether at later stages some differences would emerge. We performed the same set of analyses at DIV 14 and also in this case the two genotypes were comparable (**FIG.2 I-J**),

even though the number of crossings was increased respect to DIV 7, indicating that neurons mature physiologically under our culturing conditions.

To exclude that the absence of phenotypes observed in Ts65Dn cortical neurons was due to the efficient attachment to a substrate different from those physiologically used by cells *in vivo* (Poly-L-lyisine), we studied dendritogenesis after plating cells on laminin (**FIG.2 B**). In this condition, we appreciated a slight decrease of the number of intersections at radii 35 and 40 (**FIG.2 F**). However, although this phenotype was statistically significant, the observed effect is dramatically lower as compared to the one described in the *in vivo* condition (Dierssen et al., 2003). Moreover, the dendritic pattern, the total number of crossings and the dendritic length are similar between the two analyzed genotypes (**FIG.2 G-H**).

Together, these data indicate that dendritic arborization defects previously seen in pyramidal neurons *in vivo*, cannot be attributed to intrinsic neuronal inability to properly differentiate, but rather to complex environmental defects present within the Ts65Dn cortex, that must be further investigated.

4.3 Dendritic spines of cultured Ts65Dn neurons show an immature morphology

Neuronal maturation peaks with the generation of dendritic spines, the main post synaptic compartments of neuronal cells. Previous works demonstrated that both in DS patients and in mouse models, dendritic spines are reduced in number and show more immature characteristics (a spine is referred to as “mature” when it resembles the shape of a mushroom, with a clear distinction between the neck and head) (Benavides-Piccione et al., 2004; Dierssen et al., 2003; Popov et al, 2011; Suetsugu & Mehraein, 1980). This feature in common to several neurodevelopmental disorders (von Bohlen und Halbach, 2010).

To quantitatively investigate the morphology of dendritic spines produced by Ts65Dn neurons *in vitro,* we classified them according to Hering & Sheng, 2001 (**Fig.3 A**) and counted the number of spines falling in different classes in both euploid and Ts65Dn after 14 days in culture. We first detected a reduction in the overall number of dendritic spines (**FIG.3 B-C**) (P-value= 0,07). Moreover, from the comparison between the different spine classes, it emerged that mature mushroom spines were reduced in number, in good agreement with in vivo studies. In contrast, no alterations in filopodia or stubby spines were observed (**FIG. 3 C**). Together, these data indicate that the reduction of the number, and in particular, the decrease of mushroom spines that we observe in *ex vivo* conditions, might contribute to the synaptic defects already published in vivo (Dierssen et al., 2003), thus suggesting that synaptic alterations that characterize the disease might arise from both cell intrinsic defects (detected in vitro) and cytoarchitectural alterations (for example, cellular composition imbalance) within the cortex.

4.4 TTC3 downmodulation reverts the dendritic spine defect of Ts65Dn neurons

Considering the previous involvement of TTC3 in actin cytoskeleton remodeling and the crucial role of actin in dendritic spines, we tested whether TTC3 could contribute to the cell-autonomous alteration of spine maturation that characterizes this model.

We therefore evaluated the effects of TTC3 downregulation in Ts65Dn cortical neurons at the stage of spine development. We transfected the neurons at DIV14 with shRNA designed against TTC3 and after three days we counted the number of spines and classified them by shape (**Fig.3 A**). With this timing we only took in account the formation and maintainance of spines and not the generation of dendrites, that in turn could affect dendritic spine sprouting.

In TTC3-depleted Ts65Dn cortical neurons, we observed a reduction in the number of filopodia and an increase of mushroom spines. The measured values were similar to those observed in the euploid cultures obtained from littermate mice transfected with scramble shRNA (**FIG.3 D-E**). Importantly, TTC3 downregulation in disomic cultures did not affect *per-se* the development of dendritic spines. Thus, we can conclude that the reduction of TTC3 levels in Ts65Dn neurons is able to rescue the cell intrinsic defect of dendritic spines maturation.

4.5 TTC3 expression during development

To study the potential molecular mechanism elicited by TTC3 in dendritic spines, we characterized its expression and we performed Western blot analysis on several tissues obtained from wild type mice. To reveal TTC3 we used a homemade antibody generated in rabbit. The predicted molecular weight of the protein is 230 kDa, however the TTC3 antibody detects several bands: in most cases we can see a doublet at 230kDa and a higher band. We do not know the characteristics of these bands, but we can speculate about post translational modifications or specific isoforms. The protein was well detectable in total brain (forebrain) and in cerebellum, while its expression was ill-detectable in non neuronal tissues (**FIG.4 B**). We then analyzed TTC3 expression in brains of Ts65Dn mice: due to the difficulty to sacrifice female mice (the breeding and maintenance of the colony is hard and expensive), we decided not to evaluate TTC3 expression in Ts65Dn mice during fetal stages, and concentrated our tests on postnatal day 0 (P0). As expected from the increased dosage of TTC3 and from RNA expression studies conducted on mouse DS models, quantitative analysis of the western blots showed in Ts65Dn cortices a significant increase (around three folds) of TTC3 levels, if compared to control littermates (**FIG.4 C-D**).

We next asked whether TTC3 levels are actually increased also in tissues of DS patients. To address this issue we took advantage of a publicly available transcriptome dataset, (Olmos-Serrano et al., 2016) generated from DS and control brains collected from mid-fetal developmental stages to adulthood. Specifically, we measured and mediated the levels of TTC3 expression in specific cortical regions (dorsolateral prefrontal cortex, orbital prefrontal cortex, ventrolateral prefrontal cortex, medial prefrontal cortex, primary somatosensory cortex, posterior inferior parietal cortex, posterior superior temporal cortex, inferior temporal cortex, primary visual cortex). In both conditions (disomic vs trisomic), levels of TTC3 mRNA decrease with a similar kinetic during development. However, in all the available stages (except from ages between six months and twenty years) TTC3 levels appeared to be significantly higher in DS individuals than in age/sex matched controls (**FIG.4 E**).

4.6 TTC3 expression in dendritic spines

To further investigate the role of TTC3 in the alteration of dendritic spines observed in 4.3, we evaluated the abundance of TTC3 within synapses, performing synaptosome preparations from brain cortices (see further details on materials and methods).

We first set-up the conditions of our analysis in wild type mice, until we could detect a good TTC3 signal in the crude synaptosomal fraction. We then measured TTC3 levels synaptosomal fractions of adult euploid and Ts65Dn mice. In contrast to the increased TTC3 levels observed in total brain homogenate, synaptosomal TTC3 levels of Ts65Dn mice were comparable to euploid levels (**FIG.5 A-B**).

4.7 RhoA activation is not altered in Ts65Dn brain

In previous works, we demonstrated that TTC3 overexpression leads to hyperactivation of the small GTPase RhoA (Berto et al, 2014), with consequent abnormal actin polymerization and

inhibition of neuronal differentiation. To investigate whether the milder increase of TTC3 levels produced by trisomy may similarly affect active RhoA levels in Ts65Dn cortices, we performed an ELISA-based RhoA activity assay on cortices homogenates of Ts65Dn mice and euploid controls (see further details on materials and methods).

This analysis did not reveal significant differences between the two genotypes. With the exception of one outlier sample, just small fluctuations appeared from both groups (**FIG.6 A**). To exclude any bias caused by differences in total RhoA amount, we measured RhoA expression in the same samples and also in this case no alterations were detected (**FIG.6 B-C**). To further confirm this result, we analyzed the levels of a brain specific downstream effector of RhoA, Profilin IIa (PIIa) and its phosphorylated form, p-Profilin IIa (p-PIIa). Specifically, PIIa is phosphorylated by RhoA through the Rho Associated Protein Kinase (Rock) (Witke et al., 2001). We then evaluated the ratio of p-PIIa on PIIa in both euploid and Ts65Dn protein extracts. However, also in this case we didn't observe any difference between the two genotypes (**FIG.6 D-E**).

4.8 TTC3 interactors

To shed light on the possible TTC3 interactors that can collaborate to its function in dendritic spines, we explored, on a collaborative basis, the yeast two hybrid (Y2H) screen data generated by Hybrigenics in a systematic project funded by the Jérôme Lejeune Foundation. The "bait" of the screening was a fragment of TTC3 , while the library of the putative "preys" was generated from adult human brain. From the Y2H assay a total of 62 preys emerged, but only 6 of them had a statistically significant *p-value*: AKT2 (id: 208), FBXO7 (id: 25793), SPTAN1 (id: 6709), WAC-1 (id:51322), HERC2 (id: 8924), NUFIP1 (id: 26747) **(FIG.6 F)**.

- AKT2 is a AKT isoform expressed quite ubiquitously, with a prominent role in vascular diseases (Yu et al., 2015) and cancer development (Wang et al., 2017). Previous data

(Suizu et al., 2009) already demonstrated an interaction between TTC3 and AKT2 and also indicated the latter as a E3 ubiquitin target of the former. The three AKTs isoforms are required for neuronal survival and proper differentiation of the neurons (Diez et al., 2012), however the isoform mainly implicated in central nervous system is AKT3. (For further details about AKTs role in different biological process see (Cohen et al., 2013)).

- FBXO7 (F-Box Only Protein 7), also called PARK15, is an E3 ubiquitin ligase protein. It is a member of the F-box proteins that constitute one of the four subunits of the ubiquitin protein ligase complex called SCFs (SKP1-cullin-F-box), which function is the phosphorylation-dependent ubiquitination of substrates. Mutations of *FBXO7* are found in patients with multiple system atrophy and in a specific manifestation of Parkinson's disease, the parkinsonian-pyramidal syndrome (PDD) (Conedera et al., 2016).
- SPTAN1 (Alpha II-spectrin), also called alfa fodrin, is a member of the spectrin protein family, that play important roles in scaffolding and maintenance of plasma membrane integrity and cytoskeletal structure. Specifically, SPTAN1 is abundant in brain (Riederer, Zagon, & Goodman, 1986) and it interacts with NMDA receptor and modulate the localization or accessibility of glutamate receptors while cross linking the receptor with microfilaments (van Rossum & Hanisch, 1999). Mutations of *SPTAN1* were identified in two patients with intellectual disability, infantile spasms, hypomyelination, and brain atrophy (Hamdan et al., 2012).
- WAC-1 (WW Domain Containing Adaptor With Coiled-Coil 1) is a regulator for transcription-coupled histone H2B ubiquitination, through the targeting of E3 ligases RNF20/40 to RNA polymerase II complex for H2B ubiquitination at active transcription sites. Moreover, WAC-dependent transcription is important for cell-cycle checkpoint activation in response to genotoxic stress (Zhang & Yu, 2011). Patients carrying mutations

in WAC1 present dysmorphic features, developmental delay and hypotonia (DeSanto et al., 2015).

- HERC2 (HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 2) is a E3 ubiquitin protein ligase. Genetic variations in this gene are associated with skin/hair/eye pigmentation variability (see an example in (Ulivi et al., 2013)). Moreover, it controls DNA damage response through the regulation of the deubiquitinase enzyme USP16 (Zhang et al., 2014). Mutations of *HERC2* result in a neurodevelopmental disorder with phenotypic similarities to Angelman syndrome (Harlalka et al., 2013).
- NUFIP1 (Nuclear fragile X mental retardation-interacting protein 1) is a Fragile X Mental Retardation Protein (FMRP) interacting protein. In particular, it is highly expressed in cortex, hippocampus and Purkinje cells and it shuttles between nucleus and cytoplasm regulating both transcription and translation of messenger RNAs (Bardoni et al., 2003).

Among the statistically significant TTC3 interactors, 4 out of 6 are mutated in neurodevelopmental/neurodegenerative disorders, while 3 out of 6 are implicated with ubiquitin pathway, both directly (HERC2 and FBXO7) or indirectly (WAC-1).

Stemming from TTC3 E3 ubiquitin ligase activity, the Y2H data support a key role of this protein in ubiquitin related pathways both in physiological and pathological conditions.

We tried to validate some of these interactions, but with no positive results, mostly due to technical problems, such as the molecular weight of the candidates and the partial toxicity of TTC3 when overexpressed in cells. In particular we tried reciprocal co-immunoprecipitation between TTC3 and AKT and between TTC3 and HERC2. In the latter condition we witnessed several problems due to the high molecular weight of HERC2 (527kDa). Further improvements of the techniques and experiments are needed to definitely validate these interactions and to assess whether some of them may be direct substrates of TTC3 biochemical activity and may mediate TTC3 effects.

(5) Discussion

Down syndrome is a genetic disorder characterized by a large cohort of symptoms that greatly vary in both penetrance and severity, but the only hallmark common among all DS individuals is ID (Chapman & Hesketh, 2000).

In the first part of this work we decided to investigate more in depth DS related ID and specifically the cellular basis of cortical structure alterations that characterize DS brain. One conceivable hypothesis is that the overall altered cytoarchitecture and composition of the cortex could somehow halt the cell autonomous program of differentiation and that neurons would be actually able to properly differentiate *per se* if grown in an isolated environment. To validate this hypothesis, we cultured cortical neurons from Ts65Dn and euploid age matched controls *in vitro,* to highlight only the phenotypes deriving from cell intrinsic alterations. We analyzed all the stages of neuronal differentiation, from the establishment of polarity to synaptogenesis.

We first evaluated the ability of cortical neurons to undergo the first stages of differentiation and to polarize the axon. We didn't observe any impairment neither in the progression through the stages (see introduction for extended explanation), nor in the number of primary neurites emerging from cell soma. Moreover, also axonal polarization and length were comparable between the two genotypes, indicating that the first stages of the differentiation program are well conserved. We observed the same trend also with the analysis of cortical neurons obtained from another mouse model, the Ts2Cje. Our data are apparently in contrast with previous work (Sosa et al., 2014), in which an increased length of the axon in Ts65Dn cultures was described. However the two experimental conditions are quite different: first of all, we have cultured cortical neurons while they have analyzed hippocampal neurons, the matrix on which neurons are cultured is different (poly-L-lysine vs laminin) and finally the

timing of the analysis differs: we have evaluated stage progression and axonal length after 72hrs after plating, while in the cited work they analyzed the cells after 24hrs in culture.

Additionally, in the previous work an increased area of the growth cone (GC) was described: this phenotype was present also in our cultures together with the presence of a peculiar pattern of apparently looping microtubules. Microtubules are usually found in the Central domain of GC in parallel bundles. In Ts65Dn neurons, microtubules were found to form looping structures inside the Central domain. Looping microtubules in the growth cone are often associated with paused growth cones (Lee et al., 2004). The fact that we did not detect any difference in the axonal length, but we observed the presence of putative paused growth cones, may lead to the hypothesis that Ts65Dn neurons undergo fast and rapid growth movements, followed by pauses. Another conceivable explanation is that the paused GCs are not able to properly sense the environmental guidance cues and then show defects in axon guidance, rather than in growth. Live imaging of the neurons during axonogenesis will be necessary to properly address this issue.

The apparently different phenotypes that have emerged in the two works could also be explained by different responses of the different cell types (cortical and hippocampal neurons) to the genome imbalance caused by DS. In addition, we were not able to observe the enlarged growth cone phenotype in all the obtained cultures, which suggests a variable penetrance of this phenomenon among animals.

We then moved to the investigation of dendritogenesis, that is the maturation of the neurites into functional input outposts for the neurons. Reduced dendritic arborization has already been observed both in humans (Becker et al., 1991) and in Ts65Dn (Dierssen et al., 2003). We have grown the neurons up to 7 days and analyzed the complexity of the dendritic arbor with Sholl analysis. In contrast with the data obtained *in vivo,* we did not detect any difference between the two genotypes neither in the arbor complexity nor in the total dendritic length

(calculated inside the Sholl' area) both on poly-L-lysine and on laminin (except from subtle differences at two central radii).

In infants with DS, normal or even increased area occupied by the dendritic arbor within brain parenchyma has been observed up to six months of age. After this developmental stage, the dendritic arbor is reduced respect to disomic age matched controls and this is true for the rest of the life (Becker et al., 1991): this suggests that, at least for a specific time window during development, DS neurons behave normally. The generation of the dendritic field (i.e. the region occupied by the dendrites of a neuron that determines the extent of sensory or synaptic inputs to the neuron) is obtained through the balance of several phenomena, such as the ability of the neuron to cover the area that encompasses its synaptic inputs, a branching pattern suitable for data processing and a high degree of plasticity in order to respond to environmental changes and experience (Jan & Jan, 2010). Our data, showing that the intrinsic aspects of axonal and dendritic differentiation program occur similarly in control and trisomic neurons, support and extend these observations. Stemming from these findings, the apparent discrepancy between the *in vivo* and *in vitro* data can be explained by several hypothesis. First of all, neurons of Ts65Dn are in a peculiar condition in which, as the brain matures, they are not surrounded by the physiological (=disomic) cortical environment and cannot connect and coordinate with the correct cellular targets, thus resulting in the formation of an impaired dendritic field (the increased number of interneurons and the consequent imbalance of the excitation/inhibition tone also plays a role in the shaping and maintenance of the dendritic arborization). Secondly, we can also hypothesize that the altered circuitry in Ts65Dn cortices alters the response of dendrites to adjustments both during development and in response to experience. Indeed, Ts65Dn mice raised in an environmental enrichment (EE) condition did not display an increased number of dendritic branches respect to Ts65Dn mice raised in normal environmental conditions (on the contrary, euploid mice showed a dramatic increase

of dendritic field upon EE) (Dierssen et al., 2003). It is then conceivable to think that Ts65Dn neurons possess the intrinsic possibility to properly elongate dendrites and to form a correct dendritic field (in vitro data), but the cortical alterations (cellular composition, extracellular cues, etc) induce defects in maintenance and/or premature shrinkage of the dendritic field.

The last step of differentiation that we analyzed was the development of dendritic spines. In this case, we have observed a reduction in the total number of dendritic spines and specifically, mushroom spines were the mostly affected. This result is comparable to the data obtained in vivo, thus demonstrating that the synaptic defects are at least in part cell autonomous, although we cannot exclude that the altered dendritic development and connectivity present *in vivo* can exacerbate the phenomenon.

To explore more in depth the molecular mechanisms underlying the cell autonomous impairment of neuronal cells to generate and maintain dendritic spines when in a Ts65Dn background we focused on TTC3 andmore specifically we have evaluated its contribution to dendritic spine development in Ts65Dn cortical neurons. As indicated in the “results” section, we downregulated TTC3 at DIV14 and analyzed the neurons at DIV17. With this timing, we were able to induce a phenotypical rescue respect to Ts65Dn cultures treated with scramble shRNA: in fact, we reduced the percentage of filopodia and increased the number of mushroom (to levels comparable to euploid). Moreover, the downregulation of TTC3 did not seem to affect euploid cultures development. These data suggest that high levels of TTC3 could impair the correct generation of dendritic spines. In order to obtain a formal proof of this concept, we tried several times to overexpress a plasmid encoding a cDNA of TTC3, but we were not able to find any vital cell after the transfection. We hypothesized that while it is quite tolerable for the cells to overexpress TTC3 at first stages of development, it is not compatible with vitality at later time points

However, it should be discussed that when we analyzed neurons at DIV 14 the most prominent changes were at the level of mushroom and not of filopodia like spines, while when we observed the same components three days later, at DIV 17 (as in the case of the rescue experiments) we saw a shift toward less mushroom, together with an increased number of filopodia (in scramble shRNA condition). We can speculate that this discrepancy is mainly due to timing. In fact, being the generation and the maintenance of dendritic spines a highly dynamic process, the events observable at the initial steps of the phenomenon could be just the beginning of a more dramatic alteration with a peak at DIV 17 (when also filopodia spines are affected).

It should be also taken in consideration the effect of the transfection of the plasmid, that in the former condition takes place around DIV 5 (GFP empty vector), while in the latter at DIV 14. The different timing can affect differently the biological processes within the cell that could, in turn, reflect the different behavior of the neurons in terms of dendritic spines. Further analysis are required to dissect the divergences between the two developmental ages.

We have then analyzed TTC3 expression in synaptosomes and we evidenced that the protein was present in this specific cellular compartment, but differently from what we expected, Ts65Dn expressed the same amount of protein than euploid controls (while in total homogenates TTC3 levels were higher in Ts65Dn). This result, apparently controversial, can be discussed in light of a work (Fernandez et al., 2009) that analyzed the composition of synapses in Ts65Dn mice. The authors of this work, indicated a normal protein composition of synapses and suggested that trisomy induces a shift mainly in the activity of both phosphatases and kinases within the neuron, more than a global alteration of synaptic protein levels. Additionally, they suggested that probably small changes in the synaptic circuits contribute to cognitive impairment in people with DS more than a global alteration in synaptic protein composition. Accordingly, we propose that the unchanged levels of TTC3 in our

synaptosome preparations could be due to some yet unknown control mechanism that prevent an abnormal localization of TTC3 within synapses. We have seen that TTC3 localizes in actin rich dendritic outposts that can be identified as synapses (data not shown), however the signal to noise ratio of our anti TTC3 antibody is not suited enough to quantitatively analyze small differences in local protein abundance.

To better define the molecular mechanisms at the basis of the rescue at dendritic spines level, we evaluated the activity of the small GTPase RhoA, a previously detected partner of TTC3. Our previous work (Berto et al., 2007), demonstrated that TTC3 induces RhoA activation at least in HEK293 and in PC-12 cells. Stemming from these data, together with the increased expression of TTC3 in Ts65Dn brain, we evaluated the levels of RhoA activation in our mice. We did not detect any statistically significant difference between the two genotypes neither in the directly measured RhoA activity nor in the phosphorylation levels of one of its substrates, Profilin. Although no strong global activation was evidenced from our analysis, it is conceivable to think that local changes may occur, for example at dendritic spine level. This hypothesis is supported by the previously mentioned work in which they show that only subtle changes in the overall amount of proteins occur at synaptic level, suggesting that our approach might have been too broad to detect this kind of small fluctuations that may take place not only regionally, but also in a temporally controlled manner. FRET sensors such as "*Raichu*" (Nakamura et al., 2006) would be a powerful tool to locally track RhoA activity in both time and space. However it is also reasonable that the complex genetic scenario occurring in DS is able to compensate with other mechanisms the RhoA activation elicited by TTC3, thus resulting in normal levels of RhoA activity.

Finally, we searched for possible TTC3 molecular partners that could help us clarifying the molecular basis of its role in spines, and more generally its function in DS disease. From a systematic yeast-two-hybrid assay data (as described in section 4.8 of results), we obtained

six statistically significant partners: the previously reported AKT2 (Suizu et al., 2009a), as well as FBXO7, SPTAN1, WAC-1, HERC2 and NUFIP1. The most striking evidence that emerged from a gene onthology analysis is that three out of six interactors are related to ubiquitin pathway. In fact, FBXO7 and HERC2 are both E3 ubiquitin ligases, while WAC-1 regulates E3 ligases RNF20 and RNF40 during histone ubiquitination. These data are in line with the E3 ligase activity of TTC3, suggesting that all these proteins could act in a supramolecular complex, as it often occurs with proteins involved in ubiquitination.

Interestingly, an interaction between HERC2 and the chromosome 21 encoded histone 2A deubiquitinase USP16 (Zhang et al., 2014) has previously been described. In particular, it was shown that the two proteins physically and functionally interact during DNA double strand breaks repair process. Increased levels of USP16 induce a less effective response to ionizing radiations mediated DNA damage. A megakaryocytic cell line derived from a Down syndrome individual (CMK cells), showed a clearly reduced response to DNA damage (reduced number of ubiquitin foci), suggesting a possible implication of the ubiquitin pathway in the disease. However, the precise cross regulation between the two proteins is still unknown. Moreover, USP16 was found (Adorno et al., 2013) playing a key role in stem cell renewal in Down syndrome: indeed, it has been demonstrated that high USP16 levels (as in DS) induce reduced proliferation of stem cells, in particular of neural progenitors and fibroblasts, through a mechanism involving an altered de-ubiquitination of histones. Additionally, USP16 and TTC3 are the only two genes whose expression is altered in a large cohort (Guedj et al., 2016) of samples obtained both from humans (amniotic fluid cells, amniocytes, iPSCs, neurons, fetal brain) and from different mouse models (E15.5 forebrain of Dp16, Ts65Dn and Ts1Cje). We can then speculate that TTC3 and USP16 might interact through HERC2 and control stem cell self renewal. TTC3 role in cellular proliferation has been poorly investigated. The few available data (Dey-Guha et al., 2015) indicate that slow proliferating cancer stem cells

(poorly responding to treatments) derived from colon cancer are characterized by high TTC3 levels, that in turn induce low AKT (due to the latter ubiquitin dependent degradation by the former). We can hypothesize that TTC3 might regulate stem cell proliferation at least through three main mechanisms: the first (already supported by data) is post translational and implicates E3 ubiquitin dependent AKT degradation, thus resulting in reduced cell proliferation. The second hypothesis is that TTC3, together with USP16 and HERC2, may act on the regulation of gene transcription, tuning the ubiquitination of histones, thus modulating the expression of cell proliferation genes. The third is strictly connected to the interaction of TTC3 with Citron Kinase (CITK). Previous data (Berto et al., 2007) revealed that, at least in HEK293 cells, TTC3 and CITK physically interact. The latter has been demonstrated being implicated in cell division of HeLa cells and neuronal progenitors (Gai et al., 2016). When depleted of CITK, cells show an increased angle of division plane, impaired abscission during last steps of mitosis (Gai et al., 2011) and the mouse model KO for CITK exhibits a shift between proliferation and differentiation of neurons, tilted towards the latter (Gai et al., 2016). Then, we can speculate that TTC3 might be implicated in cellular proliferation also through this mechanism.

In conclusion, we propose that further analysis on the TTC3 interactors listed in this work may increase our understanding of DS, an essential achievement for the development of new possible therapies.

(6) Materials and methods

Mouse colony maintenance and mating

Ts65Dn colony was established in the pathogen-free space of the animal facility, under the control of our veterinary service and in full respect of the Italian regulations. The breeding was performed as described in Supplementary figure 1. Briefly, Bl6/C57 mice were crossed with C38 mice (Jackson Lab id: 003648): the derived lineage, called C37 was then crossed with Ts65Dn females (males are not fertile). C38 were maintained in homozigosity. Pups obtained from C37/Ts65Dn crossing were screened by PCR with primers spanning the fusion region between chromosome 16 and 17 (Fw17:GTGGCAAGAGACTCAAATTCAAC; Rev16: TGGCTTATTATTATCAGGGCATTT).

Neuronal primary cell culture and transfection

Mouse cortical neurons were isolated from Ts65Dn pups and euploid litters on the day of birth (P0) as previously described (Beaudoin et al., 2012). Briefly, P0 were killed and brain was extracted from the skull. Meninges were removed, the two emispheres were separated, cortices were isolated and transferred into 1ml of pre-warmed 2,5% trypsin (Sigma) for 15 minutes at 37 °C. Cortices were then washed five times with HBSS (Thermo Fisher), DNAseI (Promega) was added to the last wash at a concentration of 1:1000, and incubated at 37°C for 10 minutes. Subsequently, cells were carefully disgregated with a glass pasteur eight to ten times and neurons were plated in Mem Horse medium (Mem 1X (Lifetechnologies), 20% Horse Serum, 2mM L-glutamine (Gibco), 2% glucose) on poly-L-lyisine (Sigma. 1mg/ml.) or laminin (Invitrogen. 100µg/ml) pre-coated coverslips. Neurons were plated with a density of (33 x 10^3)/ cm^2. After 4 hours, medium was changed into Neurobasal medium (Thermo Fisher) supplemented with 0,5% B27 (Thermo Fisher) and 2mM L- glutamine (Gibco). A pre

cultured feeding layer of astrocytes was not used, but neurons were flipped upside down onto astrocytes obtained from the same culture.

Plasmids and transfection protocol

The shRNA constructs were based on the pCMV-GIN (Zeo) lentiviral vector (G. Berto et al., 2007). The control ("human") corresponds to nucleotides 1540-1562 of the human RefSeq TTC3 sequence (GGGTGTGCAATATAAAGATTATA) and shows two mismatches to the mouse and rat sequences. The perfect match plasmid correspond to nucleotides 2164-2186. Plasmid pEGFP-C1 (Clontech) was used to better visualize neuronal morphology in dendritogenesis and dendritic spines analysis. Cells were transfected using Lipofectamine LTX (Thermo Fisher) according to manufacturer's indications. If transfection was performed on neurons older than 12 days, Nupherin (Enzo Life Sciences) was added to transfection mixture according to manufacturer's indications.

Immunofluorescence, image acquisition and analysis

Neurons were fixed with 4% paraformaldehyde in PBS for 10 minutes, quenched with 50mM NH_4Cl/PBS for 15 minutes and permeabilized with 0.1% Triton X-100/PBS for 5 minutes. Immunofluorescence (IF) was performed using the described antibodies, followed by incubation with appropriate Alexa Fluor-conjugated secondary antibodies (Molecular Probes). Polymeric F-actin was detected with Tritc or Fitc phalloidin (Sigma).

Images were acquired with ViCo (Nikon) or ApoTome (Zeiss) microscopes equipped with 40x or 60x objectives. All analysis were performed with Fiji (ImageJ) software. Digital reconstructions were performed with NeuronJ plugin for Fiji. Sholl analysis was performed manually on Fiji platoform: traces of neurites were sketched with NeuronJ plugin for FiJi. In brief, Z-stacks of GFP transfected neurons were projected on one plane (tool "maximum

projection") and traces were drawn with a line. Concentric circles were centered on cell soma and the number of intersections was counted manually. Total dendritic length was measured with FiJI "segmented line" tool. Dendritic spines were counted manually on 10µm dendritic segments, 20 µm far from cell soma. At least two segments per cell were analyzed. Spines were classified as "filopodia" when no head was detectable, as "thin" when the head of the protrusion was small and far from the shaft, as "mushroom" when a clear neck-head morphology was visible, and as "stubby" in all other cases.

Western blot and synaptosome fractioning

Whole brain cortex proteins were extracted with lysis buffer (1% SDS, 25mM Tris–HCl pH 6.8, 1X protease inhibitors (Roche, Basel, Switzerland), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1mM sodium vanadate, 1mM sodium fluoride). Equal amount of proteins were loaded, resolved by reducing SDS-PAGE (Biorad precast gels) and blotted to nitrocellulose or PVDF filters (Millipore), saturated with BSA (Sigma) or non fat milk (Sigma) and incubated with antibodies and developed using the ECL system (Millipore) with a Biorad Gel Doc instrument.

Synaptosome fractions were isolated from adult mouse brain as it follows: brain was weighted and 4g/vol of buffer A (0.32 M sucrose, 1mM $MgCl_2$, 0.5 mM $CaCl_2$, 1mM $NaHCO_3$, 1mM EGTA, 1mM Dithiotheitol, 1mM PMSF, protease inhibitors (Roche)) was added. Tissue was then manually homogenized and 10g/vol buffer A was added. Samples were spinned at 1400xg for ten minutes. Supernatant was kept in ice and pellet was again homogenized with 4vol/g of buffer A. Pellet homogenate was spinned at 710xg for ten minutes. The obtained pellet was considered the nuclear fraction. The supernatant derived from this last step was combined with the one obtained before and spinned at 13,800xg for fifteen minutes. The supernatant was then spinned down at 100,000xg for one hour. The resulting pellet was the

crude synaptosomal fraction, while the supernatant was the cytosolic fraction. Synaptosomes were resuspended in buffer B (0.32 M sucrose, 1mM $NaHCO_3$, 1mM EGTA, 1mM Dithiotheitol, 1mM PMSF, protease inhibitors (Roche)). All fractions were collected and protein concentration was measured with Bradford protocol (Biorad) and samples analyzed by SDS-PAGE. Procedures were all performed at 4°C.

RhoA activation assay

RhoA G-LISA (Cytoskeleton) was performed according to manufacturer's instructions. Briefly, frozen cortices from both Ts65Dn and euploid adult mice were lysed and incubated in a Rho-GTP affinity plate for thirty minutes (to bind active Rho). Washing steps were performed to remove inactive GDP-bound Rho. The bound active RhoA was detected with a RhoA specific antibody and revealed by chemiluminescence.

Statistics

All analysis were performed with Excel (Microsoft Office) and with Graphpad Prism.

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"My kind Antonio, I can no other answer make but thanks, and thanks, and ever thanks"

("The Twelfth night", William Shakespeare).

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Infine, grazie anche a chi, a distanza, è stato presente ogni giorno di questo viaggio.

(9) Figures and Tables

9.1 Figure legend

FIGURE 1

(A) Representative images of euploid (upper panel) and Ts65Dn (lower panel) cortical neurons after 3 days in culture (DIV). Left panels: filamentous actin stained with phalloidin. Right panels: tubulin. Arrowheads indicate the axon. **(B)** Representative images of abnormally enlarged growth cone with looping microtubules detected in Ts65Dn neurons. Filamentous actin stained with phalloidin, tubulin and merge. **(C)** Percentage of neurons in stage II and stage III at 3 DIV. **(D)** Number of primary neurites emerging from cell soma after 3 DIV (primary neurites). **(E)** Axonal length (in micrometers) measured at 3 DIV. (**F-G-H**) The same as in (C-D-E), performed on cortical neurons obtained from Ts2Cje mouse model. Bars are mean ± s.e.m. Statistics: Student's t test *<0,05 and Chi-squared test. Eu n=8, Ts n=5. Scale bar represents 10 um (in A) or 5um (in B).

FIGURE 2

(A) Representative images of cortical neurons plated on Poly-L-Lysine after 7 days in vitro (DIV7). Skeletonization of the acquired image was performed with Fiji and Sholl analysis circles were superimposed manually. Left panel: euploid, right panel: Ts65Dn. **(B)** Representative images of cortical neurons plated on Laminin at 7 DIV. Left panel: euploid, right panel: Ts65Dn. **(C)** Distribution of the number of intersections, **(D)** total number of crossings and **(E)** total dendritic length within the Sholl area of neurons in (A). **(F-G-H)** same as in (C-D-E) performed on neurons in (B). **(I)** Representative images of cortical neurons plated on Poly-L-Lysine after 14 days in vitro. Left panel: euploid, right panel: Ts65Dn. **(J)** Distribution of the number of intersections of neurons in (I). Bars are mean ± s.e.m. Statistics. Eu =8, Ts n=5 for poly-L-lysine, Eu n=3, Ts n=2 for laminin. Scale bar represents 10 um.

FIGURE 3

(A) Graphic representation of the different classes of dendritic spines **(B)** Quantification of total number of spines calculated as the density of spines on ten micrometers segments. **(C)** Quantification of the number of different classes of spines calculated as the density of spines on ten micrometers. **(D)** Representative images of dendritic spines from Euploid (upper panels) and Ts65dn (lower panels) transfected with scramble (shRNA ctrl, left)) or TTC3 targeting shRNA (right). **(E)** Combined analysis of number and classification of dendritic spines in cultures obtained from euploid and Ts65Dn transfected with scramble or TTC3 directed shRNAs. Bars are mean ± s.e.m. Statistics: Student's t test *<0,05, **<0,01. At least two independent dendritic segments for each neuron were counted for more than ten cells for genotype. Segments were analyzed at a distance of 20 um from cell soma. Scale bar represents 2 um.

FIGURE 4

(A) TTC3 protein structure. **(B)** Western blot analysis of TTC3 protein expression in different tissues from adult wild type mouse. Tubulin was used as internal loading control**. (C)**. Western blot analysis and **(D)** quantification of TTC3 expression in P0 pups from euploid and Ts65Dn mice. GAPDH was used as internal loading control. Eu n=5, Ts n=6. Statistics: Student's T test. **(E)** TTC3 messenger RNA expression in human brain samples (for details see text) both from disomic and HSA21 trisomic individuals, at different developmental stages. Arrow indicates birth time. Pcw= post conception weeks, m=months, y=years. Bars are mean± s.d., Student's t test *<0,05.

FIGURE 5

(A) Western Blot of total homogenates and of crude synaptosomal fraction. PSD95 is a marker of synapses. Input is the total loaded protein amount. **(B)** Quantification of (A). TTC3 levels were normalized on input. N=3 for both genotypes. Statistics: Student's t test *<0,05.

FIGURE 6

(A) Luminescence intensity detected from cortical samples from both euploid and Ts65Dn. **(B)** Western blot analysis of total RhoA levels in the same samples analyzed for RhoA activation. Input is the total loaded protein amount. **(C)** Quantification of (B). **(D)** Western blot analysis of total Profilin, phosphorylated Profilin and Vinculin (used as loading control) of samples in (A). **(E)** Quantification of (D). **(F)** Table containing yeast two hybrid assay top scoring TTC3 binding partners: official gene name, extended name, NCBI identifier number, chromosomal location.

SUPPLEMENTARY FIGURE 1

(A) Schematic representation of HSA21 orthologs on MMU10,16 and 17. **(B)** Breeding and maintenance of the mouse colony. C38 mice are maintained in homozygosis. Male C38 are crossed with female C57. Males of the resulting strain (C37) are crossed with Ts65Dn females. **(C)** Mouse strain, Jackson Laboratories identification name and abbreviation of the animals used in this work.

FIG.1

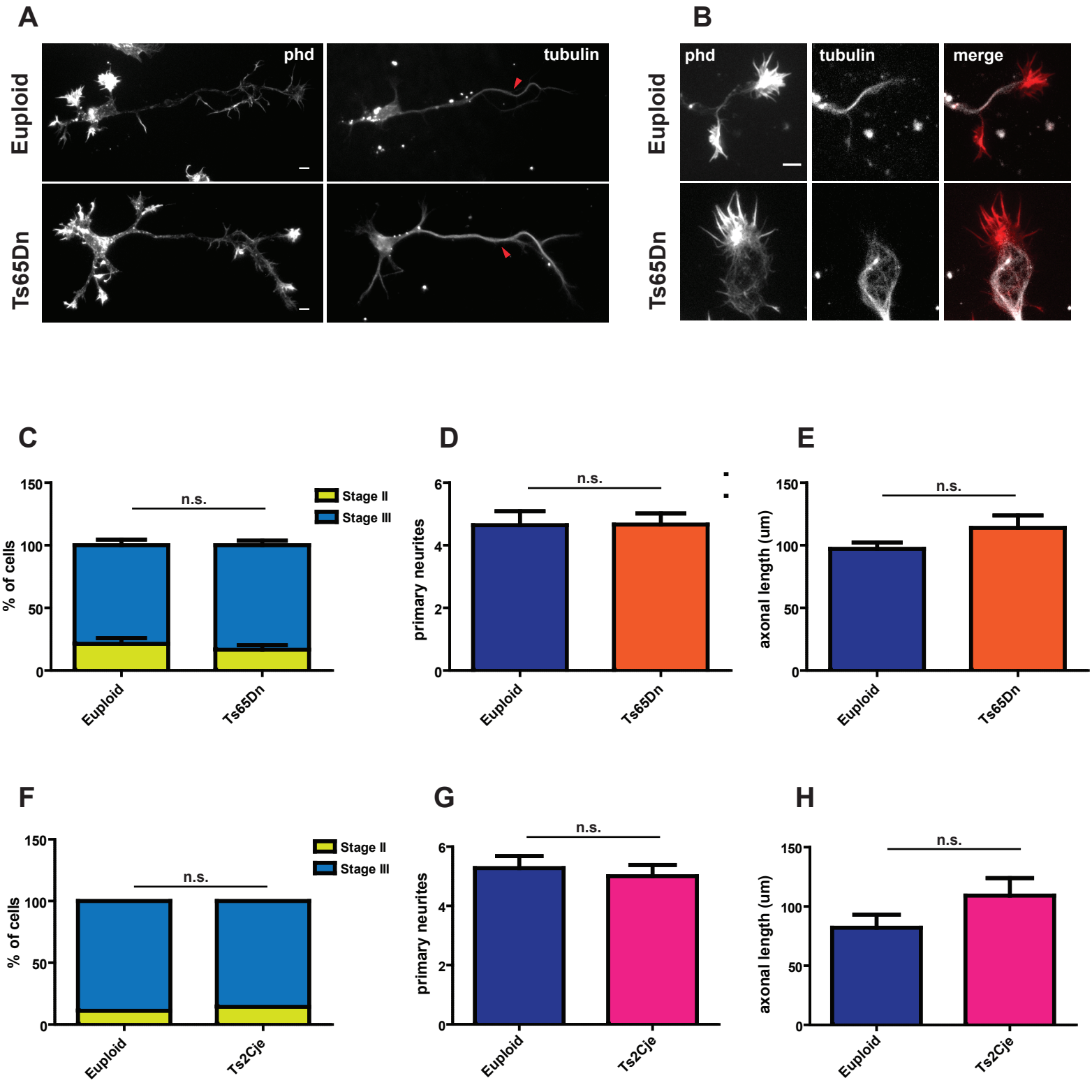

A
Euploid
Ts65Dn
phd
tubulin
B
Euploid
Ts65Dn
phd
tubulin
merge
C
% of cells
150
100
50
0
n.s.
Stage II
Stage III
Euploid
Ts65Dn
D
primary neurites
6
4
2
0
n.s.
Euploid
Ts65Dn
E
axonal length (um)
150
100
50
0
n.s.
Euploid
Ts65Dn
F
% of cells
150
100
50
0
n.s.
Stage II
Stage III
Euploid
Ts2Cje
G
primary neurites
6
4
2
0
n.s.
Euploid
Ts2Cje
H
axonal length (um)
150
100
50
0
n.s.
Euploid
Ts2Cje

FIG.2

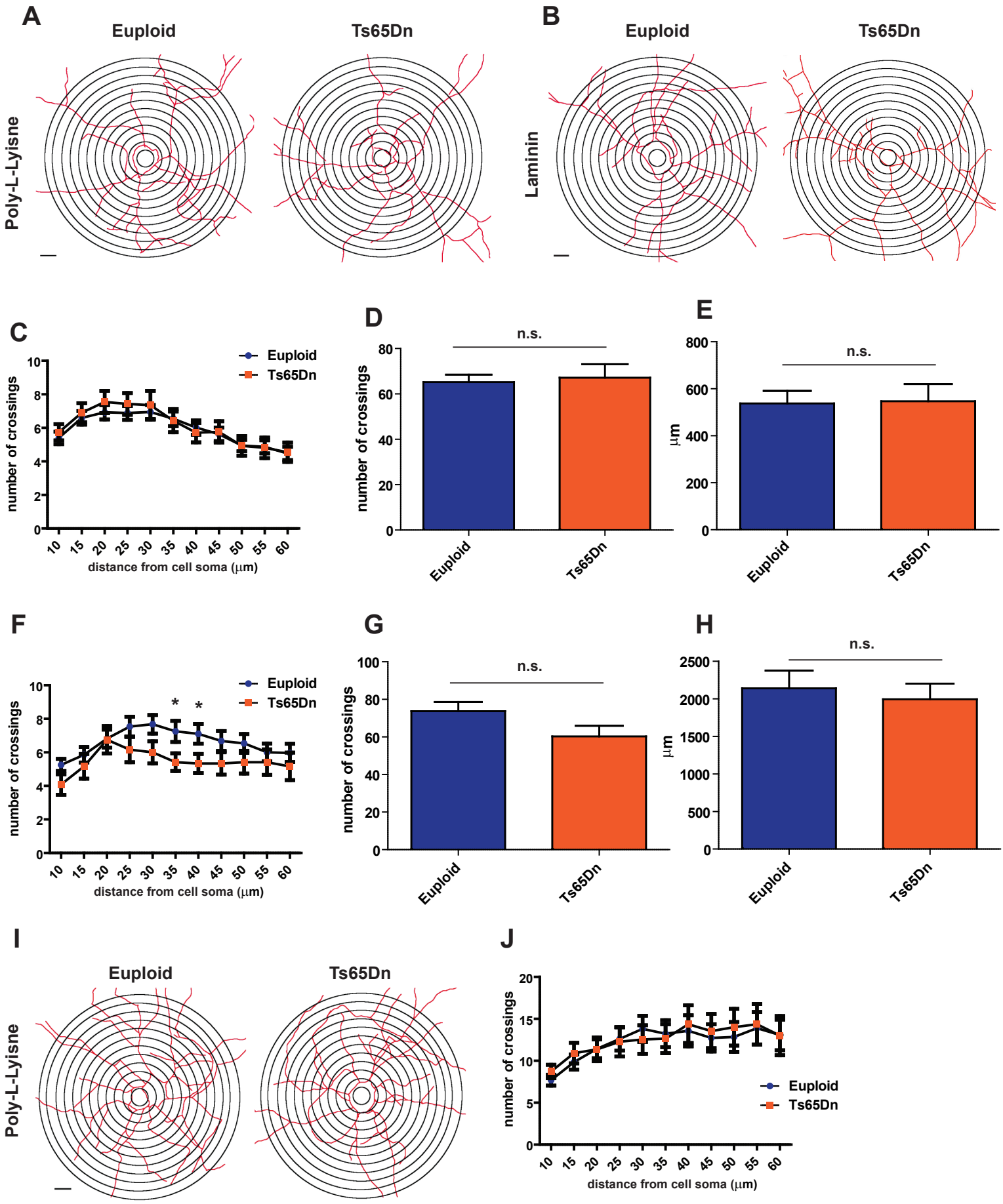

A
Euploid
Ts65Dn
Poly-L-Lyisne
B
Euploid
Ts65Dn
Laminin
C
Euploid
Ts65Dn
number of crossings
distance from cell soma (μm)
D
n.s.
number of crossings
Euploid
Ts65Dn
E
n.s.
μm
Euploid
Ts65Dn
F
Euploid
Ts65Dn
number of crossings
distance from cell soma (μm)
G
n.s.
number of crossings
Euploid
Ts65Dn
H
n.s.
μm
Euploid
Ts65Dn
I
Euploid
Ts65Dn
Poly-L-Lyisne
J
number of crossings
Euploid
Ts65Dn
distance from cell soma (μm)

FIG.3

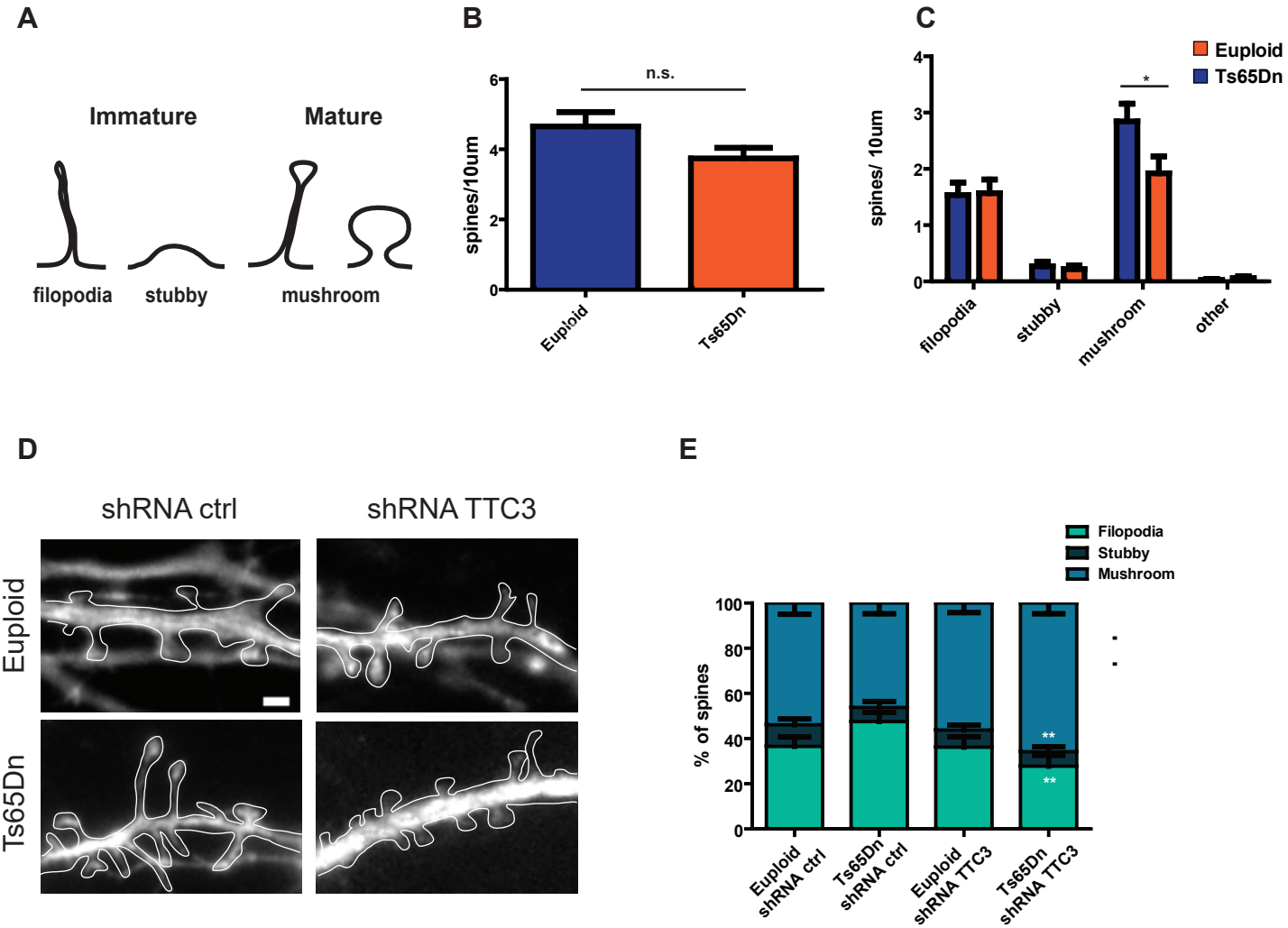
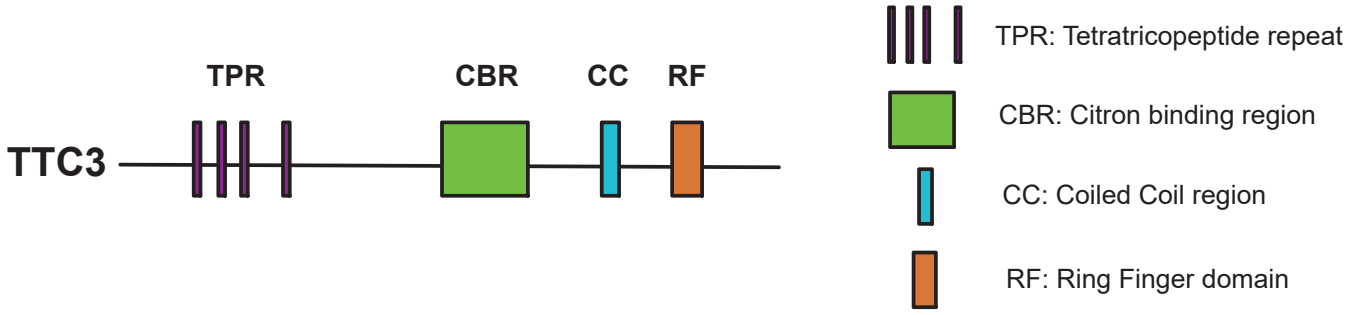
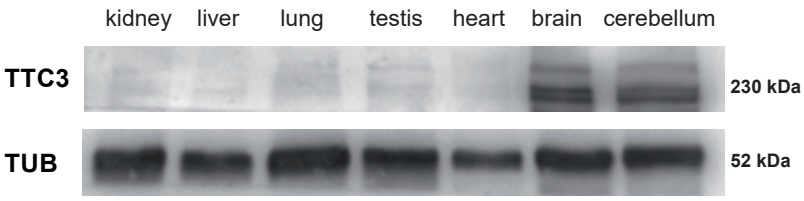

A
Immature
Mature
filopodia
stubby
mushroom
B
n.s.
spines/10um
Euploid
Ts65Dn
C
Euploid
Ts65Dn
spines/ 10um
*
filopodia
stubby
mushroom
other
D
shRNA ctrl
shRNA TTC3
Euploid
Ts65Dn
E
Filopodia
Stubby
Mushroom
% of spines
**
**
Euploid shRNA ctrl
Ts65Dn shRNA ctrl
Euploid shRNA TTC3
Ts65Dn shRNA TTC3

FIG.4

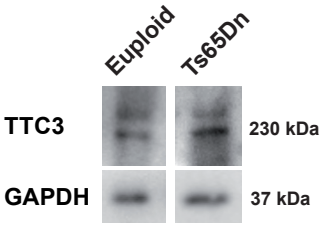
A


TPR
CBR
CC
RF
TTC3
TPR: Tetratricopeptide repeat
CBR: Citron binding region
CC: Coiled Coil region
RF: Ring Finger domain

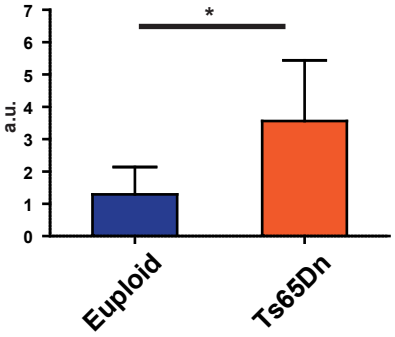
B


kidney
liver
lung
testis
heart
brain
cerebellum
TTC3
230 kDa
TUB
52 kDa

C


Euploid
Ts65Dn
TTC3
230 kDa
GAPDH
37 kDa

D


*
a.u.
0
1
2
3
4
5
6
7
Euploid
Ts65Dn

E

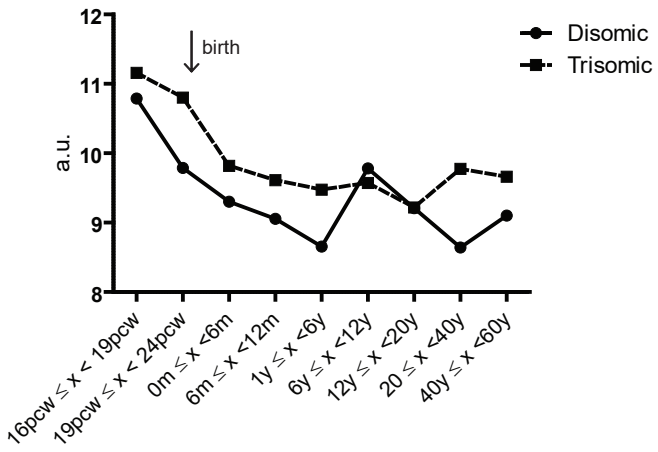

birth
Disomic
Trisomic
a.u.
8
9
10
11
12
16pcw ≤ x < 19pcw
19pcw ≤ x < 24pcw
0m ≤ x <6m
6m ≤ x <12m
1y ≤ x <6y
6y ≤ x <12y
12y ≤ x <20y
20 ≤ x <40y
40y ≤ x <60y

FIG.5

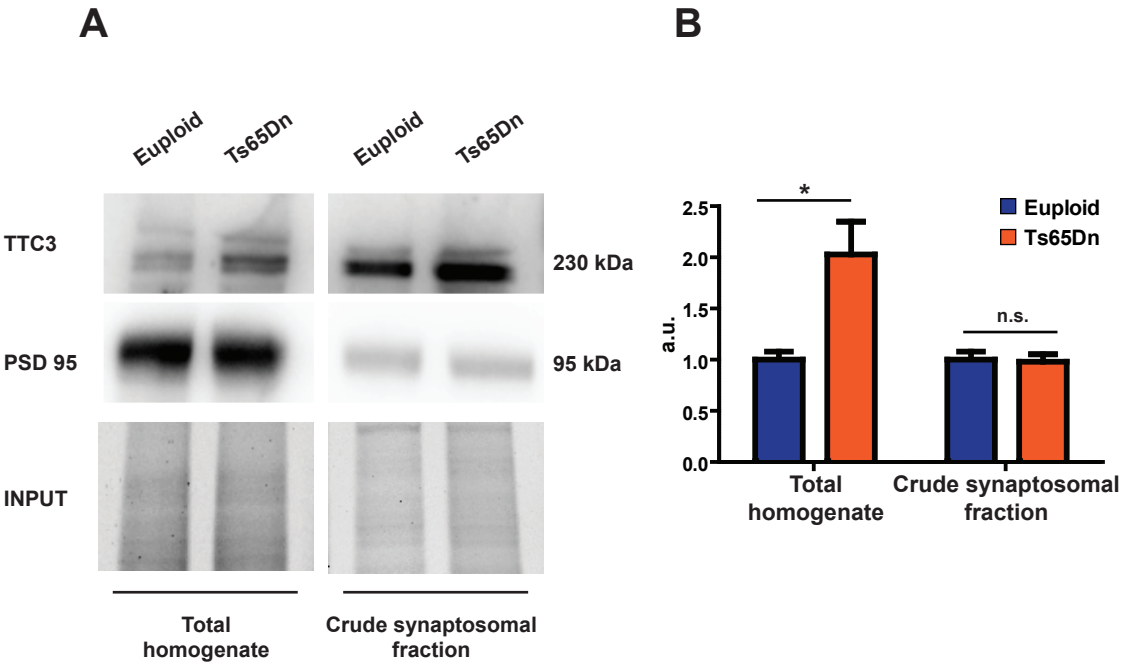
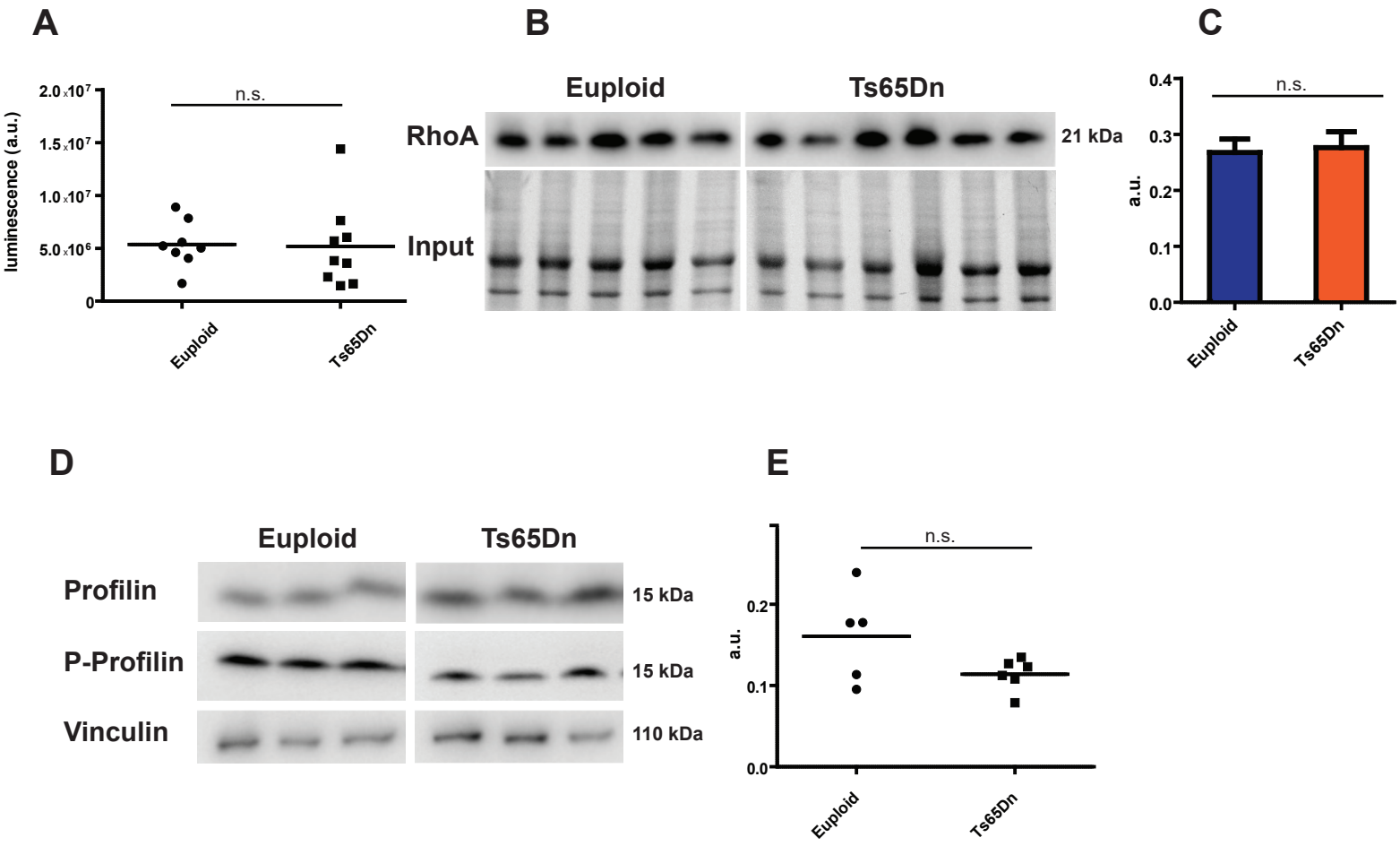

A
Euploid
Ts65Dn
Euploid
Ts65Dn
TTC3
PSD 95
INPUT
230 kDa
95 kDa
Total homogenate
Crude synaptosomal fraction
B
*
n.s.
Euploid
Ts65Dn
a.u.
2.5
2.0
1.5
1.0
0.5
0.0
Total homogenate
Crude synaptosomal fraction

FIG.6

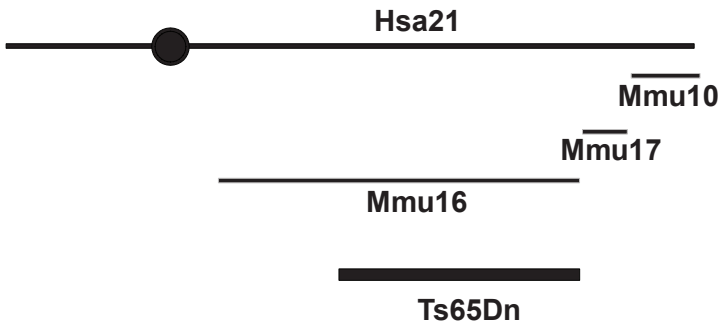


F

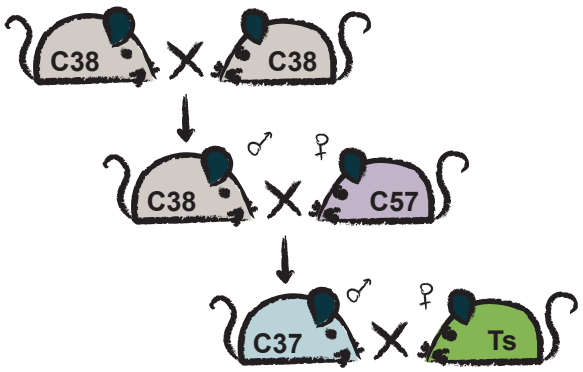
Gene	Extended name	PMID	Chromosome
AKT2	AKT serine/ threonine kinase 2	208	19q13.2
FBOX7	F-box protein 7	25793	22q12.3
SPTAN1	Spectrin alpha, non-erythrocytic 1	6709	9q34.11
WAC-1	WW containing ada-ptor with coiled-coil	51322	10p12.1
HERC2	HECT and RLD domain containing E3 ubiquitin protein ligase 2	8924	15q13.1
NUFIP1	FMR1 interacting protein 1	26747	13q14.12

SUPPL. FIG.1

A



B



C

STRAIN	JAX LAB ID	ABBREVIATION
B6EiC3Sn.BLiA-Ts(1716)65Dn/DnJ	*005252*	*Ts65Dn (or Ts)*
B6EiC3Sn.BLiA-F1/J	*003647*	*C37*
C3Sn.BLiA-Pde6b+/DnJ	*003648*	*C38*
C57BL/6J	*000664*	*C57*