# The TRRAP-HAT-Sp1 axis maintains brain homeostasis

by **Bo-Kun Yin**

born on **Apr.15th, 1992** in **Beijing, China**

To my dearest parents,

to my dearest brother,

and to my most respectful teacher, Professor Wang Zhao-Qi


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## Abstract in English

The homeostasis of the brain is tightly controlled by the viability and functionality of various cell types, including neurons and glial cells, like oligodendrocytes, astrocytes as well as microglia. Defects of neurogenesis and maintenance of neural cells are associated with multiple neuropathologies, such as Intellectual Disability (ID) and Autism Spectrum Disorders (ASD) among other diseases. HAT and HDAC modulate brain functionality, e.g. memory formation, cognitive function, and neuroprotection, whereas the disturbance of the acetylation profiles has been related to multiple neuropathological diseases. However, how epigenetic regulation participates in the neurodevelopmental, neural differentiation and neurodegenerative processes remains largely unknown. In our studies, we have chosen the HAT adaptor, Trrap, to investigate how the disturbance of acetylation would affect brain functionality. We show that Trrap deletion in post-mitotic neurons results in neurodegeneration. In addition, Trrap deficiency in adult neural stem cells compromises their self-renewal and differentiation. With integrated transcriptomics, epigenomics, and proteomics we identify Sp1 as the master regulator controlled by Trrap-HAT and demonstrate that the Trrap-HAT-Sp1 axis ensures the proper expression of genes involved in microtubule dynamics. We find that Trrap mediates Sp1 binding through the maintenance of the acetylation profile on Sp1 and that acetylation of Sp1 plays an important role, dependent and independent of Trrap, in its transcription activation. Taken together, we demonstrate that Trrap, through its mediated acetylation, is involved in neuroprotection and neural differentiation via the regulation of Sp1 activity. My dissertation provides a novel insight into the role of epigenetic regulation of transcription factors in the maintenance of brain homeostasis and preventing neurodegeneration.

## Abstrakt auf Deutsch

Die Homöostase des Gehirns wird streng durch die Lebensfähigkeit und Funktionalität verschiedener Zelltypen, einschließlich Neuronen und Gliazellen, wie Oligodendrozyten, Astrozyten sowie Mikroglia, kontrolliert. Defekte der Neurogenese und Aufrechterhaltung von Nervenzellen sind mit mehreren Neuropathologien verbunden, wie z. B. geistiger Behinderung (ID) und Autismus-Spektrum-Störungen (ASD). HAT und HDAC modulieren die Gehirnfunktion, z. B. Gedächtnisbildung, kognitive Funktion und Neuroprotektion, während die Störung der Acetylierungsprofile im Gehirn mit mehreren neuropathologischen Erkrankungen in Verbindung gebracht wurde. Wie die epigenetische Regulation an neurologischen Entwicklungs-, neuronalen Differenzierungs- und neurodegenerativen Prozessen beteiligt ist, bleibt jedoch weitgehend unbekannt. In unseren Studien haben wir den HAT-Adapter Trrap ausgewählt, um zu untersuchen, wie sich die Störung der Acetylierung auf die Gehirnfunktion auswirkt. Wir zeigen, dass die Trrap-Deletion in postmitotischen Neuronen zu einer Neurodegeneration führt. Darüber hinaus beeinträchtigt ein Trrap-Mangel in adulten neuralen Stammzellen deren Selbsterneuerung und Differenzierung. Mit integrierter Transkriptomik, Epigenomik und Proteomik identifizieren wir Sp1 als den Hauptregulator, der von Trrap-HAT kontrolliert wird, und zeigen, dass die Trrap-HAT-Sp1-Achse die richtige Expression von Genen gewährleistet, die an der Mikrotubuli-Dynamik beteiligt sind. Wir finden, dass Trrap die Sp1-Chromatin-Bindung durch die Aufrechterhaltung des Acetylierungsprofils auf Sp1 vermittelt und dass die Acetylierung an Sp1 eine wichtige Rolle, entweder abhängig oder unabhängig von Trrap, bei seiner Transkriptionsaktivierung spielt. Zusammengenommen zeigen wir, dass Trrap durch seine vermittelte Acetylierung an der Neuroprotektion und neuronalen Differenzierung über die Regulation der Sp1-Aktivität beteiligt ist. Meine Dissertation bietet einen neuartigen Einblick in die Rolle der epigenetischen Regulation von Transkriptionsfaktoren bei der Aufrechterhaltung der Homöostase des Gehirns und der Verhinderung von Neurodegeneration.

# 1. Introduction

## 1.1 The brain, embryonic, and adult neurogenesis

The expansion and elaboration of the cerebral neocortex enable humans to perform complex activities and to have higher cognitive functions during evolution [1]. Considered to be the smartest creature in the world [2], the human brain contains $10^{11}$-$10^{12}$ neurons and at least double the number of glial cells [3]. Neuronal and glial cells orchestrate the actions from simple tasks to complicate activities in a synchronized manner [4]. Newborn neurons derive from neural stem cells (NSCs) during the process termed neurogenesis. Locating at the central nervous system (CNS), NSCs give rise to neurons and glial cells in multiple steps [5]. During the embryonic stage in mammals, embryonic neural stem cells (eNSC) in the ventricular zone (VZ) of the neural tube generates a series of cell types required for CNS construction [6]. eNSCs in the VZ maintain the stem cell pool by self-renewal and gave rise to progenitor cells, which then migrate to the subventricular zone (SVZ) for limited proliferation and generation of newborn neurons [7] (Fig I).

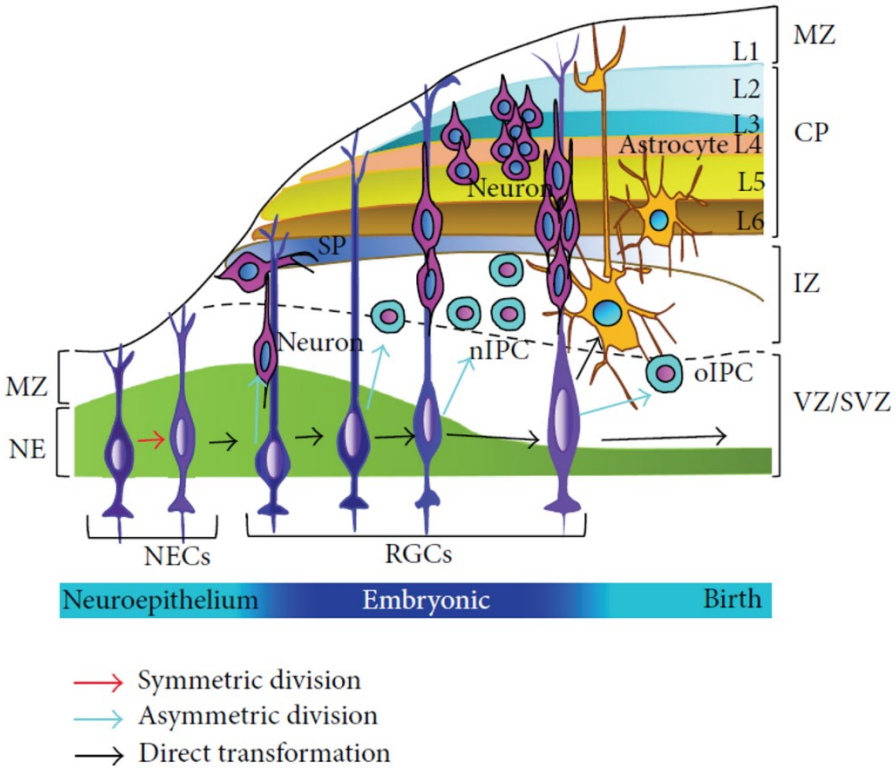


Fig I: Illustration showing the process of mammalian embryogenesis. Beginning with the neuroepithelial cells (NECs, or NSCs) symmetric division and conversion to radial glial cells (RGCs, neuroprogenitors) through its scratching, different cell types are generated through asymmetric division of RGCs. These include radial glial cells, neurogenic intermediate progenitor cells (nIPC), oligogenic intermediate progenitor cells (oIPC), neurons, and astrocytes. The RGCs represent the eNSCs mentioned in the text. MZ: Mantle layers; NE: Neuroepithelium; SP: Subplate; CP: Cortical plate; L1–6: Layers 1–6; IZ: Intermediate Zone. Graph originated from [8].

Neurogenesis in the mammalian adult brain mainly occurs in two regions: the dentate gyrus (DG) located in the subgranular zone (SGZ) in the hippocampus and the subventricular zone (SVZ) of the lateral ventricle (LV) [9]. This so-called adult neurogenesis is performed by adult neural stem cells (aNSCs) (Fig II).

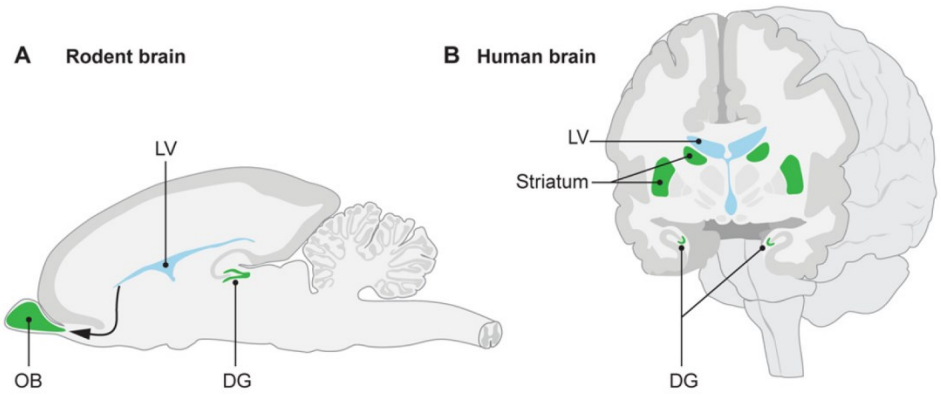



Figure II: Illustration presenting neurogenesis in the adult rodent and human brain. Green areas show the location of newborn neurons and blue areas indicate the aNSC in LV. (A) In rodents' DG, newborn neurons were generated by hippocampal aNSC. aNSCs that are generated in the LV migrate to the olfactory bulb (OB), where they produce interneurons. (B) In humans, aNSCs in DG give rise to newborn neurons like rodents. aNSC in LV generates neurons integrating into the adjacent striatum. Graph originated from [10].

Like eNSCs, aNSCs harbor self-renew capacity and are able to differentiate to produce multiple cell lineages, e.g. neurons, astrocytes, and oligodendrocytes (Fig III).

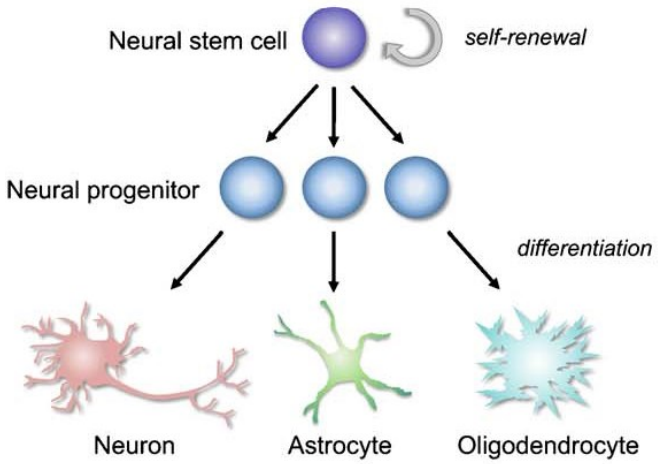



Figure III: Schematic illustration presenting the fate decision of aNSC. aNSCs can proliferate and give rise to neural progenitor cells, which then produce specific cell types of the brain: neurons, astrocytes, and oligodendrocytes. Graph originated from [11].

The hippocampal neurogenesis in the human adult brain is related to multiple brain functions, for instance, memory consolidation, emotional function, and social behavior [12]. The defect of aNSC niche maintenance and neural differentiation has been shown to diminish the functional integrity of both adult human and adult mouse brains [13]. In rodents, impairment of adult hippocampal neurogenesis has been related to the etiology of ID and/or ASD as well [14-16].

Fragile-X Syndrome (FXS) is considered to be the most common form of inherited ID and also a very important genetic cause of ASD. Hippocampal volume changes [17, 18] and impaired hippocampal function were observed in FXS patients [19, 20]. FXS mouse model also shows the abnormal hippocampal function associated with aNSC proliferation deficiency and impaired hippocampal neurogenesis [21-23]. Molecularly, FXS results most often from the loss of fragile X mental retardation protein, which then leads to increased MDM2 levels and reduced P53 levels in NSCs. In FXS mouse models, the neurogenic and cognitive deficits were successfully rescued through the administration of Nutlin-3, a clinical drug activating p53 by antagonizing MDM2 [15, 24]. These studies based on mouse models demonstrate the importance of proper adult hippocampal neurogenesis in preventing ID/ASD.

Another neurogenic region in the adult brain, the SVZ, has shown altered cytoarchitecture in autism patients. The patients had a dramatic decline of cell density

in an age-dependent manner in the septal SVZ hypocellular gap, indicative of an adult neurogenesis impairment [25]. A similar observation has been confirmed in ASD/ID mice with defective adult neurogenesis in SVZ [26-28]. These studies associate the impairment of SVZ adult neurogenesis with the etiology of ASD/ID.

### 1.2 Neurodevelopmental disorders (NDD)

The development of the brain is highly dependent on variable genetic and environmental factors. An aberration of these factors could cause NDD [29]. With over 2000 human genes affected, ID and ASD are two of the major neurodevelopmental disorders. An individual with ID is characterized by a congenital deficit in intellectual function and adaptive behavior reflected by disrupted social interaction and mental and practical abilities [30-32]. A significant cohort of ID patients also possesses peculiar facies, specific physical signs, and/or an abnormal growth pattern, classified as “syndromic” ID patients [33-35], whereas the patients without malformations are diagnosed with “non-syndromic” ID [36-38]. ASD patients also have a disruption in social interactions and communication like ID patients. Additionally, many of them are characterized by repetitive behaviors and/or limited interests [39-41]. Many children with ID also possess autistic actions, which implies the commonality of a deficient neuron population responsible for ID and ASD occurrence [4]. Studies have suggested aberrant microglia and astrocyte immune activation as a hallmark in ASD patients [42]. Moreover, the impairment of adult neurogenesis has been linked to the occurrence of ASD/ID [14-16, 25-28]. Deficits in the interneuron characterized by a reduced cell number, reduction in intrinsic cellular excitability, or weaker synaptic connectivity, have been shown in the ASD mouse model [43].

### 1.3 Neurodegenerative disease (ND)

Apart from NDD, the proper functionality of the brain could be also affected by another category of vital diseases, neurodegenerative diseases (ND). The ND describes a wide range of pathological conditions which is strongly associated with

aging [44]. One of the NDs, Alzheimer's disease (AD), and its related neurodegenerative conditions have become one of the most crucial clinical burdens affecting the elderly [44]. Common NDs include AD, Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), which are characterized by the progressive loss of specific neuronal cell populations [3]. Neurodegenerative hallmarks include progressive neuronal loss [45], synaptic dysfunction, disturbed circuits in certain neural subpopulations, and abnormal network activities [46]. Multiple cellular pathways are disrupted in neurodegenerative diseases, and these include protein quality control [47], the autophagy-lysosome pathway [48], mitochondria homeostasis [49], and synaptic toxicity and network dysfunction [50, 51].

### 1.4 Lysine acetylation and HAT

The chromatin dynamics can be modulated through diverse post-translational modifications, including phosphorylation [52], methylation [53], acetylation [54], SUMOylation [55], ADP-ribosylation [56], UFMylation [57], serotonylation [58]. Lysine acetylation can be catalyzed on histone tails, leading to a relaxed chromatin structure that grants accessibility of transcription factors and transcription machinery to the promoters of genes [59]. Lysine acetylation is mainly carried out by histone acetyltransferase (HAT or KAT, thereafter refer to HAT). Reversibly, the lysine residue on acetylated protein can be removed, namely deacetylated, by histone deacetylases (HDAC or KDAC, thereafter refer to HDAC). HAT complexes consist of various subunits, including HATs, HAT adaptors, interactors of the transcription machinery, and also other post-translational modification enzymes, e.g., for histone deubiquitination [60]. Currently, the biological function of those subunits still remains largely unknown, but it is believed that adaptors may dictate the function of individual HAT complexes.

### 1.4.1 Role of lysine acetylation on neuropathology

Epigenetic processes, such as DNA methylation and histone modification, have been shown to maintain proper brain development, whereas the misregulation of which is related to multiple neurological disorders, including ASD, ID, as well as NDs like AD, Huntington's disease, and amyotrophic lateral sclerosis [61-64]. Notably, lysine acetylation has been shown to play an important role in brain functionality, including memory formation and neuroprotection [63, 65, 66]. The disturbance of acetylation homeostasis is involved in the etiology of multiple neuropathological diseases, for instance, Huntington's disease [67], Parkinson's disease [68], and Alzheimer's disease [69]. In clinics, HDAC inhibitors (HDACi) or HAT activators are employed to treat not only the neurological symptoms of neurodegenerative diseases and psychiatric disorders but also autism and impairment of memory and cognitive function [62, 66, 70, 71]. These applications emphasize the role of HAT in the etiology of ND. However, despite mounting evidence showing that histone acetylation/deacetylation is involved in neurodegenerative disorders [72, 73], their role in adult brain homeostasis remains to be discovered.

## 1.5 TRRAP is a member of the PIKK family

The phosphatidylinositol 3-kinase-related kinases (PIKK) family consists of six Serine/Threonine protein kinases, which have a structural commonality and similar identity in protein domains. Each PIKK possesses the HEAT repeat, FAT domain, and FATC motif (Fig IV). Yet, the function of PIKKs is very diverse. DNA-PKcs, ATM, and ATR are involved in the DNA damage response (DDR) and DNA repair [74]. mTOR is involved the cellular metabolism by acquiring nutrients from the environment and intracellular sources, which then induces ATP production [75]. SMG1 is an important factor in mRNA decay, which reduces the accumulation of toxic RNA species with premature stop codon or aberrant 3'-UTR [76]. TRRAP, abbreviated as Transformation and transcription-associated protein, is a HAT scaffold protein and an essential coactivator in gene transcription [77, 78].


Fig IV: The entire PIKK family and one PI3K member. Different protein domains are labeled by the indicated colors. PIK3C3 belongs to phosphoinositide 3-kinases (PI3K), which serves as a comparison to the PIKK family. The black numbers show the amino acid sequence of respective domains, whereas blue numbers around TRRAP indicate the TRRAP-interacting region with p53, c-Myc, and LXR. The dashed line on the C terminal indicates the N-terminal border of the PIK-like/PIK domain. Graph originated from [59] (Manuscript II).

Initially, TRRAP was discovered as an essential coactivator of c-Myc and E2F1 [79]. Unlike other PIKKs, TRRAP does not have enzymatic activity due to the lack of three motifs in the kinase domain for PIKK kinase activity: The ATP-binding motif VAIK, the catalytic motif HRD, and the divalent cation-binding motif [79, 80]. Interestingly, TRRAP is shown to be highly conserved through the whole eukaryotic clades according to phylogenetic analysis and is also considered the ancestral member of all PIKKs [81]. Studies have identified TRRAP and its orthologs Tra1/Tra2 as adaptors for HAT enzymes. TRRAP interacts with two major HAT families: the general control non-derepressible 5 (GCN5)-related N-acetyltransferase (GNAT) HAT family (including Gcn5 and PCAF) and the MOZ, Ybf2/Sas3, Sas2, Tip60-related (MYST) HAT family (including TIP60) [78]. In Spt-Ada-Gcn5 acetyltransferase complex (Abbreviated as SAGA both in yeasts and mammals) and the nucleosome acetyltransferase of H4 complex (Abbreviated as NuA4 in yeasts and TIP60 in mammals), TRRAP is a common subunit within these megamolecular complexes [81]. Functionally, SAGA mainly catalyzes the acetylation of H3, whereas the NuA4 complex is responsible for the H4 acetylation [82-84]. Although the biochemical function of each subunit within the HAT complexes remains largely elusive, TRRAP/Tra1 seems to be the only subunit

interacting with transcription activator, like Tra1 with Gal4 and Gcn4p in yeast, and also the only shared component among different HAT complexes, for instance within SAGA and TIP60 complexes [85, 86]. These indicate a non-redundant role of TRRAP among different HAT complexes in different species.

### 1.5.1 Role of TRRAP in transcription

The study in the 1960s revealed that actively transcribed genes possessed hyperacetylated histones [87]. Acetylated histones have been identified as transcription factor docking sites [88-91]. The HAT adaptor TRRAP also interacts with multiple variable transcription factors during the transcription [79, 92-96]. Therefore, a plethora of studies has focused on the role of TRRAP-associated HAT activity and its mediated transcription activation: TRRAP knockdown inhibits the oncogenic transformation by c-Myc- and E1A, showing that TRRAP is a coactivator of c-Myc and E2F [79]. TRRAP also activates mdm2 transcription by recruiting p53 and promoting histone acetylation on the mdm2 promoter [92]. LXRα, a transcription factor regulating lipid metabolism, is co-activated by TRRAP, which then leads to the expression of its targets genes, including ATP-binding cassette transporter A1 and G1 (ABCA1 and ABCG1), stearoyl-CoA desaturase (SCD), and high density lipoprotein binding protein (HBP) [96]. TRRAP-TIP60 complex activates the transcription of histone H2B and H4 mediated by NPAT during the G1/S-phase transition [97]. TRRAP-TIP60 also co-activates the expression of the mitotic gene Top2A, which then promotes the proliferation of hepatocellular carcinoma cells [98]. Studies based on mouse models have also demonstrated the role of Trrap in the transcription activation of specific genes. In mouse embryonic fibroblasts (MEFs) Trrap-deletion abolished the HAT binding on the promoter of Mad1 and Mad2, and thus lowers their transcription [99]. In Trrap-deleted murine embryonic stem cells (ESCs), the active chromatin markers (AcH4, H3K4me2) on stemness genes Nanog, Oct4, and Sox2 was comparably lower than control cells, which is in accordance with a decreased transcription of these genes [100]. In embryonic neural stem cells (eNSCs), Trrap deletion abolished the binding of

E2F1 and HAT on the promoter of cell cycle regulators Cdc25A, Mad2, CycA2, and Top2A, which decreased the acetylation of the histone and thus the expression of these genes [101]. As a summary of the studies mentioned, TRRAP recruits HAT and transcription factors to target promoters on chromatin, leading to histone hyperacetylation and the subsequent transcription of the target genes.

### 1.5.2 The biological function of TRRAP in cellular and animal models

A plethora of studies revealed that Trrap is involved in different cellular processes, ranging from cell cycle progression, cell stemness maintenance, and differentiation, to brain development and neuroprotection [99-103]. Tra1 deletion in S. cerevisiae resulted in cell lethality [104]. The tra1 deletion in the fission yeast was viable, possibly due to the compensatory effect of tra2, which was formed by the duplication of tra1 [80, 105]. In mammals, Trrap is also essential for cell viability. Trrap null blastocysts had proliferation defects due to mitotic checkpoint catastrophe, causing peri-implantation lethality of mouse embryos, indicating that Trrap is needed for mouse embryonic development [102]. Trrap knockout in MEFs leads to variable cell cycle defects, including chromosome missegregation, mitotic exit failure, and compromised mitotic checkpoint [99]. In these cells, the chromatin-binding of TIP60 and PCAF at the promoters of Mad1 and Mad2 genes is abolished, decreasing the level of mitotic checkpoint protein Mad1 and Mad2 [99]. These studies demonstrated the involvement of TRRAP in mitosis progression. In embryonic stem (ES) cells, Trrap deficiency results in unscheduled differentiation [100], likely through regulating the expression of the stemness marker genes, Nanog, Oct4 and Sox2, showing the importance of TRRAP on the stemness and differentiation of ESCs [100]. In hematopoietic stem cells (HSCs), Trrap deletion causes apoptosis [106], indicative of the role of Trrap in HSCs maintenance, as well as in the homeostasis of the hematopoietic system. In murine CNS, a variety of HAT or HDAC mutations have been related to neurological dysfunction and/or brain developmental defects [107-109]. Trrap-HAT-mediated histone acetylation affects brain development by regulating the expression of cell cycle

factors and proteins involved in NSC differentiation [101]. During embryonic neurogenesis, Trrap is necessary for the proper differentiation of apical neuroprogenitors to basal progenitors and neurons, whereas Trrap deletion lengthens the cell cycle of apical neuroprogenitor cells, leading to the premature differentiation of neural progenitors and thus malformation of brain structure [101].

Clinical studies have identified 83 individuals with TRRAP variants [110-112]. In one of those studies, individuals aged between 2 and 29 years possessed 17 distinct TRRAP variants [110]. All of these 24 individuals were diagnosed with developmental delay and most of them have a malformation of diverse organs, including the brain, cerebellum, heart, kidney, or urogenital malformations. Intriguingly, although about half of them do not have an obvious malformation of brain architecture, these patients were characterized with ASD and/or ID with variable severity. These clinical reports highlight the importance of TRRAP in organ development and brain homeostasis.

### 1.6 Sp1 is a ubiquitous transcription factor

Sp1 expresses ubiquitously in all mammalian cells [113]. Originally, the protein was termed based on its purification method, the sephacryl and phosphocellulose columns, yet later renamed after specificity protein 1 [114, 115]. As one of the first cloned mammalian transcription factors, Sp1 was identified as a specific activator at the SV40 early promoter, which binds directly at the 70-110 bp upstream of the transcription initiation site, namely the GC-rich “tandem 21 bp repeats” [116]. Sp1 was identified to regulate housekeeping genes [117], yet mounting evidences indicate its tissue-specific role in transcription [118]. One of the most studied functions of Sp1 is the transcription in tumorigenesis [119]. Overexpressed Sp1 has been detected in multiple cancer types, including human glioma, breast cancer, gastric cancer, pancreatic ductal adenocarcinoma, and thyroid tumors [120-124]. Among the hallmarks of cancer progression, many of them are Sp1 targets, for instance, Bcl-2 (Promoting apoptosis) [125, 126], TSP-1 (Involved in angiogenesis) [127], and MMP9 (Supporting metastasis) [128, 129]. Sp1 has been linked to both activation and

suppression of the expression of several essential oncogenes and tumor suppressors. Therefore, Sp1 has been strongly linked with a plethora of cancer research.

### 1.6.1 The structure of the Sp1 protein

As a 785aa protein with 105 kD [115, 130], Sp1 contains three protein domains: The Sp box on the N-terminus, the Buttonhead Domain (BTD), and the Zinc finger domain on the C-terminus [113]. The Sp box is considered to lead to Sp1 degradation due to the presence of the endoproteolytic cleavage site inside this domain [131, 132]. Studies on the BTD domain considered that it promotes the Sp1 transactivation [133, 134], yet another study in Drosophila showed that the BTD domain is not necessary for the expression of the Sp1 targets and the normal organism development [135]. The Zinc finger domain is also a DNA binding domain, which consists of three adjacent Cys2His2-type zinc finger motifs and recognizes the GC boxes (GGGGCGGGG) and GT/CACC boxes (GGTGTGGGG) of the genomic sequence [114, 136-138]. Another Sp1 domain definition is based on the transcriptional activity of diverse Sp1 truncation mutants [133]: Transactivation domains A, B, C, D. Domains A, and B are serine/threonine- and glutamine-rich, which are responsible for the major transcriptional activity of Sp1 [139]. Domain C mediates the DNA binding of Sp1 and domain D supports the Sp1 multimerization, both of which promote Sp1 activity[133]. (Fig V)

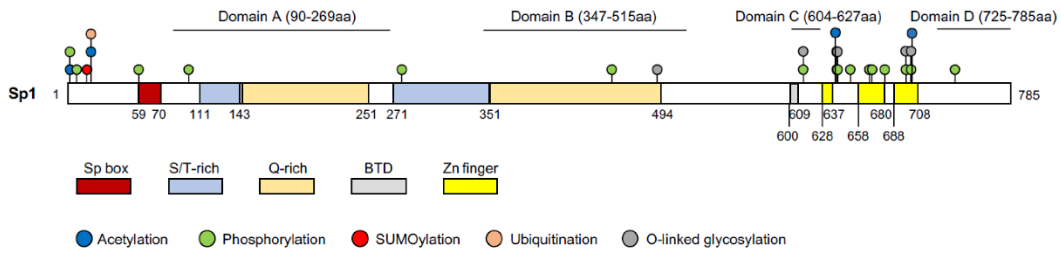



Fig V: Sp1 structure with activation domains and post-translational modifications. The domains are marked with the respective color inside Sp1. Domains A, B, C, and D are transactivation domains required for Sp1 activity. color-Coded circles indicate the post-translational modifications, among which the characterized acetylation sites are marked with blue circles. Numbers under the protein indicate the position of the amino acids at the domain border. S/T-rich: serine/threonine-rich domain; Q-Rich:

glutamine-rich domain; BTD: Buttonhead domain; Zn finger: Cys2His2-type zinc finger domain. Graph originated from [59] (Manuscript II).

### 1.6.2 Transcription initiation and transactivation by Sp1

Sp1 plays an important role in transcription initiation. It specifically interacts with the general human TBP-associated factors II 130 (hTAFII130), which is a subunit within the general transcription factor IID (TFIID) complex [140, 141]. The TFIID complex then initiates the formation of the pre-initiation complex and induces transcription. Moreover, Sp1 also interacts with TATA-binding protein (TBP) in the TFIID complex through the glutamine-rich transactivation domain [142, 143], implying the involvement of Sp1 in the transcription initiation. Particularly, Sp1 also interacts with other transcription factors, including E2F1, AP2, Oct-1, and Sp1 itself [144-147], to express its transcriptional activity in a greater-than-additive way. This effect is termed synergy in transcription. On one hand, Sp1 forms multimers through interaction with other activators (including Sp1 itself) via the transactivation domains A, B, and D to elevate its transcriptional activity [148-150]. This conclusion is driven by the observation that overexpression of truncated Sp1 lacking the DNA-binding domain still increases the transcription activity on the full-length Sp1 [148, 149]. On the other hand, the artificial addition of the Sp1-binding site also increases its transcription activity [148]. Furthermore, an additional Sp1-binding site located in the enhancer regions also promotes the transcription activity of target genes [149], implying that Sp1 functions also in an enhancer-dependent manner.

### 1.6.3 Regulation of Sp1 activity via post-translational modifications (PTMs)

The activity of Sp1 is regulated by variable PTMs, including phosphorylation [151], acetylation [152], ubiquitination [153], and SUMOylation [154]. Phosphorylation either increased or decreased the transcriptional activity of Sp1. In mammalian cells, ERK1/2 phosphorylated Sp1 on Thr453 and Thr739 after FGF2 treatment, repressing the expression of its target gene PDGFRα [155]. In connective tissues, phosphorylation on Sp1 by p38 induces the filamin-A expression [156]. Comparably, there are relatively

fewer studies explaining the role of acetylation on Sp1 activity. [157]. Currently, several acetylation residues on Sp1 have been identified: K703 [157], K639, K624, K685, K693. [158] and K19 [159], with K703 most studied. PCAF, p300, and TIP60 are known HATs to conduct acetylation on Sp1 [157, 158, 160]. As an example, p300 facilitated Sp1-chromatin binding through its interaction with Sp1 [152, 161], yet Sp1 acetylation conducted by p300 did not alter Sp1-chromatin binding on DNA [152]. TIP60 acetylated Sp1 at K639, thus impairing the Sp1-chromatin binding on the target promoter and thereby repressing its activity [158]. To date, it is not known whether Sp1 acetylation affects Sp1 stability. Acetylome study has claimed that both acetylation and ubiquitination can emerge on the same residue (K19) [159]. This implied that acetylation might stabilize Sp1 by competing against proteasomal degradation mediated by ubiquitin, as described in a similar study [162]. In this regard, SUMOylation on K16 led to proteasomal degradation of Sp1 [163], suggesting that PTMs, such as acetylation, ubiquitination, and SUMOylation and their crosstalk, on specific residues might influence the stability of Sp1.

### 1.6.4 Role of Sp1 in the nervous system and neuropathies

Sp1 regulates a plethora of target genes, most of which are involved in cell proliferation and tumorigenesis [118, 164, 165]. In addition, studies in the central nervous system (CNS) evidenced the role of Sp1 in the etiology of multiple neurological diseases, including neurodegeneration, and neural development. GWAS analysis implicated that Sp1 transcriptional activity is aberrant in AD and Parkinson’s disease (PD) patients [166]. In patients bearing Huntington's disease (HD), the mutation of huntingtin protein disrupted the interaction between Sp1 and its coactivator TAFII130, thus repressing the transcription of the dopamine D2 receptor, which is a hallmark of HD [167]. A study in the HD mouse model identified the zinc transporter 3 (ZnT3) as another Sp1 target [168], whose transcription is inhibited through mutant huntingtin. The down-regulation of ZnT3 further led to the loss of synaptic vesicular zinc molecule in the neurons located at the hippocampus, cortex, and striatum. These studies

highlight the involvement of Sp1 in HD pathology [168]. Studies on the neural models demonstrated that Sp1 regulates multiple neural genes by binding to their promoters, e.g. Slit2 [169], P2X7 [170], and Reelin [171]. Studies on the NSCs showed that Sp1 played an important role in the NSC differentiation process by affecting cell cycle factor cdkn1b [172]. To summarize the above, in addition to cancer progression, multiple studies have also linked the functionality of Sp1 with neurodegenerative diseases and neuronal maintenance.

### 1.7 Aim of the study

Neuronal functionality and survival play a fundamental role in adult brain homeostasis, whereas dysregulation of these processes leads to neurological deficits, including neurodegeneration. HATs/HDACs play important roles in regulating transcription; however, acetylation and deacetylation affect the functionality and the survival of adult neurons, as well as the adult neurogenesis process remains largely unknown. Functionally, TRRAP regulates gene expression via its scaffolding function on HATs and transcription factors in a wide range of cell types. TRRAP may also facilitate the acetylation of transcription factors, which directly alters the transcription factor activity and/or stability. Therefore, it is still possible that TRRAP-HAT can mediate PTMs of transcription factors to promote transcription in addition to its modification on chromatin.

In my thesis, I aim to investigate how the epigenetic regulator affects the homeostasis of the brain.

To address this question, I set up the following objectives for my study:

1. To examine the function of Trrap-HAT in preventing neurodegenerative progression and supporting proper neuron formation (Manuscript I).

2. To investigate how Trrap or Trrap-HAT regulates Sp1 in adult neurogenesis (Manuscript II, III).

## 2. Overview of the manuscripts

### 2.1 HAT cofactor TRRAP modulates microtubule dynamics via SP1 signaling to prevent neurodegeneration

Alicia Tapias†, David Lazaro†, Bo-Kun Yin†, Seyed Mohammad Mahdi Rasa, Anna Krepelova, Erika Kelmer Sacramento, Paulius Grigaravicius, Philipp Koch, Joanna Kirkpatrick, Alessandro Ori, Francesco Neri, Zhao-Qi Wang*

†: These authors contributed equally to this work

Published: February 17, 2021, in ***eLife***

Major aspects of the manuscript:

In this study, we demonstrated that the HAT adaptor Trrap maintains neuronal homeostasis and prevents neurodegeneration of post-mitotic Purkinje cells (PCs). Mouse-bearing Trrap deficiency in PCs showed impaired motor coordination and age-dependent neurodegeneration during adult and late life. Through the integration of transcriptomic, epigenomic, and proteomic data in the Trrap-deleted cortex, striatum, and *in vitro* cultured aNSCs, we have discovered Sp1 as the major regulator mediated by Trrap. We showed that the Trrap-HAT-Sp1 axis regulates the expression of Stathmins 3 and 4 (Stmn3, Stmn4), which are important modulators of microtubule dynamics. Trrap facilitated the hyperacetylation and Sp1 binding on the promoter of Stmn3 and Stmn4, thus promoting the transcription of Stmn3/4. Moreover, through *in vitro* neuronal studies we have proven that ectopic expression of Stmn3, and Stmn4 could rescue the neuronal defect in Trrap knockdown primary neurons.

**2.2 Beyond HAT Adaptor: TRRAP Liaisons with Sp1-Mediated Transcription**

Bo-Kun Yin and Zhao-Qi Wang

Published: 18 November 2021 in IJMS (in full)

Major aspects of the manuscript:

In this review, we summarized the current knowledge and cellular function of TRRAP in cell cycle control, cell stemness maintenance, and differentiation, as well as neural homeostasis. We also described the molecular mechanism of TRRAP-HAT in transcription regulation. Moreover, we reviewed the biological function of Sp1 and the post-translational modification of Sp1. Finally, we focused on the action mode of TRRAP and discussed how TRRAP-HAT regulates the transactivation of Sp1-governing biological processes, including neurodegeneration.

### 2.3 TRRAP-mediated acetylation on Sp1 regulates adult neurogenesis

Bo-Kun Yin, David Lazaro, and Zhao-Qi Wang

The manuscript is submitted to the *Computational and Structural Biotechnology Journal*

Major information of the manuscript:

In this manuscript, we investigated the role of Trrap in supporting adult neurogenesis. We deleted Trrap in aNSCs in mouse and cellular models and studied the proliferation and differentiation of aNSCs *in vivo* and *in vitro*. We found that Trrap-deficiency impairs the quiescence, expansion, and neural differentiation capacity of hippocampal aNSCs under Trrap-deletion *in vivo*. In addition, the decline of neuronal differentiation correlates with greatly increased astrocytes, indicative of differentiation defects of Trrap-deleted aNSCs. In consistence with the aNSC phenotypes observed *in vivo, in vitro* culture of aNSC also revealed impaired differentiation and proliferation of Trrap-deleted aNSCs. Trrap deficiency caused cell cycle arrest in the G2/M phase and compromised the differentiation capacity. We have also demonstrated that Trrap-mediated acetylation at K639 on Sp1 regulates Sp1 transactivation activity, which dictates the role of Sp1 in aNSC differentiation.

# 3. List of the manuscripts

### 3.1 Manuscript I

#### 3.1.1 Author contribution:

**Title:** HAT cofactor TRRAP modulates microtubule dynamics via SP1 signaling to prevent neurodegeneration

**Authors:** Alicia Tapias, David Lazaro, Bo-Kun Yin, Seyed Mohammad Mahdi Rasa, Anna Krepelova, Erika Kelmer Sacramento, Paulius Grigaravicius, Philipp Koch, Joanna Kirkpatrick, Alessandro Ori, Francesco Neri, Zhao-Qi Wang

**Bibliographic information** (if published or accepted for publication: Citation):

Chromosomes and Gene Expression Neuroscience

**The candidate is** (Please tick the appropriate box.)

☐ First author, x Co-first author, ☐ Corresponding author, ☐ Co-author.

**Status** (if not published; "submitted for publication", "in preparation".): Published

**Authors' contributions (in %) to the given categories of the publication**

| Author | Conceptual | Data analysis | Experimental | Writing the manuscript | Provision of material |
|---|---|---|---|---|---|
| Bo-Kun Yin | 20% | 20% | 30% | 10% | 10% |
| Alicia Tapias | 30% | 40% | 30% | 50% | 60% |
| David Lazaro | 20% | 15% | 10% | 10% | 30% |
| Others | 30% | 25% | 30% | 30% | |
| Total: | 100% | 100% | 100% | 100% | 100% |

Contribution of authors to the manuscript:

Bo-Kun Yin: Confirm the Sp1 activity after Trrap deletion with luciferase assay in aNSC; validating the ChIP-seq through ChIP analysis in aNSC; confirming Sp1 regulates STMN3/4 expression by detecting STMN3/4 level in tissues and Sp1-knocked down aNSC; establishment *in vitro* culture of primary neurons and knock

down Trrap with siRNA; construction of STMN3/4 plasmid and transfect them into primary neurons; validating the neuronal defect through immunofluorescence and IncuCyte assay; investigation; writing - original draft; writing - review and editing.

Alicia Tapias: Conceptualization of the story; establishment of the Trrap-PCΔ mouse line and study of the motor coordination of these mice; validating the PC degeneration and defect through immunofluorescence and Sholl analysis; establishment of the Trrap-FBΔ mouse line; performing RNA-seq, proteomics; analysis of the transcriptomic, ChIP-seq, and proteomic data; confirming the expression of STMN3/4 by qPCR; writing - original draft

David Lazaro: Conceptualization of the story; validating the Trrap-FBΔ; establishment of the Trrap-aNSCΔ mouse line; ChIP-seq analysis on mutant aNSC and analyze the data; investigation; Writing - original draft

Seyed Mohammad Mahdi Rasa: Analysis of the ChIP-seq data

Anna Krepelova: Supporting the ChIP technical requirement

Erika Kelmer Sacramento: Formal analysis; investigation

Paulius Grigaravicius: Formal analysis; investigation; methodology

Philipp Koch: Formal analysis

Joanna Kirkpatrick: Data curation; formal analysis; investigation; methodology

Alessandro Ori: Conceptualization; investigation; methodology

Francesco Neri: Conceptualization; data curation; formal analysis

Zhao-Qi Wang: Conceptualization; data curation; supervision; funding acquisition; investigation; writing - original draft; project administration; writing - review and editing

### 3.1.2 Manuscript

# HAT cofactor TRRAP modulates microtubule dynamics via SP1 signaling to prevent neurodegeneration

**Alicia Tapias[1†], David Lázaro[1†], Bo-Kun Yin[1†], Seyed Mohammad Mahdi Rasa[1], Anna Krepelova[1], Erika Kelmer Sacramento[1], Paulius Grigaravicius[1], Philipp Koch[1], Joanna Kirkpatrick[1], Alessandro Ori[1], Francesco Neri[1], Zhao-Qi Wang[1,2*]**

[1]Leibniz Institute on Aging – Fritz Lipmann Institute (FLI), Jena, Germany; [2]Faculty of Biological Sciences, Friedrich-Schiller-University of Jena, Jena, Germany

**Abstract** Brain homeostasis is regulated by the viability and functionality of neurons. HAT (histone acetyltransferase) and HDAC (histone deacetylase) inhibitors have been applied to treat neurological deficits in humans; yet, the epigenetic regulation in neurodegeneration remains elusive. Mutations of HAT cofactor TRRAP (transformation/transcription domain-associated protein) cause human neuropathies, including psychosis, intellectual disability, autism, and epilepsy, with unknown mechanism. Here we show that Trrap deletion in Purkinje neurons results in neurodegeneration of old mice. Integrated transcriptomics, epigenomics, and proteomics reveal that TRRAP via SP1 conducts a conserved transcriptomic program. TRRAP is required for SP1 binding at the promoter proximity of target genes, especially microtubule dynamics. The ectopic expression of Stathmin3/4 ameliorates defects of TRRAP-deficient neurons, indicating that the microtubule dynamics is particularly vulnerable to the action of SP1 activity. This study unravels a network linking three well-known, but up-to-date unconnected, signaling pathways, namely TRRAP, HAT, and SP1 with microtubule dynamics, in neuroprotection.

## Introduction

Neurodegenerative diseases are a range of incurable and debilitating conditions strongly linked with age, which represent a social and economic burden given the burgeoning elderly population. The key features of the brain are its adaptability and plasticity, which facilitate rapid, coordinated responses to changes in the environment, all of which require delicate brain functionality and maintenance. Progressive neuronal loss, synaptic deficits, disintegration of neuronal networks due to axonal and dendritic retraction, and failure of neurological functions are hallmarks of neurodegeneration (***Palop et al., 2006***; ***Gan et al., 2018***). Molecular causes of neurodegeneration are believed to include protein misfolding and degradation, neuroinflammation, oxidative stress, DNA damage accumulation, mitochondrial dysfunction, as well as programmed cell death (***Palop et al., 2006***; ***Gan et al., 2018***; ***Kurtishi et al., 2019***; ***Burté et al., 2015***; ***Chi et al., 2018***).

Epigenetic mechanisms, including DNA methylation, histone modifications, non-coding or small RNAs, have been linked to brain development and neurological disorders, such as autism, intellectual disability (ID), and epilepsy, as well as neurodegenerative processes (***Meaney and Ferguson-Smith, 2010***; ***Berson et al., 2018***; ***Christopher et al., 2017***; ***Tapias and Wang, 2017***). Histone acetylation, which is modulated by a range of histone acetyltransferase (HAT) families, is a major epigenetic modification controlling a wide range of cellular processes (***Tapias and Wang, 2017***; ***Choudhary et al., 2014***). HATs and histone deacetylases (HDACs) maintain a proper acetylation kinetics of histones, yet also other protein substrates, which can coordinate histone dynamics over

large regions of chromatin to regulate the global gene expression as well as target gene-specific regions or promoters (**Vogelauer et al., 2000**; **Nagy and Tora, 2007**). HAT-HDAC-mediated histone modifications have been suggested to play a role in brain functionality, including memory formation, mood, drug addiction, and neuroprotection (**Meaney and Ferguson-Smith, 2010**; **Berson et al., 2018**; **Christopher et al., 2017**; **Delgado-Morales et al., 2017**; **Levenson and Sweatt, 2005**; **Renthal and Nestler, 2008**). For example, the pharmacological inhibition of HDACs has been used for their anti-epileptic, anti-convulsive, and mood-stabilizing effects (**Chiu et al., 2013**). In addition, HDAC inhibitors or HAT activators have been employed in clinics to treat the neurological symptoms of neurodegenerative diseases and psychiatric disorders, as well as autism, memory loss, and cognitive function, although not always successfully (**Christopher et al., 2017**; **Delgado-Morales et al., 2017**; **Selvi et al., 2010**; **Ganai et al., 2016**). Genome-wide approaches have revealed global and local changes in multiple histone marks; yet, the impact and meaning of these alterations in the pathophysiological processes are obscure. A major hurdle is the lack of specificity of these pharmacological interventions causing adverse side effects in clinical treatment. Interestingly, alterations of the acetylation profiles have been found in neurodegenerative disorders including Huntington's disease (HD) (reviewed in **Valor, 2017**), Amyotrophic lateral sclerosis (ALS) (reviewed in **Garbes et al., 2013**), spinal muscular atrophy (SMA) (**Kernochan et al., 2005**), Parkinson's disease (PD) (**Harrison et al., 2018**; **Park et al., 2016**), and Alzheimer's disease (AD) (**Klein et al., 2019**; **Marzi et al., 2018**). These findings highlight the involvement of HATs in the etiology of neurodegenerative processes, yet through various mechanisms (**Cobos et al., 2019**). Although widely discussed (**Saha and Pahan, 2006**; **Konsoula and Barile, 2012**), the role of histone acetylation and deacetylation in the adult central nervous system (CNS) and brain homeostasis remains largely unknown.

To gain insight into how the alteration of histone acetylation maintains neuronal homeostasis and prevents neurodegeneration, we used mouse and cellular models, in which the HAT essential cofactor TRRAP is deleted, so that the general HAT activity is disturbed. TRRAP (transformation/transcription domain-associated protein) interacts with E2F1 and c-Myc at gene promoters and is a critical component shared by several HAT complexes, including those from the GNAT and MYST families, which facilitates the recruitment of HAT complexes to target proteins for acetylation (**Tapias and Wang, 2017**; **Knutson and Hahn, 2011**). The complete deletion of Trrap in mice and cells is incompatible with the life of proliferating cells and mouse development, because of severe defects in the spindle checkpoint and cell cycle control (**Herceg et al., 2001**; **Dhanalakshmi et al., 2004**). A tissue specific deletion of Trrap in embryonic neural stem cells leads to a dysregulation of the cell cycle length which drives the premature differentiation of neuroprogenitors (**Tapias et al., 2014**). In humans, missense variants of TRRAP have been recently reported to associate with neuropathological symptoms, including psychosis, ID, autism spectrum disorder (ASD), and epilepsy (**Cogné et al., 2019**; **Mavros et al., 2018**). These basic and clinical studies point to a potential involvement of TRRAP in the manifestation of these neuropathies in humans. Because the known function of TRRAP in the mitotic checkpoint and cell cycle control does not apply to postmitotic cells, i.e., neurons, how TRRAP and its mediated HAT regulate adult neuronal fitness and affect neurodegeneration remains elusive.

In this study, we attempt to elucidate the molecular pathways that are governed by Trrap-HAT in postmitotic neural tissues. We find that Trrap deletion in the mouse model (*Mus musculus*) causes an age-dependent loss of existing neurons leading to neurodegeneration. We show that Trrap-HAT specifically regulates the Sp1 pathway that controls various neural processes, among which microtubule dynamics is particularly affected. Our study discloses the Trrap-HAT-Sp1 axis as a novel regulator of neuronal arborization and neuroprotection.

## Results

### *Trrap* deletion in Purkinje cells results in cerebellar degeneration

To study the role of histone acetylation and Trrap in postmitotic neurons, we generated two mouse models. First we crossed mice carrying the *Trrap* floxed allele ($Trrap^{f/f}$) (**Herceg et al., 2001**) with *Pcp2-Cre* mice (Tg(Pcp2-cre)2Mpin) (**Barski et al., 2000**), to delete *Trrap* in Purkinje cells (Trrap-PCΔ). $Trrap^{+/f}$ mice with the Cre transgene, or $Trrap^{f/f}$ without the Cre transgene, were phenotypically normal, and thus they were used as controls. Trrap-PCΔ mice were born healthy and exhibited

normal (in rotarod tests) or mild defective (in beam balance) motor coordination at young age (1–2 months). However, they displayed an evident miscoordination at mid age (3–6 months), which became more severe after 9 months (old group) (***Figure 1A***). By the age of 1 year, Trrap-PCΔ mice developed an age-dependent locomotor dysfunction characterized by signs of ataxia, namely impaired coordination and unsteady gait (data not shown).

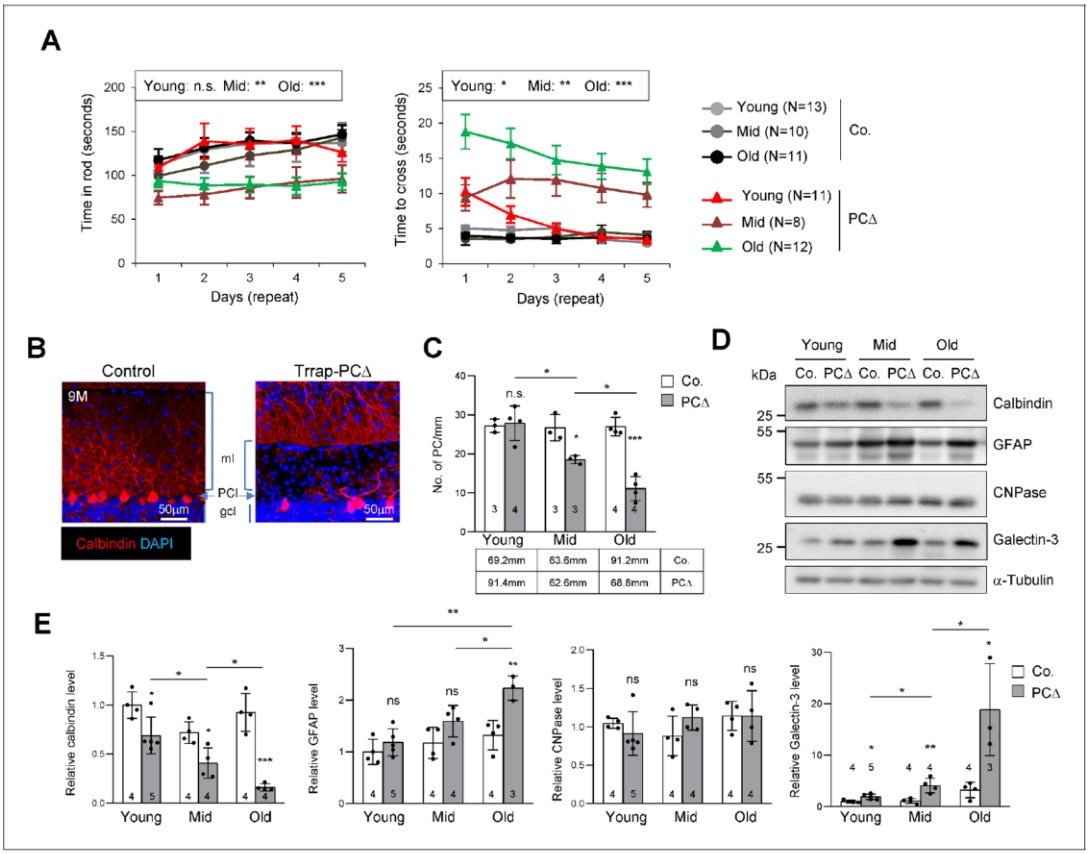



**Figure 1.** Deletion of Trrap in Purkinje cells causes neurodegeneration. (**A**) The rotarod test and the beam balance were used to assess the motor coordination of control and Trrap-PCΔ mice. The left panel depicts the time that the mice stayed in the rod before falling off. The right panel shows the quantification of the time taken by mice to cross the beam. (N) Indicates the number of mice analyzed. Young: 1–2 months; mid age: 3–6 months; old: 9–12 months. (**B**) Immunostaining of the cerebellar sections of 9-month-old mice using an antibody against calbindin (red, Purkinje cells). ml: molecular layer; gcl: granule cell layer; PCl: Purkinje Cell layer. (**C**) The quantification of the number of Purkinje cells in the cerebellum at the indicated ages. The mm of the Purkinje cell layer analyzed are indicated in the table. Young: 1–2 months; mid age: 3–6 months; old: 9–12 months. (**D**) The representative of the western blot analysis for markers for Purkinje cells (calbindin), astrocytes (GFAP), oligodendrocytes (CNPase), and activated microglia (Galectin-3). (**E**) The quantification of western blots of the cerebellum (from **D**). Signal intensities are normalized to α-Tubulin. The numbers inside the bars indicate the number of mice analyzed. Young: 1 month; mid age: 4 months; old: 9 months. Co.: control; PCΔ: Trrap-PCΔ. Mean ± standard error of the mean is shown. Two-way ANOVA and Holm–Sidak test was performed for statistical analysis in (**A**), (**B**) and Student's t-test or one-way ANOVA for (**C**), (**E**). n.s.: not significant. *p≤0.05, **p≤0.01, ***p≤0.001.

The online version of this article includes the following figure supplement(s) for figure 1:

**Figure supplement 1.** Deletion of Trrap in Purkinje cells causes neurodegeneration.

Immunostaining of the Purkinje cell marker calbindin revealed a progressive loss of Purkinje cells in Trrap-PCΔ cerebella – starting from 3 months old mice – which became severe at 9 months (***Figure 1B,C***). Western blot analysis of mutant brains of different ages confirmed a progressive Purkinje cell loss, as judged by the downregulation of calbindin (***Figure 1D,E***). While there was no significant change in the expression of the oligodendrocyte marker CNPase, a progressive increase of the astrocyte marker GFAP, namely astrogliosis, and the activated microglia marker Galectin-3 were evident in mutant cerebella, both of which are hallmarks of neurodegeneration (***Figure 1D,E***). Immunostaining of the cerebella of 2-month-old and 9-month-old mice confirmed a loss of Purkinje cells and a great increase of astrocytes (GFAP+ signals), a sign of astrogliosis, in Trrap-PCΔ cerebella at old age, whereas Trrap-PCΔ cerebella of young mice were normal (***Figure 1—figure supplement 1a,b***). Also, TUNEL staining detected more cell death in all cerebellar lobes of old mice (***Figure 1—figure supplement 1a,b***). The microglia activation and astrogliosis could be due to a homeostatic response to Purkinje cell loss, but they closely resemble neurodegeneration.

## *Trrap* deleted Purkinje cells exhibit age-dependent axonal swellings and dendrite retraction

To examine the neurodegenerative process, we analyzed *Trrap*-deleted Purkinje cells during early postnatal life. Immunostaining of brain sections using antibodies against calbindin and myelin-binding protein (MBP) detected axonal swellings of Trrap-deleted Purkinje cells readily at 1 month of age, prior to Purkinje cell loss (***Figure 2A***). Axonal swellings were generally myelinated at this age (***Figure 2A***), although a loss of myelination was observed occasionally in a few severe cases (data not shown). Furthermore, transmission electron microscopy (TEM) revealed the myelination index of Purkinje cell axons as normal in young (1-month-old) mice, but significantly lower, as judged by a higher g-ratio, in mid age (6-month-old) Trrap-PCΔ mice compared to controls (***Figure 2B,C***).

To quantify the morphological changes of *Trrap*-deleted Purkinje cells, we generated Trrap-PCΔ mice expressing a Confetti transgene (B6.Cg-Tg(Thy1-Brainbow1.0)HLich/J) (thereafter Trrap-PCΔ-Confetti), which enables individual Purkinje cells to stochastically express one of four fluorescent proteins upon Cre expression (***Snippert et al., 2010***). This allows distinguishing single neuron morphology from adjacent cells and reconstructing the dendritic trees of individual Purkinje cells (***Figure 2D***). A Sholl analysis (***Figure 2—figure supplement 1a***) of *Trrap*-deleted Purkinje cells at young age (1–4 months) and at old age (10 months) showed a progressive decrease in the size of their dendritic trees without great effects on their complexity as judged by the critical value (***Figure 2E,F***, ***Figure 2—figure supplement 1b***). Consistent with the Sholl analysis data, the molecular layer became thinner at older age compared to young age (***Figure 2G***, ***Figure 1—figure supplement 1b***). These observations indicate that Trrap deletion does not affect arborization, but rather causes a retraction of already formed dendrites of neurons.

## Trrap deletion changes transcriptional programs in neurons

The scarcity of Purkinje cell neurons in the Trrap-PCΔ cerebellar model limited the searching for the molecular mechanism related to the Trrap-HAT function. To gain a feasible approach, we devised another mouse model in which Trrap was deleted in a large subset of neurons, which would facilitate the molecular analysis of the HAT function in the brain. To this end, we crossed *Trrap*$^{f/f}$ mice with *Camk2-Cre* transgenic mice (Tg(Camk2a-cre/ERT2)2Gsc) to generate mice with a Trrap deletion in pyramidal neurons in the cortex and striatum of the forebrain (designated as Trrap-FBΔ). Trrap-FBΔ brains were normal and had an efficient deletion of Trrap already at day 10 of the postnatal life (P10) (***Figure 3A***, see below for protein analysis in ***Figure 4A,B***, and for qPCR analysis ***Figure 4—figure supplement 1f***). We then carried out RNA-seq and proteomic analyses using cortices and striata from P10 Trrap-FBΔ and control mice. Trrap deletion resulted in highly reproducible changes in the transcriptome of cortices and striata with 5090 and 4389 differentially expressed genes (DEGs) respectively (cutoff adjusted, $p<0.05$) (***Figure 3B***, ***Figure 3—figure supplement 1a***, ***Supplementary file 1***). The Trrap-FBΔ cortex and striatum shared 2695 common DEGs, corresponding to 52.9% and 61.4% of the respective tissues. The directionality of the changes was conserved in 99.3% of the genes (***Figure 3C***, ***Supplementary file 1***). Among the common DEGs, 1122 upregulated and 1554 downregulated genes were overlapping between these two parts of the brain (***Figure 3C***). These results strongly suggest that similar mechanisms operate in the neurons from

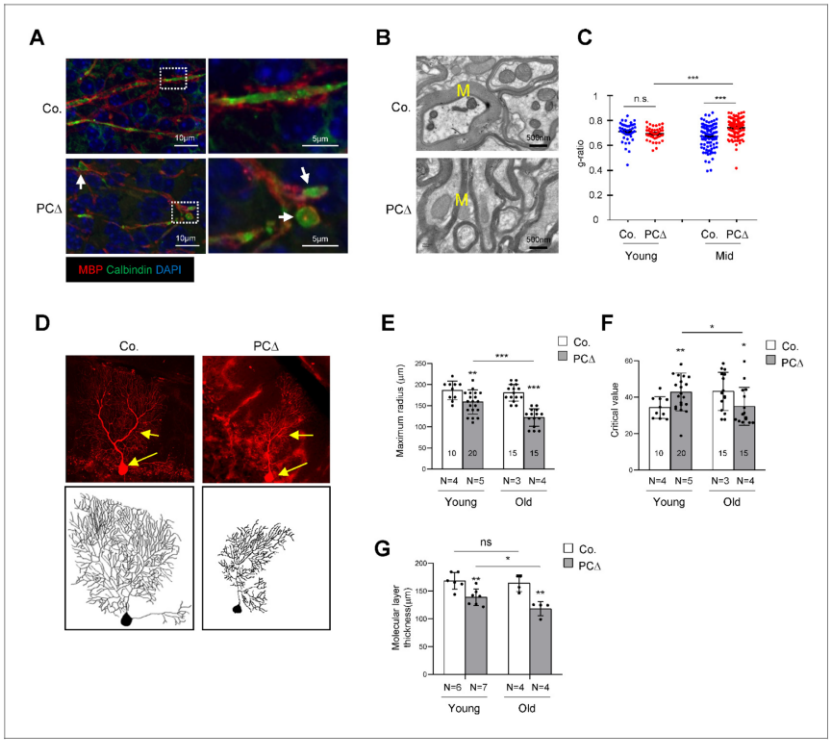



**Figure 2.** Deletion of Trrap in Purkinje cells leads to defects in their axons and dendrites. (**A**) Cerebellar sections from 1-month-old mice were stained with antibodies against Calbindin (green, Purkinje cells) and myelin-binding protein (MBP, red, Myelin sheets) and counterstained with DAPI. White arrows indicate axonal swellings. (**B**) Representative images of electromicrographs showing axon myelination in the cerebellar white matter of 6-month-old control and Trrap-PCΔ mice. M: Myelin sheet. (**C**) The quantification of the myelination index at the indicated ages by g-ratio, which is measured by ImageJ as ag-factor (the square-root of the area of the inner surface of an axon divided by the area of the outer surface including the myelin). Thus, a high g-ratio indicates a low myelination index. (**D**) Single Purkinje cells were analyzed by tracing the expression of the Confetti transgene (RFP). Representative Purkinje cell images of maximum intensity projection (MIP) from Z-stacks (upper panel) of 10-month-old mice are shown after reconstruction (lower panel) based on RFP expression in Trrap-PCΔ mice. (**E**) The quantification of the maximum radius after Sholl analysis, at the indicated ages, demonstrating that Purkinje cells retract their dendrites in Trrap-PCΔ mice. Young: 1–4 months; old: 10 months. (**F**) The graph shows the critical value measured by the Sholl analysis, at the indicated ages, indicative of the complexity of Purkinje cells. (**G**) The quantification of the molecular layer thickness of all cerebellar lobes. Young: 1–4 months; old: 10 months. Co.: control; PCΔ:Trrap-PCΔ. N: the number of mice analyzed. The numbers inside the bars indicate the number of cells analyzed. Mean ± standard error of the mean is shown. Student's t-test or one-way ANOVA was performed for statistical analysis. n.s.: not significant. *p≤0.05, **p≤0.01, ***p≤0.001.

The online version of this article includes the following figure supplement(s) for figure 2:

**Figure supplement 1.** Sholl analysis of degeneration of Purkinje cells of Trrap-PCΔ mice.

both brain regions. Gene ontology (GO) analyses of the common DEGs in RNA-seq data sets of both the cortex and striatum revealed alterations in multiple signaling pathways important for neuronal processes (***Figure 3D***, ***Source data 1A***, ***Supplementary file 1***). Intriguingly, about a half of the Top50 pathways were linked with microtubule dynamics and its related cellular processes (***Figure 3D***).

Whole proteomic analysis of P10 Trrap-FBΔ cortices identified 122 out of 6919 proteins to be significantly altered after Trrap deletion (cutoff, $q < 0.1$) (***Figure 3B***, ***Figure 3—figure supplement 1b***, ***Supplementary file 2***). Notably, a comparison between RNA-seq and proteomics data sets showed

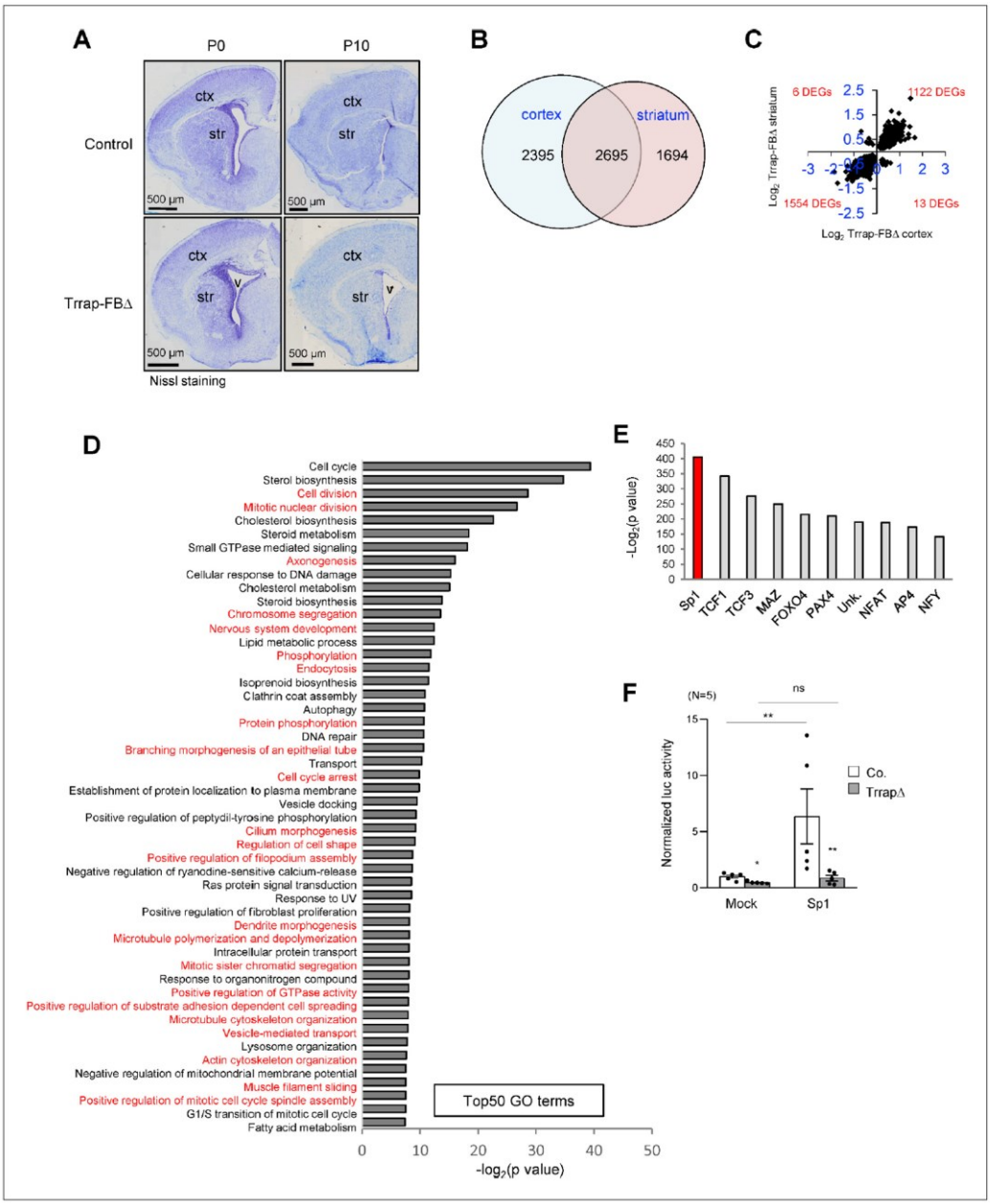



**Figure 3.** Deletion of Trrap in pyramid neurons of the forebrain results in a progress degeneration of the cortex and striatum. (A) Nissl staining of the coronal session of Trrap-FBΔ brain at postnatal day 0 (P0) and 10 (P10). Ctx: cortex; str: striatum; v: ventricle. (B) The Venn diagram depicts the overlap between the differentially expressed genes (DEGs) measured by RNA-seq in the cortex and striatum. The numbers refer to the DEGs in the indicated data sets. (C) $Log_2$ of the fold changes of the 2695 common DEGs in Trrap-FBΔ cortex and striatum. (D) Top50 GO terms of the 2695 overlapping hits

*Figure 3 continued on next page*

*Figure 3 continued*

identified in the RNA-seq data set of the cortex and striatum. Note that microtubule dynamics related processes are highlighted in red. (**E**) Transcription factor binding site (TFBS) enrichment analysis of the 1261 common DEGs in aNSCs, the cortex, and the striatum identified by RNA-seq. (**F**) Luciferase assays using a Sp1-responsive construct. The graph shows the luciferase activity normalized by Bradford assay. N: the number of cell lines analyzed; Mock: empty vector, Sp1: overexpression; luc: luciferase. Co.: control; aNSCsΔ: Trrap-aNSCsΔ. n: the number of cell lines analyzed. Mean ± standard error of the mean is shown. Unpaired t-test was performed for statistical analysis. n.s.: not significant; *p≤0.05, **p≤0.01.
The online version of this article includes the following figure supplement(s) for figure 3:

**Figure supplement 1.** Trrap deletion leads to transcriptome and proteome changes.
**Figure supplement 2.** Comparative Omics analysis of Trrap deleted aNSCs with Trrap-FBΔ brains.

that 85% of the proteins altered by Trrap deletion, i.e., 33 upregulated and 71 downregulated genes, were altered in the same way at the RNA level, which resembled 100% directionality (***Figure 3B***, ***Figure 3—figure supplement 1c***, ***Supplementary file 2***). These results indicate that most changes in the proteome were due to changes in the transcriptome.

## Trrap deletion alters Sp1 pathway

TRRAP is a cofactor interacting with various transcription factors and recruits HAT activity to their target gene promoters. To understand through which transcription factors Trrap was mediating its function, we performed a transcription factor binding site (TFBS) enrichment analysis on the common DEGs in Trrap-deleted cortices and striata. We found that most of these transcriptional changes after Trrap deletion were mediated mainly by limited transcription factors, among which transcription factors Sp1 and TCFs appeared to be the most relevant upstream factors (***Figure 3E***, ***Supplementary file 3***). They mediated Trrap-dependent changes not only upstream of the common DEGs, but also upstream of the DEGs from each data set of cortices and striata (***Supplementary file 3***). Transcription factors TCF1, TCF3, and NFAT are known effectors of the Wnt signaling pathways (***Cadigan and Waterman, 2012***) and were indeed downstream of the Sp1-mediated transcription regulation upon Trrap deletion (***Supplementary file 1***). Hence, our data suggest that Sp1 is likely the main regulator for all these changes in Trrap-deleted brains.

## The Sp1 pathway is a conserved transcriptomic network in Trrap-deleted neural cells

Sp1 is a key transcription factor capable of regulating many cellular processes, including proliferation, cell differentiation, apoptosis, immune responses, DNA damage responses, and chromatin remodeling (***Li and Davie, 2010***). We attempted to analyze the transcriptional activity of Sp1 in the absence of Trrap. To achieve this, we had to adopt an in vitro culture approach and used replicating adult neural stem cells (aNSCs) from *Trrap*$^{f/f}$ mice expressing the CreER$^{T2}$ transgene (*Rosa26*-CreER$^{T2}$ Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj}$) (designated as Trrap-iΔ). Addition of 4-hydroxytamoxifen (4-OHT) in cultured Trrap-iΔ NSCs induced an efficient deletion of Trrap (***Figure 3—figure supplement 2a,b***). We first validated the transcriptome of aNSCs in comparison with that of Trrap-FBΔ cortex and striatum and found 1261 common DEGs among these three samples. The directionality is very conserved in 93.1% between the cortex and aNSCs (***Figure 3—figure supplement 2c,d***, ***Supplementary file 1***). Intriguingly, TFBS analysis revealed a remarkable commonality of DEGs among Trrap-deleted cortices, striata, and aNSCs as the transcriptional targets of Sp1 (***Figure 3—figure supplement 2e***, ***Supplementary file 3***). Thus, Trrap-HAT regulates a very conserved transcriptomic network in different brain regions as well as in NSCs. Using these cells, we then performed a luciferase reporter assay to investigate whether Sp1 is directly regulated by Trrap and detected a dramatic decrease in Sp1 activity in Trrap-deleted NSCs, compared to control cells. Strikingly, ectopic overexpression of Sp1 in Trrap-deleted cells failed to activate the Sp1-reporter (***Figure 3F***). These data indicate that Trrap is indeed required for Sp1-mediated transcriptional activation.

## Alteration of Sp1-targets after Trrap deletion

RNA-seq analysis suggests that Trrap ablation leads to changes of the Sp1-dependent transcription regulation of its downstream targets in various neurological processes (***Source data 1A***). We then

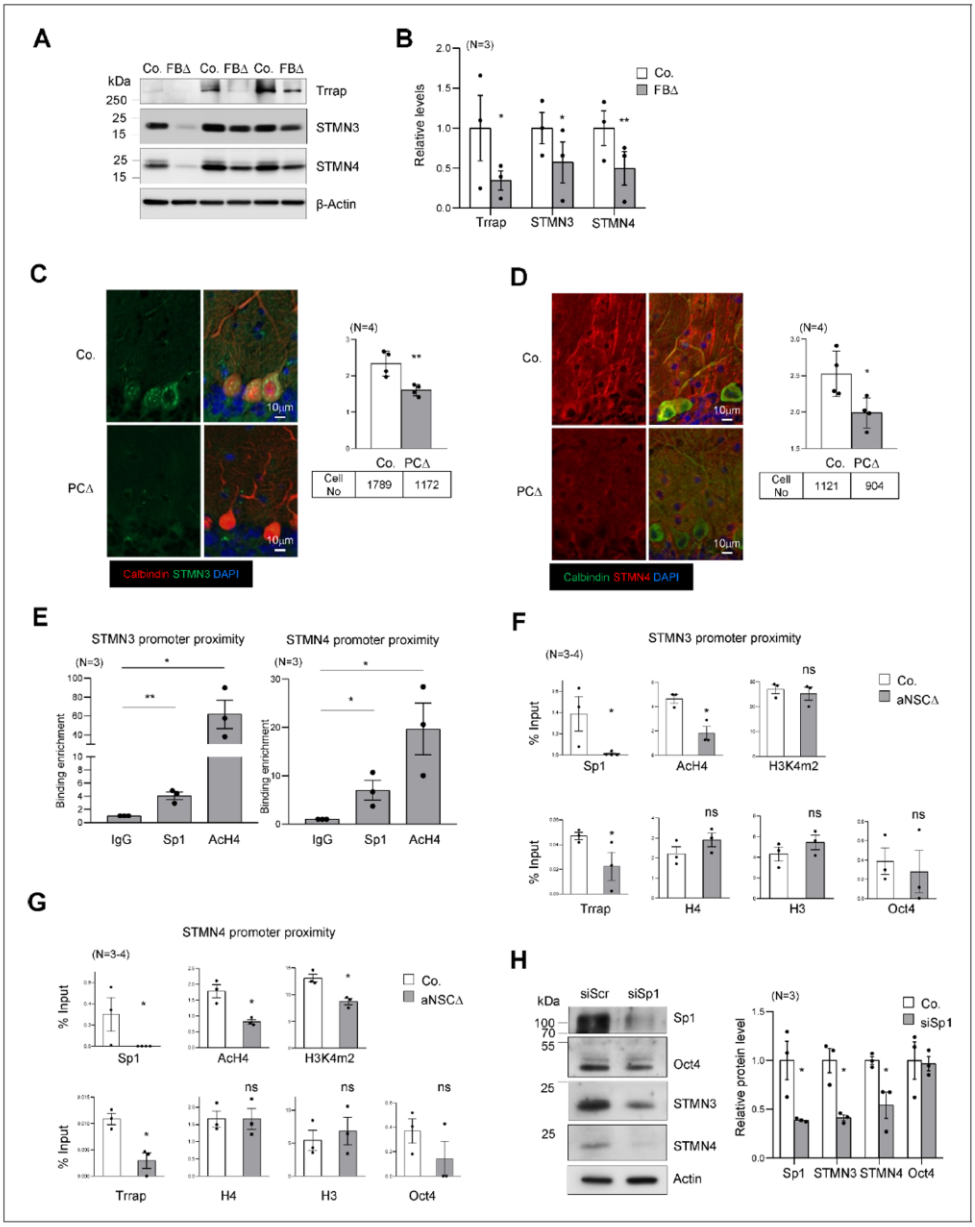



**Figure 4.** Trrap regulates the expression of STMNs via Sp1. (A) Western blot analysis of the Trrap deletion and expression of STMNs in the forebrain of indicated genotype at postnatal day 10 (P10). β-actin is a loading control. Co: control; FBΔ: Trrap-FBΔ. (B) The quantification of the expression of the indicated proteins in mutant forebrains measured by western blots are related value to adjacent controls after normalization to β-actin. N: the number of mice analyzed. The error bar presents the standard error. Paired t-test was used for statistical analysis. *p≤0.05, **p≤0.01. (C and D) Sagittal sections

*Figure 4 continued on next page*

*Figure 4 continued*

of 4-month-old Trrap-PCΔ mice were stained against STMN3 (green, **C**) and STMN4 (green, **D**), the Purkinje cell marker Calbindin (red) and counterstained with DAPI (blue). The right panel shows the average intensity of STMN3 or STMN4 in Purkinje cells normalized by the intensity in the neighboring cells (not Trrap-deleted). n: the number of cells analyzed; N = 4 mice analyzed. (**E**) ChIP analysis on the STMN3 and STMN4 promoters in control striata using antibodies against Sp1, AcH4, and IgG. qPCR analysis was performed to quantify the binding of the indicated factors to the promoter. The binding enrichment was calculated as fold enrichment over IgG. N = 3 mice analyzed. The primers that contain the Sp1 site for ChIP assays are marked in ***Figure 4—figure supplement 1g***. (**F and G**) ChIP analysis on the proximity of STMN3 (**F**) and STMN4 (**G**) promoters in control and TrrapΔ aNSCs. Protein binding value is presented in percentage of input. The large error bars in Oct4 ChIP are due to an inclusion of a high value from one pair of samples. N = 3–4 mice analyzed. (**H**) Western blot analysis of STMN3 and STMN4 expression after siRNA-mediated knockdown of Sp1 in aNSCs. Oct4 is an Sp1 independent transcription factor control and β-actin controls loading. (**C–H**) Mean ± standard error of the mean is shown. Unpaired t-test was performed for statistical analysis. *p≤0.05, **p≤0.01.

The online version of this article includes the following figure supplement(s) for figure 4:

**Figure supplement 1.** Expression and HAT binding analysis of STMNs in Trrap-FBΔ brains.

compared the Sp1 targets identified in our transcriptome (DEGs) analyses (***Supplementary file 1***) with Sp1 targets identified by ChIP-seq from the Harmonizome database (***Rouillard et al., 2016***) (https://amp.pharm.mssm.edu/Harmonizome/gene_set/SP1/ENCODE+Transcription+Factor+Targets). We found that a high degree of the DEGs from Trrap-deleted brains as well as aNSCs were putative Sp1 targets (***Figure 3—figure supplement 2f***, ***Supplementary file 4***). GO analysis of the common DEGs revealed that more than 30% of the Top50 pathways under the control of Sp1 were linked with microtubule dynamics-related cellular processes (***Figure 3—figure supplement 2g***, ***Supplementary file 4***).

To further examine how Trrap affects HATs at target gene promoters, we performed a ChIP-seq study of acetylated histones H3 and H4 using Trrap-deleted aNSCs. Depletion of Trrap led to a downregulation of 2274 AcH3 and 1355 AcH4 peaks that were associated with coding genes (equivalent to 10% of the most depleted regions in TrrapΔ versus controls) (***Figure 4—figure supplement 1a***, ***Supplementary file 5***). Only 12.6% and 10.2% respectively of these depleted peaks correlated with changes in the RNA level of the corresponding genes in Trrap-FBΔ brains (***Figure 4—figure supplement 1a***, ***Supplementary file 5***). ChIP-seq analyses revealed no significant difference of AcH3 on Sp1-site between control and Trrap-deleted aNSCs, whereas H4Ac on Sp1-site in control (mean = 12.37) is slightly lower than in Trrap mutants (mean = 12.54) (***Figure 4—figure supplement 1b***). Interestingly, the downregulated genes had a significantly lower acetylation level of H3 and H4 in the Sp1 promoter area in Trrap deleted cells (***Figure 4—figure supplement 1c***). Twenty-two genes exhibited downregulated histone H3 and H4 acetylation and were also downregulated in the RNA-seq from brains. Among them, 11 genes were Sp1 targets according to the Harmonizome database (***Supplementary file 5***). The microtubule dynamics proteins STMN3 (SCLIP) and STMN4 (RB3) are of special interest (***Figure 4—figure supplement 1d,e***), because microtubule dynamics have been proposed to be involved in brain homeostasis (***Chauvin and Sobel, 2015***; ***Dubey et al., 2015***) and defects in microtubule dynamics often cause axonal swellings and dendrite retraction in postmitotic neurons (***Dubey et al., 2015***; ***Voelzmann et al., 2016***). Together with the high incidence of the dysregulated processes associated with microtubule dynamics, which are regulated by Sp1 (***Figure 3E***, ***Figure 3—figure supplement 2f***), the finding of these two microtubule destabilizing proteins postulates this particular cellular process as the main route affected by Trrap deletion in the brain.

## Trrap-HAT mediates Sp1 transcriptional control of microtubule dynamic genes

The microtubule destabilizing proteins STMN3 and STMN4 are members of the Stathmin protein family (***Chauvin and Sobel, 2015***). STMNs 3 and 4 were found within the Top30 changes in our RNA-seq and proteomics data sets of brain samples (***Source data 1B and C***, ***Supplementary file 2***). qPCR analysis confirmed a great downregulation of these genes in Trrap deleted cells of forebrain tissues (***Figure 4—figure supplement 1f***). Western blot analysis also confirmed a great reduction of both STMN3 and STMN4 proteins in Trrap-deleted forebrains at P10 (***Figure 4A,B***). We next turned our analysis to our neurodegeneration model Trrap-PCΔ mice. Co-staining of STMN3 or STMN4 with calbindin in the brain sections detected a significant decrease of both proteins in Trrap-deleted

Purkinje cells compared to controls (***Figure 4C,D***). To explore the mechanism, ChIP-seq was performed and showed that the level of AcH3 and AcH4 at the promoters of these genes was greatly reduced (***Figure 4—figure supplement 1g***). To validate the RNA-seq and ChIP-seq data, we performed ChIP experiments in brain samples and found both Sp1 binding and histone H4 acetylation were enriched at the STMN3 and STMN4 promoters in controls (***Figure 4E***). Upon Trrap deletion there was a dramatic decrease in Sp1 binding, as well as in the acetylation of histone H4 in the STMN3 and STMN4 promoters (***Figure 4F,G***). We also noted that Trrap deletion did not change the $H3K4^{m2}$ level in the STMN3 promoter, yet decreased mildly in the STMN4 promoter. Moreover, Trrap deficiency did not compromise binding of Sp1-unrelated transcription factor Oct4 at the promoter proximities of these STMNs genes (***Figure 4F,G***). These data together indicate an essentiality of Trrap for loading Sp1 and HATs to the promoters of these Sp1 target genes. Furthermore, siRNA-mediated Sp1 knockdown indeed decreased expression of STMN3 and STMN4 proteins (***Figure 4H***). These results demonstrate that the Trrap-HAT-Sp1 axis directly controls the expression of STMN3 and STMN4 in neurons.

### Functional test of STMNs in neuronal defects by Trrap deficiency

Stathmin family proteins STMN3 and STMN4 are mostly or exclusively expressed in the nervous system where they control microtubule dynamics, an essential process for neuronal differentiation, morphogenesis, and functionality (***Chauvin and Sobel, 2015***; ***Dubey et al., 2015***). To investigate if Trrap deficiency especially affecting neuronal homeostasis was indeed mediated by STMNs, first we knocked down Trrap by siRNAs in primary neurons isolated from wild-type E16.5 cortex (***Figure 5—figure supplement 1a***), co-transfected with GFP or STMNs 3- or 4-expressing vectors at day 6 in vitro culture (DIV6), designated day 0 post transfection (DPT0), and analyzed the neuronal phenotype at DPT6 (***Figure 5—figure supplement 1b***). Immunofluorescence analysis revealed that the Trrap knockdown evidently reduced the neurite length and the branching numbers of neurons (***Figure 5A–C***). This was also confirmed by the IncuCyte assay at DPT6 (***Figure 5—figure supplement 1c,d***), which allows scoring a large number of cells. These findings indicate that Trrap deficiency also compromises neuronal arborization in vitro. To examine whether the downregulation of STMNs is indeed responsible for the neuropathies of Trrap deleted neurons, we ectopically expressed STMN3 in Trrap knockdown neurons (***Figure 5—figure supplement 1e***). Intriguingly, ectopic expression of STMN3 is sufficient to rescue these neuronal defects caused by Trrap knockdown (***Figure 5A,B***). Similarly, ectopic expression of STMN4 also corrected the neurite length and branching defects in Trrap knockdown primary neurons (***Figure 5A,C***). Interestingly, we note a co-upregulation of both STMNs when either STMN3 or STMN4 was overexpressed (***Figure 5—figure supplement 1e***), which suggests a co-stabilization or cooperative action of both STMNs in microtubule dynamics in the brain; yet the underlying mechanism requires further investigation. Taken together, these experiments demonstrate that Trrap prevents neuropathy by regulating Stathmin associated with microtubule dynamics.

## Discussion

The maintenance of neuron function and numbers are important for proper adult brain homeostasis. A loss of control of these processes prompts age-related neurological deficits, including neurodegeneration. Various mechanisms, including protein folding/stability, neuroinflammations, or DNA damage accumulation, have been implicated in the maintenance of brain homoeostasis and functionality. The acetylation modulations of proteins have been proposed to be important for the maintenance and function of neural cells (***Tapias and Wang, 2017***) as well as in neurodegenerative disorders, including HD, AD, PD, and ALS, yet through different mechanisms (***Cobos et al., 2019***). These studies highlight the involvement of HATs in the etiology of neurodegenerative processes. Despite the assumption that HAT/HDAC conducts a general regulatory function in transcription, how acetylation and deacetylation modulate the functionality, fitness, and even the survival of adult neurons is largely unknown. The current study shows that the HAT cofactor TRRAP is vital for preventing neurodegeneration of the Trrap-PCΔ mouse model. Trrap is an essential gene in proliferating cells because a Trrap null mutation causes lethality in cells and mice (***Herceg et al., 2001***). Unexpectedly, the deletion of Trrap in postmitotic neural cells (i.e., Purkinje neurons) is compatible with life, but elicits a full range of age-dependent neurodegenerative symptoms – axonal

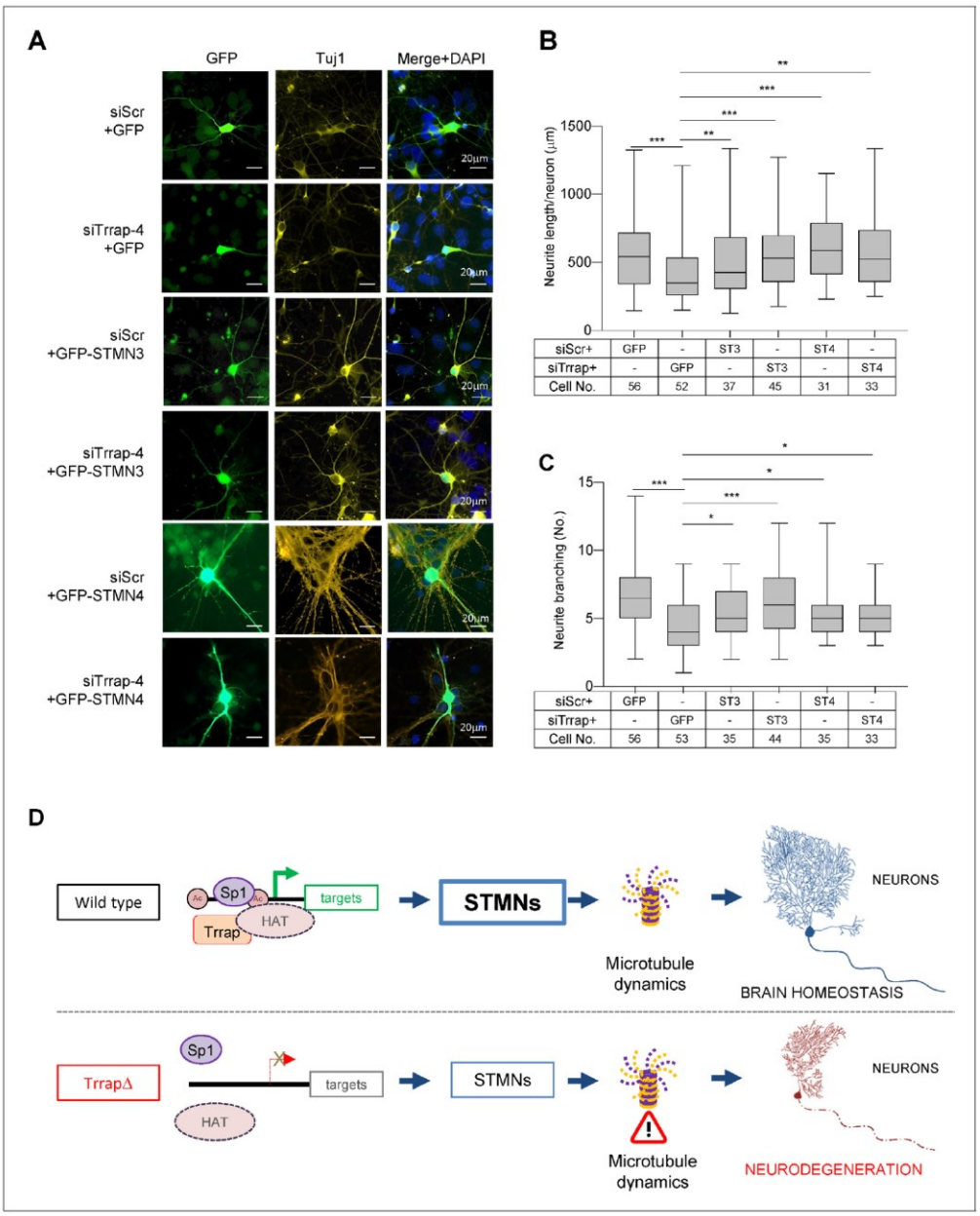



**Figure 5.** Trrap deletion causes neuronal defects in vitro that can be rescued by ectopic expression of STMN3. (A) Immunofluorescent images of primary neurons isolated from E16.5 forebrains at 6 days post co-transfection of siRNA (siScramble, siTrrap-4) with GFP, or with the GFP-STMN3, or with GFP-STMN4 expressing vector. (B) The neurite length after the Trrap knockdown and rescue by the STMN3 or STMN4 overexpression was analyzed at 6 days post co-transfection of the indicated siRNA with the GFP-, STMN3-, or STMN4-expressing vector. The neurite length is measured with NeuronJ

*Figure 5 continued on next page*

*Figure 5 continued*

(ImageJ plug-in). Only GFP-positive neurons (indicative of transfection) were analyzed. Each bar represents the data from four to six mouse embryos; the experiments were repeated more than three times. Unpaired t-test was performed for statistical analysis. **p≤0.01, ***p≤0.001, n.s., not significant. (**C**) The neurite branching after the Trrap knockdown and rescue by STMN3 or STMN4 overexpression was analyzed at 6 days post co-transfection of the indicated siRNA with the GFP, STMN3-, or STMN4-expressing vector. The neurite length is measured with NeuronJ (ImageJ software). Only GFP-positive neurons were scored and are shown. Each bar represents the data from four to six mouse embryos; the experiments were repeated more than three times. Unpaired t-test was performed for statistical analysis. *p≤0.05, **p≤0.01, ***p≤0.001, n.s., not significant. (**D**) Working model of Trrap-HAT-Sp1 in brain homeostasis and neurodegeneration. The Trrap deletion compromises HAT to acetylate histones resulting in insufficient binding of Sp1 and the subsequent downregulation of target genes involved in microtubule dynamics (STMNs). The dysregulation of STMNs provokes the axonal swelling, declines of neurite lengths and branching of postmitotic neurons, ultimately, leading to defective neuronal homeostasis and neurodegeneration.

The online version of this article includes the following figure supplement(s) for figure 5:

**Figure supplement 1.** Ectopic expression of STMNs rescues defects of Trrap knockdown neurons.

demyelination, dendrite retraction, progressive neuronal death, reactive astrogliosis, and the activation of microglial cells. Trrap deletion in non-dividing neurons, avoiding lethality, allows the identification of Sp1 as a specific master regulator, which is under the control of Trrap-HAT, to ensure proper neuronal arborization and to prevent neuron loss (***Figure 5D***). Although our omics studies detect a range of genes that have been altered, microtubule dynamics seems to be particularly vulnerable to Trrap deletion, because the major changes in the Trrap deleted brains are the processes involving microtubule dynamics and that ectopic expression of microtubule destabilizing proteins STMNs can largely ameliorate the arborization defects of Trrap-deficient neurons.

The TFBS enrichment analysis of these commonly dysregulated genes in Trrap-deleted brains points to selective transcription programs of the Trrap-HAT downstream. Our integrated omics analyses revealed a remarkable commonality of Trrap-HAT-regulated genes via Sp1 in the neurons of the cortex and striatum, and even with neural stem cells. Intriguingly, the Sp1 pathway is on the top of the changes by Trrap deletion and Trrap is required for the Sp1 transcriptional activity. The action of Trrap-HAT in postmitotic neurons is to regulate, via Sp1, the expression of neuroprotection and brain homeostasis genes, among which microtubule dynamics is most affected (***Dubey et al., 2015***; ***Noelanders and Vleminckx, 2017***). Although we have not completely confirmed that all these molecular pathways governed by Trrap-HAT-Sp1 in pyramidal neurons (Trrap-FBΔ mice) would be identical in Purkinje neurons (due to the technical limitation), STMN3/4 were indeed downregulated in Trrap-PCΔ models. In agreement with the ChIP data, knockdown of Sp1 repressed STMN3/4 expression. Thus, it is likely that the Sp1-mediated specific transcriptome could also function in the prevention of neurodegeneration.

Sp1 is a master transcription factor binding to the GC box of promoters and can also regulate by itself. It has many downstream target genes, among which the regulation of cancer and cell proliferation have been well studied (***Li and Davie, 2010***; ***Vizcaíno et al., 2015***; ***Suske, 2017***). However, the upstream regulatory mechanism of Sp1 has not been defined previously. Here we show that Trrap-HAT is upstream of Sp1 and has a specific regulatory role in the Sp1-mediated transcription. Although Sp1 has been reported to bind to the promoter of some genes in neural cells, such as Slit2 (***Saunders et al., 2016***), P2 × 7 (***García-Huerta et al., 2012***), and Reelin (***Chen et al., 2007***), it has not been linked directly with neural development and degeneration. Our transcriptome analyses reveal that many neurological processes are indeed regulated by Sp1 downstream targets, many of which are connected with microtubule dynamics (see ***Figure 3D***, ***Figure 3—figure supplement 2g***, ***Source data 1A***). For example, Trrap facilitates Sp1 binding to the gene loci of the microtubule destabilizing proteins STMN3 and STMN4. These findings are particularly interesting, because Sp1 has been implicated in neurodegeneration disorders; yet previously published data are often controversial. A GWAS analysis detected Sp1 among candidates mediating transcriptional activity changes in AD and PD patients (***Ramanan and Saykin, 2013***). Sp1 seems to prevent neurotoxicity in HD (***Dunah et al., 2002***), whereas others showed that a downregulation is protective in HD development (***Qiu et al., 2006***). Sp1 is found upregulated in AD patients and also in an AD mouse model (***Citron et al., 2008***); however, when it was chemically inhibited, memory deficits were even enhanced in AD transgenic mice (***Citron et al., 2015***), ruling out an instrumental role of Sp1 in AD. These controversial findings are perhaps not surprising, because the expression changes of the Sp1

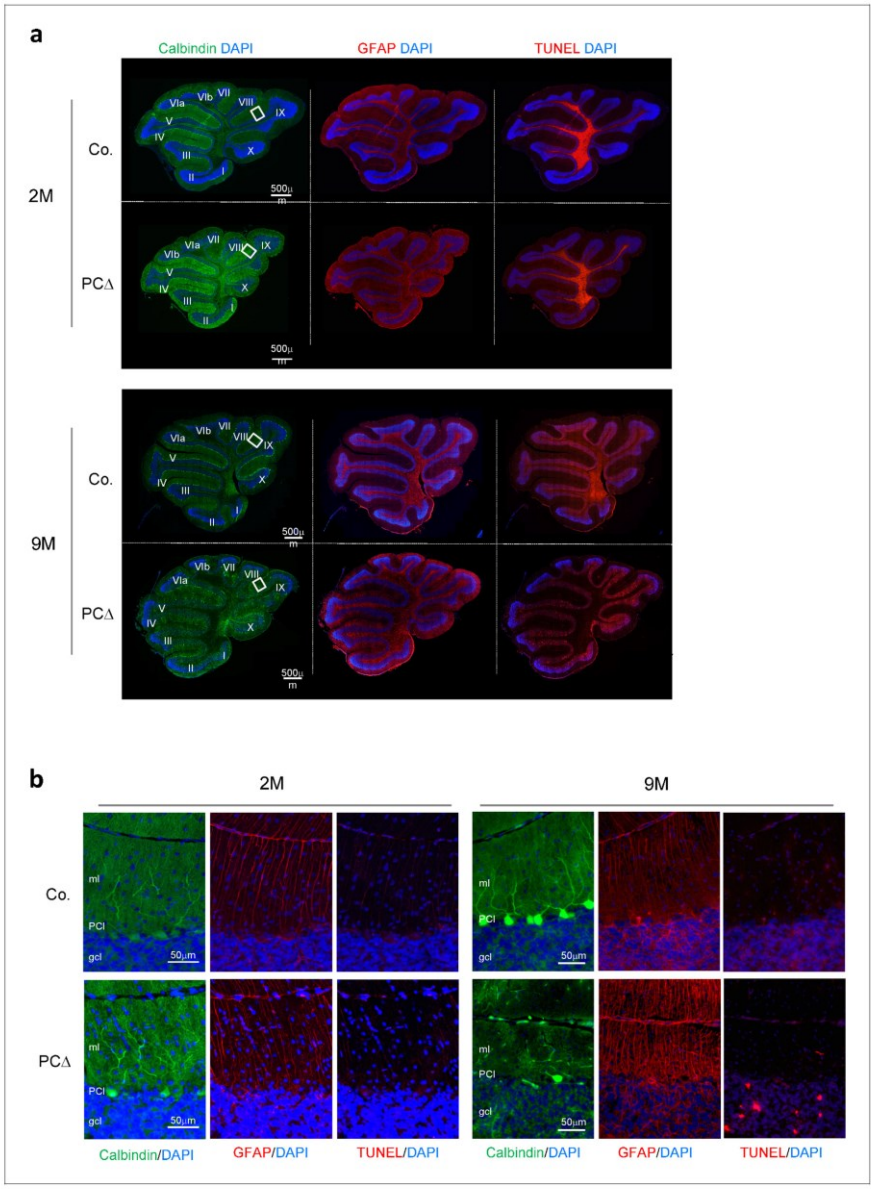



**Figure 1—figure supplement 1.** Deletion of Trrap in Purkinje cells causes neurodegeneration. (**a**) Immunostaining of sagittal sections using TUNEL reaction (red, cell death) and antibodies against calbindin (green, Purkinje Cells) and GFAP (red, astrocytes), and counterstained with DAPI. A representative image from 9-month-old mice shows a marked reduction in Purkinje cell numbers (green) in Trrap-PCΔ samples accompanied by reactive astrogliosis (red) in all layers of the cerebellar lobes and an increase in cell death in all cerebellar areas. The cerebellar lobes are numbered I–X. (**b**) High

*Figure 1—figure supplement 1 continued on next page*

*Figure 1—figure supplement 1 continued*

magnification of the areas highlighted in (**a**). Note a thinner molecular layer of 9-month-old cerebella (see quantification in ***Figure 2G***). PCl: Purkinje cell layer, ml: molecular layer, gcl: granule cell layer. Co.: control; PCΔ: Trrap-PCΔ.

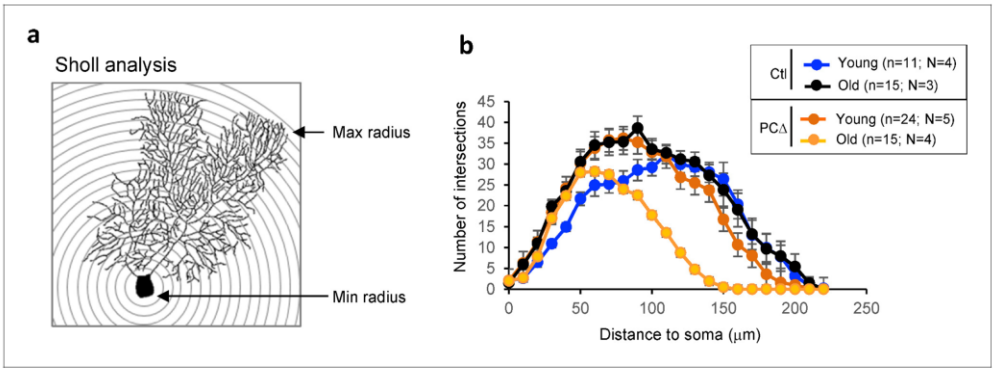
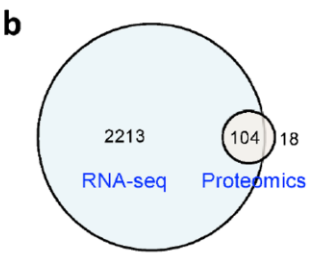



**Figure 2—figure supplement 1.** Sholl analysis of degeneration of Purkinje cells of Trrap-PCΔ mice. (**a**) Scheme of Sholl analysis. Concentric circles are drawn around a given Purkinje cell and the number of times the cell crosses the circles is scored. (**b**) The graph shows the results of the Sholl analysis indicating that Purkinje cells at indicated age (old, 10 months; young, 1–4 months) retract their dendrites in Trrap-PCΔ mice. N: number of mice; n: cells.

**a**

| Analysis | Model | Tissue | Changes | Up-regulated | Down-regulated |
|---|---|---|---|---|---|
| RNA-seq | Trrap-FBΔ | Cortex | 5090 DEGs | 2259 | 2831 |
| RNA-seq | Trrap-FBΔ | Striatum | 4389 DEGs | 2050 | 2339 |
| Proteomics | Trrap-FBΔ | Cortex | 127 proteins | 38 | 89 |



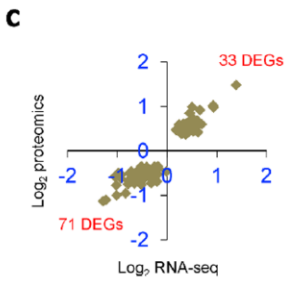
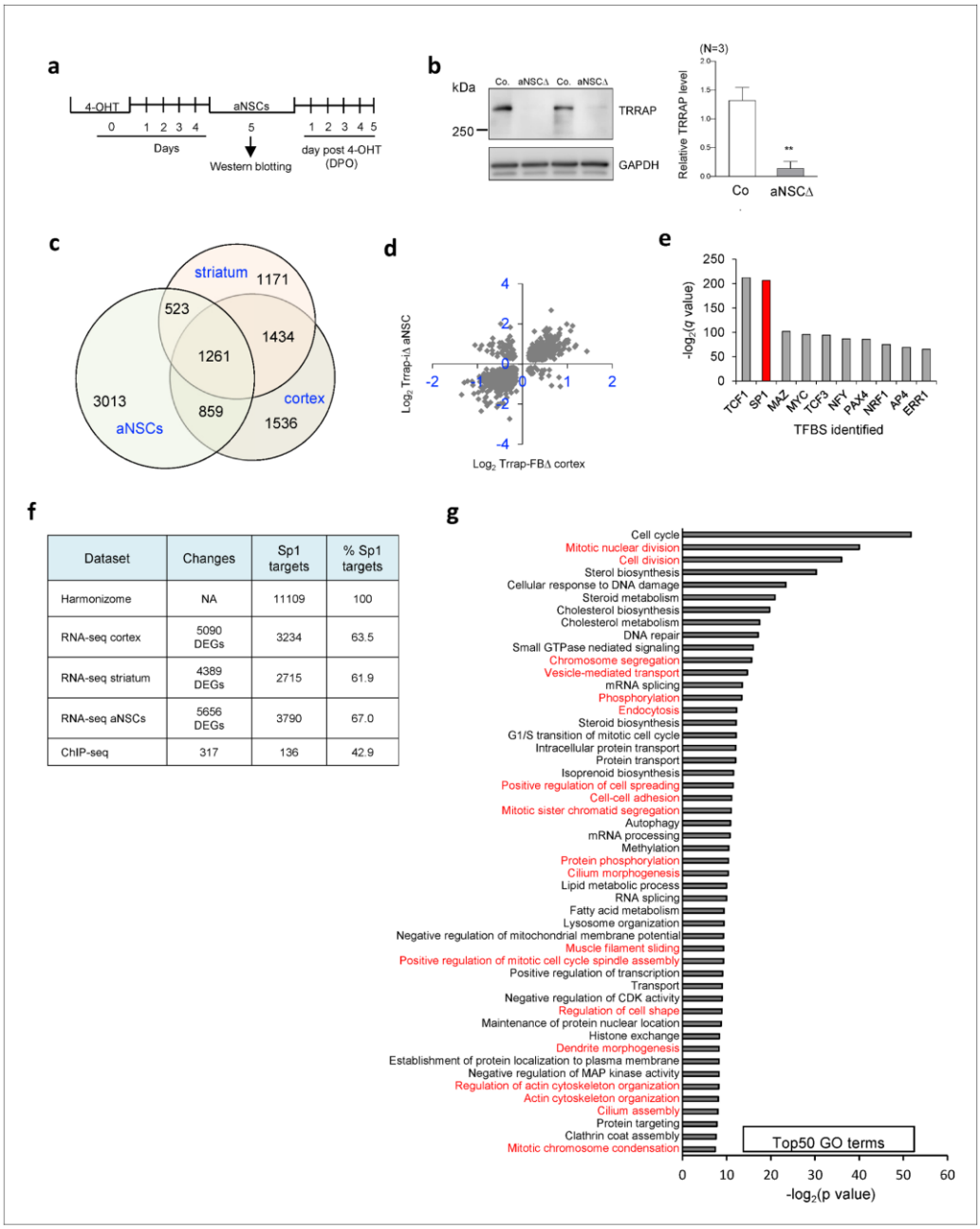




**Figure 3—figure supplement 1.** Trrap deletion leads to transcriptome and proteome changes. (**a**) The table summarizes the different genome-wide approaches used to analyze the function of Trrap in different cell types and the general results obtained. For all analyses, four to five biological replicates were used. (**b**) The Venn diagram depicts the overlap between the differentially expressed genes (DEGs) measured by RNA-seq and protein changes measured by mass spectrometry (cutoff, $q \leq 0.1$) in Trrap-FBΔ cortices. The numbers refer to common DEGs/DEP (differentially expressed proteins) in the indicated data sets. (**c**) The Venn diagram depicts the $\log_2$ of the fold changes of the 104 common DEGs/proteins in Trrap-FBΔ cortices.




| Dataset | Changes | Sp1 targets | % Sp1 targets |
|---|---|---|---|
| Harmonizome | NA | 11109 | 100 |
| RNA-seq cortex | 5090 DEGs | 3234 | 63.5 |
| RNA-seq striatum | 4389 DEGs | 2715 | 61.9 |
| RNA-seq aNSCs | 5656 DEGs | 3790 | 67.0 |
| ChIP-seq | 317 | 136 | 42.9 |

**Figure 3—figure supplement 2.** Comparative Omics analysis of Trrap deleted aNSCs with Trrap-FBΔ brains. (**a**) The experimental workflow of the Trrap deletion in aNSCs in culture by 4-OHT followed by experiments at indicated day post 4-OHT treatment (DPO). (**b**) Western blot analysis of the

*Figure 3—figure supplement 2 continued on next page*

*Figure 3—figure supplement 2 continued*

Trrap deletion in aNSC at 5DPO. GAPDH is a loading control. Quantification of the Trrap protein levels from three animals (N = 3) after normalization to GAPDH is shown on the left. Co.: control; aNSCΔ: Trrap-aNSCΔ. (**c**) The Venn diagram depicts the overlap between the differentially expressed genes (DEGs) measured by RNA-seq in the cortex, striatum, and aNSCs. The numbers refer to DEGs in the indicated data sets. (**d**) $Log_2$ of the fold changes of the 2120 common DEGs in Trrap-FBΔ cortex and Trrap-aNSCΔ aNSCs. (**e**) Transcription factor binding site (TFBS) enrichment analysis of the 1261 common DEGs in aNSCs, cortex, and striatum identified by RNA-seq. (**f**) A list of the Sp1 targets based on published ChIP-seq results was obtained from the Harmonizome database (***Rouillard et al., 2016***) and compared with our RNA-seq results to determine the amount of Sp1 targets in each data set. (**g**) Top50 GO terms of the processes regulated by Sp1. Note that microtubule dynamics related processes are highlighted in red.

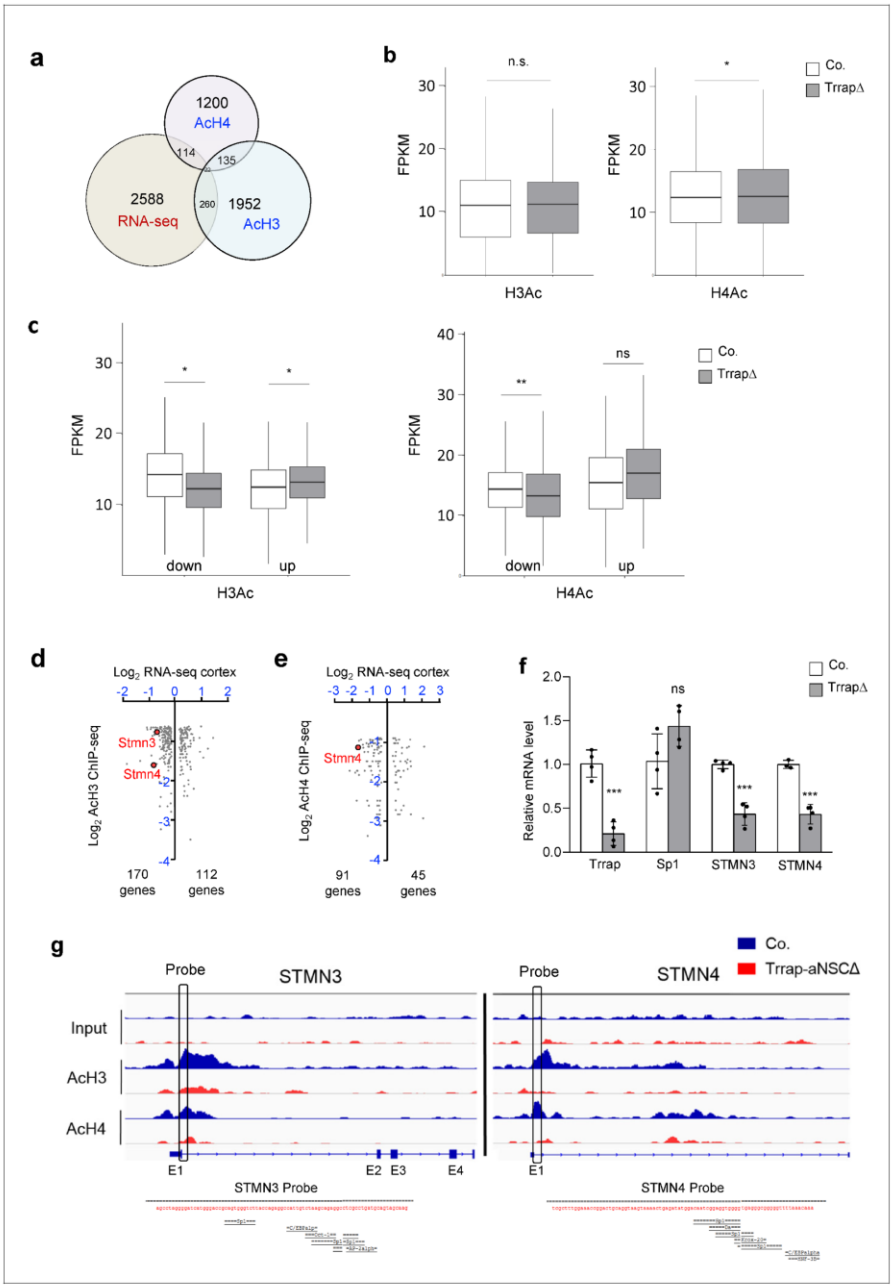



**Figure 4—figure supplement 1.** Expression and HAT binding analysis of STMNs in Trrap-FBΔ brains. (**a**) The scheme depicts the overlap between the common differentially expressed genes (DEGs) measured in the RNA-seq analyses of the cortex and striatum of Trrap-FBΔ mice and acetylated histone

*Figure 4—figure supplement 1 continued on next page*

*Figure 4—figure supplement 1 continued*

depleted peaks in aNSCs. The numbers refer to common genes identified in the indicated data sets. (**b**) Level of histone acetylation across SP1-motif in the whole genome. Box plot shows the distribution of lower quartile 25% and upper quartile 75%. RPKM: Fragment per kb pair region per million sequence reads. Whiskers present minimal to maximal value. Unpaired t-test was performed for statistical analysis. *p≤0.05. (**c**) Level of histone acetylation across SP1-motif in the genome at Trrap DEGs. Box plot shows the distribution of lower quartile 25% and upper quartile 75%. Whiskers present minimal to maximal value. 'Up' indicates the acetylated histone peak among genes that are upregulated after Trrap-deletion, 'down' indicates those which are downregulated. Unpaired t-test was performed for statistical analysis. *p≤0.05, **p≤0.01. (**d**) Genes overlapping in RNA-seq from cortices and striatum and AcH3 ChIP-seq in aNSCs. The $log_2$ of the fold changes in RNA-seq of cortices and AcH3 ChIP-seq of the 282 genes where decreased AcH3 was observed concomitant with changes in RNA levels. STMN3 and STMN4 are targets analyzed in this study and related to neuronal homeostasis. (**e**) Genes overlapping in RNA-seq from cortices and striatum and AcH4 ChIP-seq in aNSCs. $Log_2$ of the fold changes in RNA-seq of cortices and AcH4 ChIP-seq of the 136 genes where decreased AcH4 was observed concomitant with changes in RNA levels. STMN4 is a target analyzed in this study and related to neuronal homeostasis. (**f**) Quantitative PCR (q-PCR) analysis of Sp1 target genes in Trrap-FBΔ cortex and striatum. N = 4, the number of mice analyzed. Unpaired t-test was performed for statistical analysis. *p≤0.05; ***p≤0.001; n.s.: not significant. (**g**) Genomic view of the *Stmn3* and *Stmn4* gene showing the levels of histone H3 and H4 acetylation in control and mutant aNSCs from ChIP-seq data sets (***Supplementary file 5***). The primers used for ChIP assays are located in the promoter proximity of respective genes. Lower panels depict the sequence of both ChIP probes, in which Sp1 consensus is shown.

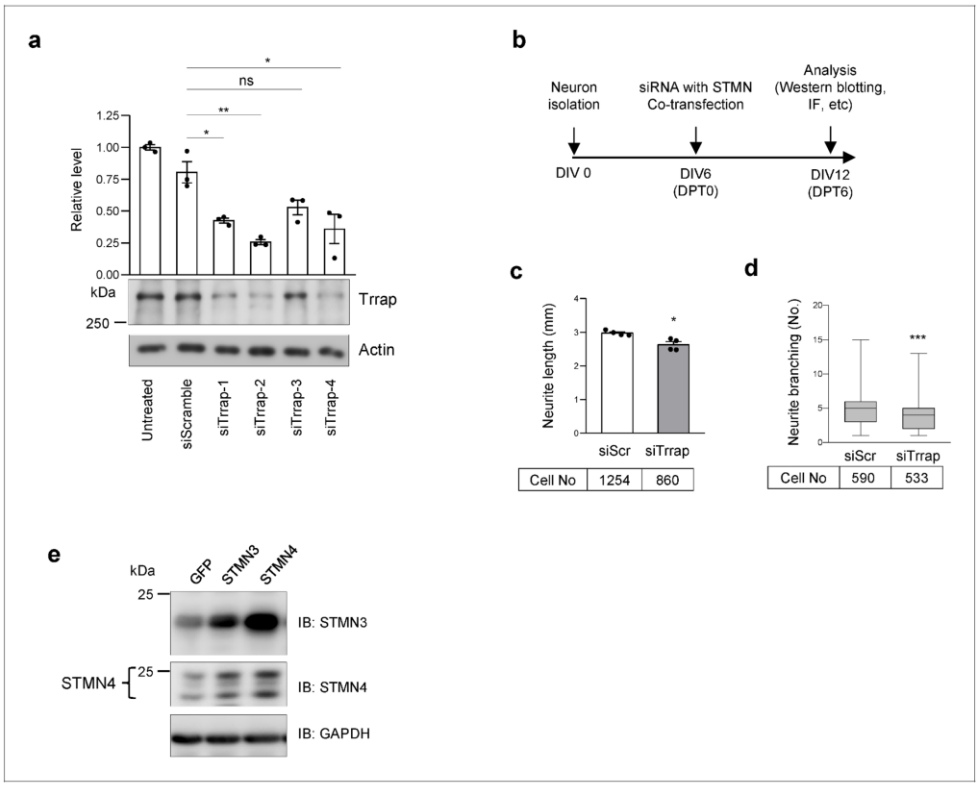



**Figure 5—figure supplement 1.** Ectopic expression of STMNs rescues defects of Trrap knockdown neurons. (**a**) Western blot analysis of the siRNA mediated Trrap knockdown in N2A cells. Various siRNAs against Trrap (−1,–2, −3,–4) and siScramble (as a control) are shown. The efficiency of the Trrap knockdown was determined by ImageJ software and is shown on the top of the blot. The Trrap level is normalized to the non-treated sample after correction to GAPDH. The error bar presents the standard error. Unpaired t-test was used for statistical analysis. *p≤0.05, **p≤0.01. (**b**) Scheme of primary neuron transfection and analysis. The primary neurons were isolated from E16.5 murine cortical tissue and cultured for 6 days (DIV) prior to transfection. Six days post-transfection (DPT), the neuronal culture was subject to analysis. (**c**) The neurite length was acquired and analyzed by IncuCyte at DPT6. The total neurite length (mm) was divided by the number of the cell-body cluster in the whole culture plate. Only GFP-positive (indicative of transfection) neurons were scored. The mouse embryo number: N = 6. The error bar represents the standard deviation. Unpaired t-test was used for a statistical analysis. *p≤0.05. (**d**) The neuronal culture was imaged by IncuCyte at DPT6 and the neurite branching number per cell-body cluster is shown. The neurite branching was scored manually on all GFP-positive only neurons. The mouse embryo number: N = 6. The error bar represents the standard deviation. Unpaired t-test was used for statistical analysis. ***p≤0.001.

## Materials and methods

### Key resources table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|---|---|---|---|---|
| Gene (*M. musculus*) | Trrap | Genebank | MGI:MGI:2153272 | |
| Gene (*M. musculus*) | Sp1 | Genebank | MGI:MGI:98372 | |
| Gene (*M. musculus*) | STMN3 | Genebank | MGI:MGI:1277137 | |
| Gene (*M. musculus*) | STMN4 | Genebank | MGI:MGI:1931224 | |

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| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|---|---|---|---|---|
| Strain, strain background (*M. musculus*) | Trrapf/f; Pcp2-Cre | This paper | | Trrap deletion in Purkinje cells; *M. musculus*, male and female; Please refer to 'Materials and methods' in the paper, Section 'Mice' |
| Strain, strain background (*M. musculus*) | Trrapf/f; Camk2-Cre | This paper | | Trrap deletion in forebrain; *M. musculus*, male and female; Please refer to 'Materials and methods' in this paper, Section 'Mice' |
| Strain, strain background (*M. musculus*) | Trrapf/+; Rosa26-CreERT2 Trrapf/f; Rosa26-CreERT2 | This paper | | Trrapf/+ acts as a control to Trrapf/f Trrap deletion in adult neural stem cells; *M. musculus*, male and female; Please refer to 'Materials and methods' in this paper, Section 'Mice' |
| Strain, strain background (*M. musculus*) | B6.Cg-Tg(Thy1-Brainbow1.0)HLich/J (R26R-Confetti); Trrapf/f; Pcp2-Cre | This paper | | Tracing of the single Purkinje cells; *M. musculus*, male and female; Please refer to 'Materials and methods' in this paper, Section 'Mice' |
| Genetic reagent (*M. musculus*) | Lipofectamine 2000 | Invitrogen | Cat#: 11668027 | siTrrap and Plasmid co-transfection; *M. musculus* |
| Genetic reagent (*M. musculus*) | Lipofectamine RNAiMAX | Invitrogen | Cat#: 13778075 | siSp1 transfection; *M. musculus* |
| Cell line (*M. musculus*) | Trrap-aNSC | This paper | | Primary cell line; *M. musculus*; Please refer to 'Materials and methods' in this paper, Section 'aNSC cell culture'. |
| Cell line (*M. musculus*) | E16.5 cortical neuron | This paper | | Primary cell line; *M. musculus*; Please refer to 'Materials and methods' in the paper, Section 'Isolation and culture of murine primary neurons'. |
| Cell line (*M. musculus*) | Neuro-2a Neuroblastoma cells | PMID:4534402 | ATCC CCL-131 | Cell line; *M. musculus* |
| Transfected construct (*M. musculus*) | ON-TARGETplus siRNA Reagents -Mouse (siScramble) | Horizon Discovery | Cat#: D-001810-10-05 | UGGUUUAC AUGUCGACUAA; *M. musculus* |
| Transfected construct (*M. musculus*) | siTrrap-1 | Horizon Discovery | Cat#: LQ-051873-01-0005 | CAAAAGUAG UGAACCGCUA; *M. musculus* |
| Transfected construct (*M. musculus*) | siTrrap-2 | Horizon Discovery | Cat#: LQ-051873-01-0005 | CCUACAUUG UGGAGCGGUU; *M. musculus* |

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| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|---|---|---|---|---|
| Transfected construct (*M. musculus*) | siTrrap-3 | Horizon Discovery | Cat#: LQ-051873-01-0005 | GCCAACUGUC AGACCGUAA; *M. musculus* |
| Transfected construct (*M. musculus*) | siTrrap-4 | Horizon Discovery | Cat#: LQ-051873-01-0005 | CGUACCUGG UCAUGAACGA; *M. musculus* |
| Antibody | Anti-Calbindin (Mouse Monoclonal) | Sigma | Cat#:C9848 RRID:AB_476894 | IF:1:300 WB: 1:1000 |
| Antibody | Anti-GFAP (Mouse Monoclonal) | Agilent | Cat#:G3893 RRID:AB_477010 | IF:1:300 WB: 1:1000 |
| Antibody | Anti-MBP (Mouse Monoclonal) | Millipore | Cat#:MAB384 RRID:AB_240837 | IF:1:300 |
| Antibody | Anti-GFP (Rabbit Monoclonal) | Cell Signaling Technology | Cat#:2956 RRID:AB_1196615 | IF: 1:200 |
| Antibody | Anti-GFP (Mouse Monoclonal) | Santa Cruz | Cat#:sc-390394 | IF:1:200 WB: 1:400 |
| Antibody | Anti-Sp1 (Mouse Monoclonal) | Santa Cruz | Cat#:sc-17824 RRID:AB_628272 | IF: 1:50 |
| Antibody | Anti-Sp1 (Rabbit Polyclonal) | Millipore | Cat#:07–645 RRID:AB_310773 | WB:1:1000 ChIP: 1:80 |
| Antibody | Anti-STMN3 (Rabbit Polyclonal) | Proteintech, | Cat#:11311–1-AP RRID:AB_2197399 | IF:1:100 WB:1:1000 |
| Antibody | Anti-STMN4 (Mouse Monoclonal) | Santa Cruz | Cat#:sc-376829 | IF:1:100 WB:1:1000 |
| Antibody | Anti-Tuj1 (Mouse Monoclonal) | Covance | Cat#: MMS-435P RRID:AB_2313773 | IF:1:400 |
| Antibody | Anti-CNPase (Mouse Monoclonal) | Sigma | Cat#: SAB4200693 | IF:1:1000 |
| Antibody | Anti-Galectin3 (Rat Monoclonal) | eBioscience | Cat#:14-5301-82 RRID:AB_837132 | WB:1:1000 |
| Antibody | Anti-a-tubulin (Mouse Monoclonal) | Sigma | Cat#:sc-32293 RRID:AB_628412 | WB: 1:5000 |
| Antibody | Anti-TRRAP (Mouse) clone TRR-2D5 | Euromedex | ID: IG-TRR-2D5 | WB:1:1000 |
| Antibody | Anti-TRRAP (Mouse) clone TRR-1B3 | Euromedex | ID: IG-TRR-1B3 | ChIP: 1:40 |
| Antibody | Anti-β-actin (Mouse Monoclonal) | Sigma | Cat#:A5441 RRID:AB_476744 | WB:1:3000 |
| Antibody | Anti-AcH3 (Rabbit Polyclonal) | Millipore | Cat#:06–599 RRID:AB_2115283 | ChIP: 1:150 |

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| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|---|---|---|---|---|
| Antibody | Anti-AcH4 (Rabbit Polyclonal) | Millipore | Cat#:06–866 RRID:AB_310270 | ChIP: 1:150 |
| Antibody | Anti-H3K4me2 (Rabbit Polyclonal) | Abcam | Cat#: ab7766 RRID:AB_2560996 | ChIP: 1:100 |
| Antibody | H3 (Rabbit Monoclonal) | Abcam | Cat#: ab1791 RRID:AB_302613 | ChIP: 1:150 |
| Antibody | H4 (Rabbit Polyclonal) | Abcam | Cat#: ab7311 RRID:AB_305837 | ChIP: 1:150 |
| Antibody | Oct-4 (Rabbit Monoclonal) | Cell Signaling | Cat#: 2840 RRID:AB_2167691 | ChIP: 1:80 WB:1:1000 |
| Antibody | IgG (Rabbit Polyclonal) | Sigma | Cat#: I8140 RRID:AB_1163661 | ChIP: 1:1500 (2 µg antibody) |
| Recombinant DNA reagent | EF1a-GFP-P2A-STMN3-Poly(A) (plasmid) | This paper | | STMN3 overexpression plasmid; *M. musculus*; Please refer to 'Materials and methods' in this paper, Section 'Construction of STMNs expression vectors'. |
| Recombinant DNA reagent | EF1a-GFP-Poly(A)-EF1a-STMN4-Poly(A) (plasmid) | This paper | | STMN4 overexpression plasmid; *M. musculus*; Please refer to 'Materials and methods' in this paper, Section 'Construction of STMNs expression vectors'. |
| Recombinant DNA reagent | −111 hTF m3 | Addgene | Cat#: 15450 | Sp1 activity reporter; *H. sapiens* |
| Recombinant DNA reagent | pN3-Sp1FL | Addgene | Cat#: 24543 | Sp1 overexpression reporter; *H. sapiens* |
| Sequence-based reagent | Sp1 primer | PrimerBank | ID 7305515a1 | Fwd, 5'-GCCGCCT TTTCTCAGACTC-3'; Rev, 5'-TTGGGTGACT CAATTCTGCTG-3' |
| Sequence-based reagent | STMN3 primer | PrimerBank | ID 6677873a1 | Fwd, 5'-CAGCACCG TATCTGCCTACAA-3'; Rev, 5'-GTAGATGGT GTTCGGGTGAGG-3'; |
| Sequence-based reagent | STMN4 primer | PrimerBank | ID 9790189a1 | Fwd, 5'-ATGGAAGT CATCGAGCTGAACA-3'; Rev, 5'-GGGAGGCATT AAACTCAGGCA-3'. |
| Sequence-based reagent | STMN3 promoter primer | This paper | | Fwd, 5'-CTTGCTACTG CATCAGGCGA-3'; Rev, 5'-AGCCTAGGG GATCATGGGAC-3'; |
| Sequence-based reagent | STMN4 promoter primer | This paper | | Fwd, 5'-TCGCTTTGG AAACCGGACTG-3'; Rev, 5'-TTTGTTT AAAACCCCCGCCC-3'. |

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| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|---|---|---|---|---|
| Commercial assay or kit | Incucyte S3 | Sartorius AG | Product Code: 4695 | For neurite detection and quantification |
| Commercial assay or kit | RNeasy Lipid Tissue Mini Kit | Qiagen | Cat #: 74804 | |
| Commercial assay or kit | RNAeasy Mini Kit | Qiagen | Cat #: 74104 | |
| Commercial assay or kit | LightCycler 480 Real-Time PCR System | Roche | Product No. 05015243001 | |
| Commercial assay or kit | RNA 6000 nano kit | Agilent | Cat #: 5067–1511 | |
| Commercial assay or kit | TruSeq Stranded mRNA Kit | Illumina | Cat #: 20020594 | |
| Commercial assay or kit | Dual-Glo Luciferase Assay System | Promega | Cat# E2920 | |
| Commercial assay or kit | QiaQuick PCR Purification Kit | Qiagen | Cat# 28106 | |
| Commercial assay or kit | Fragment Analyzer | Agilent | Cat#: M5310AA | |
| Commercial assay or kit | NextSeq500 platform | Illumina | RRID:SCR_014983 | |
| Commercial assay or kit | TruSeq ChIP Sample Preparation Kit | Illumina | Cat#: IP-202–1024 | |
| Chemical compound, drug | Epoxy resin 'Epon' | SERVA | | Glycid ether 100 for electron microscopy |
| Chemical compound, drug | cOmplete, Mini, EDTA-free | Roche | Cat#: 04693159001 | Protease Inhibitor |
| Chemical compound, drug | PhosSTOP | Roche | Cat#: PHOSS-RO | Phosphatase Inhibitor |
| Chemical compound, drug | protein-A-conjugated magnetic beads | Invitrogen | Cat#: 10003D | |
| Chemical compound, drug | protein-G-conjugated magnetic beads | Invitrogen | 10001D | |
| Chemical compound, drug | Platinum SYBR Green qPCR SuperMix-UDG | Qiagen | 11733046 | |
| Software, algorithm | NeuronJ Plug-in by ImageJ software | National Institutes of Health | | Neurite tracing and quantification |
| Software, algorithm | Fiji plugins Simple Neurite Tracing | National Institutes of Health | | Sholl analysis |
| Software, algorithm | bcl2FastQ | Illumina | RRID:SCR_015058 | Version 1.8.4 |

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| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|---|---|---|---|---|
| Software, algorithm | STAR | PMID:23104886 | RRID:SCR_015899 | Version 2.5.4b; RNA sequence mapping parameters: `–alignIntronMax` 100000 `–outSJfilterReads` Unique `–outSAMmultNmax` 1 `–outFilterMismatch NoverLmax` 0.04 |
| Software, algorithm | FeatureCounts | PMID:24227677 | RRID:SCR_012919 | Version 1.5.0; parameters: metafeature mode, stranded mode '2', Ensembl 92 annotation |
| Software, algorithm | ENSEMBL annotation | PMID:31691826 | RRID:SCR_002344 | Release 92 for *Mus musculus* |
| Software, algorithm | MultiQC | PMID:27312411 | RRID:SCR_014982 | Version 1.6; RNA sequence quality assessment of the raw input data, the read mapping and assignment steps |
| Software, algorithm | R package DESeq2 | PMID:25516281 | RRID:SCR_015687 | Version 1.20.0; Analysis of differential expressed genes in pairwise comparisons. |
| Software, algorithm | R package VennDiagram | PMID:21269502 | RRID:SCR_002414 | Version 1.6.20 |
| Software, algorithm | Database for Annotation, Visualization and Integrated Discovery (DAVID) programs | https://david.ncifcrf.gov/home.jsp | | DAVID v6.7; Gene ontology (GO) and KEGG pathway enrichment analyses |
| Software, algorithm | TFBS enrichment analysis | UC San Diego, Broad Institute, USA | GSEA 4.1.0 | Based on GSEA database or Harmonizome database for Sp1 targets |
| Software, algorithm | Ingenuity Pathway Analysis (IPA) program | Qiagen | | Analysis of Sp1 targets affected by Trrap deletion |
| Software, algorithm | R package AnnotationDbi | Bioconductor | DOI: 10.18129/B9.bioc.AnnotationDbi | Version 1.42.1 |
| Software, algorithm | R package org.Mm.eg.db | Bioconductor | DOI: 10.18129/B9.bioc.org.Mm.eg.db | Version 3.6.0 |
| Software, algorithm | FastQC | Babraham Bioinformatics, UK | RRID:SCR_014583 | Version 0.11.5 |
| Software, algorithm | Bowtie | http://bowtie-bio.sourceforge.net | RRID:SCR_005476 | Version 1.1.2 |
| Software, algorithm | MACS14 | https://bio.tools/macs | RRID:SCR_013291 | |
| Software, algorithm | R | https://www.r-project.org/ | RRID:SCR_001905 | Version 3.4.4 |

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| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|---|---|---|---|---|
| Other | Beam walking | Homemade | | |
| Other | Mouse Rota-rod | Ugo Basile | Cat#: 47600 | |
| Other | DAPI stain | Invitrogen | Cat#: D1306 | 1:5000 |
| Other | Bioruptor | Diagenode | N/A | Sonication |
| Other | vibrating microtome HM 650 V | Thermo Scientific Microm | | Sagittal section cutting |
| Other | Reichert Ultracut S | Leica | | Ultrathin section cutting |
| Other | JEM 1400 electron microscope | JEOL | | Electron microscopic imaging |
| Other | Orius SC 1000 CCD-camera | GATAN | | Electron microscopic imaging |
| Other | Bioanalyzer 2100 | Agilent | | Quality check and quantification of RNA |

## Mice

Mice carrying the conditional (floxed; *Trrap*$^{f/f}$) allele (***Herceg et al., 2001***) were crossed with *Pcp2*-Cre transgenic mice (Tg(Pcp2-cre)2Mpin) (***Barski et al., 2000***), *Camk2*-Cre (Tg(Camk2a-cre/ERT2) 2Gsc), or *Rosa26*-CreER$^{T2}$ Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj}$, to generate mice with a specific deletion in Purkinje cells (Trrap-PCΔ) and forebrain glutamatergic neurons (Trrap-FBΔ), or an inducible deletion in all tissues (Trrap-iΔ). To trace the single cell morphology of Purkinje cells, B6.Cg-Tg(Thy1-Brainbow1.0)HLich/J (R26R-Confetti) knock-in mice were crossed with Trrap-PCΔ mice. The double-fluorescent reporter *mT/mG* knock-in mice (***Muzumdar et al., 2007***) were intercrossed with Trrap-aNSCΔ mice, to identify Trrap-deleted cells. The Trrap, Cre, mT/mG, and Confetti genotypes of mice were determined by PCR on DNA extracted from tail tissue, as previously described (***Loizou et al., 2009***). Animal experiments were conducted according to German animal welfare legislation, and the protocol is approved by Thüringen Landesamt für Verbraucherschutz (TLV) (03-042/16), Germany.

## Histology

Tissues for histology were fixed in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose and frozen in Richard-Allan Scientific Neg-50 Frozen Section Medium (Thermo Scientific, Waltham, MA, USA). The sections (thickness of 5–20 μm) were later used for immunofluorescence staining.

## Construction of STMNs expression vectors

The EF1a-GFP-P2A-STMN3-Poly(A) plasmid was generated by subcloning the RV-Cre2A-GFP (kindly provided by Xiaobing Qing) and the STMN3 protein coding region into the EF1a-GFP construct (***Li et al., 2015***). For the EF1a-GFP-Poly(A)-EF1a-STMN4-Poly(A) vector, EF1a-promoter, STMN4 protein coding region, and Poly(A) sequence were subcloned into the EF1a-GFP construct. The DNA fragments were assembled with Gibson Assembly Master Mix (New England Biolabs, Massachusetts, USA). The STMN3 and STMN4 protein coding regions were amplified from cDNA library of the murine 10 days postnatal forebrain samples.

## siRNA sequences

### Isolation and culture of murine primary neurons

Murine neurons were isolated from mouse embryos at embryonic stage E16.5 (E16.5). The cortex was removed and was first incubated with 0.05% trypsin under 37˚C for 15 min. The tissue was then

mechanically disintegrated with 1 ml Eppendorf pipettes in an incubation medium (Eagle's minimal essential medium (MEM) supplemented with 1× FCS, 1× B-27 Supplements, 500 µM L-glutamine, 1 mM sodium pyruvate, 1× penicillin–streptomycin, 10 mM HEPES). The suspension was filtered through a cell strainer (40 µm porosity). After centrifugation (630 rpm for 5 min) the neurons in supernatant were seeded into poly-L-lysine coated multiple well plates at the indicated number ($6 \times 10^4$ cells/well in 24-well plate, $3 \times 10^5$ cells/well in 6-well plate) and cultured in the Neurobasal medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 1 × B-27 Supplements, 500 µM L-glutamine, and 10 mM HEPES until further use.

## Motor coordination tests

Beam walking: Mice were trained to run along a 1 m long beam (3 cm thick) to their home cage. The test was performed on five consecutive days on a 2 cm thick beam, with three runs each day. The mice were video-taped and timed crossing the beam.

Rotarod test: Mice were habituated to the test situation by placing them on a rotarod (Ugo Basile, Gemonio, Italy) with constant rotation (5 rpm) for 5 min the day prior to the test. In the test phase, two trials per mouse were performed with accelerating rotation (2–50 rpm within 4 min) and maximum duration of 5 min, with the time measured until mice fell off the rod.

## N2A cell culture

N2A cells were cultured in DMEM supplemented with 1× FCS, 1× penicillin–streptomycin, and 10 mM HEPES. When the N2A culture reached ~70% confluency, the cells were trypsinized and the cell suspension was centrifuged. The cells were seeded in $1.5 \times 10^5$ cells/well onto 6-well plate.

## Transfection of primary neurons or N2A cells

Primary neurons were transfected on day 6 in vitro (DIV6) and N2A cells on day 1 after passage using lipofectamin 2000 in Opti-MEM (0.4 µg plasmid + 0.8 µl lipofectamine 2000 in 100 µl Opti-MEM/well in 24-well plate, 1.2 µg plasmid + 2.4 µl lipofectamine 2000 in 300 µl Opti-MEM/well in 6-well plate, and/or 25 µM siRNA). After 30 min incubation under 37°C with the Neurobasal medium supplemented with 500 µM L-glutamine (300 µl/well in 24-well plate, 1.2 ml/well in 6-well plate), the plasmid/siRNA-Lipofectamine mix was replaced by the neuronal culture medium.

## IncuCyte quantification

The primary neuron culture was placed into Incucyte S3 (Sartorius AG, Göttingen, Germany) for imaging acquisition of phase contrast and GFP signals (10× magnification, 36 images/well in 24-well plate, and 144 images/well in 6-well plate). The image analysis and the neurite detection parameter were determined for each plate separately through IncuCyte NeuroTrack Software Module for S3 or ZOOM.

## Immunofluorescent staining and quantification

Prior to immunostaining, primary neurons on coverslips were fixed with 4% PFA and incubated with 0.7% Triton in PBS for 15 min. The fixed samples were incubated with primary antibodies under 4°C overnight. After incubation with secondary antibody in 1:5000 DAPI, the samples were conserved by ProLong Gold Antifade reagent (Thermo Fisher Scientific). Images were captured by the ApoTome microscope (Zeiss Jena, Germany) under 20× or 40× objectives. The neurite branching, neurite length, and axonal swelling were then scored with NeuronJ Plug-in by ImageJ software and validated manually.

## qRT-PCR analysis

The total RNA was isolated from tissues or aNSCs using the RNeasy Lipid Tissue Mini Kit (Qiagen) and an RNAeasy Mini Kit (Qiagen) respectively and following the manufacturer's instructions. cDNA was synthesized using the SuperScript III Reverse Transcriptase (Thermo Fischer Scientific). qPCRs were performed using Platinum SYBR Green qPCR SuperMix-UDG (Thermo Fischer Scientific) and a LightCycler 480 Real-Time PCR System (Roche). The Trrap and Actin primers used for amplification were previously described (***Tapias et al., 2014***). The remaining primer sequences were obtained from the PrimerBank (***Spandidos et al., 2010***; ***Spandidos et al., 2008***; ***Wang, 2003***) and were as

follows: Sp1 (PrimerBank ID 7305515a1): Fwd, 5′-GCCGCCTTTTCTCAGACTC-3′; Rev, 5′-TTGGG TGACTCAATTCTGCTG −3′; STMN3 (PrimerBank ID 6677873a1): Fwd, 5′- CAGCACCGTATCTGCC TACAA-3′; Rev, 5′-GTAGATGGTGTTCGGGTGAGG-3′; STMN4 (PrimerBank ID 9790189a1): Fwd, 5′-ATGGAAGTCATCGAGCTGAACA-3′; Rev, 5′- GGGAGGCATTAAACTCAGGCA-3′. Quantification of the qPCR data was performed by the ΔΔCp method using actin as an internal control. Gene expression values were expressed relative to the gene expression in control tissues or aNSCs.

## TUNEL reaction and immunofluorescence staining in brain sections

Immunofluorescence and TUNEL staining were performed on cryosections prepared from PFA-fixed brains of the indicated ages, as previously described (***Tapias et al., 2014***), using the following antibodies: mouse anti-Calbindin (1:300, Sigma), rabbit anti-GFAP (1:300, Agilent, Santa Clara, USA), mouse anti-MBP (1:300, Millipore, Burlington, USA), rabbit anti-Calbindin (1:300, Swant, Marly, Switzerland), rabbit anti-GFP (1:200, Cell Signaling Technology, Danvers, USA) mouse anti-GFP (1:200, Santa Cruz), rabbit anti-Sp1 (1:50, Santa Cruz), mouse anti-STMN4 (1:100, Santa Cruz), rabbit anti-STMN3 (1:100, Proteintech, Rosemont, USA), and mouse anti-Tuj1 (1:400, Covance).

## Immunoblot analysis

Total protein lysates were prepared from brain tissue or aNSCs using the RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF), and complete mini protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany). N2A cells or neuron lysates were prepared as follows: the culture was treated with 0.25% trypsin for 5 min under 37˚C, resuspend with DMEM medium supplemented with 1× FCS and centrifuged under 1100 rpm for 5 min. The resulting pellets were washed once with PBS and lysed with the RIPA buffer. Immunoblotting was performed as described previously (***Tapias et al., 2014***), using the following antibodies: mouse anti-Calbindin (1:1000, Sigma), rabbit anti-GFAP (1:1000, Dako-Agilent), mouse anti-CNPase (1:1000, Sigma), rat anti-galectin3 (1:1000, eBioscience, Affymetrix, Santa Clara, USA), mouse anti-α-tubulin (1:5000, Sigma), mouse anti-TRRAP (1;1000, Euromedex, Souffelweyersheim, France), rabbit anti-Sp1 (1:1000, Millipore), mouse anti-RB3/STMN4 (1:1000, Santa Cruz), rabbit anti-STMN3 (1:1000, Proteintech), mouse anti-GAPDH (1:1000, Sigma), mouse anti-β-actin (1:5000, Sigma), mouse anti-GFP (1:400, Santa Cruz), and rabbit anti-Oct4 (1:1000, Cell Signaling).

## Dendritic tree analysis

The tissues were fixed in 4% paraformaldehyde (PFA) and embedded in 4% low-melting agarose. 200 µm sagittal sections were obtained using a vibrating microtome HM 650 V (Thermo Scientific Microm) and mounted in slides. Imaging was performed using a Zeiss LSM 710 Confocal three microscope (Zeiss) and the Sholl analysis (***Kroner et al., 2014***; ***Sholl, 1953***) achieved using the Fiji plugins Simple Neurite Tracing (***Longair et al., 2011***).

## Transmission electron microscopy

Mice were sacrificed using $CO_2$ and perfused intracardially with cold fixative (3% glutaraldehyde, 1% paraformaldehyde, 0.5% acrolein, 4% sucrose, 0.05 M $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.3). The cerebellum was isolated and postfixed for a minimum of 1 day. For a secondary fixation, the samples were incubated in 2% $OsO_4$/1% potassium ferrocyanide in 0.1 M cacodylate buffer for 3 hr at 4˚C in the dark, followed by dehydration in an ascending water/acetone series – then embedded in epoxy resin 'Epon' (glycid ether 100, SERVA, Heidelberg, Germany). The resin was allowed to polymerize for 2 days at 60˚C in flat embedding molds. Ultrathin sections (50 nm) were produced using an ultramicrotome (Reichert Ultracut S; Leica, Wetzlar, Germany) and electron micrographs taken on a JEM 1400 electron microscope (JEOL, Akishima, Japan), using an accelerating voltage of 80 kV coupled with Orius SC 1000 CCD-camera (GATAN, Pleasanton, USA).

## Transcriptomics

The total RNA was isolated from tissues or cultured aNSCs using an RNeasy Lipid Tissue Mini Kit (Qiagen, Venlo, The Netherlands) and an RNAeasy Mini Kit (Qiagen) respectively, per manufacturer's instructions. The sequencing of RNA samples was done using Illumina's next-generation sequencing methodology (***Bentley et al., 2008***) – the quality check and quantification of the total RNA were

completed using the Agilent Bioanalyzer 2100 in combination with the RNA 6000 nano kit (Agilent Technologies). For library preparation 3 µg of tissue total RNA or 800 ng of aNSC total RNA were introduced to the TruSeq Stranded mRNA Kit (Illumina, San Diego, USA), per manufacturer's description. The quantification and quality check of the libraries were conducted using the Agilent Bioanalyzer 2100 in combination with the DNA 7500 kit. For sequencing of tissues, pools of four libraries were compiled and each pool was loaded on one lane of a HiSeq2500 machine running in 51cycle/single-end/high-output mode. For the sequencing of aNSCs, all libraries were pooled and loaded on three lanes of a HiSeq2500 machine running in 51 cycle/single-end/high-output mode. The sequence information was extracted in FastQ format using Illumina's bcl2FastQ v1.8.4. The sequencing resulted in around 55mio and 37mio reads per sample for tissues and aNSCs, respectively.

The reads of all samples were mapped to the mouse reference genome (GRCm38) with the Ensembl genome annotation (Release Ensembl 92) using STAR (version 2.5.4b; parameters: `–alignIntronMax` 100000 `–outSJfilterReads` Unique `–outSAMmultNmax` 1 `–outFilterMismatchNoverLmax` 0.04) (***Dobin et al., 2013***). Reads mapped uniquely to one genomic position were assigned to the gene annotated at this position with FeatureCounts (version 1.5.0; meta-feature mode, stranded mode '2', Ensembl 92) (***Liao et al., 2014***). A quality assessment of the raw input data, the read mapping and assignment steps, was performed using MultiQC (version 1.6) (***Ewels et al., 2016***), with the respective results provided in ***Supplementary file 6***.

Read counts per gene were subjected to the R package DESeq2 (version 1.20.0) (***Love et al., 2014***), to test for differential expressions in pairwise comparisons as follows: Cultured aNSCs: five mutants contrasted to five controls; Cortex: four mutants contrasted to four controls; Striatum: four mutants contrasted to four controls. For each gene and comparison, the p-value was calculated using the Wald significance test. The resulting p-values were adjusted for multiple testing with Benjamini and Hochberg correction. Genes with an adjusted $p<0.05$ (false discovery rate, FDR) are considered differentially expressed. The log2 fold changes (LFC) were shrunk with lfcShrink from the DESeq2 package, to control for a variance of LFC estimates for genes with low read counts. The overlaps of all three pairwise DEG lists were calculated and visualized using the R package VennDiagram (version 1.6.20).

GO and KEGG pathway enrichment analyses were performed by supplying the gene lists of DEG overlaps into the database for annotation, visualization, and integrated discovery (DAVID) programs (***Huang et al., 2009a***; ***Huang et al., 2009b***). TFBS enrichment analysis was performed by supplying the different lists of DEGs into the gene set enrichment analysis (GSEA) database (***Subramanian et al., 2005***; ***Mootha et al., 2003***). A list of the Sp1 targets was extracted from the Harmonizome database (***Rouillard et al., 2016***) and compared with the RNA-seq data sets. The lists of Sp1 targets affected by the Trrap deletion was then analyzed using the Ingenuity Pathway Analysis (IPA) program (Qiagen).

## Sample preparation for MS proteomics

First, homogenates of the cortex tissues were prepared using the bead-beating device (24 tissue homogenizer) from Precellys (Montigny-le-Bretonneux, France). Frozen tissue was transferred on ice to bead-beating tubes (Precellys CKMix, 0.5 ml) containing ice-cold PBS with Protease and a Phosphatase Inhibitor cocktail (Roche) and beaten for 2 cycles of 20 s at 6000 rpm, with a 30 s break at 4˚C. Homogenates were prepared at an estimated protein concentration of 10 µg/µl; based on 5% protein content of fresh brain tissues by weight. A volume of homogenate corresponding to approximately 500 µg protein was transferred to 1.5 ml Eppendorf tubes and taken for lysis. Lysis was carried out by resuspension of the homogenate in lysis buffer (final concentration 4% SDS, 0.1 M HEPES [pH 8], 50 mM DTT) to a final protein concentration of 1 µg/µl, followed by a sonication in a Bioruptor (Diagenode, Seraing, Belgium) (10 cycles, 1 min ON/30 s OFF, 20˚C). The samples were heated (95˚C, 10 min), and sonication steps repeated. The lysates were clarified by brief centrifugation, incubated with iodacetamide, (15 mM) at RT, in the dark. Each sample was treated with four volumes ice-cold acetone to precipitate the proteins (overnight, −20˚C). The samples were centrifuged at 20,800 g (30 min, 4˚C). The supernatant was removed and the pellets washed twice with 400 µl of ice-cold 80% acetone/20% water. The pellets were air-dried before dissolving in a digestion buffer (3 M urea in 0.1 M HEPES, pH 8) at 1 µg/µl. A 1:100 w/w amount of LysC (Wako, Richmond, USA; sequencing grade) was added to each sample before incubation (4 hr, 37˚C, 1000 rpm).

The samples were diluted 1:1 with milliQ water and incubated with a 1:100 w/w amount of trypsin (Promega, Madison, USA; sequencing grade) (overnight, 37˚C, 650 rpm). The digests were acidified with 10% trifluoroacetic acid and desalted with Waters Oasis HLB µElution Plate 30 µm (Waters, Milford, USA) in the presence of a slow vacuum, to manufacturer's instructions. The eluates were dried down with the speed vacuum centrifuge. Peptide labeling with TMT and subsequent high pH fractionation and LC-MS were conducted as detailed previously (***Buczak et al., 2018***). Briefly, the peptide samples obtained from the digestion were labeled with TMT-10plex isobaric mass tags (Thermo Fischer Scientific) per manufacturer's instructions. Equal amounts of the labeled peptides from the 10 samples (five replicates each condition) were mixed, desalted, and pre-fractionated into 16 fractions using high pH reverse phase fractionation on an Agilent Infinity 1260 HPLC, then each fraction was measured individually by nano-LC-MS on an Orbitrap Fusion Lumos employing SPS-MS3 data acquisition (Thermo Fischer Scientific). Subsequently, the fraction data were searched together in Mascot 2.5.1 (Matrix Science, Boston, USA) using Proteome Discoverer 2.0 (Thermo Fischer Scientific) against the Swissprot *Mus musculus* database (2016; 16,756 entries) and a list of common contaminants. Reporter ion intensity values for the PSMs were exported and processed using in-house written R scripts to remove common contaminants and decoy hits. Only PSMs having reporter ion intensities above $1 \times 10^3$ in all the relevant TMT channels were retained for a quantitative analysis, as described in ***Buczak et al., 2018***. Briefly, the reporter ion (TMT) intensities were $\log_2$-transformed and normalized. Peptide-level data were summarized into their respective protein groups by taking the median value. For differential protein expression, the five replicates of the two conditions respectively within the TMT10-plex were taken together. Protein ratios were calculated for all protein groups quantified with at least two peptides. To compare DEP in the cortex obtained by RNA-seq to protein DEP (differentially expressed proteins) obtained by mass spectrometry, Ensembl gene IDs were mapped to Uniprot IDs with the R packages AnnotationDbi (1.42.1) and org.Mm.eg.db (3.6.0), while only genes/proteins present in both analyses were considered. When for a single Uniprot ID multiple Ensembl IDs are known, the proteomics measurement is duplicated and all different transcriptomics results assigned to this entry.

## aNSC cell culture

The SVZ of 2–4 months old mice were isolated, minced, and digested with DMEM/F-12 medium supplemented with 20 U/ml papain, 240 µg/ml cysteine, and 400 µg/ml DNAse I type IV. After 1 hr, the digestion was stopped by ovomucoid trypsin inhibitor. The homogenized aNSCs were then cultured in suspension medium (DMEM/F-12 medium supplemented with 1× B-27 Supplements, 1× penicillin–streptomycin, 20 ng/ml EGF, 20 ng/ml bFGF). To induce Trrap deletion, aNSCs were treated with 1 µM 4-hydroxytamoxifen (4-OHT) for 3 days, followed by incubation in fresh medium for another 2 days.

## Transfection, Sp1 knockdown, and luciferase assay

$2 \times 10^5$ aNSCs were plated in 50 µg/ml PLL and 10 µg/ml laminin pre-coated 24-well plates in Neurobasal Medium (NEM) supplemented with 1× B-27 Supplements, 2 mM L-glutamine, 1× N-2 Supplement, 1× penicillin–streptomycin, 20 ng/ml EGF, and 20 ng/ml bFGF. The transfection was performed after overnight culture using Lipofectamine 2000. For luciferase assay of Sp1 activity, the vector −111 hr TF m3 was used as Sp1 reporter plasmid – gifted by Nigel Mackman (Addgene plasmid # 15450; http://n2t.net/addgene:15450; RRID:Addgene_15450). Guntram Suske gifted the vector pN3-Sp1FL used to overexpress Sp1 (Addgene plasmid # 24543; http://n2t.net/addgene:24543; RRID:Addgene_24543). 24 hr later, transfection cells were collected to measure the luciferase activity using a Dual-Glo Luciferase Assay System (Promega), per manufacturer's instructions. For Sp1 knockdown, aNSC in adherent conditions was supplemented with 30 nM siRNA against Sp1 mixed with RNAiMAX reagent (Thermo Fischer Scientific). After 48 hr, transfection cells were collected for immunoblot analysis.

## Chromatin preparation for ChIP and ChIP-seq

$2 \times 10^6$ aNSCs were cross-linked by adding formaldehyde 1% for 10 min at room temperature, quenched with 0.125 M glycine for 5 min at room temperature, then washed three times in phosphate-buffered saline (PBS) before freezing. Pellets were suspended in 0.25 ml SDS lysis buffer (50

mM HEPES-KOH, 140 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1% sodium deoxycholate, 1% SDS, 10 mM NaB, and protease inhibitors), incubated on a rotator for 30 min at 4˚C, sonicated for 20 min at 4˚C, then centrifuged at 14,000 rpm for 10 min at 4˚C. Supernatants were diluted 10-fold with a ChIP dilution buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, 10 µM NaB, and protease inhibitors) (25 µl retained as input) and incubated overnight in gentle rotation at 4˚C with 4 µg of antibody. The following antibodies were used: rabbit anti-SP1 (Millipore), rabbit anti-acetyl-Histone 3 (Millipore), mouse anti-TRRAP (Euromedex), rabbit anti-acetyl-Histone 4 (Millipore), rabbit anti-H3K4me2 (Abcam), rabbit anti-H3 (Abcam), rabbit anti-H4 (Abcam), rabbit anti-Oct4 (Cell Signaling), and rabbit anti-IgG (Sigma). After that, 40 µl of preblocked protein-G-conjugated magnetic beads (DYNAL, Thermo Fischer) were added and incubated for 2 hr in a rotator at 4˚C. The immunoprecipitated complexes were washed three times in low-salt wash buffer (0.1% SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), once in high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl) and once in TE buffer. The complexes were eluted by adding 0.2 ml of Elution buffer (TE 1×, 1% SDS, 150 mM NaCl, 5 mM DTT) for 30 min in rotation at room temperature. The de-cross-linking was performed overnight at 65˚C. The de-cross-linked DNA was purified using a QiaQuick PCR Purification Kit (Qiagen) according to the manufacturer's instruction.

### ChIP-seq

For the library preparation, approximately 10 ng of purified ChIP DNA was end-repaired, dA-tailed, and adaptor-ligated using the TruSeq ChIP Sample Preparation Kit (illumina), to manufacturer's instructions. The size of the library was checked using Fragment Analyzer (Agilent) and the library sequenced on the NextSeq500 platform (illumina). The Fastq files quality check was performed with FastQC (v0.11.5). Fastq files mapping to mm9 genome was performed by using Bowtie (v1.1.2) with `–best –strata` -m one parameters. Duplicate reads were removed using a custom script. For peak calling, macs14 (v1.4.2) was used with `–nolambda` parameter and two different p-value cutoffs (1e-3 for histone modifications and 1e-5 for SP1). Other downstream analyses were done using R (v3.4.4). For a RPM (Read Per Million) calculation, the peaks were merged using the Peakreference function (TCseq_1.2.0 package). The merged peaks were used as the reference for the calculation of RPM for each sample by using a custom script. 10% or 30% of the most depleted regions in mutant versus control samples for histone modifications and Sp1 respectively were used as cutoff for defining differentially regulated regions. Differentially regulated regions were assigned to the nearest gene (ENSEMBL annotation), where the distance of the region was less than ±5 Kb to the TSS (Transcription Start Site).

ChIP qRT-PCR was performed using the Platinum SYBR Green qPCR SuperMix-UDG (Thermo Fischer Scientific) and a LightCycler 480 Real-Time PCR System (Roche). All experiment values were subtracted by those obtained with a rabbit nonimmune serum (IgG) and divided by input, as indicated in the literature (***Neri et al., 2012***). The following primers were used for amplification: STMN3: Fwd, 5'-CTTGCTACTGCATCAGGCGA-3'; Rev, 5'-AGCCTAGGGGATCATGGGAC-3'; STMN4: Fwd, 5'-TCGCTTTGGAAACCGGACTG-3'; Rev, 5'-TTTGTTTAAAACCCCCGCCC-3'.

| siRNA | Sequence (5'→ 3') |
|---|---|
| siScramble | UGGUUUACAUGUCGACUAA |
| siTrrap-1 | CAAAAGUAGUGAACCGCUA |
| siTrrap-2 | CCUACAUUGUGGAGCGGUU |
| siTrrap-3 | GCCAACUGUCAGACCGUAA |
| siTrrap-4 | CGUACCUGGUCAUGAACGA |
| siSp1 | GGAUGGUUCUGGUCAAAUAtt |


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## Author contributions

Alicia Tapias, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review and editing; David Lázaro, Conceptualization, Data curation, Formal analysis, Investigation, Writing - original draft; Bo-Kun Yin, Data curation, Formal analysis, Investigation; Seyed Mohammad Mahdi Rasa, Anna Krepelova, Erika Kelmer Sacramento, Formal analysis, Investigation; Paulius Grigaravicius, Formal analysis, Investigation, Methodology; Philipp Koch, Formal analysis; Joanna Kirkpatrick, Data curation, Formal analysis, Investigation, Methodology; Alessandro Ori, Conceptualization, Investigation, Methodology; Francesco Neri, Conceptualization, Data curation, Formal analysis; Zhao-Qi Wang, Conceptualization, Data curation, Supervision, Funding acquisition, Investigation, Writing - original draft, Project administration, Writing - review and editing

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# Additional files

## Supplementary files

- Source data 1. SP1-regulated molecular pathways. (**A**) Top30 nervous system processes targets of Sp1. DEGs in all three RNA-seq data sets were compared with the list of the Sp1 targets from the Harmonizome database (***Rouillard et al., 2016***) and the resulting list was analyzed using IPA to find the disease process associated with the DEGs (cutoff, $p<0.05$). (**B**) Top30 differentially expressed Sp1 targets. DEGs in the RNA-seq data sets were compared with the list of Sp1 targets from the

Harmonizome database and the Top30 DEGs (cutoff, p<0.05) are indicated. (**C**) Top30 protein changes of Sp1 targets. Proteins from the forebrain, whose expression changed after the Trrap deletion and correlated with the changes in RNA-seq, were compared with the list of Sp1 targets obtained from the Harmonizome database. The Top30 results based on the *q*-value are summarized.

- Supplementary file 1. The list of up- and downregulated genes (adjusted p-value <0.05) in different data sets. The list includes the DEGs in Trrap-FBΔ cortices (**A**), Trrap-FBΔ striata (**B**), and Trrap-deleted aNSCs (**C**). The list also includes comparisons between Trrap-FBΔ cortices and striata (**D**) and Trrap-FBΔ cortices, striata, and Trrap-deleted aNSCs (**E**). Moreover, it includes the GO (**F**) and KEGG (**G**) terms obtained from the list in (**D**), statistical information, and the list of genes in each group.

- Supplementary file 2. The list of protein changes in Trrap-FBΔ cortices. (**A**) The list of protein changes after Trrap deletion. (**B**) The comparison between protein changes (proteomics, $q < 0.1$) and mRNA changes (transcriptomics, adjusted p-value <0.05).

- Supplementary file 3. The results of TFBS enrichment analysis in different data sets. The list includes the results from the TFBS enrichment analysis using the following lists as a template: (**A**) DEGs in D. (**B**) First 2940 DEGs from the list in A sorted by adjusted p-value. (**C**) DEGs 2941 to 5090 from the list in A sorted by adjusted p-value. (**D**) First 2940 DEGs from the list in B sorted by adjusted p-value. (**E**) DEGs 2941 to 4741 from the list in B sorted by adjusted p-value. (**F**) DEGs in E.

- Supplementary file 4. Changes in Sp1 targets in different data sets. (**A**) A list of known Sp1 targets was obtained from the Harmonizome database (***Dubey et al., 2015***). The common gene names were transformed to Ensembl gene IDs using the online conversion tool from the DAVID database. (**B**) A comparison between the DEGs in Suppl. File 1A and the known Sp1 targets listed in (**A**). (**C**) A comparison between the DEGs in Suppl. File 1B and the known Sp1 targets listed in (**A**). (**D**) A comparison between the DEGs in Suppl. File 1D and the known Sp1 targets listed in (**A**). (**E**) The GO terms obtained from the list in (**D**), statistical information, and the list of genes in each group. (**F**) The KEGG terms obtained from the list in (**D**), statistical information, and the list of genes in each group. (**G**) The list includes the results of the Sp1 ChIP-seq in aNSCs. 30% of the most depleted regions in Trrap-D versus control aNSCs for Sp1 were used as cutoff for defining differentially regulated regions. (**H**) A comparison between the ChIP-seq results in (**G**) and the known Sp1 targets listed in (**A**). (**I**) The overlaps between the ChIP-seq results in (**G**) and the DEGs in Suppl. File 1D. (**J**) A comparison between the DEGs in (**I**) and the known Sp1 targets listed in (**A**).

- Supplementary file 5. Changes in acetylation after Trrap deletion. (**A**) The list includes the results of AcH3 ChIP-seq of aNSCs. The 10% most depleted regions in Trrap-Δ versus control aNSCs for AcH3 are summarized. (**B**) The list includes the results of the AcH4 ChIP-seq of aNSCs. The 10% most depleted regions in Trrap-Δ versus control aNSCs for AcH4 are summarized. (**C**) The overlaps between the genes mapped in (**A**) and (**B**). (**D**) The overlaps between the DEGs in D and the genes mapped in (**A**). (**E**) The overlaps between the DEGs in D and the genes mapped in (**B**). (**F**) The overlaps between the DEGs in D and the genes mapped in (**C**). (**G**) A combined list from (**D**) and (**E**) was created and compared with the list of Sp1 targets in ***Supplementary file 4A***. The list contains genes where the acetylation of H3 or H4 was decreased, whose expression was altered after Trrap deletion and which are reported Sp1 targets.

- Supplementary file 6. Quality assessment of RNA-seq raw input data. The table provides the results of the read mapping and assignment steps performed using MultiQC (version 1.6) (***Ewels et al., 2016***).

- Transparent reporting form

## Data availability

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through the GEO Series accession numbers GSE131213 (RNA-seq aNSCs), GSE131283 (RNA-seq brain tissues) and GSE131028 (ChIP-seq aNSCs). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository, with the dataset identifier PXD013730.

The following datasets were generated:

| Author(s) | Year | Dataset title | Dataset URL | Database and Identifier |
|---|---|---|---|---|
| Kirkpatrick J, Ori A, Wang ZQ | 2021 | The mass spectrometry proteomics of different brain areas of Trrap conditional knockout *Mus musculus*. | https://www.ebi.ac.uk/pride/archive/projects/PXD013730 | PRIDE, PXD013730 |
| Groth M, Koch P, Pellón DL, Wang ZQ | 2021 | RNA-seq of murine primary adult stem cells of Trrap inducible knockout *Mus musculus* with and without 4-OHT treatment | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131213 | NCBI Gene Expression Omnibus, GSE131213 |
| Groth M, Koch P, Pellón DL, Soler AT, Wang ZQ | 2021 | RNA-seq of different brain tissue areas of Trrap conditional knockout *Mus musculus* | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131283 | NCBI Gene Expression Omnibus, GSE131283 |
| Krepelova A, Rasa SMM, Neri F, Wang ZQ | 2021 | ChIP-seq of adult neuro-stem cells of Trrap inducible knockout *Mus musculus* with and without 4-OHT treatment. | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131028 | NCBI Gene Expression Omnibus, GSE131028 |

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### 3.2 Manuscript II

#### 3.2.1 Author contribution

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| **Author** | **Conceptual** | **Writing the manuscript** |
|---|---|---|
| Bo-Kun Yin | 50% | 70% |
| Zhao-Qi Wang | 50% | 30% |
| Total: | 100% | 100% |

Contribution of authors to the manuscript:

Bo-Kun Yin: Writing - original draft; writing - review and editing

Zhao-Qi Wang: Supervision; writing - original draft, writing - review and editing

### 3.2.2 Manuscript

*Review*

# Beyond HAT Adaptor: TRRAP Liaisons with Sp1-Mediated Transcription

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**Abstract:** The members of the phosphatidylinositol 3-kinase-related kinase (PIKK) family play vital roles in multiple biological processes, including DNA damage response, metabolism, cell growth, mRNA decay, and transcription. TRRAP, as the only member lacking the enzymatic activity in this family, is an adaptor protein for several histone acetyltransferase (HAT) complexes and a scaffold protein for multiple transcription factors. TRRAP has been demonstrated to regulate various cellular functions in cell cycle progression, cell stemness maintenance and differentiation, as well as neural homeostasis. TRRAP is known to be an important orchestrator of many molecular machineries in gene transcription by modulating the activity of some key transcription factors, including E2F1, c-Myc, p53, and recently, Sp1. This review summarizes the biological and biochemical studies on the action mode of TRRAP together with the transcription factors, focusing on how TRRAP-HAT mediates the transactivation of Sp1-governing biological processes, including neurodegeneration.

**Keywords:** TRRAP; HAT; Sp1; transcription; neuro-development; neuodegeneration

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## 1. The PIKK Family and TRRAP

Phosphatidylinositol 3-kinase-related kinases (PIKK) are a protein family consisting of six serine/threonine protein kinases that transfer signals to a variety of protein substrates to control multiformities of biological processes. PIKK members share a structural commonality and identity in protein domains. Each PIKK member contains the HEAT repeat, FAT domain, and FATC motif (Figure 1). Although the catalytic domain shares homology between the PIKK and the PI3K family, the PIKK catalytic motif varies from PI3K in the structure of the ATP-binding motif, VAIK, and the divalent cation-binding motif, DFG. Functionally, PIKKs are not involved in lipid phosphorylation, as is the major task of PI3K [1] (Figure 1). Despite similar biochemical function of the PIKK members, the biological functions of these members are very diverse. DNA-PKcs, ATM, and ATR are considered to be important regulators in DNA damage response (DDR) and DNA repair [2]. mTOR is a nutrient-responding kinase, which regulates pathways in metabolism and cell growth [3]. SMG1 is involved in mRNA decay, a process of preventing an accumulation of toxic RNA species due to a premature stop codon or the presence of aberrant 3′-UTR [4]. TRRAP, a short term for transformation/transcription domain-associated protein, is the only pseudokinase in this family, which was initially identified as a protein involved in oncogenic transformation and plays an essential role in gene transcription [5]. Generally, the kinase domain without its catalytic activity, if conserved throughout evolution, is considered the fundamental importance of the non-catalytic function of the protein [6].

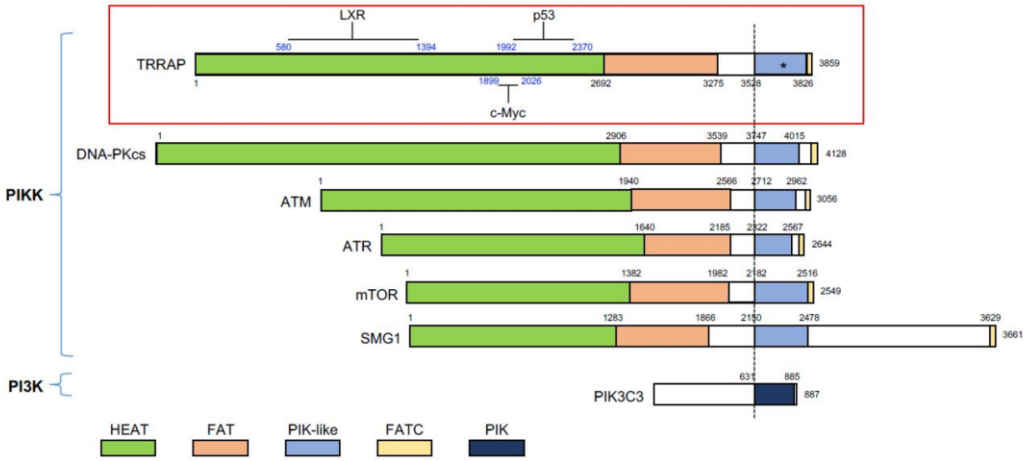


**Figure 1.** Protein domains of the phosphatidylinositol 3-kinase-related kinase (PIKK) family members. All members contain the PIK-like domain that confers kinase activity, except TRRAP that lacks the key motifs (marked with asterisk). The PIK-like domain is flanked by the FAT domain and the FATC domain. PIK3C3, a member of phosphoinositide 3-kinases (PI3K), serves as a comparison. The known regions (blue numbers) in TRRAP, which interact with p53, c-Myc, and LXR (liver X receptor), are shown. Many of the proteins have been reported to associate with TRRAP; however, if there are no exact regions mapped, these proteins are not shown. The black numbers indicate the amino acid sequence of TRRAP. All of the proteins were aligned with the dashed line, which indicates the N-terminal border of the PIK-like/PIK domain. PIKK, phosphatidylinositol 3-kinase-related kinase; PI3K, phosphoinositide 3-kinases; PIK3C3, phosphatidylinositol 3-kinase catalytic subunit type 3.

TRRAP is a huge protein consisting of 3859 amino acids [1] of 434 kD molecular weight. Initially, TRRAP was identified as an essential co-activator of c-Myc and E2F1 [7]. Later studies showed that all orthologs and paralogs of TRRAP (yeast ortholog Tra1) lack the enzymatic activity. Three motifs for the PIKK kinase activity are missing in the PI3K-like kinase domain in TRRAP: the ATP-binding motif VAIK, the catalytic motif HRD, and the divalent cation-binding motif (DFG) [7,8]. Phylogenetic analysis claims that TRRAP/Tra1 is highly conserved through the eukaryotic clades and considered it as the ancestral member of the PIKK family [1].

## 2. TRRAP and HAT in Transcription

### *2.1. TRRAP as an Adaptor Protein of HAT*

Lysine acetylation on histone tails leads to the relaxation of chromatin structure, granting accessibility of transcription factors (TFs) and transcription machinery to the chromatin. The chromatin dynamics are influenced through various post-translational modifications, including phosphorylation, methylation, acetylation, SUMOylation [9], ADP-ribosylation, UFMylation, and serotonylation. Acetylation of the lysine residue on histones is mainly carried out by the activity of histone acetyltransferase (HAT or KAT, thereafter refer to HAT) and is a major epigenetic modulation of chromatin remodeling and gene expression. In a reversible way, the acetylated protein can be deacetylated through histone deacetylases (HDAC or KDAC, thereafter refer to HDAC). The different subunits of the HAT complexes include acetyltransferases, HAT adaptors, interactors of transcription machineries, and also other post-translational modification enzymes, e.g., for histone deubiquitination [10]. The biological function of the subunits within each HAT complex still remains largely unknown, but it is believed that adaptors may dictate the function of individual HAT complexes.

A plethora of studies identified orthologs and paralogs of TRRAP as a shared component of several HAT complexes in eukaryotes, from yeast to humans. TRRAP is in

association with two major HAT families: the general control nonderepressible-related (GCN5) acetyltransferase (GNAT) HAT family (including Gcn5 and PCAF) and the MOZ, Ybf2/Sas3, Sas2, Tip60-related (MYST) HAT family (including Tip60) [11]. TRRAP is a subunit of two megamolecular complexes: the Spt-Ada-Gcn5 acetyltransferase complex (named SAGA both in yeasts and mammals) and the nucleosome acetyltransferase of H4 complex (named NuA4 in yeasts and TIP60 in mammals) [1]. Although TRRAP-containing HAT complexes consist of different subunits and acetylate different substrates, TRRAP is likely to be the only shared component among different HAT complexes, for instance within SAGA and TIP60 complexes [12].

### 2.2. The Role of TRRAP in Transcription

In mammals, TRRAP has been shown to interact with multiple TFs, including E2F1, c-Myc, p53, LXR, and $\beta$-catenin, leading to the transcription activation of their target genes [7,13–16] (Figure 1). Histone acetylation has later been demonstrated to form docking sites for TFs [17]. Although there is no direct evidence showing that TRRAP interacts with chromatin in a direct manner, a new study on the 3D structure of the yeast TRRAP ortholog, Tra1, suggested that TRRAP owns a DNA binding domain [18]. Although the 3D structure of murine TRRAP has also been mapped (UniProt ID: Q80YV3), whether the DNA binding region affects the interaction between TRRAP-chromatin and their recruitment of TFs, is currently unknown.

As TRRAP is an adaptor protein in HAT complexes, many studies have focused on the TRRAP-related HAT activity and transcription activation. Early studies showed that TRRAP-containing HAT complexes are recruited to an activator-interacting nucleosome to initiate transcription [19,20]. Mechanistically, TRRAP recruits HAT and TFs to chromatin, leading to the hyperacetylation of the histone and activates the transcription of the target genes. Depending on the model of the studies, the target genes can be responsible for different cellular processes, depending on the cell types (Table 1).

**Table 1.** TRRAP target genes and their respective involvement in cellular processes.

| TRRAP Target Genes | Cell Type | Cellular Process and Reference |
|---|---|---|
| ABCA1, ABCG1, SCD, HBP | Hepatic cell lines | Lipid metabolism [16] |
| APOC3 | Hepatocytes | Triglyceride metabolism [21] |
| Cdc25A, CycA2, TopA2 | Neural progenitors | Stem cell differentiation [22] |
| CyclinD2, ID2, MCM7 | Hematopoietic stem cells | Maintenance of the hematopoietic stem cell pool [23] |
| H2B, H4 | HEK293T cells | G1/S-phase transition [24] |
| Mad1, Mad2 | Embryonic fibroblasts | Cell cycle progression [25] |
| MCIDAS, CCNO, MYB | Airway epithelial cells | Multiciliated cell formation [26] |
| NANOG | Ovarian cancer cells | Tumorigenic potential of ovarian cancer stem cells [27] |
| Nanog, Oct4, Sox2 | Embryonic stem cells | Maintenance of cell stemness [28] |
| STMN3, STMN4 | Postmitotic neurons | Microtubule dynamics [29] |
| TOP2A | Hepatocellular carcinoma cells | Proliferation of tumor cells [27] |

TRRAP is an essential co-activator of c-Myc and E2F and the knockdown of TRRAP inhibits the c-Myc- and E1A-mediated oncogenic transformation [7]. In cooperation with p53, TRRAP activates MDM2 transcription through the recruitment of p53 and by increasing histone acetylation on the MDM2 promoter [13]. $\beta$-catenin associates with TRRAP and mixed-lineage-leukemia (MLL1/MLL2) SET1-type chromatin-modifying complexes, which lead to H3K4 trimethylation on the Wnt target gene c-Myc and the transactivation of $\beta$-catenin [15]. TRRAP interacts with and co-activates LXR$\beta$, an activator controlling lipid metabolism, which then induces the expression of LXR$\alpha$ targets: ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1), stearoyl-CoA desaturase (SCD), and

high-density lipoprotein binding protein (HBP) [16]. TRRAP is a component of the PGC-1$\beta$ transcriptional complex to regulate the APOC3 expression and thereby, the lipoprotein metabolism [21]. The co-activator of histone transcription, NPAT, recruits the TRRAP-Tip60 complex to the promoter of H2B and H4 and activates the transcription of histone genes during the G1/S-phase transition [24]. As it is upstream of the transcriptional coregulator Multicilin, TRRAP regulates the expression of MCIDAS, CCNO, and Myb, which are involved in multiciliated cell differentiation [26]. TRRAP is upregulated in ovarian cancer cells and is shown to regulate the NANOG expression that maintains the stemness-like characteristics of cancer stem cells, yet in a less studied mechanism [27]. TRRAP-Tip60 co-activates the expression of the mitotic gene Top2A and promotes hepatocellular carcinoma cell proliferation [30].

Studies using mouse models have demonstrated that TRRAP is important for the transcription activation of specific genes. In mouse embryonic fibroblasts (MEFs), TRRAP recruits HAT onto the Mad1 and Mad2 promoters and thus promotes their transcription [25]. In TRRAP-deleted murine embryonic stem cells (ESCs), a low level of active chromatin markers (AcH4, H3K4me2) and a decreased transcription of stemness genes *Nanog*, *Oct4*, and *Sox2*, were detected [28]. A study using TRRAP knockout in mouse neuro-stem cells revealed that TRRAP-deletion abolished the binding of E2F1, HAT, and AcH3 onto the promoter of the cell cycle regulators Cdc25A, Mad2, CycA2, and Top2A [22]. Recently, we discovered that TRRAP mediates the binding of Sp1, a key TF, to chromatin, which promotes the hyperacetylation of the promoter region and thus induces the transcription of Sp1 target genes [29]. All of these studies demonstrate an essential role of TRRAP in the recruitment of HAT enzymes and TFs onto the target promoter to modulate proper transcription.

### *2.3. The Biological Function of TRRAP in Different Cellular and Animal Models*

Studies from different model systems give a broad insight into the function of TRRAP in transcription activation and its involvement in various cellular processes. TRRAP is essential for cell viability in mammals. TRRAP null mouse blastocysts could not proliferate due to mitotic checkpoint catastrophe, resulting in a peri-implantation lethality of mouse embryos [31]. In addition, TRRAP knockout MEFs showed chromosome missegregation, mitotic exit failure, and compromised a mitotic checkpoint. TRRAP-deletion abolished the chromatin-binding of Tip60 and PCAF at the promoters of the *Mad1* and *Mad2* genes [25]. The deletion of TRRAP in ESCs resulted in an unscheduled differentiation of ESCs, likely through TRRAP's role in HAT-mediated chromatin remodeling and the expression of the stemness marker genes, *Nanog*, *Oct4*, and *Sox2* [28]. In addition, TRRAP- deletion-induced apoptosis of the hematopoietic stem cells (HSCs) and the downregulation of c-Myc, a well-known interactor of TRRAP [23], scoring the importance of TRRAP in the homeostasis of the hematopoietic system. Clinical studies identified individuals carrying missense variants of TRRAP, who show developmental delay and neuropathological symptoms, including a variable degree of intellectual disability, autism, ASD, and epilepsy. Some of the individuals also exhibit a malformation of the cerebellum, heart, kidney, and urogenital tracks [18,32]. These case reports highlight the importance of TRRAP in organ development and neuronal functionality.

It is known that a variety of HAT or HDAC mutations lead to neurological dysfunctions and brain developmental defects in mouse models [33,34]. Using mouse models, we showed that TRRAP-HAT-mediated histone acetylation plays a vital role in brain development by regulating the expression of genes related to cell cycle progression and neural stem cell differentiation [22]. TRRAP is required for proper differentiation of neuroprogenitors during neocortical neurogenesis because its deletion lengthened the cell cycle of apical neuroprogenitor cells, rendering to a premature differentiation of neural progenitors [22]. Despite having no effects on the E2F1 protein level, TRRAP-deletion decreased the chromatin binding of E2F1 and HAT and repressed the active chromatin mark AcH3, on the promoter of cell cycle regulators, thus reducing the expression of these factors [22].

Surprisingly, when TRRAP was specifically deleted in post-mitotic Purkinje cells (PC) in a mouse model, these mutant neuronal cells were viable and contributed normally to cerebellogenesis, which nevertheless caused a progressive neurodegeneration in aged animals [29]. The integrated transcriptomic, proteomic, and epigenomic analyses identified that Sp1 is a novel TF under the control of TRRAP. We found that the TRRAP-HAT-Sp1 transactivation activity regulates the expression of genes involved in microtubule dynamics, specifically Stmn3 and Stmn4. TRRAP-deletion results in a hypoacetylation of, and simultaneously an insufficient Sp1 binding on, Stmn3 and Stmn4 promoter proximity [29]. These findings thus identify an important role of the TRRAP-HAT-Sp1 axis in the protection against neurodegeneration via the regulation of microtubule dynamics. However, whether the acetylation of the Sp1 protein per se by TRRAP-mediated-HATs directly changes Sp1 activity on the target promoter, deserves future investigations.

## 3. Sp1 Is a Ubiquitous TF

### *3.1. Overview*

Sp1 is a TF that is expressed ubiquitously in all mammalian cell types [35]. It was named after its purification method, the sephacryl and phosphocellulose columns, but later renamed after specificity protein 1 [36,37]. Initially, Sp1 was considered to regulate housekeeping genes, yet later studies revealed the tissue-specific function of the Sp1-mediated transcription [38]. Perhaps, the most studied function of Sp1 is in tumorigenesis [39]. The overexpression of Sp1 emerges in various cancer types, e.g., human glioma, breast cancer, gastric cancer, pancreatic ductal adenocarcinoma, and thyroid tumors. Many Sp1 targets are the hallmarks of cancer progression, including EGF on sustained proliferation/immortality [40], Bcl-2 on apoptosis [41], TSP-1 on angiogenesis [42], MMP9 on metastasis [43], and BRCA1 on DNA damage/stress response [44].

### *3.2. Sp1 Structure*

Sp1 is a 785aa protein with a molecular weight of 105 kD and its structure has been well mapped [35] (Figure 2). Sp1 contains three domains: the Sp box on the N-terminus, the Buttonhead domain (BTD), and the Zinc finger domain on the C-terminus [35]. The Zinc finger domain (the DNA binding domain), consisting of three adjacent Cys2His2-type zinc finger motifs, recognizes the GC boxes (GGGGCGGGG) and the GT/CACC boxes (GGTGTGGGG) of the DNA sequence. The BTD domain is suggested to express the Sp1 activity [35]. However, a recent study showed that the absence of this domain in *Drosophila* did not affect the expression of the Sp1 targets nor fly development [45]. The Sp box contains an endoproteolytic cleavage site, which is implied in protein degradation. Sp1 has four transactivation domains; domain A and B are serine/threonine- and glutamine-rich, which are responsible for most of the transcriptional activity of Sp1. The highly charged domain C promotes the transactivation and the DNA binding of Sp1, whereas domain D supports Sp1 multimerization [35].

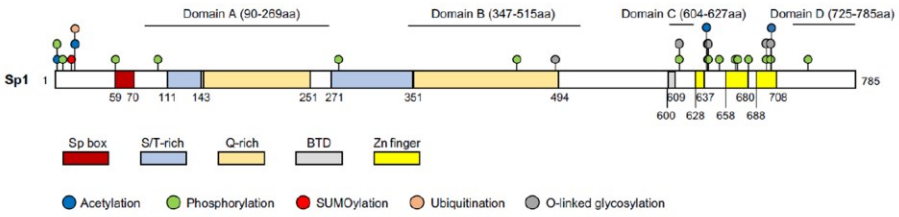


**Figure 2.** Protein structure of Sp1 with indicated domains and post-translational modifications. Domains A, B, C, and D indicate transactivation domains. All of the other domains are indicated in boxes with different colors. Putative post-translational modifications are labelled in color-coded circles. The numbers under the protein indicate the position of the amino acid at the border of the domains. S/T-rich: serine/threonine-rich domain; Q-Rich: glutamine-rich domain; BTD: Buttonhead domain; Zn finger: Cys2His2-type zinc finger domain. UniProt ID: P08047 and references [46–48].

### 3.3. Transcription Initiation and Transactivation by Sp1

Sp1 induces the initiation of transcription through the recruitment of the basal transcription machinery to the chromatin. It specifically interacts with general human TBP and TBP-associated factor II 130 (hTAFII130) in the general transcription factor IID (TFIID) complex, which then initiates the formation of the pre-initiation complex and thus, the transcription initiation [49,50]. Sp1 expresses its activity in a synergistic way with other TFs, including E2F1, AP2, Oct-1, and Sp1 itself [51–54]. Sp1 interacts with other activators and forms the multimers through the glutamine-rich transactivation domains A and B, as well as D [55]. Even an additional binding site outside of the promoter, but in the enhancer regions, can promote the transcription activity of target genes [46]. Another evidence of the functional synergy of Sp1 at the regulatory element is that the assembly of multiple tetramers of Sp1 increases its activity [46,56].

### 3.4. Regulation of Sp1 Activity

Sp1 has been shown to interact with epigenetic modifiers to induce or repress the transcription of target genes. Sp1 recruits HAT to stimulate Sp1 transactivation. p300, as a HAT enzyme, is an interaction partner of Sp1, which facilitates the binding of Sp1 to chromatin, to initiate the transcription [57,58]. Another study focused on the effect of NGF on neuronal differentiation, unraveled the cooperation between Sp1 and p300. p300 co-activates Sp1, leading to the transcription of p21 and promoting neuronal differentiation [59]. Sp1 also harbors an inhibitory function in transcription. Sp1 recruits the repressor HDAC1 to the GM2-synthase promoter as a part of the repressor complex and thus inhibits the expression of GM2-synthase [60]. However, Sp1-mediated transcription can be complex in a way that the activating or repressing effect of Sp1 on gene regulation could be switched in a temporal–spatial manner, depending on the partners or interactors at the promoter of the target genes. For example, Sp1 regulates the transcription of 12(S)-lipoxygenase, which participates in the epidermal and epithelial inflammation, through the recruitment of HDAC1 and p300 to the promoter [61]. Upon treatment by phorbol 12-myristate 13-acetate (PMA), Sp1 is constitutively acetylated by yet unknown HATs and recruits HDAC1 to the chromatin, to deacetylate Sp1 and subsequently dissociates HDAC1 from Sp1. The Sp1 deacetylation would allow the interaction of p300 with Sp1 and the recruitment of p300 to the target promoter which then catalyzes histone acetylation and leads to target expression [61]. This study has conceptualized that the recruitment of a post-translational modifier to TFs at certain chromatin regions dictates the transcriptional activity of TFs.

### 3.5. Post-Translational Modification (PTM) on Sp1

The activity of Sp1 can be affected by various PTMs: phosphorylation [62], acetylation [57], glycosylation [63], ubiquitination [64], SUMOylation [65], and poly(ADP-ribosyl)ation [66]. The temporal–spatial interaction mode between these PTMs complexes the regulation of Sp1 activity. Depending on the kinase, phosphorylation can either increase or decrease the transcriptional activity of Sp1. Under the FGF2 treatment in mammalian cells, for instance, ERK1/2 phosphorylate Sp1 on Thr453 and Thr739, thus repressing the expression of its target, PDGFR$\alpha$ [67]. p38 kinase phosphorylates Sp1 and induces the filamin-A expression in connective tissues [68]. Compared to phosphorylation, very few HATs are known to conduct acetylation on Sp1 and the results are often inconsistent. It has been shown that Sp1 acetylation, catalyzed by p300, does not affect Sp1 binding on DNA [57]. In contrary to this, another study showed that p300 acetylates Sp1 on K703, which reduces the interaction between Sp1 and p300 and represses its transcriptional activity [61]. To date, not many acetylation residues on Sp1 have been identified, other than K703 [61], K639, K624, K685, K693 [48], and K19 [47], with K703 being the most studied. However, which residues of Sp1 are acetylated by other HATs and what the biological functions of the respective acetylation sites are, remain unknown. SUMOylation and acetylation can affect protein activity in an antagonistic manner. Moreover, SUMOylation and ubiquitination both affect Sp1 stability [69]. SUMOylation of Sp1 at K16 increases

the ubiquitination and degradation of Sp1. However, phosphorylation of Sp1 inhibits the SUMOylation on K16 and thereby stabilizes Sp1 [69]. Generally, ubiquitination competes against acetylation on the common lysine residue and determines the stability. A study showed that Sp1 can be acetylated and ubiquitinated on K19 [47]; although, whether the Sp1 acetylation on K16 and/or K19 can affect Sp1 protein stability or its activity, was not reported.

### *3.6. Novel Functions of Sp1 in the Nervous System and Diseases*

As a master transcription factor, Sp1 has a plethora of downstream target genes, yet, the upstream regulatory mechanism of Sp1 remained unknown until recently in a study showing that TRRAP-HAT is upstream of Sp1, to regulate the Sp1-mediated transcription [29]. A well-documented function of Sp1 is in cell proliferation of cancer [39]. Moreover, Sp1 has been linked with the neuropathologies of the nervous system, albeit some controversies. The GWAS analysis implicates that Sp1 mediates transcriptional activity changes in patients suffering from the neurodegenerative disorders, Alzheimer's disease (AD) and Parkinson's disease (PD) [70]. Sp1 is upregulated in AD patients and AD mouse models; however, the chemical inhibitor of Sp1 even led to severe memory deficits in AD transgenic mice [71]. The occurrence of Huntington's disease (HD) results from the mutation of the huntingtin protein, which contains an aberrant poly-glutamine tract. The mutant huntingtin was shown to disrupt the interaction between Sp1 and the co-activator TAFII130, thereby inhibiting the transcription of the dopamine D2 receptor, a hallmark of HD [72]. Further studies in the HD mouse model demonstrated that mutant huntingtin inhibits ZnT3 transcription through the interaction with Sp1, causing a loss of the synaptic vesicular zinc molecule in the neurons of the CA1, CA2, and CA3 region of the hippocampus, cortex, and striatum [73]. Synaptic vesicular zinc modulates synaptic transmission and the maintenance of cognitive capacity in the prevention of the HD pathology [73]. However, it is also reported that a downregulation of Sp1 is protective in HD development [74]. These controversial findings, although confirmed a role of Sp1 in neuropathies, can be explained by the complex mechanism of Sp1 activity regulation, such as transcriptional regulation, epigenetic, and posttranslational modifications [38]. It is also possible that Sp1 behaves differently in the manifestation of the disease processes in a very heterogenous genetic background in human studies.

Despite the controversy, Sp1 has been shown, in neural model studies, to bind to the promoters of neural genes, e.g., Slit2 [75], P2X7 [76], and Reelin [77]. Our transcriptome studies identified various neurological processes that are regulated by Sp1 downstream targets [29]. Many of these processes are linked with microtubule dynamics, which are closely related to neuronal homeostasis and neurodegenerative processes [78]. Specifically, Sp1 binds to the promoter of genes encoding the microtubule destabilizing proteins Stmn3 and Stmn4, and in the absence of TRRAP, Sp1 activity in the expression of Stmn3/4 was greatly compromised [29]. These findings disclose the involvement of Sp1 in neurodegenerative processes and also implicate that the miss-regulation of Sp1 activity by upstream modulators can be an etiological mechanism of neuropathological phenotypes.

## 4. Outlook

TRRAP has been shown to control gene expression in different cell types, via its role in recruiting and activating the HAT-mediated TF activity. In different cell types, TRRAP regulates different TFs that control cell cycle progression and cell differentiation. However, whether all of these involve Sp1, is unclear. It has been shown that Sp1 is upstream of c-Myc, a partner of TRRAP. E2F1 is another TRRAP interactor and has been shown to interact with Sp1 to grant its transcriptional activity [79,80]. Thus, the TRRAP-Sp1 axis might be the upstream regulator on all of the observed phenotypes in different cell lineages.

TRRAP is required for strengthening the binding of E2F1 and HATs on chromatin. This is analogous to the situation of p300, which is required for the Sp1 binding on the chromatin. However, TRRAP can act as a scaffold to accommodate TFs and HATs onto the

chromatin but may also mediate the acetylation directly on TFs which may affect the Sp1 binding on chromatin, or simply alter the TF activity. This hypothesis may be supported by observations showing that lysine acetylation affects the stability and activity of TFs [81]. In addition, the TRRAP-HAT-mediated acetylation of Sp1 could compete against other PTMs, for example, ubiquitination or SUMOylation, and thus, affects Sp1 stability. Despite some of the molecular hits, the significance and function of these PTMs of Sp1 have not been well studied. Finally, targeting the TRRAP-HAT-Sp1 axis would be a potential strategy for pharmaceutical intervention, aiming at the prevention and treatment of neurodegenerative diseases.

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### 3.3 Manuscript III

#### 3.3.1 Author contribution

**Title:** TRRAP-mediated acetylation on Sp1 regulates adult neurogenesis

**Authors:** Bo-Kun Yin, David Lazaro, and Zhao-Qi Wang

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**Authors' contributions (in %) to the given categories of the publication**

| **Author** | **Conceptual** | **Data analysis** | **Experimental** | **Writing the manuscript** | **Provision of material** |
|---|---|---|---|---|---|
| Bo-Kun Yin | 40% | 60% | 80% | 70% | 40% |
| David Lazaro | 10% | 20% | 20% | 10% | 60% |
| Zhao-Qi Wang | 50% | 20% | | 20% | |
| Total: | 100% | 100% | 100% | 100% | 100% |

Contribution of authors to the manuscript:

Bo-Kun Yin: Conceptualization of the story; establishment of the aNSC differentiation and proliferation protocol *in vitro*; performing cell cycle profile assay and analysis by immunoblotting the cell cycle factor regulated by Trrap; constructing the Sp1 truncation and point mutation; investigating the Sp1-Trrap interaction, Sp1-chromatin binding and Sp1 transcriptional activity from different Sp1 construct; Writing - original draft

David Lazaro: Establishing the Trrap-aNSCΔ *in vivo* and studying the adult neurogenesis by immunofluorescence.

Zhao-Qi Wang: Conceptualization; funding acquisition; investigation; project administration; writing - review and editing Supervision; writing - original draft; writing - review and editing.

### 3.3.2 Manuscript

TRRAP-mediated acetylation on Sp1 regulates adult neurogenesis

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Running title: Trrap-mediated acetylation on Sp1 improves adult hippocampal neurogenesis

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**Abstract**

The adult hippocampal neurogenesis plays a vital role in the function of the central nervous system (CNS), including memory consolidation, cognitive flexibility, emotional function, and social behavior. The deficiency of adult neural stem cells (aNSCs) in exiting quiescence, self-renewal and differentiation capacity is detrimental to the functional integrity of neurons and cognition of the adult brain. Histone acetyltransferase (HAT) and histone deacetylase (HDAC) have been shown to modulate brain functionality and are important for embryonic neurogenesis via regulation of gene transcription. We showed previously that Trrap, an common adaptor for several HAT complexes, is required for Sp1 transactivation, maintaining the microtubule dynamics in neuronal cells. Here, we show that Trrap deletion compromises self-renewal and differentiation of aNSCs in mice and *in vitro* cultures. We found that the acetylation status of lysine residues K16, K19, K703 and K639 all fail to overcome Trrap-deficiency-incurred instability of Sp1, indicating a scaffold role of Trrap. Interestingly, the deacetylation of Sp1 at K639 and K703 greatly increases Sp1 binding to the promoter of target genes, which antagonizes Trrap binding, and thereby elevates Sp1 activity. However, only deacetylated K639 is refractory to Trrap deficiency and corrects the differentiation defects of Trrap-deleted aNSCs. We demonstrate that the acetylation pattern at K639 regulated by Trrap-HAT dictates the role of Sp1 in the regulation of adult neurogenesis.

**Introduction**

Normal brain development tightly depends on complex genetic and environmental processes, an aberration of which could lead to neurodevelopmental disorders [29]. The synchronized function among neuronal and glia cells orchestrates variable actions ranged from simple tasks to complicate activities [4]. Neurogenesis is a process of generating newborn neurons from neural stem cells (NSCs). In adult human, neurogenesis only occurs in two regions: the subgranular zone (SGZ) in hippocampus and subventricular zone (SVZ) of the lateral ventricle [9]. The hippocampus mainly

consists of the Cornu Ammonis fields (CA1, CA2 and CA3) and the dentate gyrus (DG) [173] and is responsible for memory and learning [174]. The adult neurogenesis in the SGZ is mainly derived from adult neural stem cells (aNSCs) that differentiate into excitatory DG neurons and integrate into the inner circuitry of the hippocampus, connecting mainly to the CA3 pyramidal neurons, mossy cells and hilar interneurons [175]. The adult hippocampal neurogenesis is considered to play a vital role in multiple functions in the central nervous system (CNS), including memory consolidation, cognitive flexibility, emotional function, and social behavior [176-178].

The activation of quiescent aNSCs, the proper self-renewal and differentiation capacity of aNSCs are important for the functional adult brain [13, 179, 180]. Studies have shown that the impaired adult hippocampal neurogenesis leads to Intellectual Disability (ID) and Autism Spectrum Disorders (ASD) [14-16]. Human Fragile-X Syndrome (FXS), as is the most common form of inherited ID and the most important genetic cue of ASD, exhibits hippocampal volume changes [17, 18] and compromised hippocampal function [19, 20]. FXS mouse model studies show abnormal hippocampal functions due to defects of proliferation of aNSCs and neurogenesis [21-23]. Many mouse models of ASD or ID demonstrated their link with adult neurogenesis defects in the SVZ [26-28]. These clinical and laboratory studies highlight the impairment of adult neurogenesis as the etiology of ASD/ID. These studies highlight the impairment of SVZ adult neurogenesis in the etiology of ASD/ID.

Histone acetyltransferase (HAT) and histone deacetylase (HDAC) conducts protein acetylation, originally described for histones, but also for other proteins [61]. HAT and HDAC have been shown to modulate the brain functionality, including memory formation and neuroprotection [63, 65, 66]. The disturbance of the acetylation profiles has been related to multiple neuropathological diseases, for instance, Huntington's disease [67], Parkinson's disease [68] and Alzheimer's disease [69]. TRRAP, as abbreviated for Transformation/transcription domain-associated protein, is a member of Phosphatidylinositol 3-kinase-related kinases (PIKK) family. As the only pseudokinase (lacking the critical motifs required for ATP binding and catalysis,

thereby missing the kinase activity) in the PIKK family, TRRAP acts as a scaffold protein mediating transcription regulation and protein acetylation [59]. TRRAP is a cofactor for two major HAT families: the general control nonderepressible-related (GCN5) acetyltransferase (GNAT) HAT family (e.g. Gcn5 and PCAF) and MOZ, Ybf2/Sas3, Sas2, Tip60-related (MYST) HAT family (e.g. TIP60) [78]. TRRAP is required for HAT activity, for instance TIP60 and PCAF [97-99] and co-activates the target gene transcription. Through interacting with transcription factors, TRRAP facilitates the binding of HATs to promoter regions of target genes, leading to acetylation of histone [103]. This action enables the relaxation of chromatin conformation and facilitates the transcription process [181-183]. Depending on the cell type examined, the function of TRRAP targets genes covers a wide range of cellular processes, including stem cell differentiation, hematopoietic stem cell pool maintenance, cancer progression and lipid metabolism [96, 101, 106, 184].

Since Trrap deletion leads to peri-implantation lethality in mice [102], TRRAP null mutation is believed to cause embryonic lethality in humans. Recent studies identified 83 TRRAP variants in humans [110-112]. 17 distinct TRRAP variant were identified in patients with developmental delay and malformation of diverse organs, including brain, cerebellum, heart, kidney, or urogenital tracks [110]. Intriguingly, nearly half of the patients exhibit ASD and/or ID with variable severity, yet, lacking obvious malformation of brain architecture [110]. Using mouse models, we previously showed that Trrap deficiency causes premature differentiation of embryonic neuroprogenitors (NPCs) by disrupting cell cycle progression during neocortical neurogenesis [101]. While dissecting Trrap function in post-mitotic neurons, we found that Trrap is not required for development of Purkinje cells, but prevents its neurodegeneration in adult life [103]. These studies demonstrate the involvement of Trrap in the maintenance of embryonic neurogenesis and post-mitotic neurons. Molecularly, Trrap recruits Sp1 to chromatin to ensure the expression of microtubule-destabilizing phosphoproteins STMN3 and STMN4 to maintain proper microtubule dynamics in neurons [103]. We found that Trrap facilitates the Sp1 binding to promoters of these targets and is required for Sp1

transcriptional activity. These studies demonstrate the involvement of Trrap in the maintenance of embryonic neurogenesis and postmitotic neurons. Yet, how Trrap regulates Sp1 remains elusive.

Sp1 is a transcription factor that regulates the expression of genes involved in various cellular processes [113]. Sp1 activity is regulated by multiple pathways, for instance, by the PKC and MAPK cascades [185] and MEK-1 [186]. Importantly, multiple post-translational modifications (PTMs) have been shown to regulate Sp1 activity, including phosphorylation [151], acetylation [152], SUMOylation [163] and ubiquitination [153]. p300 acetylates Sp1 on residue K703 to repress Sp1 transcriptional activity [157]. TIP60 acetylates Sp1 on K639 impairing its binding on the promoter of target genes, thereby repressing its activity [158]. SUMOylation on K16 leads to proteasomal degradation of Sp1 [163]. It is also known that both acetylation and ubiquitination can modify the same residue (K19) [159], suggesting that acetylation may stabilize Sp1 through competing against ubiquitin-mediated proteasomal degradation [162]. Therefore, PTMs at these lysine residues may crosstalk and modulate the activity and stability of Sp1.

In this study, we investigated the possible role of Trrap-dependent acetylation or its scaffold function on Sp1's transactivation in respect to adult neurogenesis. We identified that deacetylation at K639 residue on Sp1 ensures the aNSC differentiation process and Trrap, via its role in modifying Sp1 activity, is required for proper cell proliferation and differentiation of aNSCs.

## Material and Method

### Inducible Trrap-deletion *in vivo* and *in vitro*

Mice carrying the conditional (floxed, *Trrap*$^{f/f}$) allele [102] were crossed with mice carrying the transgene *Nestin*-CreER$^{T2}$ [187] (*Trrap*$^{f/f}$; *Nestin*-CreER$^{T2}$) or CreER$^{T2}$ [188] (*Trrap*$^{f/f}$;*Rosa26*-CreER). All experiments were conducted according to German animal welfare legislation, and the protocol was approved by Thüringen Landesamt für Verbraucherschutz (TLV) (03-042/16), Germany.

**Truncation and mutagenesis of Sp1 constructs**

Human Sp1 was cloned into the pFLAG-CMV-2 plasmid using HindIII and XbaI restriction enzyme. Sp1 sequence was subcloned originally from the pcDNA3.1-V5-His-Sp1 plasmid kindly provided by Professor Xiaozhong Peng (Peking Union Medical College, Beijing, China). Truncation design followed the Sp1 truncation from previous study [133]. Mutagenesis on Sp1 construct was performed following the instruction of the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, United States).

**Histology and immunofluorescence staining**

Tissues for histology were fixed in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose and frozen in Richard-Allan™ Scientific Neg-50™ Frozen Section Medium (Thermo Fisher Scientific, Waltham, MA, USA). Sections with thickness of 5–20 µm were used for immunofluorescence staining. Following antibodies/reagent were used for immunofluorescence staining: Rabbit anti-GFAP (1:300, Agilent), rabbit anti-Ki67 (1:200, Thermo Fisher Scientific), goat Sox2 (1:200, Santa Cruz Biotechnology, Dallas, United States), goat anti-doublecortin (DCX) (1:200, Santa Cruz), mouse anti-GFP (1:200, Santa Cruz), donkey anti-rabbit Cy5 (1:200 – 1:400, Jackson ImmunoResearch, West Grove, United States), rabbit anti-goat IgG Cy3 (1:200 – 1:400, Sigma-Aldrich, St. Louis, USA), Donkey anti-mouse Cy2 (1:200 – 1:400, Jackson ImmunoResearch) and DAPI (1:3000 - 1:5000, Thermo Fisher Scientific).

**Inducible Trrap-deletion in aNSCs *in vivo* and *in vitro***

The genotypes of Trrap and Cre in mice were determined by PCR on DNA extracted from tail tissue, as previously described [106]. To induce *Trrap* deletion in Trrap-aNSCΔ mice, Tamoxifen (100 µg/g) (TAM, Sigma-Aldrich) was injected peritoneally during 5 consecutive days. To knockout *Trrap in vitro*, adult stem cells (aNSC) were isolated as previously described [103] and aNSCs were treated with 1µM

4-hydroxytamoxifen (4-OHT, Sigma-Aldrich) for 3 days to induce Trrap deletion, followed by additional 2 days incubation in fresh medium.

**aNSC cell culture and transfection**

aNSCs were cultured in aNSC culture medium consisting of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Thermo Fisher Scientific), 1 x B-27™ Supplements (B27, Thermo Fisher Scientific), 1 x penicillin–streptomycin, 20 ng/ml Animal-Free Recombinant Human EGF (EGF, Peprotech, Rocky Hill, United States), and 20 ng/ml Recombinant Human FGF-basic (bFGF, Peprotech). Prior to aNSCs seeding, 24-well culture dishes were coated with 50 mg/ml Poly-L-Lysine, (PLL, Sigma-Aldrich) and 10 mg/ml Laminin (Sigma-Aldrich). 3 x $10^5$ aNSCs treated with 4-OHT were plated each well in Neurobasal™ Medium (NEM, Thermo Fisher Scientific) supplemented with 1 x B27, 2 mM L-glutamine, 1 x N-2 Supplement (N2, Thermo Fisher Scientific), 1 x penicillin–streptomycin, 20 ng/ml EGF, and 20 ng/ml bFGF. On the next day, transfection was performed on monolayer aNSCs. The Sp1 reporter (-111 hTF m3, Addgene plasmid # 15450), exogenous FLAG-Sp1 and CMV-GFP was mixed with Lipofectamine™ 2000 Transfection Reagent (Lipo2000, Thermo Fisher Scientific) and transfected into aNSCs. 24 hours later, aNSC cultures were then subjected to luciferase assay. For *in vitro* differentiation, aNSCs were transfected using Lipo2000 mixed with plasmids in NEM supplemented with 500 μM L-glutamine (0.27 μg plasmid + 0.5 μl Lipo2000 in 370 μl/well in 24-well plate). After 30 min incubation, the plasmid-Lipo2000 mix was replaced by the original differentiation medium with supplement.

**N2A cell culture and plasmid transfection**

Neuroblastoma cell line N2A was cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) supplemented with 1 x FCS, 1x penicillin-streptomycin, and 1mM sodium pyruvate (Thermo Fisher Scientific). Cells were passaged every 2-3 days in 1:12 ratio when the N2A culture reached ~70% confluency.

For transfection, cells were seeded in 2.8 x $10^5$ cells/well onto 6-well plate. The transfection of N2A cells was performed with polyethylenimine (PEI, Polyscience, Eppelheim, Germany) at a ratio of 1 µg plasmid per 3 µg PEI. 24 h after transfection, the cells were harvested for co-immunoprecipitation (co-IP), ChIP, or immunoblot.

**Trrap knockdown and transfection in N2A cells**

siRNA against Trrap (Cat#: LQ-051873-01-0005, Horizon Discovery, Waterbeach, United Kingdom) was mixed with Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific) and transfected into N2A cells. After 24 hours transfection, Sp1 construct was then mixed with PEI and transfected into N2A. N2A cells were then incubated for another 24 hours and subjected to co-IP analysis.

**Proliferation assay by IncuCyte**

The aNSC culture was seeded 5 x $10^4$ per well in 50ml/ml PLL- and 10 mg/ml Laminin-coated 24-well plate and incubated in IncuCyte S3 (Sartorius AG, Göttingen, Germany) for imaging acquisition of phase contrast (10x magnification, 36 images/well in 24-well plate). The confluency of aNSCs was determined through Incucyte® Live-Cell Analysis.

**Cell cycle profiling**

aNSC were fixed in 4% paraformaldehyde (PFA), followed by permeabilization in 0.3% Triton-X. Cells were then labelled with 5 µg/ml DAPI diluted in 0.1% Triton-X for 20 minutes under room temperature. Cells were then sorted using a BD FACSCanto™ II Cell Sorter to detect DNA content with UV or violet laser (370/405 nm) and blue emission filter (450/500nm).

***In vitro* aNSC differentiation**

2-4 x $10^4$ aNSCs were resuspended with aNSC plating medium (DMEM/F-12 medium supplemented with 1 x N2 (Thermo Fisher Scientific) and 20 ng/ml bFGF and

plated onto 50 µg/ml Poly-L-Orthinine- (PLO, Sigma-Aldrich) and 10 µg/ml laminin-coated 12 mm glass coverslips. After 24 hours, the medium was replaced by the aNSC differentiation medium (DMEM/F-12 with 1 x B27 Supplements, 1 x N2 Supplement, 2 mM L-glutamine, 0.5% FBS, 20 ng/ml bFGF and 0.5 µM retinoic acid). Half of the medium was refreshed once every 2 days. The aNSCs were processed for immunostaining at 1-, 4-, 5- or 9- day-post-differentiation (DPD). The aNSC transfection during *in vitro* differentiation was performed at 2 DPD.

**Immunofluorescent staining and quantification**

aNSCs were fixed with 4% PFA and permeabilized with 0.7% Triton diluted in DPBS, no calcium, no magnesium (PBS, Thermo Fisher Scientific) for 15 min. Samples were then incubated with primary antibodies (resuspended in PBS supplemented with 1% BSA, 5% donkey serum and 0.1% Triton) under 4 °C overnight. After being washed with PBS, the samples were then further incubated with secondary antibodies (resuspended in PBS supplemented with 7% BSA and 1:1000 DAPI) for 1 hour. The samples were then conserved by ProLong™ Gold Antifade reagent (Thermo Fisher Scientific). Following antibody/reagent were used for immunofluorescence staining: mouse anti-Tubulin βIII (Tuj1, 1:400, Covance, Princeton, United States), rabbit anti-GFAP (1:300, Agilent), sheep anti-mouse IgG Cy3 (1:400, Sigma-Aldrich) and DAPI (1:3000). The coverslips were then imaged by the ZEISS Apotome 3 (Carl Zeiss AG, Jena, Germany) under the 20x or 40x objectives. The neuron population was then scored with ImageJ software and numerated manually.

**Luciferase assay**

24 hours after transfection, aNSCs were then lysed and cell lysates were subjected to activity assay according to the instruction by Dual-Glo® Luciferase Assay System (Promega, Madison, United States). The luciferase activity was then normalized by the total cell protein concentration measured with the Pierce™ BCA Protein Assay Kit (BCA assay, Thermo Fisher Scientific).

**Immunoblot analysis**

Total protein lysates were prepared from aNSCs with the RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF), and cOmplete™ Protease inhibitor-Cocktail (protease inhibitor, Roche, Basal, Switzerland). Protein was quantified using the BCA Assay. Immunoblotting was performed as described previously [103], using the following antibodies: mouse anti-TRRAP (1;1000, Euromedex, Souffelweyersheim, France), mouse anti-Cdc25A (1:500, Santa Cruz), rabbit anti-Cyclin D1 (1:500, Santa Cruz), mouse anti-Mad2 (1:1000, BD Biosciences, Franklin Lakes, United States), mouse anti-FLAG (1:1000, Sigma-Aldrich), rabbit anti-Sp1 (1:1000, Merck Millipore, Burlington, United States ), mouse anti-b-actin (1:5000, Sigma-Aldrich), mouse anti-GFP (1:400, Santa Cruz) and rabbit anti-Plk1 (1:1000, Abcam, Cambridge, United Kingdom).

**Co-IP**

For co-IP, cells were harvested with NET-N buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and protease inhibitor. Approximate 1 mg of total lysate was incubated with the Dynabeads™ Protein A Immunoprecipitation Kit (Thermo Fisher Scientific) and 2ug mouse anti-FLAG antibodies at 4°C overnight. The precipitates were then washed with the NET-N buffer with fresh protease inhibitor, followed by elution with SDS buffer lysis buffer (50 mM HEPES-KOH, 140 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1% sodium deoxycholate, 1% SDS, 10 mM NaB, and protease inhibitor) and immunoblot analysis. Immunoblotting was performed using the following antibodies: mouse anti-TRRAP (1;1000, Euromedex, Souffelweyersheim, France), mouse anti-FLAG (1:1000, Sigma-Aldrich), mouse anti-□-actin (1:5000), goat anti-TIP60 (1:500 Santa Cruz), goat anti-mouse HRP (1:3000, Agilent), goat anti-rabbit HRP (1:3000, Agilent) and rabbit anti-goat HRP (1:3000, Agilent).

**Chromatin immunoprecipitation (ChIP) assay**

Transfected N2A cells were wash with PBS and collected with cell scraper, followed by the same protocol as previously described [103].

**Statistical analysis**

The statistical analysis was performed through GraphPad (Dotmatics, Boston, United States). Paired/unpaired t-test or Two-Way ANOVA were performed on the respective data labelled in the figure legends.

**Result**

**Deletion of *Trrap* abrogates adult neurogenesis**

To study the role of Trrap-HAT in aNSCs, we first generated TAM-inducible and traceable deletion of *Trrap* in mice. For this, *Trrap*$^{f/f}$ mice [102] were crossed with mice carrying the transgene *Nestin*-CreER$^{T2}$ [187] to produce *Trrap*$^{f/f}$; *Nestin*-CreER$^{T2}$ mice, which upon TAM treatment generates inducible Trrap deletion in neural stem cells in adult mice (designated Trrap-aNSCΔ). To trace Trrap-deleted cells *in vivo*, Trrap-aNSCΔ mice were subsequently crossed with double-fluorescent reporter *mT/mG* knock-in mice [189]. This reporter allows monitoring of Trrap-deleted cells by GFP expression, after switching from Tomato by Cre recombination in the aNSCs in the DG and SVZ. *Trrap*$^{f/+}$ mice with the Cre transgene showed no detectable abnormalities and were used as controls.

We analyzed mutant mice at 1- and 5-months post-tamoxifen (TAM) injection (MPT). Co-immunostaining demonstrated that *Trrap* deletion yielded a great decrease in aNSC proliferation (judged by GFP$^+$Ki67$^+$) and an increase in cell death (GFP$^+$TUNEL$^+$), as early as 1 MPT (Fig 1A-C). Co-staining of GFP with the aNSC markers GFAP and Sox2 in Trrap-aNSCΔ brain detected a progressive decrease of aNSCs (GFAP$^+$Sox2$^+$ localized in the subgranular area of the DG) during 1 MPT and 5 MPT (Fig 1D-E). Intriguingly, we found a great increase of GFAP$^+$Sox2$^+$ cells localized outside the subgranular area of the DG, characterized by a protoplasmic-spongiform morphology (Fig 1D, 1F). In addition, co-staining of GFP together with

newborn neuron marker DCX showed a great reduction of newborn neurons ($GFP^+DCX^+$) at 1 MPT and a complete absence of newborn neurons at 5 MPT (Fig 1G-H). These results indicate that Trrap deletion results in a progressive loss of the self-renewal capacity of aNSCs. The decline of neuronal differentiation correlates with greatly increased astrocytes, suggesting a differentiation defect of Trrap-deleted aNSCs.

**Trrap deletion compromises proliferation and differentiation of aNSCs *in vitro***

To further investigate the intrinsic role of TRRAP in the proliferation and differentiation of aNSCs, we generated a mouse line *Trrap*$^{f/f}$; *Rosa26*-cre$^{ER}$ (Trrap$^{f/f}$-CreER) by crossing *Trrap*$^{f/f}$ mice with *Rosa26*-cre$^{ER}$ mice [103] and isolated aNSCs from these mice at the age of 2-3 months. *Trrap*$^{f/+}$-CreER with 4-OHT treatment were used as control as previously described [103].

To monitor the proliferation, we used the IncuCyte assay following and measuring in live the confluency of aNSC monolayer culture. We detected a slower confluency of mutants than controls, indicative of a lower proliferation rate (Fig 2A). We also examined the cell cycle profile of aNSCs by FACS (Fig S1A). The proportion of G2/M of Trrap-aNSCΔ was about 40% more than controls, indicative of an accumulation of G2/M cells (Fig S1B). Western Blotting revealed a higher level of the mitotic checkpoint protein Mad2 in mutant aNSCs compared to controls, indicating a blockage of mitosis (Fig 2B-C). Moreover, the mutant aNSCs contained higher Cyclin D1, a trigger of G1/S transition, than control cells, indicating that cells entering S phase and more cells replicating.

Next we analysed differentiation of aNSCs using an adjusted *in vitro* differentiation assay [190] (Fig 1D). The identity of the cell mixture was monitored by immunostaining with lineage markers at different DPD (Fig 2E, Fig S1C). The neuron population ($Tuj1^+$) in control aNSCs was increased by DPD1 and reached about 15% in the culture between DPD4-5, which then remained stable until DPD9. In contrast, Trrap-deleted aNSCs gave rise to a significantly lower number of neurons compared to control (Fig

2E). The mutant neuron population occupied less than 10% of the cultures at DPD4, followed by a drop to 4% at DPD9. The findings indicate an impaired differentiation capacity, which is lost faster, in Trrap-deficient aNSCs. Thus, Trrap is required for proper proliferation and differentiation of aNSCs.

**Sp1 interacts with Trrap**

We showed previously that Trrap, via HAT-mediated histone acetylation, facilitates Sp1 binding to promoters to initiate transcription of its target genes, e.g., STMN3/4 [103]. Sp1 has been shown to interact with diverse coactivators to enhance its transcriptional activity [191-194]. We questioned if the present of Trrap itself is necessary for the Sp1 activity. To this end, we performed an interaction study between Sp1 and Trrap. . We constructed various truncation mutants of Sp1 based on the previously defined transcription activation domains [133] (Fig 3A). Co-IP in neuroblastoma N2A cell line after transfection of indicated mutant Sp1 vectors revealed various interaction strength between Sp1 and endogenous Trrap, but with a strong interaction via the transactivation domains A and B (Fig 3A-B), which have been shown to facilitates Sp1 transcription activity [148] .

**Sp1 stability in Trrap mutant cells is not affected by the acetylation status of K16 and K19 on Sp1**

Previously, we found that Sp1 activity was greatly reduced in Trrap deficient cells while the Sp1 mRNA level was unaltered in these cells [103]. Western blotting detected a very low Sp1 level in Trrap-aNSCΔ cells compared to controls (Fig 3C). K16 and K19 residues have been hinted for Sp1 stability. It is assumed that acetylation of these residues, potentially competes against the SUMOylation [163] and ubiquitination processes [159], interferred the stabilization of Sp1 [162]. Therefore, we reasoned that Trrap-deletion impaired acetylation thereby facilitated ubiquitination and SUMOylation of these lysines, which might render Sp1 undergoing proteasomal degradation.

To further understand how Sp1 stability is affected by Trrap, we next analyzed lysine acetylation of Sp1. To this end, we constructed Sp1 mutants by replacing both

K16 and K19 with either an acetylation-incompetent residue arginine (R) (Sp1-K16R, -K19R, or Sp1-K16R, K19R) or a mimicking acetylation glutamine (Q) (Sp1-K16Q, K19Q). We transfected the respective vectors together with GFP vector (which monitors transfection efficiency) into aNSCs. We detected slightly lower, albeit not significant, GFP levels in Trrap-aNSCΔ cells compared to control cells. Strikingly, all Sp1 vectors, regardless of wildtype or mutant, could not be expressed efficiently in Trrap mutant cells compared to control cells (Fig 3C). After normalizing with the level of GFP, Sp1-K16R, K19R and Sp1-K16Q, K19Q mutations increased the Sp1 stability, albert not significantly, in comparison to wildtype Sp1 (Fig 3C), suggesting that although both lysine residues have slightly conferred a stability to Sp1, their stability are still severely compromised in the Trrap mutant background. Next, we analyzed the activity of ectopically expressed Sp1 proteins after normalization with the exogenous Sp1 level, all Sp1 constructs expressed a lower activity in Trrap-mutant cells (Fig 3D) and the Sp1-K2R and Sp1-K2Q construct both had lower, activity in mutant cells. Interestingly, Sp1-K16R/K19R increased the Sp1 activity in control cells but not in mutant cells. Taken altogether, both residues do not seem to be involved in acetylation-related Sp1 stability, which however depends on the presence of Trrap to activate Sp1 albeit in an unknown manner.

**Acetylation of K639, but not K703, affects Trrap-dependent Sp1 activity**

Sp1 can be acetylated at K703 residue and the deacetylation of K703 increases Sp1 activity [157]. Next, we transfected two mutants (Sp1-K703Q, Sp1-K703R) together with GFP into aNSCs and analyzed their stability and activity. Western blotting revealed no obvious differences of expression between mutant and wildtype Sp1 vectors (Fig 4A). However, all these vectors expressed significantly lower in Trrap mutant cells compared to wildtype controls (Fig 4A). Analog to previous findings [157], acetylation incompetent Sp1-K703R had a higher activity compared to Sp1-WT. Of note, the K703R mutant elevated the Sp1 activity in mutant cells to the level of Sp1-WT transfected wildtype cells (Fig 4B), indicative of rescuing Sp1 activity defect

incurred by Trrap knockout. Sp1-K703R had a 4.6 folds higher activity in control cells compared to mutant cells, although the ratio of Sp1 activity between control and mutant cells was similar in WT-Sp1 and Sp1-K703R transfected cells (3.5 folds vs 2.8 fold). In contrast, the acetylation mimic Sp1-K703Q reduced the Sp1 activity in both wildtype and Trrap mutant aNSC cells (Fig 4B). In mutant cells, K703Q has even much lower activity compared to control, suggesting that Ac-K703 indeed modulates Sp1 activity, but likely independent of Trrap.

Next, we investigated another acetylated lysine residue K639, which can be acetylated by the Trrap interacting partner TIP60 [158]. We found an interaction between Sp1 and TIP60 in N2A cells through co-IP assay, which was abolished by Trrap knockdown (Fig 4C), consistent with the known interaction of Trrap and TIP60 [195]. To further examine whether Trrap mediates proper acetylation of K639 on Sp1, we transfected WT and acetylation deficient K639R mutant Sp1 vectors into control and mutant aNSCs. Western blot analysis detected a lower expression of Sp1-WT and Sp1-K639R in Trrap mutant cells than that in control cells (Fig 5A), demonstrating that K639 deacetylation does not overcome Sp1 destabilization incurred by Trrap-deletion. Strikingly, the overexpressed K639R increased Sp1 activity to a similarly high level in both control and mutant aNSCs (Fig 5B). The activity of exogenous Sp1-K639R in mutant cells, after normalization with the FLAG level (i.e., amount of exogenous Sp1), was even comparable, if not more, than control (Fig 5C), indicating that K639R overrides Trrap deficiency-incurred low Sp1 activity. This result lets us conclude that Trrap mediates the acetylation status on K639 on Sp1.

**Chromatin binding of K639 and K703 mutant Sp1 at target gene promoters**

We further explored how the acetylation at these lysine residues affects Sp1 binding to promoters of target genes. We transfected Sp1 mutant vectors into N2A cells and investigated by chromatin immunoprecipitation (ChIP) their binding onto two Sp1 targets, p21 [196] and STMN3 [103]. We found that both deacetylation mutant K639R and K703R increased the Sp1 binding on its promoter correlating well with their

increased activity (Fig 5D). Although K703Q abolished Sp1 activity, the promoter-binding is not affected (Fig 5D). Intriguingly, co-IP analysis revealed that Sp1-K639R or Sp1-K703R had lower Sp1-Trrap interaction, while acetylation mimic Sp1-K703Q did not significantly reduce this interaction, compared to Sp1-WT (Fig 5E). Taken together, these results suggest that Sp1 deacetylation at these two lysine residues facilitates its chromatin binding but prevents its interaction with Trrap.

**Acetylation at K639 of Sp1 is required for aNSC differentiation**

We found that K639 is a key acetylation site that regulates Sp1 activity under Trrap-mediated acetylation and TIP60 interaction (Fig 4C, 5C). To further study the biological meaning of K639R, we induced aNSC differentiation after transfection with Sp1-K639R. Ectopic expression of Sp1-K639R, but not Sp1-WT, increased the neuron population in Trrap mutant aNSCs after at 5 DPD (Fig 6A-B), indicating that the differentiation is indeed controlled by the K639 deacetylation on Sp1 via Trrap-mediated HATs.

**Discussion**

A wealth of evidence indicates the involvement of the HAT and HDAC complexes in brain development and the maintenance of adult neurogenesis in the brain. [65, 197, 198]. We previously showed in mouse models that Trrap is essential for embryonic neurogenesis by controlling cell cycle progression [101] and Trrap maintains homeostasis of post-mitotic neurons by regulating microtubule dynamics through modulating the activity of transcription factor Sp1 [103]. In the current study, we find that Trrap-deficiency impairs the quiescence, expansion and differentiation capacity of aNSCs *in vivo* and *in vitro*. We identify that the forced deacetylation at K639, which disrupts its binding with Trrap, increases Sp1 binding to chromatin and its transcriptional activity. Ectopic expression of this mutant Sp1 reverses the differentiation defect of Trrap-deleted aNSCs. These data unravel a novel function of Trrap-HAT in adult neurogenesis via modulation of Sp1 acetylation.

Consistent with the *in vivo* mouse model, Trrap deletion in cultured aNSCs also compromised their proliferation and neuron formation, indicating that the effect of Trrap deletion is cell-autonomous. Notably, the mutant neuronal population decreased progressively from 4-5 DPD to 9 DPD, which corroborates the progressive loss of neuronal population in Trrap-aNSC△ mouse model *in vivo*, possibly due to the loss of neuronal maintenance [103] and also the exhaustion of the neuroprogenitor pool [101]. Interestingly, we noticed a reverse correlation of the neuron production with a great increase of astrocytes, indicating a disturbed differentiation program in aNSCs without Trrap. The conversion from the neurogenic to astrocytic differentiation of aNSCs is reminiscent of a process described for aNSC aging and aging-related depletion of the adult neurogenesis [199]. We conclude that Trrap plays a fundamental role in both embryonic and adult neurogenesis.

Despite a normal transcription of Sp1 mRNA in Trrap-deleted cells [103], the Sp1 protein level is greatly reduced in Trrap deficient aNSCs due to proteasomal degradation. We find that the acetylation pattern of the documented lysine residues K16, K19, K703 and K639 cannot confer a normal protein level of Sp1 in Trrap deficient aNSCs. These results suggest that Trrap scaffold is essential for its stability, or that other to-be-discovered residues modified by Trrap-HAT stabilizes Sp1.

The deacetylation at K639 and K703 increases Sp1 activity in Trrap mutant cells, suggesting that acetylation at these two sites is inhibitory for Sp1 activity. This seems to be achieved by K639R and K703R mutations that improve the Sp1 binding at promoters its targets (e.g., p21 and STMN3). In this regard, it worth mentioning that TIP60 acetylates Sp1 at K639 that impairs its promoter binding on target genes, thereby repressing Sp1 activity [158]. In contrast to a previous study that Sp1 deacetylation leads to higher interaction with p300 [157], Sp1 deacetylation mutants (K639R and K703R) compromises Sp1-Trrap interaction, reversely correlating with their Sp1 activity. While p300 and Trrap (or its related HAT) may compete for Sp1 interaction, deacetylated Sp1 (K639R and K703R) antagonizes Trrap binding at gene promoters, which may nevertheless facilitate the recruitment of Trrap-independent

HATs for acetylation of histones at promoter regions. In supporting these hypotheses, previous work showed that deacetylated Sp1 recruits p300 to chromatin to acetylate histones around promoter regions thereby promoting transcription [157]. In addition, acetylation of different lysine residues is catalyzed by specific HATs and have different functional outcomes. For examples, K120 on p53 is acetylated by MOF, which facilitates the acetylation of histones on promoter region and activates the expression of pro-apoptotic genes PUMA and BAX [200]. On the other hand, acetylation of K382 on p53 promotes recruitment of p300 to p21 promoter, leading to increased histone acetylation on the promoter region and subsequently p21 transcription [201]. Thus, the acetylation status of lysine residues of transcription factors may modulate the selective recruitment of specific HATs to the promoters of the target genes.

Consistent with G1/S and G2/M arrest in Trrap mutant cells, we found high levels of Cyclin D1 and Mad2 (Fig. 1B). Mad2 is increased in Trrap mutant cells, indicating a strong G2/M arrest (Fig. S1A-C). Moreover, in line with our previous studies showing that Trrap deletion caused cytokinesis failure and an accumulation of cells containing greater than 4N DNA content [99, 102], we also found an increased DNA content in Trrap mutant aNSCs.

In the current study, we identify the specific lysine 639 of Sp1 to be a key modification site for Trrap-HAT mediated transcription. The deacetylation at specific K639 on Sp1 facilitates Sp1 binding to chromatin at target promoters, which is normally inhibited by the occupancy of Trrap, thus leading to hyperacetylation of the promoter region for gene transcription (as a readout of Sp1 activity). When Trrap is deleted, K639 on Sp1 is acetylated by Trrap-independent HATs, abolishing the Sp1-chromatin binding and repressing expression of Sp1 targets responsible for adult neurogenesis. Nevertheless, Trrap deletion destabilizes Sp1 and thereby impairs its transcriptional activity, which are possibly due to the deacetylation of other to-be-discovered residues modified by Trrap-HAT, or the lack of Trrap itself, whose scaffold function is necessary for Sp1 stability, consistent with the notion that TRRAP as scaffold mediates the binding of transcription factors onto target promoter [96, 97, 99, 101, 103]. Taken

together, we show that a fine-tuning of Sp1 activity via Trrap-HAT is critical for controlling the cell fate of aNSCs during adult neurogenesis.

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**Contribution of the authors**

B.-K. Y., conceived the project, performed the majority of the experiments, interpreted the data and wrote the manuscript. D.L. executed the *in vivo* study of Trrap-aNSCΔ mice. Z.-Q.W. designed the experiments, supervised the project and wrote the manuscript.

**Conflict of interest**

None.

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**Figure Legend**

**Figure 1 Deletion of Trrap in aNSCs abolishes the adult neurogenesis.**

a. Distribution of proliferative and apoptotic cells in dentate gyrus sections after tamoxifen induction. Immunostaining of the sections using antibodies against Ki67 (white, proliferative cells) and GFP (green, recombinant cells), or reagents TUNEL reaction (red, cell death) and DAPI. A representative image from 1-month-old mice after tamoxifen injection (1MPT). B-C. The quantification of the proliferative ($Ki67^{+}$) and apoptotic ($TUNEL^{+}$) cells in the dentate gyrus of control and Trrap-aNSCsΔ at 1MPT. 3 $Trrap^{f/+}$ and 3 Trrap-aNSCΔ mice analyzed. D. Immunostaining of dentate gyrus sections using an antibody against GFAP (red, stem cell marker), Sox2 (white, stem

cell marker), GFP (green, recombinant cells) and counterstained with DAPI. A representative image from 5 MPT mice is shown. The aNSCs population is identified by their somas located in the subgranular cell layer (asterisk) and the newborn astrocytes by their somas located in the granular cell layer (arrow head). E-F. The quantification of aNSCs and newborn astrocytes in the dentate gyrus at the indicated time points after tamoxifen injection. 3 $Trrap^{f/+}$ and 3 Trrap-aNSCΔ mice analyzed. G. Immunostaining of dentate gyrus sections using antibodies against DCX (orange), GFP (green, recombinant cells) and counterstained with DAPI. A representative image from 5MPT mice is shown. H. The quantification of newborn neurons ($DCX^+GFP^+$) in the dentate gyrus at the indicated time points after tamoxifen injection. 3 $Trrap^{f/+}$ and 3 Trrap-aNSCΔ mice analyzed. MPT = months post TAM injection. Co.: control; aNSCsΔ: Trrap-aNSCsΔ. The numbers inside the columns represent the number of mice analyzed. The number of cells scored are shown under each column. Mean ± standard error of mean is shown. Unpaired t-test was performed for statistical analysis. n.s.: not significant. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

**Figure 2 Deletion of Trrap reduces the differentiation potential and proliferative rate in aNSC**

A. Live-Cell confluency monitoring by Incucyte® on control and Trrap-aNSCΔ. Cells originated from 3 mice analyzed. B. Immunoblot on the samples cultured on monolayer at day 5 with quantification in (C). Cells originated from 3 mice analyzed. D. Scheme presenting the inducible differentiation experiment. Trrap deletion on aNSC was induced by addition of 4-OHT for 3 days. After 2 days incubation in fresh medium, aNSC was cultured as monolayer. On DPD0, the differentiation on aNSC was initiated and the differentiation medium was changed every 2 days. Scale bar indicates 40 μm. E. Immunofluorescent images showing the differentiation states of aNSC at 9 DPD. Cell mixture was stained against neuronal marker Tuj1 (Red), astrocyte marker GFAP (Green) and counterstained with DAPI (blue). The right panels quantify the proportion of $Tuj1^+$, $GFAP^-$ and $Tuj1^-$, $GFAP^+$ cells in percentage to whole cell population ($DAPI^+$).

Cells from 3-5 Trrap$^{f/+}$ and Trrap-aNSCΔ mice at each DPD were analyzed. Co.: control; aNSCsΔ: Trrap-aNSCsΔ. Mean ± standard error of mean is shown. Unpaired t-test was performed for statistical analysis in (C) and (E). (A) was analyzed through Two-way ANOVA. n.s.: not significant. *: p≤0.05, **: p≤0.01, ***: p≤0.001.

**Figure 3 Trrap interacts and stabilize Sp1 without affecting the K16 and K19 residue**

A. Truncation design for Sp1-Trrap interaction examination. All truncations contain Flag-label on the N-Terminal. The label of each truncation is labelled on the left side. Each color represents one characterized domain on Sp1, while white domain shows the region without being defined. Black lines among constructs label the boarders of the same domains. On the right side presents the quantification of truncated Sp1-Trrap interaction from (B). N = 4. B. Co-IP result shows the interaction between each Sp1 truncation and endogenous Trrap. N2A were transfected with different constructs and subjected to Co-IP. Pulled-down protein were then analyzed by immunoblotting. Left panel shows the input level of transfected constructs. On the right panel, the pulled-down samples was blot by Flag Trrap. The interaction between truncated Sp1 and Trrap is determined by quantifying Trrap band (The upper band in IB: Trrap) with band intensity in Flag blot. Values on the left side of (A) shows the normalized intensity of Sp1-Trrap interaction. C. Left panel shows the immunoblot analysis on the protein level of transfected Sp1 variants from control and mutant aNSC. Right panel presents the quantification of whole Sp1 level from immunoblot. Number inside the columns indicates the mouse number used for analysis. Cells from 3-5 Trrap$^{f/+}$ and Trrap-aNSCΔ mice analyzed. Co.: fl/+ aNSC; Δ: fl/fl. D. Transfected aNSCs were subjected to luciferase and immunoblotting analysis. Exogenous activity of Sp1 variants was calculated by quantifying the exogenous Sp1 activity (Subtracting the endogenous Sp1 activity from the whole Sp1 activity) by the exogenous Sp1 level (Flag). Cells from 3-4 Trrap$^{f/+}$ and Trrap-aNSCΔ mice analyzed. Mean ± standard error of mean is shown.

Paired t-test was performed for statistical analysis in (C) and (D). n.s.: not significant. *: $p \leq 0.05$.

**Figure 4 Trrap does not affect the K703 acetylation**

A. Control and mutant aNSC was co-transfected with Sp1-K703Q or Sp1-K703R variants and Sp1-reporter and were analyzed through immunoblotting. Right panels shows the quantification of whole Sp1 level (Sp1) and exogenous Sp1 level (Flag). Cells from 4-5 $Trrap^{f/+}$ and Trrap-aNSCΔ mice analyzed. B. Exogenous activity of Sp1 variants was calculated by quantifying the exogenous Sp1 activity (Subtracting the endogenous Sp1 activity from the whole Sp1 activity) by the exogenous Sp1 level (Flag). Cells from 3 $Trrap^{f/+}$ and Trrap-aNSCΔ mice analyzed. C. Co-IP analysis reveals the interaction between Sp1 and TIP60 in N2A cells. N2A cells were transfected with siRNA against Trrap, followed by 24 hr incubation and FLAG-Sp1 transfection. After another 24 hr incubation, the cells were subjected to co-IP and immunoblotting subsequently. Trrap knockdown abolished the interaction between Sp1 and TIP60. Actin blot acts as a loading control. Paired t-test was performed for statistical analysis in (B) n.s.: not significant. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

**Figure 5 Trrap-mediated acetylation acts on K639**

A. Immunoblot shows the level of exogenous Sp1 variants and total Sp1 in both control and mutant aNSCs co-transfected with FLAG-Sp1, FLAG-Sp1-K639R variants together with GFP. Actin serves a loading control. B. The total Sp1 activity from control and mutant aNSC transfected with the indicated constructs together with Sp1-reporter. Cells from 4-5 $Trrap^{f/+}$ and Trrap-aNSCΔ mice analyzed. C. The exogenous Sp1 activity is calculated according to the methods in Fig 4.B. Cells from 6 $Trrap^{f/+}$ and 6 Trrap-aNSCΔ mice analyzed. D. ChIP analysis on the promoter of p21 and Stmn3 in N2A cells transfected with FLAG-EV, FLAG-Sp1 or FLAG-Sp1 variants using anti-FLAG antibodies. qPCR analysis was performed to quantify the binding of respective Sp1 variant on target gene promoters. The binding

enrichment is presented as fold enrichment over FLAG-Sp1 binding value. The binding value of FLAG-EV, FLAG-Sp1 and FLAG-Sp1 variants was calculated in percentage of input subtracted with IgG binding value in percentage of input. E. Co-IP analysis determines the interaction between exogenous Sp1 variants and endogenous Trrap in N2A cells. The interaction strength was calculated by dividing the signal intensity of Trrap by the intensity of FLAG. N = 3. Mean ± standard error of mean is shown. Paired t-test was performed for statistical analysis in (B), (C), (E). n.s.: not significant. *: $p \leq 0.05$, **: $p \leq 0.01$.

**Figure 6 Acetylation-incompetent K639 rescues the differentiation defect in Trrap-aNSCΔ**

A. Immunofluorescent images showing the differentiation states of aNSC at 5 DPD. Cell mixture was stained against neuronal marker Tuj1 (Red) counterstained with DAPI (blue). Scale bar indicates 40 μm. B. Quantification of the proportion of $Tuj1^+$ cells in percentage to whole cell population ($DAPI^+$). Cells from 3 $Trrap^{f/+}$ and Trrap-aNSCΔ mice analyzed. Mean ± standard error of mean is shown. Unpaired t-test was performed for statistical analysis in (B). n.s.: not significant. *: $p \leq 0.05$, **: $p \leq 0.01$.

**Figure S1 Cell cycle profile and differentiation potential of aNSC with or without Trrap deficiency**

A. Representative distribution of DAPI intensity for aNSC. The first peak (Grey) represents cells with 2n DNA content (G1) and the second peak (Green) represents cells with 4n DNA content (G2/M). B. Chart represents the population of G1 and G2/M cells in percentage. C. Immunofluorescent images showing the differentiated aNSC at DPD1 (left panel) and DPD 4-5 (right panel) with neuronal marker Tuj1 (Red), astrocyte marker GFAP (Green) and with (blue). Scale bar indicates 40 μm.

## Main figures and supplementary figures

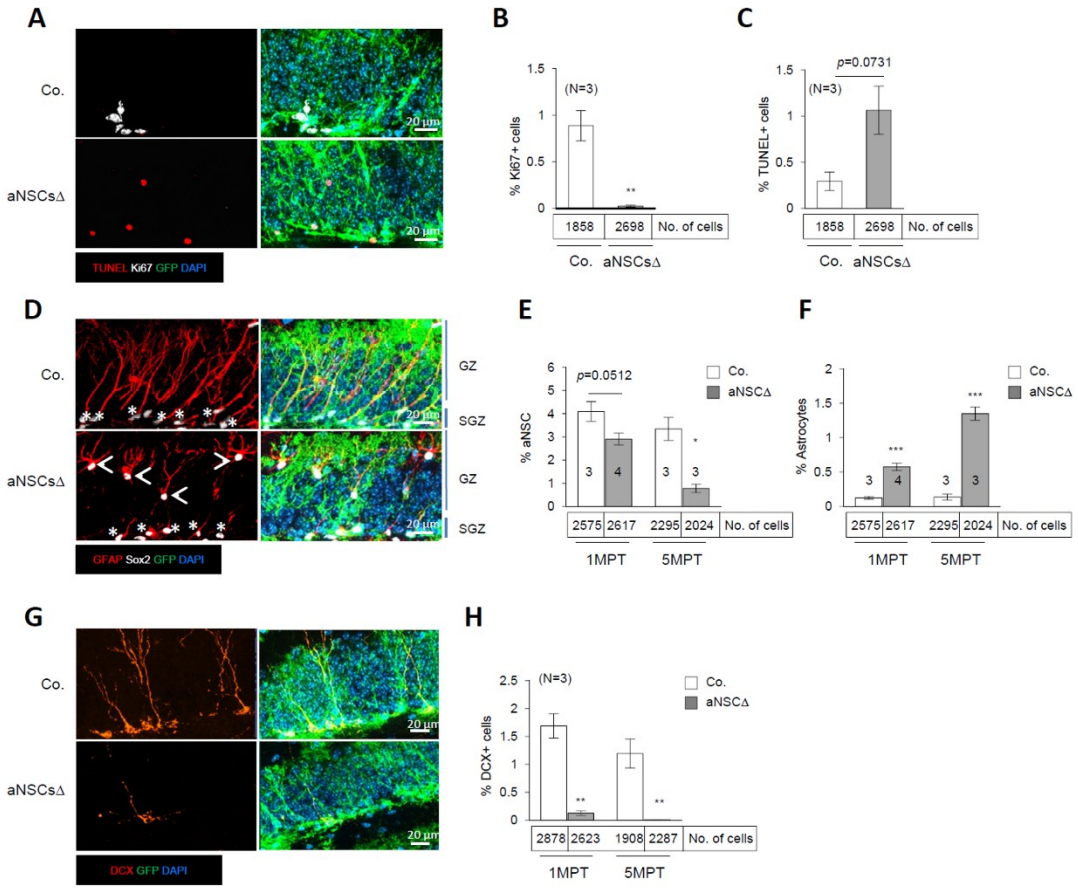
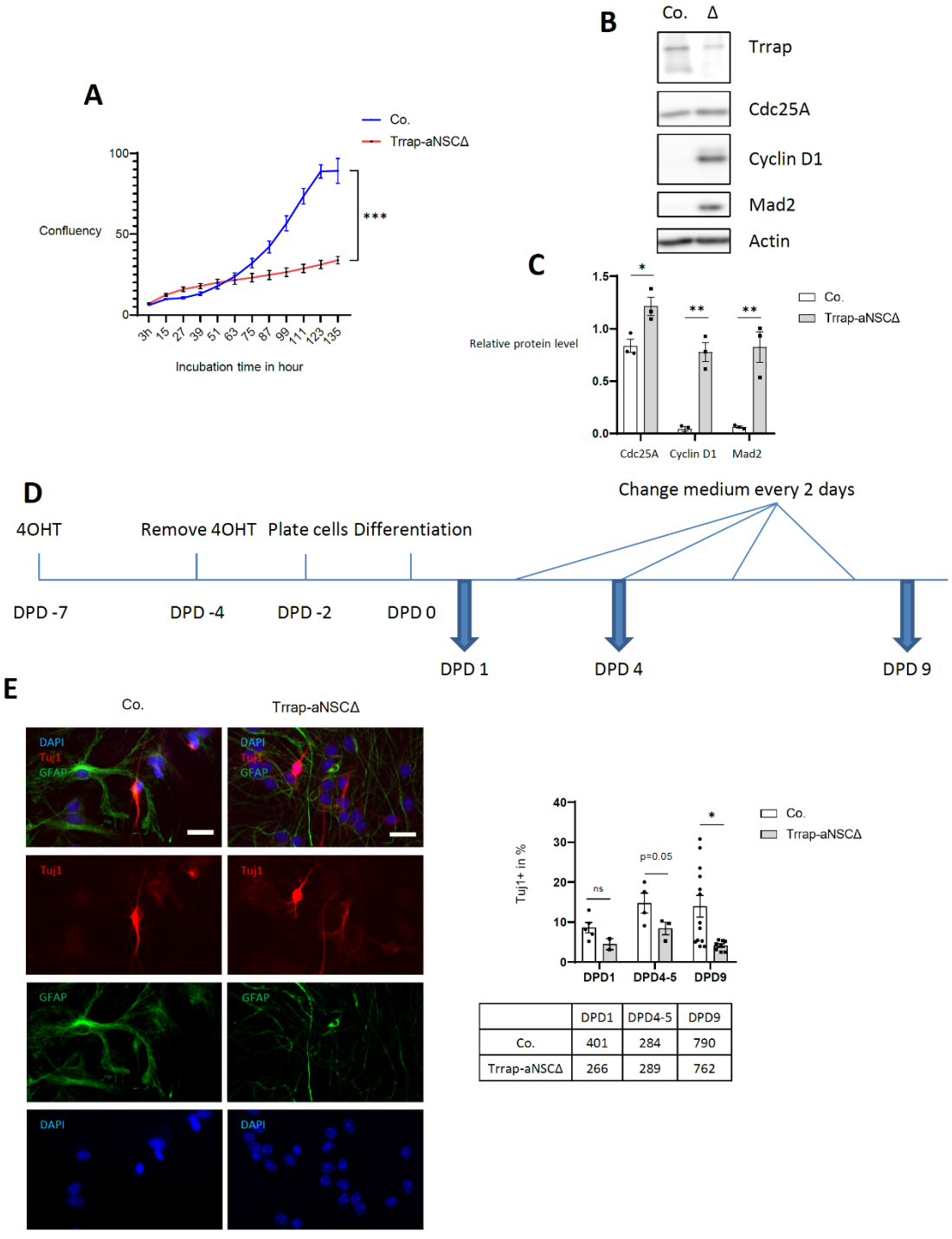



**Figure 1**




| | DPD1 | DPD4-5 | DPD9 |
|---|---|---|---|
| Co. | 401 | 284 | 790 |
| Trrap-aNSCΔ | 266 | 289 | 762 |

**Figure 2**

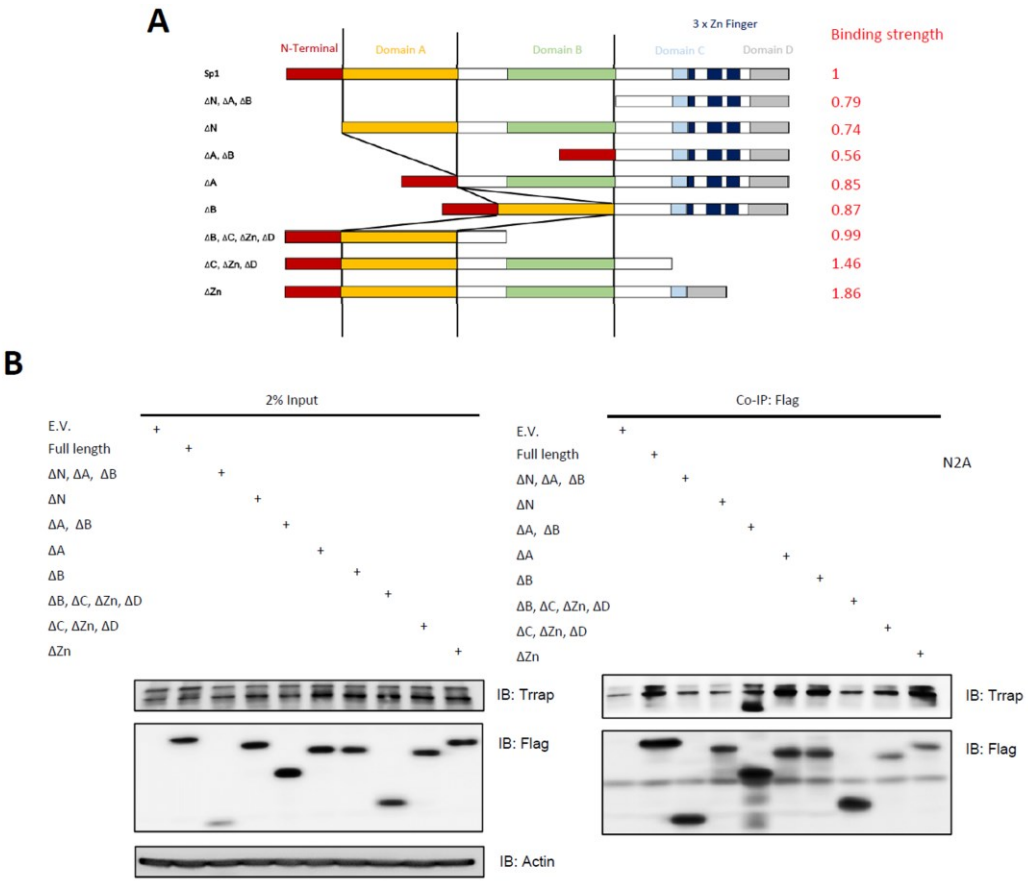

A
N-Terminal
Domain A
Domain B
3 x Zn Finger
Domain C
Domain D
Binding strength
Sp1
1
ΔN, ΔA, ΔB
0.79
ΔN
0.74
ΔA, ΔB
0.56
ΔA
0.85
ΔB
0.87
ΔB, ΔC, ΔZn, ΔD
0.99
ΔC, ΔZn, ΔD
1.46
ΔZn
1.86
B
2% Input
Co-IP: Flag
N2A
E.V.
Full length
ΔN, ΔA, ΔB
ΔN
ΔA, ΔB
ΔA
ΔB
ΔB, ΔC, ΔZn, ΔD
ΔC, ΔZn, ΔD
ΔZn
IB: Trrap
IB: Flag
IB: Actin

**Figure 3**

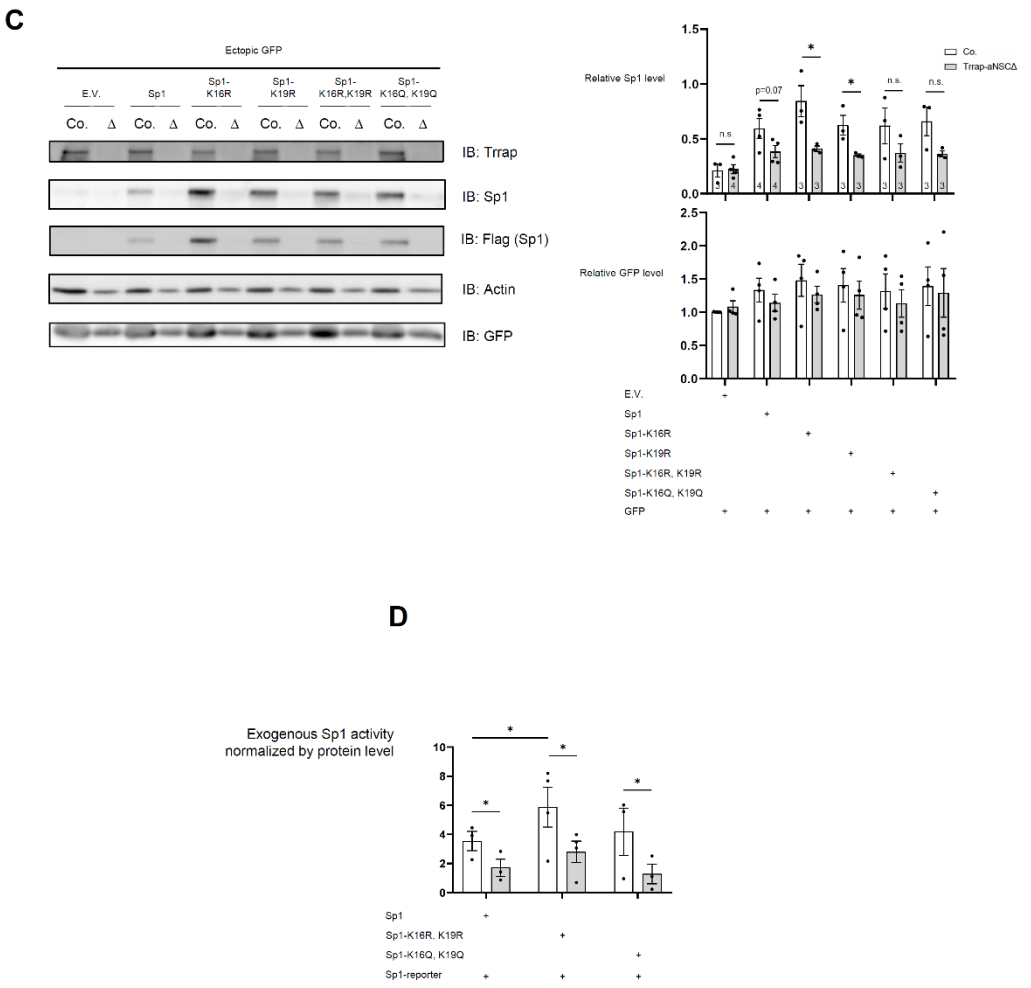

C
Ectopic GFP
E.V.
Sp1
Sp1-K16R
Sp1-K19R
Sp1-K16R,K19R
Sp1-K16Q, K19Q
Co.
Δ
IB: Trrap
IB: Sp1
IB: Flag (Sp1)
IB: Actin
IB: GFP
Relative Sp1 level
Relative GFP level
Co.
Trrap-aNSCΔ
n.s.
p=0.07
*
E.V.
Sp1
Sp1-K16R
Sp1-K19R
Sp1-K16R, K19R
Sp1-K16Q, K19Q
GFP
D
Exogenous Sp1 activity
normalized by protein level
Sp1
Sp1-K16R, K19R
Sp1-K16Q, K19Q
Sp1-reporter

**Figure 3**

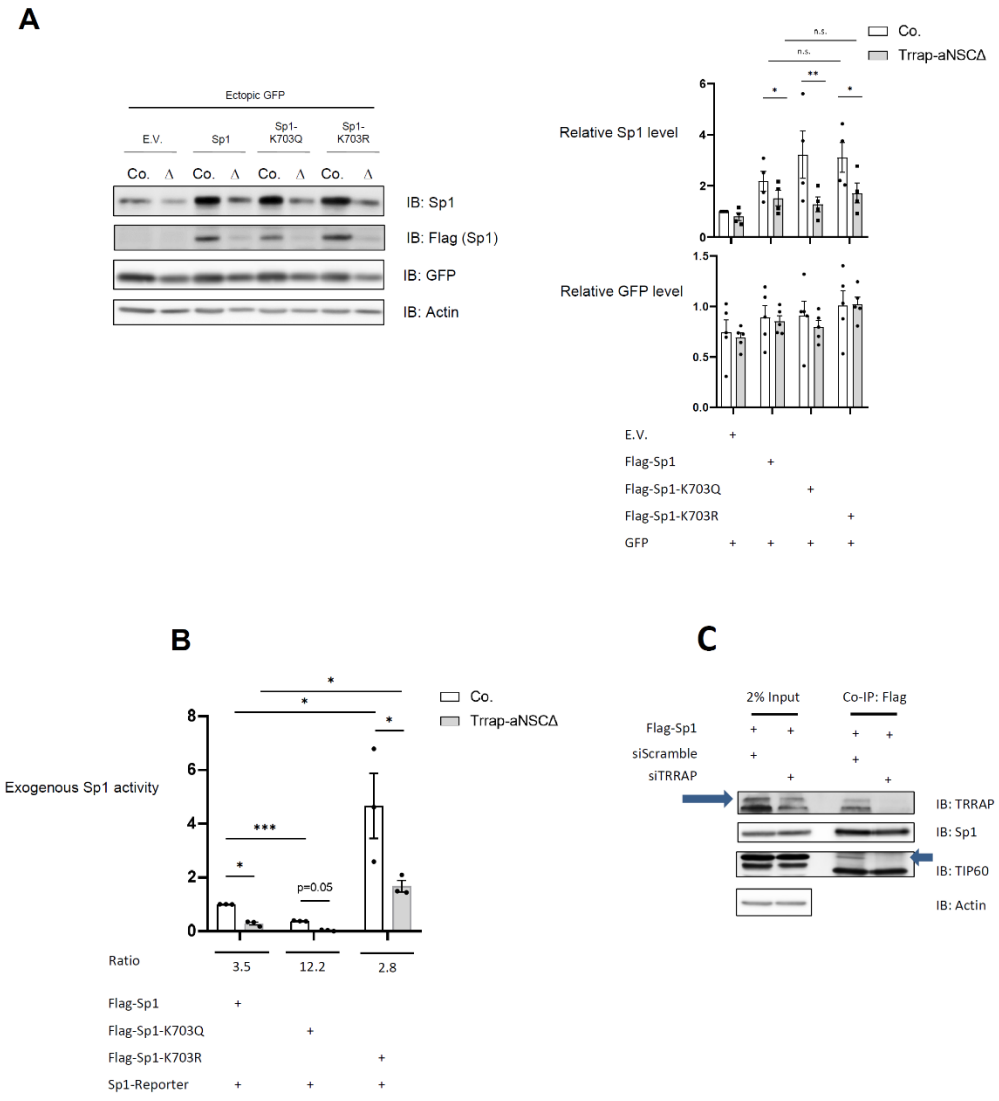

A
Ectopic GFP
E.V.
Sp1
Sp1-K703Q
Sp1-K703R
Co. Λ Co. Λ Co. Λ Co. Λ
IB: Sp1
IB: Flag (Sp1)
IB: GFP
IB: Actin
Co.
Trrap-aNSCΔ
n.s.
n.s.
**
*
*
Relative Sp1 level
Relative GFP level
E.V.
Flag-Sp1
Flag-Sp1-K703Q
Flag-Sp1-K703R
GFP
B
Co.
Trrap-aNSCΔ
Exogenous Sp1 activity
***
*
p=0.05
Ratio
3.5
12.2
2.8
Flag-Sp1
Flag-Sp1-K703Q
Flag-Sp1-K703R
Sp1-Reporter
C
2% Input
Co-IP: Flag
Flag-Sp1
siScramble
siTRRAP
IB: TRRAP
IB: Sp1
IB: TIP60
IB: Actin

**Figure 4**

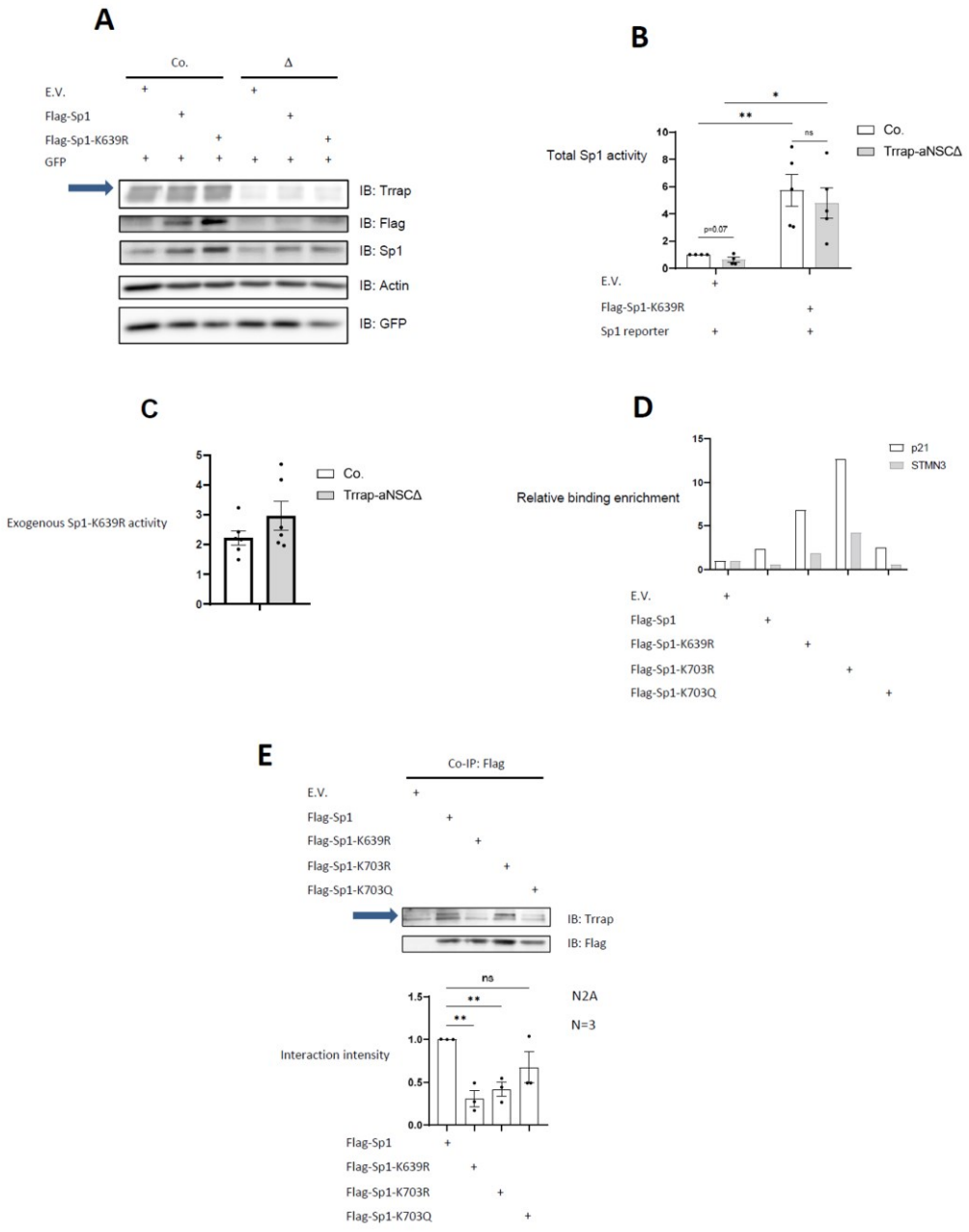
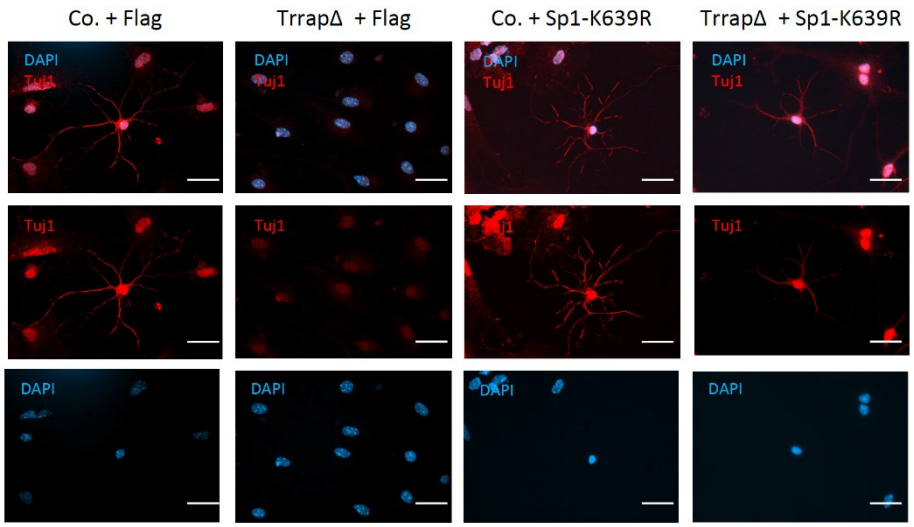
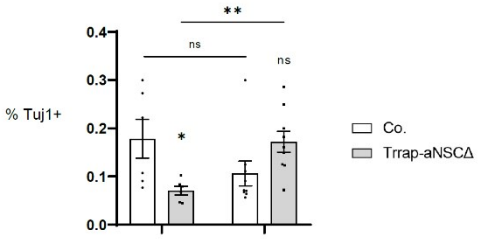

A
Co.
Δ
E.V.
Flag-Sp1
Flag-Sp1-K639R
GFP
IB: Trrap
IB: Flag
IB: Sp1
IB: Actin
IB: GFP
B
Total Sp1 activity
Co.
Trrap-aNSCΔ
p=0.07
E.V.
Flag-Sp1-K639R
Sp1 reporter
C
Exogenous Sp1-K639R activity
Co.
Trrap-aNSCΔ
D
Relative binding enrichment
p21
STMN3
E.V.
Flag-Sp1
Flag-Sp1-K639R
Flag-Sp1-K703R
Flag-Sp1-K703Q
E
Co-IP: Flag
E.V.
Flag-Sp1
Flag-Sp1-K639R
Flag-Sp1-K703R
Flag-Sp1-K703Q
IB: Trrap
IB: Flag
Interaction intensity
N2A
N=3
Flag-Sp1
Flag-Sp1-K639R
Flag-Sp1-K703R
Flag-Sp1-K703Q

**Figure 5**

**A**



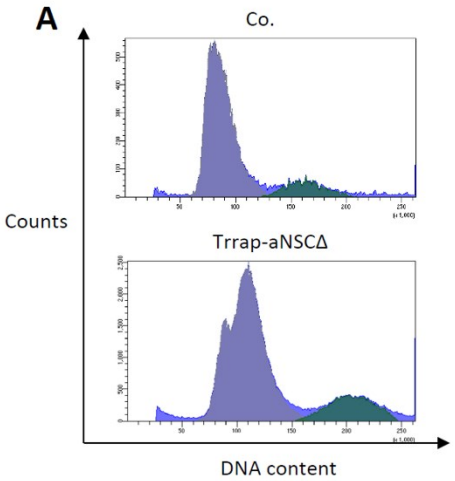

**B**




| | Flag | K639R |
|---|---|---|
| Co. | 106 | 403 |
| Trrap-aNSCΔ | 438 | 368 |

**Figure 6**

**A**




**B**

| Cell population in % | G1 | G2/M |
|---|---|---|
| WT aNSC | 56.7 | 9.4 |
| Mutant aNSC | 64.8 | 12.2 |

**C**

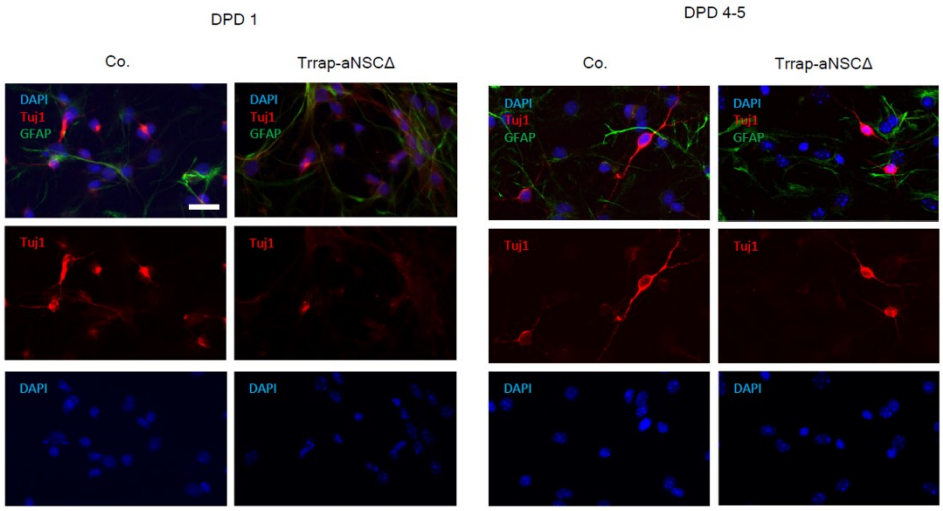



**Figure S1**

# 4. Discussion

Based on our discovery, we confirmed that Trrap is a pivotal modulator in brain homeostasis. It ensures the proper self-renewal and differentiation of aNSCs and also maintains the survival in post-mitotic neurons. Moreover, we have also unraveled the underlying mechanism behind these processes, namely the Trrap-HAT-Sp1 axis in the regulation of the target genes.

## 4.1. Sp1 as a master regulator mediated by Trrap-HAT to maintain post-mitotic neurons

To address the first objective of my thesis, we investigated the underlying mechanism of acetylation maintaining neuronal homeostasis, we altered the acetylation level in post-mitotic PC by deleting the HAT-essential adaptor Trrap (Manuscript I). We detected an age-dependent progressive PC degeneration in Trrap-PCΔ mice. By the cell amount decrease, the PCs in aged mutant animals showed regression of the size of their dendritic trees and more axonal swellings, characteristics of neurodegeneration. These morphological aberrations indicate that Trrap deletion led to the retraction of already formed neuron dendrites, as well as the defect in microtubule dynamics [202, 203]. This defect in microtube dynamics is likely due to the diminished transactivation of Sp1 under Trrap-deletion, especially affecting the transcription of Stmn3 and Stmn4. With integrated transcriptomic, proteomic, and epigenomic analysis in variable Trrap-deleted tissues and cells, we identified Sp1 as a novel transcription factor controlled by Trrap (Manuscript I). We discovered the Trrap-HAT-Sp1 axis modulating the microtubule dynamics through the regulation of Stmn3 and Stmn4. Trrap-deletion leads to a hypoacetylation and a lower Sp1-chromatin binding, on the proximity of the Stmn3/4 promoter, which then results in both lower Stmn3, and Stmn4 expression and protein levels. By functional testing *in vitro*, we have detected a neuronal defect in Trrap knockdown cortical neurons, characterized by reduced neurite length and branches. Strikingly, these neuronal arborization

deficiencies in cortical neurons were rescued through the ectopic overexpression of Stmn3/4, demonstrating the role of Trrap in maintaining neuron functionality via modulating the microtubule dynamics. Later in manuscript III, we disclosed the exact molecular mechanism of Trrap-mediated Sp1 transactivation.

The function of HATs and HDACs has been shown to play an important role in brain development. The disturbance of lysine acetylation balance has been linked to multiple neuropathogenesis and defects in brain development in human and mouse models [61]. HDACs have been shown to play a pivotal role in the pathogenesis of NDs and a series of HDACi have been considered to treat diverse NDs [204]. Moreover, the deficiency of Sp1 transactivation leads to neuronal defects in HD pathology, reflecting its role in preventing neurodegenerative progression [167, 168]. Also, Sp1 regulates the transcription of multiple neural genes [169-171], demonstrating its role in neuronal maintenance. Our observation that Trrap inhibits neurodegeneration in post-mitotic cells thereby supports its role in the maintenance of neural homeostasis as a HAT adaptor, as well as a co-activator of the master transcription factor Sp1.

STMN regulates the microtubule dynamics, thus affecting neuronal homeostasis. Defects in microtubule dynamics lead to variable neuronal defects, including axonal swellings and dendrite retraction, reminiscent of neurodegeneration [202]. Our data support the idea that ensured expression of Stmn3 and Stmn4 maintains the microtubule dynamics, thus preventing neuron abnormality and degradation.

The proper maintenance of microtubule dynamics supports neuronal activity and STMN3 and STMN4 have been related to neuronal functionality [203, 205]. Although the loss of PC impairs motor coordination (Manuscript I), it is also possible that the PC activity is repressed while the microtubule dynamic is disturbed under Trrap deficiency. Through the electrophysiological techniques, one can score the activity of Trrap-PCΔ, as well as the Trrap knockdown primary neurons *in vitro*. This experiment will answer, apart from supporting the neurite arborization, whether Stmn3/4 overexpression is also able to maintain neuronal activity when Trrap is absent.

### 4.2. Acetylation of Sp1 modulates cell fate of aNSCs

To address the second objective, we further investigated the role of Trrap in adult neurogenesis (Manuscript III). Trrap-deficiency impaired adult neurogenesis by reducing the differentiation potential of SGZ aNSCs *in vivo* and *in vitro*. The proliferation of aNSCs was also declined by Trrap-deletion *in vitro*. These results highlight the involvement of Trrap in neuronal maintenance and adult neurogenesis, providing a novel understanding of the pathology for patients with TRRAP missense variants.

Despite only giving rise to a small population of neurons, adult neurogenesis plays an indispensable role in maintaining the functionality of a great neuronal population in the human brain. The deficiency of maintaining an aNSC niche and the subsequent defect in adult neural differentiation is detrimental to the functional integrity of the adult brain [13]. Among patients with TRRAP missense variants, most of them bear the ID and/or ASD symptoms with variable severity [110]. FXS patients showed hippocampal volume changes [17, 18] and impaired hippocampal function[19, 20], indicating that the impairment of adult hippocampal neurogenesis is responsible for the etiology of ASD/ID. FXS mouse model also shows the abnormal hippocampal function associated with aNSC proliferation deficiency and impaired hippocampal neurogenesis [21-23]. These studies on ASD/ID strongly linked the neuropathogenesis of TRRAP missense variants to impaired adult neurogenesis.

Regarding the fact that only seven of these patients showed structural brain anomalies [110], however, it is also possible that other “non-syndromic” patients characterized with ID/ASD had a defect in neuronal functionality. This hypothesis is supported by the study on patients bearing non-syndromic X-linked intellectual disability [206]. A study on a mouse model with the missense mutation from those patients strongly suggested that these patients could have a defect in neurotransmitter release and synaptic plasticity in hippocampal neurons [206]. In this regard, it is plausible that a certain neuron subpopulation was dysfunctional in patients with TRRAP mutation. To this end,

future studies could investigate the effect of TRRAP mutations (human) on neuronal functionality, as well as the aNSC differentiation process.

In Manuscript I, we demonstrated that Sp1 activity is regulated by Trrap-HAT, yet how Trrap affects the binding of Sp1 and whether Trrap-mediated acetylation would affect Sp1 activity was not clear (Manuscript II). We then investigated the role of Trrap-mediated acetylation on the Sp1 stability (Manuscript III). We first detected a lower Sp1 level in Trrap-deleted aNSCs. We hypothesized that Trrap-deletion results in deacetylation and thereby conducts ubiquitination at K19 and SUMOylation at K16, leading to Sp1 proteasomal degradation. However, either acetylation (Sp1-K16Q, K19Q) or deacetylation (Sp1- K16R, K19R) failed to improve the reduced Sp1 level incurred by Trrap-deficiency. Modification on the other 2 residues, K703 [157] and K639 [207] also did not stabilize Sp1. These data suggest that Trrap might facilitate the acetylation of other to-be-discovered residues to stabilize Sp1 and is required to prevent Sp1 from degradation. In this regard, we have also attempted to detect the Sp1 acetylation pattern through liquid chromatography-mass spectrometry (LC-MS) (Data not shown). In HEK293 cells, we overexpressed Flag-tagged Sp1-WT and treated the cells with HDACi to boost the acetylation profile on Sp1. After precipitating the protein through Flag antibody, we fractionated the protein and detected the peptides through MS. We have confirmed the acetylation of K19, as published [159], and a novel lysine residue K712. However, we have not detected any other lysines to be acetylated, possibly due to the low sensitivity of the LC-MS devices utilized or the low abundance of acetylated Sp1. For further studies, it is meaningful to validate the effect of acetylation at K712, as well as at other yet-to-be-characterized lysine residues, K624, K685, and K693 [158] on Sp1 stabilization under Trrap-deletion.

To understand how Trrap regulates Sp1 activity, we confirmed the interaction of Sp1 with Trrap via transactivation domains A and B, indicative of Trrap acting as a scaffold and/or an Sp1 coactivator (Manuscript III). Domain A and B are essential domains for Sp1 transactivation, which interact with the TATA-box-binding protein (TBP)-associated factor (TAF) dTAFII110 component of the TFIID complex,

emphasizing the importance of these domains on transcriptional activation [143]. Also, domains A and B are required for the synergistic interaction of Sp1 multimerization Sp1 complexes, which facilitates its transcription activity [148]. Trrap has been shown to interact and co-activate multiple transcription factors [79, 92, 96]. The observation that Sp1 interacts with Trrap through its transactivation domains A and B implies the role of Trrap as an essential coactivator on Sp1 transcriptional activity. It is plausible that Trrap mediates the interaction between Sp1 and the transcriptional machinery. The synergistic interaction of Sp1 with other Sp1 molecules might also be facilitated via Trrap-Sp1 interaction. If Trrap is missing, Sp1 likely lacks the interaction with the transcriptional machinery, as well as the synergistic interaction with several other Sp1 molecules, promoting the reduced Sp1 activity under Trrap-deletion.

K639R mutation renders Sp1 resistant to Trrap-deletion-mediated downregulation of Sp1 activity, which therefore effectively rescued the differentiation defect of TrrapΔ-aNSC. This finding demonstrates that Sp1 deacetylation at K639R can overcome Trrap deficiency and is a molecular event to grant a full aNSC differentiation capacity. We have also introduced Sp1-WT into TrrapΔ-aNSCs (Fig.S2E). Noticeably, the neuronal population in Trrap-deleted aNSCs was not increased through Sp1 overexpression, indicating that the differentiation capacity is indeed controlled by acetylation of Sp1 and thereby its activity. In this regard, it is perhaps not surprising that Sp1-WT is insufficient to rescue the differentiation defects of Trrap-deficient aNSCs. Moreover, since the Sp1 level is maintained low during adult neural differentiation [208, 209], an excessive level of Sp1 or its activity might even inhibit neural differentiation. This demonstrates that a fine-tuning of Sp1 activity is critical for the aNSCs to determine cell fate during differentiation.

Pharmacological inhibition of HDACs or activation of HATs has been clinically utilized for the treatment of neurodegenerative diseases and psychiatric disorders [61]. We have also demonstrated the importance of the HAT adaptor Trrap on brain homeostasis. Therefore, it would be of great interest to investigate the effect of HDACs and/or HATs, which compensates for the diminished Trrap-mediated acetylation, on

Trrap-related neurodegenerative and neurodevelopmental phenotype. For instance, the administration of HDACi might ameliorate the PC loss and thus the motor incoordination of Trrap-PCΔ mice. Also, the administration of HDACi into murine hippocampus lacking Trrap might rescue the adult neurogenesis defect in mutant animals. In clinics, HDACi or HAT activators are used to treat not only neurodegenerative diseases but also autism and impairment of memory and cognitive function [62, 66, 70, 71], suggesting that HDACi might act on neuronal maintenance and hippocampal function integrity. If proven to be true, these phenotypical observations would support the clinical usage of HDACi for treating neurological dysfunction in patients with TRRAP mutation.

Rho kinases are suggested to play a regulatory role in cell proliferation, migration, and apoptosis [210, 211]. The Rho-associated kinase inhibitor (ROCKi) Y-27632 has been shown to prevent human ESCs from dissociation-induced apoptosis and increase their cloning efficiency [212]. Also, it inhibits the apoptosis of neuronal progenitors derived from stem cells following animal transplantation [213]. To investigate if the ROCK pathway is involved in the proliferation defect of TrrapΔ-aNSC, we have treated mutant cells with Y-27632 (Fig S2). We found ROCKi improved the proliferation of Trrap mutant aNSC significantly, although not reaching the control proliferation capacity (Fig S2A). Cell cycle analysis by immunoblotting showed that ROCKi ameliorated Mad2 in mutant aNSC, indicating that ROCKi released the G2/M block in Trrap mutant aNSC (Fig S2B-C). This observation is supported by the fact that ROCKi activates CDC25C/CDK1 pathway, which then activates FOXM1 and promotes G2/M transition [214]. ROCKi also increased the level of cyclin D1 in the mutant, which implies a fast G1/S transition (Fig S2D). Another study also showed that ROCKi initiates the G1/S transition by activating the CDK1/2-cyclin A [214], which then phosphorylates retinoblastoma protein (pRb) to release E2F1 and initiates the G1/S transition [215, 216]. These findings explain how ROCKi enables cells to overcome proliferation defects under Trrap deficiency. However, it is still not clear how ROCKi regulates the expression of the cell cycle factors under Trrap deficiency. Further

studies could focus on the effect of ROCKi on the Sp1-mediated transcription, especially of the corresponding cell cycle factors, e.g. Mad2 and cyclin D1.

In manuscript I, we have linked the Trrap-HAT-Sp1 with microtubule dynamics in neuroprotection, reflected by the rescuing defects of Stmn3 and Stmn4 incurred by Trrap-deletion. However, it was unknown whether the Sp1 activity was affected by its stability and acetylation profile. Our later study in manuscript III confirmed that the Trrap-mediated acetylation profile on Sp1 promotes its activity, thus supporting adult neurogenesis. Therefore, it is likely that the deacetylated status at the K693 residue can increase the expression of Stmn3/4. Based on the observation that Sp1-K639R has an increased chromatin binding on the Stmn3 promoter compared to Sp1-WT (Manuscript III) and Trrap-deletion abolishes Sp1 binding on this promoter region, overexpression of K639R might increase the STMN3/4 expression in TrrapΔ-aNSCs, thus maintaining microtubule dynamics and preventing neuronal defect.

Our study reveals Sp1 as a novel transcription factor under the control of Trrap. Sp1 interacts with E2F and is essential for the regulation of E2F target genes [194]. Sp1 has been shown to co-activate c-Myc and initiate transcription of the target gene [217]. Moreover, Trrap has been shown to mediate the activity of both E2F and c-Myc [59]. Our observation that Trrap interacts with all these transcription factors indicates that it might be necessary for the coactivation between these transcription factors and functions as a scaffold recruiting all these transcription factors to the target promoter region, thus initiating transcription.

### 4.3 Perspectives

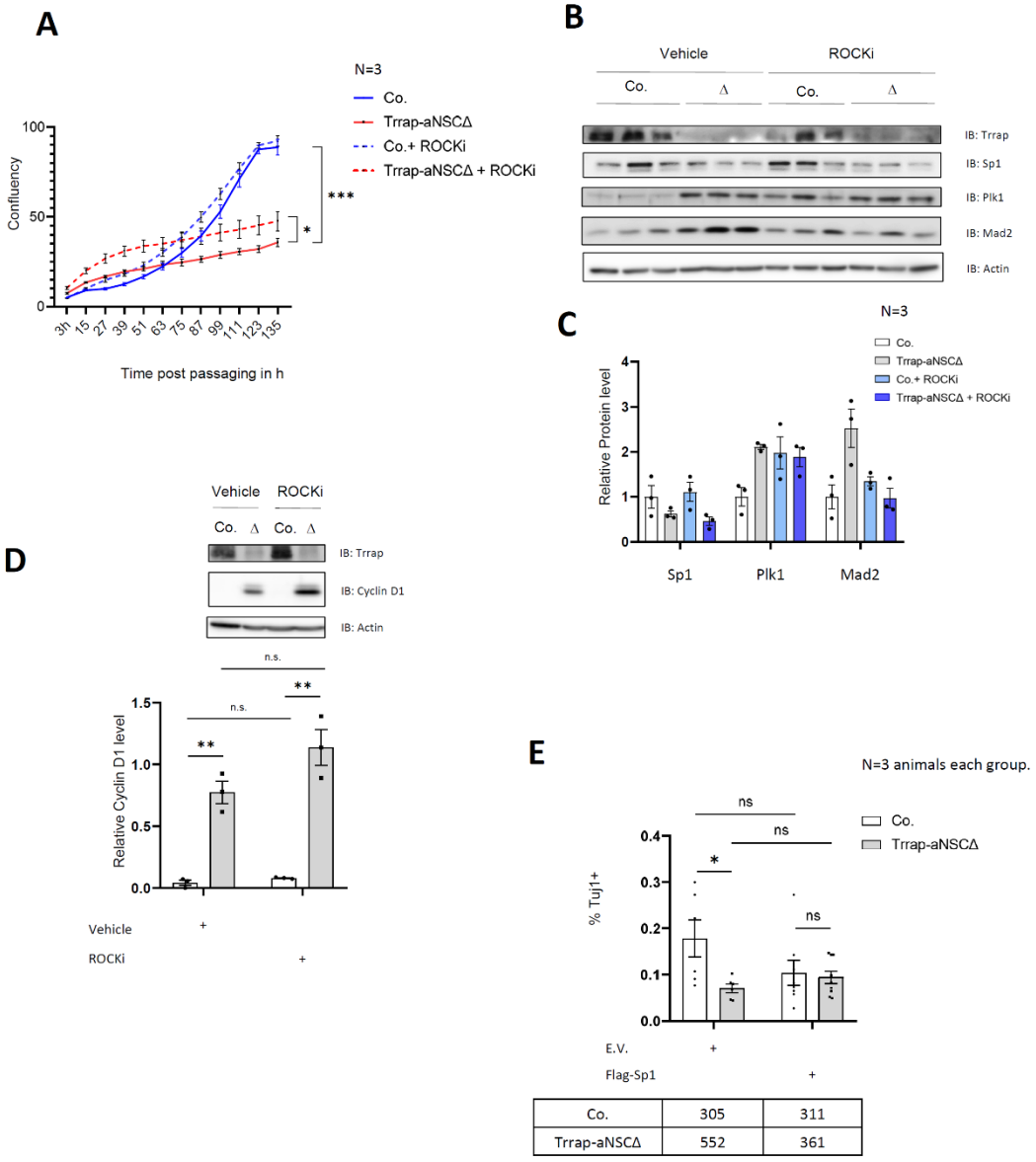
Our study discovers the novel function of TRRAP-HAT-Sp1 in brain homeostasis and provides a novel insight into the pathology of patients with TRRAP variants. Most of the patients with TRRAP variants are diagnosed with ASD/ID [110], and the impaired hippocampal adult neurogenesis is strongly linked with the etiology of ASD/ID [14-16]. Through supporting adult neurogenesis, TRRAP maintains the proliferation and differentiation of aNSC as well as the adult neurogenesis in the hippocampus

(Manuscript III). Although the function of these TRRAP variants has not been characterized, our study suggests that misregulation of Sp1 activity by TRRAP deficiency might be the etiologic factor of the developmental delay of the CNS in those patients.

Our study (Manuscript I) demonstrates the prevention of neurodegeneration by Trrap because it regulates Sp1 and its target genes STMNs. Though it has been only addressed that all of the individuals carrying TRRAP variants bear developmental delay, it is plausible that they might also have accelerated neurodegeneration of those individuals during aging, which might be expected due to the loss of neural maintenance.

Taken together, our work identifies the novel roles of TRRAP in regulating Sp1 transcriptional activity and preventing neurodevelopmental and -degenerative diseases. We provide a new link of this HAT adaptor together with a master transcription regulator Sp1 as an indispensable element in brain homeostasis.

## 4.4 Additional result




| | | |
|---|---|---|
| Co. | 305 | 311 |
| Trrap-aNSCΔ | 552 | 361 |

**Figure S2 ROCK inhibitor treatment rescued the proliferation potential in Trrap-aNSCΔ.**

A. Live-Cell confluency monitoring by Incucyte® on control and Trrap-aNSCΔ treated with or without ROCKi. B. Immunoblot on the samples cultured on monolayer at day 5. C. Quantification of immunoblot result from (B). Cells from 3 $Trrap^{f/+}$ and Trrap-aNSCΔ mice were analyzed. D. Control and mutant aNSCs were cultured with or without ROCKi for 5 days, followed by immunoblotting analysis. The right panel shows the quantification of cyclin D1 level from different conditions. E. Quantification

of Tuj1$^{+}$ population in aNSCs after transfection with FLAG-EV or FLAG-Sp1 at 5 DPD. Cells from 3 Trrap$^{f/+}$ and Trrap-aNSCΔ mice analyzed. The mean ± standard error is shown. (A) was analyzed through Two-way ANOVA. (D) was analyzed through paired t-test. (E) was analyzed through an unpaired t-test. Co.: control; aNSCsΔ: Trrap-aNSCsΔ. n.s.: not significant. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

## 5. Conclusion

Our studies have unraveled the role of TRRAP involved in brain homeostasis. Trrap prevents post-mitotic PC from degradation by maintaining the microtubule dynamics via the Trrap-HAT-Sp1 axis. Trrap supports adult neurogenesis by increasing the proliferation and differentiation potential of aNSC. We have also demonstrated that deacetylated K639 on Sp1 is refractory to Trrap deficiency and corrects the differentiation defect of Trrap-deleted aNSCs. Taken together, our study has provided a novel understanding of the role of epigenetic adaptors on brain maintenance and a new insight into the neuropathology of patients with TRRAP missense variants.

# 6. Zusammenfassung

Unsere Studien haben die Rolle von TRRAP enträtselt, die an der Homöostase des Gehirns beteiligt ist. Trrap verhindert die Degeneration von post-mitotischen PC, indem es die Dynamik der Mikrotubuli über die Achse Trrap-HAT-Sp1 aufrechterhält. Trrap unterstützt die adulte Neurogenese, indem es das Proliferations- und Differenzierungspotential von aNSC erhöht. Wir haben auch gezeigt, dass deacetyliertes K639 auf Sp1 resistent gegen Trrap-Mangel ist und die Differenzierungsdefekt von Trrap-deletierten aNSCs korrigiert. Zusammengenommen hat unsere Studie ein neuartiges Verständnis der Rolle des epigenetischen Adapters bei der Homöostase des Gehirns und einen neuen Einblick in die Neuropathologie von Patienten mit TRRAP-Mißense-Varianten neulich geliefert.

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## 8. Eigenständigkeitserklärung/ Declaration of Independence

**Auf Deutsch:**

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Zuhilfenahme der angegebenen Mittel und Literatur angefertigt habe.

Darüber hinhaus erkläre ich, dass ich mich mit der vorgelegten Arbeit an keiner anderen Hochschule um den akademischen Grade des doctor rerum naturalium (Dr. rer. nat.) beworben habe und, dass ich weder früher noch gegenwärtig die Eröffnung eines Verfahren zum Erwerb des Titels des doctor rerum naturlium an einer anderen Hochschule beantragt habe.

Ich habe keinen Promotionsberater in Anspruch genommen. Kein Dritter von mir hat direkte oder indirekte geldwerte Vorteile für Arbeiten im Zusammenhang mit der eingereichten Dissertation erhalten.

Ich habe die Dissertation nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht.

Ich habe die Namen der Personen angegeben, die mir bei der Auswahl und Analyse der Materialien geholfen und mich auch beim Schreiben des Manuskripts unterstützt haben.

Ich bestätige, dass ich das Promotionsvorhaben nicht bereits als Abschlussarbeit zu einem Staatsexamen oder einer anderen wissenschaftlichen Prüfung eingereicht habe.

**In English:**

I confirm that I'm familiar with the relevant course of examination for doctoral candidates.

I have composed and written the dissertation by myself and I have acknowledged all additional assistance, personal communication, and sources within the work.

I have not enlisted the assistance of a doctoral consultant (Promotionsberater) and no third parties have received either direct or indirect monetary benefits from me for work connected to the submitted dissertation.

I have not submitted the dissertation as an examination paper for a state or other scientific examination.

I have provided the names of the persons who assisted me in selecting and analyzing materials and also supported me in writing the manuscript.

I confirm that I have not already submitted the doctoral thesis project as my final thesis for a state examination or other scientific examination

Ort, Datum

Unterschrift

## 9. Author Contribution (Form 2)

**Manuscript No.** I

**Short reference** Tapias et al (2021), eLife

**Contribution of the doctoral candidate**

Contribution of the doctoral candidate to figures reflecting experimental data (only for original articles):

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|---|---|---|
| **Figure(s) #** 1, 2 | ☐ | 100% (the data presented in this figure come entirely from experimental work carried out by the candidate) |
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| | x | Approximate contribution of the doctoral candidate to the figure: 16%<br>Brief description of the contribution:<br>*Figure parts F* |
| ** Can refer to more than one fig. if the answer is the same* | | |

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| **Figure(s) #**<br>1-S1,<br>2-S1,<br>3-S1,<br>3-S2,<br>4-S1, | ☐ | 100% (the data presented in this figure come entirely from experimental work carried out by the candidate) |
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**FORM 2**

**Manuscript No.** II

**Short reference** Yin and Wang (2021), IJMS

**Contribution of the doctoral candidate**

Contribution of the doctoral candidate to figures reflecting experimental data (only for original articles):

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**FORM 2**

**Manuscript No.** III

**Short reference** Yin et al, submitted to the Computational and Structural Biotechnology Journal

**Contribution of the doctoral candidate**

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