Role of cystatin B in the regulation of histone H3 tail proteolysis in the mouse brain – study on the molecular mechanisms of progressive myoclonus epilepsy type 1

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Abstract

Cystatin B (CSTB) is a protein that functions as a potent inhibitor of papain-like cysteine proteases. Biallelic loss-of-function mutations in the *CSTB* gene underlie human neurological diseases of variable severity and age of onset: A total loss of CSTB expression causes a rapidly progressing microcephaly manifesting soon after birth, whereas an incomplete loss of CSTB expression leads to progressive myoclonus epilepsy type 1 (EPM1), a neurodegenerative disease with onset between ages 6 and 15 years. Previous studies have focused on identifying the phenotypes associated with CSTB-deficiency both in human patients and in a *Cstb* knockout mouse strain (*Cstb*$^{-/-}$). Despite the progress made, the exact physiological role of this protein is missing. Therefore, the molecular mechanisms downstream of CSTB-deficiency remain poorly understood.

The bulk of knowledge predicts a pivotal role for CSTB in regulating brain development, maturation and neuronal survival. Owing to its nuclear localization in immature cells, we hypothesized that CSTB acts as a regulator of lysosomal cysteine proteases moonlighting in the nucleus during normal brain development.

To investigate the neurodevelopmental role of CSTB, we established an *in vitro* model of neural stem cell renewal and differentiation derived from the embryonic mouse brain. We performed the experiments parallelly in wild type and in *Cstb*$^{-/-}$ mice. We found that new-born neurons undergo limited proteolysis of the N-terminal tail of histone H3 (H3 cleavage) between alanine 21 and threonine 22 (histone H3 cleavage site 1, H3cs1) by cysteine cathepsins B and L. CSTB-deficiency elicits premature H3 cleavage in undifferentiated neural progenitors (NPC) and enhanced H3 cleavage during neurogenesis. Histone proteolysis has been implicated in gene expression reprogramming during cell state transitions. Accordingly, *Cstb*$^{-/-}$ NPCs exhibit time-resolved transcriptional alterations with matching functional defects, highlighting delayed activation of nuclear-encoded mitochondrial genes and impaired mitochondrial respiratory function during differentiation.

To investigate the role of CSTB in the regulation of H3 cleavage during postnatal brain development and maturation, we continued by characterizing the timeline and distribution of H3cs1 in brains of postnatal wild type and in *Cstb*$^{-/-}$ mice. We found that H3cs1 is downregulated during postnatal weeks three and four, a temporal window marked by the maturation of neuronal circuits and refinement of cellular identities. Unlike controls, *Cstb*$^{-/-}$ mice display persistent H3 cleavage until adulthood, with the most prominent difference observed immediately before the symptomatic onset of the disease. The overproduction of H3cs1 has been reported in the context of cellular senescence. Accordingly, we show that the pre-symptomatic *Cstb*$^{-/-}$ mouse cerebellum recapitulates several phenotypes compatible with cellular senescence concomitant with the dysregulation of H3 cleavage. Finally, we provide evidence indicating that CSTB acts as a specific inhibitor of the chromatin-bound pool of cathepsin L *in vivo*. Collectively, our findings establish CSTB as a critical regulator of nuclear cathepsin L signalling in the mouse brain. Dysfunction of this pathway is likely to have a central role in the pathophysiology of CSTB-deficiency.

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I. **Daura E,** Tegelberg S, Yoshihara M, Jackson C, Simonetti F, Aksentjeff K, Ezer S, Hakala P, Katayama S, Kere J, Lehesjoki A-E, Joensuu T (2021). *Cystatin B-deficiency triggers ectopic histone H3 tail cleavage during neurogenesis*. Neurobiology of disease.

II. **Daura E**, Tegelberg S, Hakala P, Lehesjoki A-E, Joensuu T (2022). *Cystatin B deficiency results in sustained histone H3 tail cleavage in postnatal mouse brain mediated by increased chromatin-associated cathepsin L activity*. Frontiers in Molecular Neuroscience.

In addition, unpublished results are presented.

Publications are referred in the text by their roman numerals.

Abbreviations

53BP1	*P53-binding protein 1*
A	*Adenine and alanine*
a.a.	*Amino acid*
AD	*Alzheimer's disease*
ADP/ATP	*Adenosine di/triphosphate*
ATAC	*Assay for transposase-accessible chromatin*
BDNF	*Brain-derived neurotrophic factor*
bp	*Base pair*
BRD1	*Bromodomain-containing protein 1*
C	*Cytosine*
CB	*Cerebellum*
CDKIs	*Cyclin-dependent kinase inhibitors*
c-FLIPL	*Cellular FLICE inhibitory protein, long isoform*
CGL	*Cerebellar granular layer*
ChIP	*Chromatin immunoprecipitation*
Circ-CUX1	*CUX1 circular RNA*
CSTA	*Cystatin A, Stefin A*
CSTB	*Cystatin B, Stefin B*
CTSL (SC/DC)	*Cathepsin L (single chain/double chain)*
CTX	*Cortex*
CUX1	*Homeobox protein cut-like 1*
CXCL1/10/13	*C-X-C ligand-containing chemokine 1/10/13*
Da	*Dalton*
DCX	*Doublecortin*
DDR	*DNA repair and DNA damage response*
DEG	*Differentially expressed gene*
del	*Deletion*
DNA	*Deoxyribonucleic acid*
E	*Embryonic day*
E/I	*Excitation/inhibition*
E/IGL	*External/internal granular layer of the cerebellum*
EEG	*Electroencephalogram*
EGF	*Epidermal growth factor*
EGFP	*Enhanced green fluorescent protein*
ELANE	*Neutrophil elastase*
EMT	*Epithelial-mesenchymal transition*
EPM1	*Progressive myoclonus epilepsy type 1*
Exp	*Expansion mutation*
FGF2	*Fibroblast growth factor 2/B*

FMD	*Foot and mouth disease*
G	*Glycine and guanine*
GABA	*Gamma aminobutyric acid*
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*
GFAP	*Glial fibrillary acidic protein*
GO	*Gene ontology*
GSEA	*Gene set enrichment analysis*
H3	*Histone H3*
H3.3	*Histone variant H3.3*
H3cs1	*Histone H3 cleavage site 1 (A21-T22)*
H3K14/18ac	*Histone H3 acetylated at lysine 14/18*
H3K4/27me2/3	*Histone H3 di/trimethylated at lysine 4/27*
H3 cleavage	*Histone H3 N-terminal tail proteolysis*
ICC	*Immunocytochemistry*
IHC	*Immunohistochemistry*
ins	*Insertion*
IPs	*Intermediate progenitor cells*
K	*Lysine*
KCNC1	*Potassium Voltage-Gated Channel Subfamily C Member 1*
LAMP1	*Lysosomal-associated membrane protein 1*
MeCP2	*Methyl-CpG binding protein 2*
MEFs	*Mouse embryonic fibroblasts*
MENT	*Myeloid and erythroid nuclear termination protein*
mESC	*Mouse embryonic stem cells*
ML	*Molecular layer*
MMP-2/9	*Matrix metalloprotease 2/9*
MNEI	*Monocyte/neutrophil elastase inhibitor, Serpin B1*
NECs	*Neuroepithelial/neuroectodermal cells*
NPC	*Neural stem/progenitor cell*
OXPHOS	*Mitochondrial oxidative phosphorylation*
P	*Postnatal day*
PCA	*Principal component analysis*
PCL	*Purkinje cell layer*
PCR	*Polymerase chain reaction*
PME	*Progressive myoclonus epilepsy*
PTM	*Post-translational modification*
Q	*Glutamine*
R	*Arginine*
RNA (mRNA/tRNA)	*Ribonucleic acid (messenger RNA/transfer RNA)*
RT-qPCR	*Reverse transcription quantitative real-time PCR*
SAHF	*Senescence-associated heterochromatin foci*
SA-βGal	*Senescence-associated β-galactosidase*
seq	*Sequencing*

T	*Thymine*
TNFα	*Tumor necrosis factor alpha*
TRAIL	*Tumor necrosis factor-related apoptosis-inducing ligand*
TuJ1	*Class III beta-tubulin*
V	*Valine*
VGAT	*Vesicular GABA transporter*
wt	*Wild type*
γH2AX	*Histone variant H2AX phosphorylated at serine 139*

1. Introduction

Protein cleavage and degradation are fundamental processes in all living organisms. These biochemical reactions are mediated by six classes of enzymes collectively known as proteases. The regulation of protease activities is carried out by class-specific protease inhibitors. These proteins have been traditionally viewed as passive gatekeepers of cellular homeostasis. Indeed, protease inhibitors are the first line of defence against non-specific protein degradation, a potentially catastrophic effect of stress-induced lysosome dysfunction (Boya & Kroemer, 2008). However, the limited proteolysis of histone substrates is emerging as an important player in gene expression reprogramming, implying a previously unsuspected role for protease inhibitors in cell biology (Cheung *et al*, 2021; Dhaenens, 2021).

Histones are the packaging devices of eukaryotic genomes, and their post-translational modification is the single most important source of cellular memory and plasticity (Allis *et al*, 2007). For this reason, the amino acid sequence of histone proteins is highly conserved from unicellular eukaryotes to humans. On the contrary, proteases are characterized by their continuous diversification throughout the evolutionary tree (Neurath, 1984). Consequently, cells must have evolved a very intricate regulatory system to prevent unwanted interactions between the two (Dhaenens, 2021).

CSTB is a cysteine protease inhibitor whose deficiency results in brain disease by mechanisms that are mostly unknown. Biallelic loss-of-function mutations in the *CSTB* gene are the most common single cause of progressive myoclonus epilepsy worldwide, with an estimated prevalence of 1.5/100,000 persons in Europe and 1.91/100,000 persons in Finland (Sipila *et al*, 2020). As it is often the case with rare diseases, a specific treatment is missing, and its development awaits the discovery of CSTB function in brain physiology.

With the goal of setting a mechanistic framework that would help decipher the pathophysiology of CSTB-deficiency, I asked whether CSTB is involved in the regulation of histones. This research question led us to discover a novel layer of epigenetic regulation underlying brain development.

2. Review of the literature

2.1. Cystatin B

2.1.1. Evolutionary origins and biochemical properties

In the decade of the 1980s, the search for biologically active compounds in the chicken egg white fuelled the discovery of a protein that behaved as a specific inhibitor of papain-like cysteine proteases (Barrett, 1981). The protein that is currently referred to as chicken cystatin shared important structural analogies with over a dozen proteins isolated from mammalian tissues, suggesting their common evolutionary origin (Barrett et al, 1986; Müller-Esterl et al, 1985).

Cystatins form a superfamily of cysteine protease inhibitors that originated in bacteria and diversified within the plant and animal kingdoms (Benchabane *et al*, 2010; Kordis & Turk, 2009; Shamsi & Bano, 2017). All members of the cystatin superfamily contain at least one copy of the conserved Q-x-V-x-G sequence. The protein regions containing this amino acid motif fold into beta-hairpin loops that protrude from the protein surface and mediate protein-protein interactions. Q-x-V-x-G motifs block peptidase activities by forming hydrophobic contacts with the amino acids adjacent to the catalytic cysteine residue. Additionally, all biologically active cystatins contain two other conserved protease interaction sites: a glycine residue close to the amino terminus and a proline-tryptophan pair in the carboxy-terminal domain (Bode et al, 1988; Stubbs et al, 1990). The sum of these protein-protein interactions ultimately results in a stable occlusion of the protease active site cleft (Auerswald *et al*, 1995; Auerswald *et al*, 1994; Stubbs *et al*, 1990).

Type-1 cystatins —also called stefins— are one of the three cystatin families encoded in animal genomes. They are single-chain polypeptides of approximately 100 amino acids that fold into a five-stranded antiparallel beta sheet wrapped around an N-terminal alpha helix in a β1-α1-β2-β3-β4-β5 topology. They present a single Q-x-V-x-G motif (Q-V-A-A-G) that is found in the β2-β3 junction. Unlike the other cystatin subfamilies, stefins are devoid of disulphide bonds or carbohydrate modifications (Järvinen & Rinne, 1982; Ritonja *et al*, 1985; Turk & Bode, 1991; Turk *et al*, 2008). They are predominantly intracellular, but are also found in biological fluids such as saliva, milk or cerebrospinal fluid (Abrahamson *et al*, 1986). In mammalian cells, the main representatives of this family are cystatin A (CSTA) and cystatin B (CSTB). In mice and human, no other type 1 cystatin has been reported (Turk *et al.*, 2008). *In vitro*, both of these proteins act as potent, equimolar and non-selective inhibitors of cysteine proteases including cathepsin L, cathepsin B, cathepsin H, cathepsin S and papain (Bromme *et al*, 1991; Green *et al*, 1984). However, CSTA has a more acid isoelectric point than CSTB, with pI values ranging from 4.5 to 5.0 and 5.9 to 6.5, respectively (Turk & Bode, 1991). Additionally, CSTB has a unique cysteine residue at position three that is critical for protease binding, evidenced by *in vitro* kinetic experiments with N-terminally truncated CSTB variants (Pol & Björk, 2003; Turk & Bode, 1991). CSTB also contains a putative 14-3-3 binding site. However, in a series of co-immunoprecipitation experiments, an interaction between CSTB and the 14-3-3 family of proteins could not be detected (Sun *et al*, 2012).

Upon conformational perturbation or mutation-induced misfolding, CSTB and a few other members of the cystatin superfamily can form highly stable dimers through a well-described mechanism known

as domain swapping (Nandwani *et al*, 2019; Staniforth *et al*, 2001). Importantly, this biochemical property has been implicated in the pathogenesis of hereditary cystatin C amyloid angiopathy, leading to the conclusion that cystatins can act as amyloid-forming agents in disease (Abrahamson, 1996).

2.1.2. Biological function

CSTB is expressed in a wide variety of mammalian tissues and cell types (Joensuu *et al*, 2007; Turk & Bode, 1991). Its sub-cellular localization is both nuclear and cytoplasmic (Alakurtti *et al*, 2005; Brännvall *et al*, 2003; Maubach *et al*, 2008; Riccio *et al*, 2001), and it varies depending on the differentiation status of the cell. CSTB function has been linked with central biological processes including apoptosis (Pennacchio *et al*, 1998; Yang *et al*, 2010), oxidative stress response (Lehtinen *et al*, 2009), immune- and defense response (Korber *et al*, 2016; Okuneva *et al*, 2015; Okuneva *et al*, 2016), cell cycle progression (Ceru *et al*, 2010), synaptic function (Joensuu *et al*, 2014; Penna *et al*, 2019) and cell migration (Di Matteo *et al*, 2020). However, the molecular mechanisms underlying these effects are only partially understood.

In differentiated cells, CSTB can be detected both in the nucleus and in the cytoplasm, where it is thought to localize at the periphery of a subset of lysosomes (Alakurtti *et al.*, 2005). Indeed, intracellular cystatins are thought to protect the cell against cysteine protease leakage from damaged lysosomes (Shah & Bano, 2009). In osteoclasts, lysosome-associated CSTB inhibits cathepsin K, a critical mediator of bone reabsorption (Laitala-Leinonen *et al*, 2006). Four different *CSTB* variants linked with human brain disease failed to associate with the lysosomes (Alakurtti *et al.*, 2005; Joensuu *et al.*, 2007). These findings suggested that the neurological function of CSTB must be intimately linked with this cellular compartment (Alakurtti *et al.*, 2005; Joensuu *et al.*, 2007). In line with this hypothesis, CSTB was shown to protect brain cells against oxidative damage (Lehtinen *et al.*, 2009), a well-established source of lysosome dysfunction (Pivtoraiko *et al*, 2009). Cerebellar granule neurons cultured *ex vivo* displayed upregulation of CSTB expression specifically upon induction of oxidative stress (Lehtinen *et al.*, 2009). Accordingly, knocking down CSTB exacerbated the cytotoxic effect of oxidizing agents. This phenotype was efficiently rescued via cathepsin B knockdown, suggesting that CSTB protects neurons from oxidative stress through the inhibition of this protease activity.

CSTB is mostly localized in the cell nucleus in immature, proliferative cells (Alakurtti *et al.*, 2005; Riccio *et al.*, 2001). In T98G astrocytoma cells, CSTB co-immunoprecipitated with nuclear species of the cysteine protease cathepsin L, with canonical histones H3 and H2B and the histone variant H2A.Z (Ceru *et al.*, 2010). Nuclear cathepsin L was previously shown to shorten the cell cycle via proteolytic activation of the pro-division transcription factor Cut Like Homeobox 1 (CUX1). In accordance with these findings, overexpressing CSTB in astrocytoma cells diminished the proteolytic processing of CUX1 and delayed the progression of the cell cycle, suggesting that CSTB serves as a cell cycle modulator by protecting CUX1 from cathepsin L-mediated activation. Another study showed that knocking down CSTB expression in hepatic stellate cells boosted nuclear, but not cytoplasmic, cathepsin F activity (Maubach *et al.*, 2008). This cysteine cathepsin was shown to interact with the chromatin of hepatic stellate cells, where it mediates cell state transitions by mechanisms that are yet unknown. Finally, reducing hepatic stellate cells reprogramming by retinol

treatment boosted the expression of CSTB. These findings collectively suggested that CSTB participates in the regulation of stellate cell identity through the regulation of nuclear cathepsin F. The evidence linking cysteine protease inhibitors with the regulation of the chromatin is not unique to CSTB; Cystatin D, a type-II member of the cystatin superfamily, was shown to co-localize with chromatin markers of active transcription such as RNA polymerase II in a human colorectal carcinoma cell line (Ferrer-Mayorga *et al*, 2015). Cystatin D overexpression led to a significant alteration of the transcriptome, which was consistent with the inhibitory effects on colorectal cancer growth previously attributed to this protein (Alvarez-Díaz *et al*, 2009).

A recent study showed that CSTB can be regionally synthesized in synaptic terminals (Penna *et al.*, 2019). Peptides belonging to CSTB were also detected in the supernatant of synaptosome preparations following *in vitro* membrane depolarization, suggesting that CSTB can be secreted by neurons in physiological conditions. In line with these findings, endogenous and recombinant tagged CSTB were detected in the cerebrospinal fluid of mouse embryos and in the conditioned media of primary murine neuronal cultures, respectively (Di Matteo *et al.*, 2020). The presence of CSTB in the extracellular space was shown to stimulate interneuron migration and neural progenitor cell proliferation. However, the lack of a mechanistic basis for type-1-cystatin secretion and the existence of compelling evidence indicating that CSTB is not secreted in other cell types (Alakurtti *et al.*, 2005) call for caution when considering these findings.

Several studies in different cell types have directly or indirectly shown that CSTB has an anti-apoptotic effect (Kopitar-Jerala *et al*, 2005; Laitala-Leinonen *et al.*, 2006; Lehtinen *et al.*, 2009). Murine thymocytes lacking a functional CSTB gene displayed enhanced sensitivity to staurosporine-induced apoptosis (Kopitar-Jerala *et al.*, 2005). This phenotype was rescued by pre-treating cells with pharmacological caspase inhibitors, but not with general cysteine protease inhibitors, suggesting that the anti-apoptotic activity attributed to CSTB is independent of its basic role as a cysteine protease inhibitor. In a follow-up study, the same research group showed that overexpressing recombinant CSTB proteins in the nucleus, but not in the cytosol, protected T98G astrocytoma cells from staurosporine-induced caspase 7 activation (Sun *et al.*, 2012). Finally, CSTB was shown to block melanoma cell apoptosis by interfering with the TNF-related apoptosis-inducing ligand (TRAIL) signalling axis (Yang *et al.*, 2010). In accordance with previous findings, this mechanism did not involve inhibition of cysteine proteases cathepsins B and cathepsin L. On the other hand, CSTB was shown to stabilize an inhibitor of the TRAIL pathway (c-FLIP$_L$) by reducing its interaction with an E3 ubiquitin ligase. The exact mechanism by which CSTB mediates this effect remains unreported.

CSTB is upregulated in human cancers including ovarian carcinoma (Wang *et al*, 2014), hepatocarcinoma (Lee *et al*, 2008), bladder cancer (Feldman *et al*, 2009), mammary cancer (Butinar *et al*, 2014) and melanoma (Yang *et al.*, 2010). In two of these diseases, the expression level of CSTB has been directly correlated with tumour progression (Feldman *et al.*, 2009; Wang *et al.*, 2014). Knocking out CSTB in a mouse strain that spontaneously develops mammary cancers led to significantly reduced tumour growth correlated with increased apoptosis within the tumour (Butinar *et al.*, 2014). Therefore, resistance to apoptosis is likely to be a key mechanism downstream of CSTB expression in some forms of cancer. On the other hand, CSTB expression is downregulated in tumor tissue from oral squamous cell carcinoma patients, with the degree of CSTB loss being directly

proportional to the malignancy of the cancer and to the probability of cancer resurgence after treatment (Xu *et al*, 2021). Overexpressing CSTB in cells of the oral epithelium led to the upregulation of genes associated with keratinization and differentiation, suggesting that the anti-carcinogenic activity displayed by CSTB in oral cancer could involve activation of the epithelial differentiation program.

Finally, overexpressing CSTB in induced pluripotent stem cell-derived human brain organoids led to neural progenitor cell expansion, whereas CSTB-deficient brain organoids showed reduced proliferation and premature differentiation (Di Matteo *et al.*, 2020). The molecular basis of these mechanisms is not known.

2.1.3. Cystatin B deficiency and brain disease

Defects in CSTB expression resulting from biallelic loss-of-function mutations in the CSTB gene are a well-established cause of human brain disease (Canafoglia *et al*, 2012; Lehesjoki & Kälviäinen, 2020; Mancini *et al*, 2016; Pennacchio *et al*, 1996; Pinto *et al*, 2012). The degree of CSTB loss characteristic of each genotype has been directly implicated in the severity of the symptoms (Fig. 1A). Briefly, patients that present residual CSTB expression develop progressive myoclonus epilepsy during childhood or adolescence, whereas individuals with a biallelic CSTB-null genotype (*CSTB*$^{-/-}$) present with severe developmental encephalopathy in the neonatal period.

2.1.3.1. Molecular genetics

The human *CSTB* gene spans a 3.82 kb region of chromosome 21 (21q22.3) and encodes a polypeptide of 98-amino acid residues. The gene consists of three exons separated by two introns (Pennacchio *et al.*, 1996; Safran *et al*, 2010), contains two potential transcription start sites and is subject to alternative splicing (Joensuu *et al.*, 2007). It is under the influence of multiple enhancer and promoter sequences, found at a distance of up to ≈ 400 kb upstream and downstream of the transcription start site (Source: GeneHancer; (Fishilevich *et al*, 2017)). The promoter region is located 670 to 1 bp from the putative transcription start site (Alakurtti *et al*, 2000). It contains binding sites for SP1 and AP1 transcription factors, as well as a consensus response element for the androgen receptor. The *CSTB* promoter also contains two or three copies of the 12 bp sequence 5'-CCCCGCCCCGCG-3' (Osawa *et al*, 2003). The expansion of this dodecamer minisatellite repeat to 12 copies or more has been linked to reduced *CSTB* expression and unstable transmission to the offspring (Lalioti *et al*, 1997b). Alleles containing a minimum of 30 minisatellite copies are considered fully-penetrant disease-causing variants (Lehesjoki & Kälviäinen, 2020) and account for over 90% of the pathogenic *CSTB* alleles worldwide (Lalioti *et al.*, 1997b). Importantly, promoter expansion does not completely abolish *CSTB* expression (Joensuu *et al.*, 2007). This conclusion derives from the observation that patient cells homozygous for this mutation preserve *CSTB* mRNA and protein levels corresponding to five to ten percent of those in the healthy controls. The dodecamer repeat expansion mutation impairs *CSTB* transcription by interfering with promoter function (Alakurtti *et al.*, 2000). However, the mechanistic basis is unknown. The hypotheses formulated so far include the modification of the appropriate spacing between DNA regulatory elements (Lalioti *et al*, 1999), the formation of aberrant DNA secondary structures (Saha & Usdin, 2001) and the deposition of repressive epigenetic signatures onto the elongated promoter (Poeta *et al*, 2020).

Besides the promoter expansion, 15 disease-causing mutations have been reported to date (Assenza *et al*, 2017; Bespalova *et al*, 1997; Canafoglia *et al.*, 2012; de Haan *et al*, 2004; Joensuu *et al.*, 2007; Kagitani-Shimono *et al*, 2002; Lafreniere *et al*, 1997; Lalioti *et al*, 1997a; O'Brien *et al*, 2017; Pinto *et al.*, 2012). All of them are located in the coding region, mostly within exons but also in intron-exon boundary junctions. Four of these mutations have been found in homozygosity in at least one patient (c.10G>C, c.66G>A, c.202C>T and c.218dupT), whereas the rest have only been described in compound heterozygosity with the dodecamer repeat expansion. The c.10G>C mutation results in the substitution of a highly-conserved glycine residue in exon one by an arginine (Lalioti *et al.*, 1997a). The c.66G>A mutation triggers abnormal RNA splicing and intron retention upon protein translation (Pinto *et al.*, 2012). In both cases, the resulting protein product is thought to be partially functional, as judged by the relatively mild phenotype displayed by patients carrying these mutations in both alleles. On the contrary, the c.202C>T nucleotide change encoding an early stop codon (Alakurtti *et al.*, 2005; Mancini *et al.*, 2016), and the c.218dupT leading to frameshift in exon three (O'Brien *et al.*, 2017) cause severe neurodevelopmental defects in homozygous individuals (Mancini *et al.*, 2016; O'Brien *et al.*, 2017), and thus are considered *bona fide CSTB* null alleles.

2.1.3.2. Progressive myoclonus epilepsy

Progressive myoclonus epilepsies (PMEs) are a group of clinically-defined rare brain diseases that present with involuntary muscle twitches (myoclonus), epileptic seizures and neurodegeneration (Marseille Consensus Group, 1990). All PMEs are genetically inherited disorders. However, the underlying genetic defects and pathophysiology of the individual PME disorders is very heterogeneous (Orsini *et al*, 2019). For example, (1) Lafora's disease (EPM2) is a particularly severe PME caused by biallelic mutations in two genes involved in the metabolism of glycogen whose deficiency leads to the accumulation of intracellular aggregates with a neurotoxic effect (Sullivan *et al*, 2017); (2) Myoclonic epilepsy with ragged-red fibers (MERRF) is a multisystem disease with progressive myoclonus epilepsy caused by mutations in the mitochondrial tRNA repertoire (Hameed & Tadi, 2022) and (3) Myoclonus epilepsy and ataxia due to potassium channel mutation (MEAK, EPM7) is a PME caused by a specific heterozygous missense mutation in the *KCNC1* gene (Muona *et al*, 2015). Interestingly, other mutations in the *KCNC1* gene have been shown to cause developmental epileptic encephalopathy or intellectual disability without epilepsy (Cameron JM, 2019; Park *et al*, 2019). Despite the etiologic diversity, the characteristic combination of symptoms present in all PMEs suggest that these disorders must share a common physiological or neuroanatomical basis (Serratosa *et al*, 1999).

Progressive myoclonus epilepsy type 1 (OMIM 254800; Unverricht Lundborg disease, EPM1) is caused by biallelic loss-of-function mutations in the *CSTB* gene leading to an incomplete loss of CSTB function (Joensuu *et al.*, 2007; Pennacchio *et al.*, 1996). EPM1 is the most common form of progressive myoclonus epilepsy worldwide (Orsini *et al.*, 2019). The highest disease prevalence is found in Mediterranean and Baltic countries (Lehesjoki & Kälviäinen, 2020; Orsini *et al.*, 2019). It is more common in Finland than anywhere else in the world, with 135 diagnosed cases nationwide in the period between 1998 and 2016 and a prevalence of 2 cases in 100,000 people (Sipila *et al.*, 2020). In some countries, the incidence of EPM1 might be higher than currently estimated due to recurrent misdiagnosis with other juvenile-onset myoclonic epilepsies (de Haan et al, 2004).

The clinical symptoms associated with EPM1 manifest at ages 6 to 15 (Fig. 1A). In over half of patients, the disease begins with myoclonus (Hypponen *et al*, 2015; Kälviäinen *et al*, 2008; Lehesjoki & Kälviäinen, 2020). At first, the myoclonic jerks appear when the patient is stressed, engages in physical exercise or perceives external stimuli such as flickering lights or loud noises. The myoclonus is disabling and refractory to treatment, and it tends to aggravate during the first five to ten years after disease onset. The majority of EPM1 patients present with generalized tonic-clonic epileptic seizures, but other seizures such are absences have also been described. The seizures are more frequent during the first years after the symptomatic onset, but they may eventually cease with proper anti-epileptic medication. The subsequent disease progression is characterized by the appearance of motor incoordination (ataxia), speech difficulties and intention tremor. The severity of the symptoms and the rate of deterioration vary from patient to patient, even within members of the same family. The disease is not fatal but severely incapacitating, with one third of the patients becoming wheelchair-bound before age 30.

Antiepileptic treatment and rehabilitation, including psychosocial support, are the backbone of EPM1 patients' care (Eldridge *et al*, 1983; Kälviäinen *et al.*, 2008; Lehesjoki & Kälviäinen, 2020). Valproic acid is so far the most efficient therapeutic strategy against EPM1 symptoms. Other drugs of choice include clonazepam, levetiracetam and high doses of piracetam. Phenytoin, another antiepileptic agent, was shown to aggravate the phenotype of EPM1 patients, ultimately leading to intellectual disability and severe dyskinesia correlated with a pronounced loss of cerebellar Purkinje neurons.

Individuals compound heterozygous for *CSTB* null and promoter expansion mutations ($CSTB^{-/Exp}$) develop a more severe PME disease with earlier age of onset, more severe myoclonus, and seizures that may be drug resistant (Assenza *et al.*, 2017; Canafoglia *et al.*, 2012; Koskenkorva *et al*, 2011) (Fig. 1A). These patients may also show psychiatric symptoms and moderate to severe cognitive impairment. On the other hand, heterozygous carriers of the expansion mutation sometimes exhibit minor neurological symptoms and abnormal electroencephalogram (EEG) recordings (Koskiniemi *et al*, 1974; Norio & Koskiniemi, 1979) (Fig. 1A).

Neurophysiological and neuroimaging studies have revealed increased excitability, decreased plasticity and grey-matter degeneration in the primary motor cortex of EPM1 patients. (Danner *et al*, 2009; Danner *et al*, 2011; Koskenkorva *et al*, 2009; Koskenkorva *et al*, 2012; Mascalchi *et al*, 2002). Signs of degeneration are also present in the somatosensory, auditory and visual cortices, and to a lesser extent in the thalamus and brainstem (Koskenkorva *et al.*, 2009; Manninen *et al*, 2013; Mascalchi *et al.*, 2002). Changes in the cerebellum have been reported both in cortex and in deep nuclei (Mascalchi *et al.*, 2002). The cerebellar signs stand out in patients with a $CSTB^{-/Exp}$ genotype, presenting a more severe disease manifestation (Canafoglia *et al.*, 2012). Finally, a diffusion-tension imaging study of axonal tract organization in EPM1 patients identified widespread white matter atrophy affecting the brain as a whole, but particularly the thalamocortical circuits and the cerebellum (Manninen *et al.*, 2013).

The understanding of the disease mechanisms acting in the brain of EPM1 patients is very scarce. A study combining positron emission tomography with magnetic resonance imaging revealed increased binding of a dopamine receptor antagonist in the thalamus and striatum of EPM1 patients, suggesting

higher density of dopamine receptors and/or decreased intrasynaptic dopamine levels in these brain areas (Korja *et al*, 2007b). The authors of this study argue that EPM1 could share pathomechanisms with more common movement disorders such as Parkinson's disease. On the other hand, the results of other studies suggest that the characteristic symptoms of EPM1 could arise from underlying defects in GABAergic signalling. Signs of GABAergic dysfunction in brains of EPM1 patients include GABA-linked abnormalities in EEG recordings, statistically reduced levels of free GABA in the cerebrospinal fluid of 15 EPM1 patients and decreased immunoreactivity for the vesicular GABA transporter in brain tissue from a deceased individual with EPM1 (Airaksinen & Leino, 1982; Buzzi *et al*, 2012; Shibasaki *et al*, 1985).

Apart from the neurological phenotype, EPM1 patients display a number of skeletal changes, most commonly diffuse thickening of the cranial bones, scoliosis, enlarged sinuses and accessory ossicles on the foot (Danner *et al*, 2013; Korja *et al*, 2007a; Suoranta *et al*, 2012). Additionally, lymphoblastoid cell lines derived from blood samples from EPM1 patients exhibit elevated cysteine protease activities attributed to cathepsins B, L and S, but not to cathepsin H (Rinne *et al*, 2002).

2.1.3.3. Developmental encephalopathy

Biallelic *CSTB* null mutations were recently identified as the cause of the severe neurodevelopmental defects present in two unrelated pairs of siblings (Mancini *et al.*, 2016; O'Brien *et al.*, 2017) (Fig. 1A). Their phenotype was characterized by a combination of progressive microcephaly, profound developmental delay, epilepsy, cortical blindness, hypotonia and dyskinesia. The seizures were tonic-clonic and progressive in nature, similarly to those of EPM1 patients, and were first detected at 3 months of age. Interestingly, one of two sisters bearing the same genotype never developed signs of epilepsy (O'Brien *et al.*, 2017). Neuroimaging revealed progressive neurodegeneration in the basal ganglia and thalamus, and to a lesser extent in the brainstem and cerebellum. Diffuse and progressive hypomyelination including atrophy of the corpus callosum was also detected. Additionally, the patients exhibited dysmorphic features of the face, neck and oral cavity.

2.1.3.4. Phenotype of the *Cstb* knockout mouse

The murine Cstb gene is 77% identical to its human counterpart (Pennacchio & Myers, 1996). It also consists of three exons and two introns, and it is located in chromosome 10, in a region that is homologous to the q22 segment of human chromosome 21. In 1998, a *Cstb* knockout 129Sv mouse strain ($Cstb^{-/-}$ mouse) was generated by inserting a 1.3-kb neomycin cassette into the first exon of the *Cstb* gene (Pennacchio *et al.*, 1998). $Cstb^{-/-}$ mice express no functional *Cstb* mRNA or protein, but develop less severe symptoms than humans with a $CSTB^{-/-}$ genotype (Fig. 1A). This is consistent with the observation that other gene mutations associated with microcephaly in humans tend to cause milder neurodevelopmental defects in mice (Bedogni *et al*, 2016; Chen *et al*, 2014; Pulvers *et al*, 2010; Trimborn *et al*, 2010). On the other hand, $Cstb^{-/-}$ mice recapitulate the majority of clinical traits of human EPM1. These include spontaneous myoclonus and myoclonic seizures with onset at around postnatal day 30 (P30) and ataxia by six months of age (Pennacchio *et al.*, 1998). Of interest, the stage of murine development at which the symptomatic onset takes place coincides with that of human patients (Bell, 2018; Lehesjoki & Kälviäinen, 2020; Pennacchio *et al.*, 1998). Finally, $Cstb^{-/-}$ mice

present some unique features, including lack of tonic-clonic epileptic seizures and presence of myoclonus mostly during sleep.

The onset of the symptoms coincides with the first signs of neuronal death in the granular layer of the cerebellum (Pennacchio *et al.*, 1998) (Fig. 1B). Simultaneously, the first signs of neurodegeneration appear within the somatosensory cortex (Tegelberg *et al*, 2012). The neurodegenerative changes are significantly less pronounced in *Cstb*$^{-/-}$ mice with a mixed C57BL/6/129Sv background, indicating that unknown genetic factors protect or sensitize brain cells against CSTB-deficiency (Pennacchio *et al.*, 1998). Interestingly, a CSTB / cathepsin B double-knockout mouse strain presents with a relatively milder degree of neurodegeneration than *Cstb*$^{-/-}$ mice (Houseweart *et al*, 2003). However, this mouse strain still exhibits myoclonus and ataxia, suggesting that cathepsin B does not mediate the pathophysiology immediately downstream of CSTB-deficiency.

Cortical thinning can be detected from 2 months of age onwards, both in the cerebrum and in the cerebellum (Tegelberg *et al.*, 2012) (Fig. 1B). Simultaneously, white matter tracts in the cerebellum and thalamus begin to degenerate (Manninen *et al*, 2014b) (Fig. 1B). By 6 months of age, the *Cstb*$^{-/-}$ cerebellum has shrunk to half of the normal size, and signs of white matter atrophy can be detected in all major axonal tracts of the central nervous system (Manninen *et al.*, 2014b; Tegelberg *et al.*, 2012). In later stages of the disease, neurodegeneration is observed in many other brain areas, highlighting a subset of thalamic nuclei that receive afferents from heavily atrophied regions of the cortex.

Cortical thinning is temporally correlated with a progressive loss of GABA transporter (VGAT) (Buzzi *et al.*, 2012). This observation, together with others indicating that *Cstb*$^{-/-}$ neurons are less responsive to GABA neurotransmitter and release less GABA upon *in vitro* stimulation, add to the hypothesis that the GABAergic system is particularly vulnerable to CSTB-deficiency (Airaksinen & Leino, 1982; Buzzi *et al.*, 2012; Franceschetti *et al*, 2007). This theory suggests that the appearance of myoclonus and seizures is favoured by the state of latent hyperexcitability derived from GABAergic dysfunction.

A few studies have investigated the pathophysiology underlying neurodegeneration in the *Cstb*$^{-/-}$ mouse brain. Proteomics analysis of synaptosomes collected two weeks before the symptomatic onset revealed that 36 proteins located in mitochondria displayed increased abundance in the cerebellum of *Cstb*$^{-/-}$ mice (Gorski *et al*, 2020) (Fig. 1B). Among these proteins, there was a significant enrichment in biological processes including mitochondrial respiration and local protein translation. Accordingly, synaptic mitochondria develop a striking loss of respiratory capacity shortly after onset of myoclonus (Gorski, manuscript in preparation). Besides differences in the mitochondrial proteome, the study reported altered abundance of proteins involved in intracellular transport and protein translation, including upregulation of seven structural proteins of the ribosome (Gorski *et al.*, 2020). Finally, a gene expression array of *Cstb*$^{-/-}$ cerebellar tissue exposed alterations in a discrete set of synapse-related genes at postnatal day seven (Joensuu *et al.*, 2014). In accordance with the GABAergic hypothesis, the authors reported a 75% increase in the mRNA levels of GABAA receptor subtypes α6 and δ in the absence of CSTB. A series of electrophysiological recordings revealed decreased inhibition of Purkinje neurons, the only source of output from the cerebellum (Fig. 1B). The presence

of synapse-related changes in a neurodevelopmental stage at which cerebellar morphogenesis is still ongoing (Butts *et al*, 2014) pinpoints to defects in cerebellar maturation.

Microglia in the *Cstb*$^{-/-}$ mouse brain undergo pathological activation at postnatal day 14 (Tegelberg *et al.*, 2012) (Fig. 1B). The phenotype is anatomically widespread, but particularly striking in brain regions including the cingulate cortex, a subset of posterior thalamic nuclei and the substantia nigra. From P14 to P30, the macrophage-like phenotype of activated *Cstb*$^{-/-}$ microglia shifts from "anti-inflammatory" (M2) to "pro-inflammatory" (M1), concomitant with increased expression of inflammatory markers in the cortex (Okuneva *et al.*, 2015). Moreover, the morphology of activated microglia changes from spherical with many thin processes to elongated with very few but thickened processes (Tegelberg *et al.*, 2012). Interestingly, the latter morphology is similar to that of microglia appearing during aging and in the context of Alzheimer's disease (AD) (Davies *et al*, 2017). Cultured microglia express significantly higher levels of *Cstb* mRNA than neurons and astroglia (Okuneva *et al.*, 2015), and display alterations in cell signalling in the absence of a functional *Cstb* gene (Korber *et al.*, 2016). These observations fuelled the hypothesis that CSTB-deficiency causes neurodegeneration by directly interfering with microglial homeostasis. However, a recent study showed that silencing *Cstb* expression in microglia cultured *ex vivo* does not induce the phagocytic impairment observed in *Cstb*$^{-/-}$ brains (Sierra-Torre *et al*, 2020). Additionally, the latest developments in the field indicate that many common neurodegenerative disorders involve microglial activation and dysfunction in the prodromal stages of the disease (Harry, 2021).

Astrocytes in the *Cstb*$^{-/-}$ mouse brain also undergo pathologic activation (Franceschetti *et al.*, 2007; Manninen *et al.*, 2014b; Shannon *et al*, 2002; Tegelberg *et al.*, 2012) (Fig. 1B). The onset of the symptoms coincides with the first signs of astrocytosis, evidenced by a dramatic increase in the expression of glial fibrillary acidic protein (GFAP) (Tegelberg *et al.*, 2012). Similar to the other phenotypes, the presence of reactive astrogliosis is progressive and regionally heterogeneous (Tegelberg *et al.*, 2012).

Older mice bearing the *Cstb* null mutation in only one allele (*Cstb*$^{+/-}$) presented a unique phenotype involving poor motor performance, handling-induced seizures and mild but consistent signs of cerebellar neurodegeneration (Kaasik *et al*, 2007) (Fig. 1A). The abundance of CSTB protein in cerebella of *Cstb*$^{+/-}$ mice was only 50% lower than that of wild-type littermates, indicating that cerebellar homeostasis is critically dependent on CSTB function.

The phenotypic traits of *Cstb*$^{-/-}$ mice in other organs comprise alterations in the composition of serum including decreased levels of serotonin (Arbatova *et al*, 2005) and increased levels of pro-inflammatory chemokines CXCL1, CXCL10, CXCL13 and TNFα (Okuneva *et al.*, 2016). *Cstb*$^{-/-}$ mice also present with increased bone mineral density correlated with a reduction in the activity and number of mature osteoclasts (Manninen *et al*, 2015).

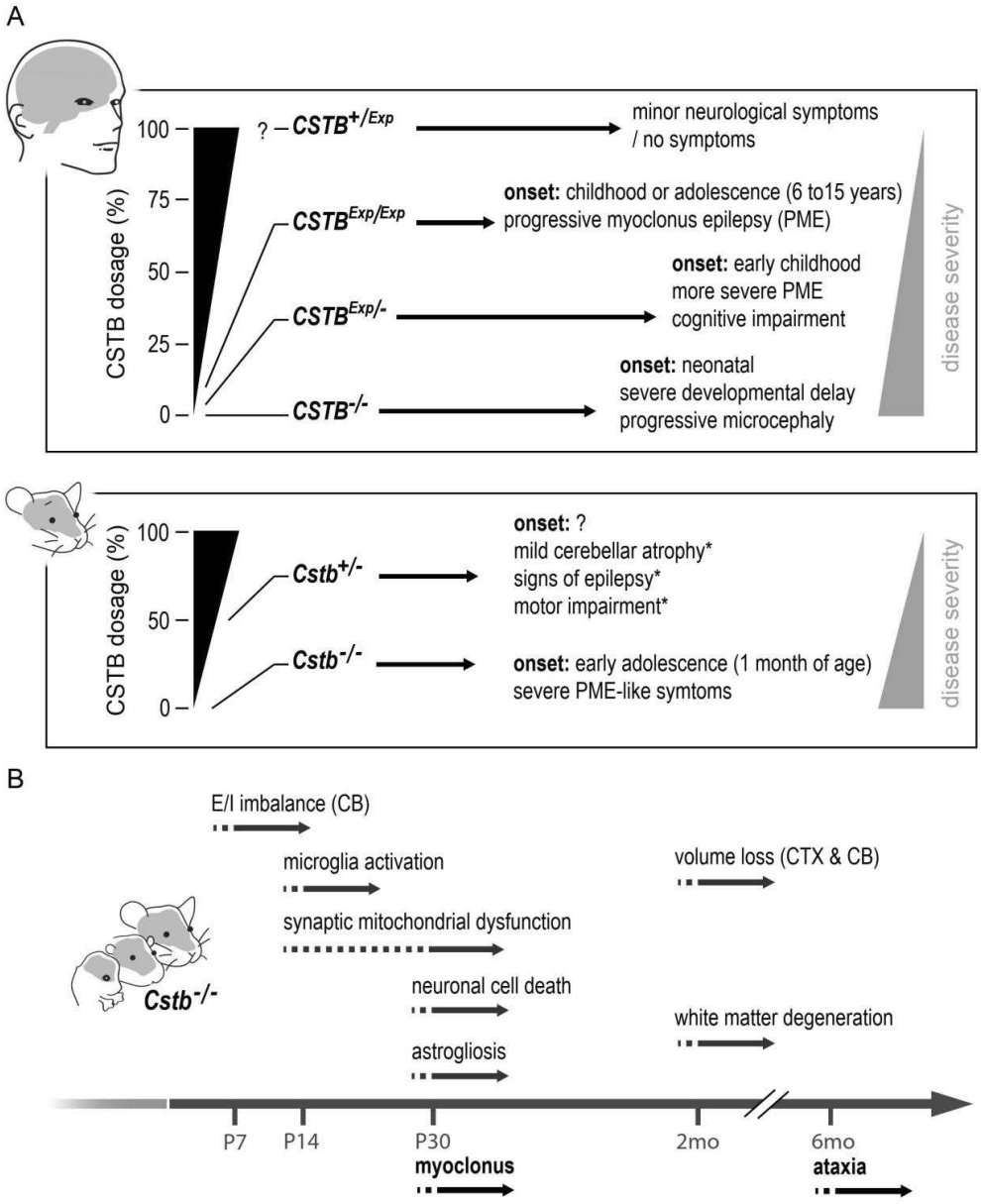


Figure 1. Phenotypes associated with CSTB-deficiency in humans and mice.
A: The degree of CSTB loss directly correlates with increased disease severity and penetrance. *CSTB*$^{+/Exp}$: promoter expansion mutation in combination with a normal allele; *CSTB*$^{Exp/Exp}$: homozygosity for the promoter expansion mutation; *CSTB*$^{Exp/-}$: promoter expansion mutation in combination with a null allele; *CSTB*$^{-/-}$: biallelic null mutations; *Cstb*$^{+/-}$: monoallelic *Cstb* deletion, * phenotypes reported in old mice (17 months of age); *Cstb*$^{-/-}$*:* biallelic *Cstb* deletion.
B: Timeline of most prominent phenotypic changes occurring in the *Cstb*$^{-/-}$ mouse brain. E/I imbalance: excitation/inhibition imbalance, CB: cerebellum, CTX: cortex.

2.2. Nuclear cathepsin L pathways

Cysteine cathepsins are an abundant group of enzymes that primarily mediate end-stage protein breakdown in the lysosomal compartment (Turk *et al*, 2012). However, certain members of the cathepsin family carry out specialized functions in extra-lysosomal locations, highlighting the activation or inactivation of proteins involved in cell signalling (Yadati *et al*, 2020). The archetypic example of a moonlighting protease is cathepsin L, whose non-canonical activities already outnumber those taking place within the endo-lysosomal system (Duncan *et al*, 2008; Goulet *et al*, 2004; Islam *et al*, 2022; Yasothornsrikul *et al*, 2003).

In murine tissues, cathepsin L is typically translated as a pre-pro-enzyme containing an N-terminal signal peptide, processed to a pro-cathepsin L of approximately 39 kDa and stored in lysosomes (Ishidoh *et al*, 1998). Once there, it is further cleaved into two catalytically active forms: single-chain cathepsin L, with a molecular mass of approximately 30 kDa, and double-chain cathepsin L, consisting on a 5-kDa light chain and a 25-kDa heavy chain connected through a disulphide bond (Fig. 2A). The translation of the cathepsin L mRNA can also be initiated at downstream AUG codons (Goulet *et al.*, 2004) (Fig. 2A). The use of these alternative in-frame start codons results in the production of shorter pro-cathepsin L isoforms that are devoid of the signal peptide. These cathepsin L molecules avoid entry into the ER lumen, and shuttle to the nucleus using importin β1 and the putative nuclear localization sequence of other proteins, including transcription factor Snail (Burton *et al*, 2017b) (Fig. 2B).

In the nucleus, cathepsin L has been shown to cleave an N-terminal fragment of a subset of histones H3 and H2A in the context of cell state transitions (Coradin *et al*, 2021; Duarte *et al*, 2014; Duncan *et al.*, 2008; Ferrari *et al*, 2021) (Fig. BA). The proteolytic removal of histone tails —particularly that of histone H3— alters the epigenetic status of the chromatin and ultimately elicits gene expression reprogramming (Cheung *et al.*, 2021). The biological implications of histone tail cleavage are reviewed in detail in section 2.3.2.

Cathepsin L is also responsible for the conversion of homeodomain transcription factor CUX1 p200 into its shorter form: CUX1 p110 (Goulet *et al.*, 2004) (Fig. 2B). This proteolytic event seems to be coordinated with the cell cycle, and results in a shortening of the G1/S transition by the recruitment of activator E2F factors to the promoter region of cell cycle-related genes (Goulet *et al.*, 2004; Truscott *et al*, 2008). The cathepsin L/CUX1 axis also promotes epithelial-mesenchymal transition (EMT) by a mechanism involving transcriptional repression of the epithelial cadherin and upregulation of Snail, which in turn induces the expression EMT-related genes (Fei *et al*, 2018). Interestingly, the mere presence of Snail is sufficient to induce cytoplasmic-to-nuclear cathepsin L shuttling. The feed-forward regulatory loop between nuclear cathepsin L and Snail is thought to confer invasive properties to gliomas, prostate cancers and mesenchymal breast cancers (Burton *et al*, 2017a; Fei *et al.*, 2018). CUX1 also supports metabolic reprogramming in neuroblastomas by stimulating the transcription of genes involved in aerobic glycolysis (Li *et al*, 2019). *Circ-CUX1*, a circular non-coding RNA originated from the alternative splicing of the *CUX1* mRNA, exerts most of the gene regulatory functions downstream of this pathway. Interestingly, glycolysis-related gene expression was abolished in cells treated with a pharmacological cysteine protease inhibitor,

suggesting that cathepsin L-dependent CUX1 protein cleavage is necessary for the activation of this transcriptional network (Li *et al.*, 2019).

Due to its high abundance and fast DNA-binding kinetics, the intact form of CUX1 does not fully qualify as a classic transcription factor (Moon *et al*, 2000). Instead, CUX1 p200 promotes oxidative DNA damage repair by directly stimulating purine base excision with its CUT domains (Ramdzan *et al*, 2014). CUX1-dependent DNA repair prevents tumour formation, but paradoxically allows cancer cells to sustain a hyperactive metabolic state while avoiding DNA-stress-induced cellular senescence (Ramdzan *et al*, 2015). Accordingly, both loss- and gain-of-function mutations in *CUX1* promote cancer progression, and CUX1 is considered both a tumour suppressor and an oncogene (Liu *et al*, 2020; Ramdzan & Nepveu, 2014). Recently, CUX1 p200 was shown to drive a vascular senescence program via transcriptional activation of the *CDKN2* loci, encoding the cyclin-dependent kinase inhibitors (CDKIs) $p16^{INK4a}$, $p14^{ARF}$ and $p15^{INK4b}$ (Jiang *et al*, 2022). Finally, the expression of CUX1 marks the pyramidal neurons that populate the upper layers of the mammalian cortex (Cubelos *et al*, 2010). There, it modulates the transcription of Kv1 voltage-dependent potassium channels, leading to cell-type-specific firing modes and connectivity patters (Rodríguez-Tornos *et al*, 2016). CUX1 is also expressed in cerebellar granule neuron precursors and migratory granule neurons, but its function in cerebellar development remains elusive (Topka *et al*, 2014).

Cathepsin L can also cleave lamin B1, a major component of the nuclear lamina, generating a distinctive C-terminal fragment of 21 kDa (Islam *et al.*, 2022) (Fig. 2B). This proteolytic event is at least partially responsible for the neuronal laminopathy observed both in murine models of amyloid-β toxicity and in hippocampal tissue samples from AD patients. Interestingly, neuroblastoma cells treated with amyloid-β displayed elevated cathepsin L activity and protein levels, leading to lamin B1 damage but also histone H3 tail proteolysis. In mouse embryonic fibroblasts (MEFs), overexpressing cathepsin L alone was sufficient to induce a nuclear lamina damage. Caspase 6 can also cleave lamin B1 in the context of AD, rendering a 46-kDa fragment (Islam *et al*, 2019). Interestingly, cathepsin L is partially accountable for the proteolytic activation of caspase 6 downstream of amyloid-β toxicity.

Finally, cathepsin L regulates the stability of p53-binding protein 1 (53BP1), one of the principal mediators of DNA double strand break signalling and repair (Gonzalez-Suarez *et al*, 2011; Grotsky *et al*, 2013) (Fig. 2B). Overexpression of cathepsin L in primary fibroblasts triggered 53BP1 degradation and accumulation of DNA aberrations. Remarkably, treatment with vitamin D rescued the phenotype without altering cathepsin L expression. The authors suggest that this effect is probably mediated by cystatin D, a cysteine cathepsin inhibitor that is transcriptionally upregulated in colorectal carcinoma cells treated with vitamin D. In MEFs, acute depletion of lamin A/C triggered cathepsin L upregulation and 53BP1 loss (Gonzalez-Suarez *et al.*, 2011). However, other studies show that the degradation of DNA damage repair machinery (including 53BP1) that occurs downstream of lamin A/C-deficiency is mediated by ubiquitin/proteasome pathways (Gonzalez-Suarez *et al*, 2009; Johnson *et al*, 2004). Therefore, it remains unclear whether nuclear cathepsin L regulates 53BP1 stability via direct interaction or through other, more complex mechanisms.

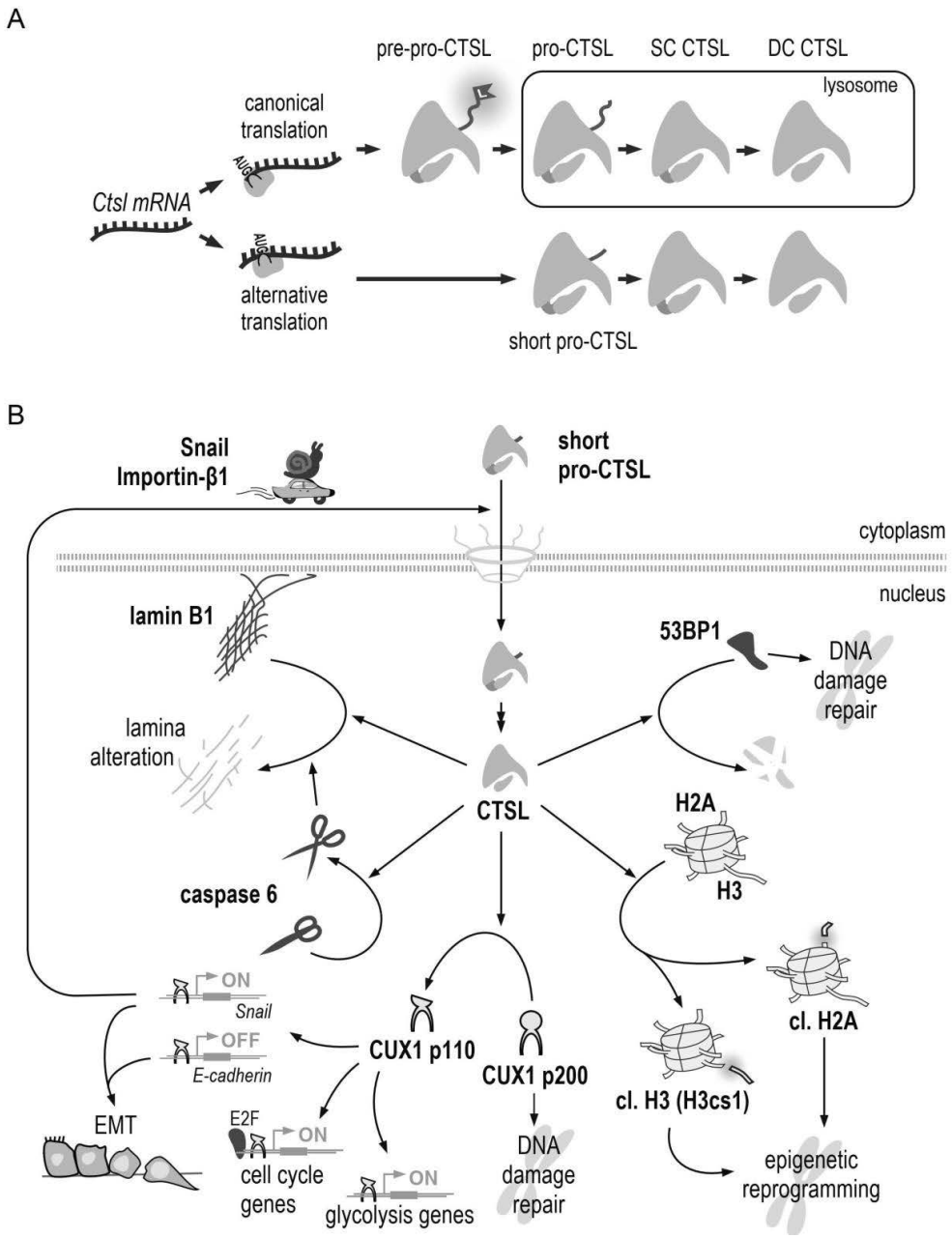


Figure 2. Synthesis and molecular biology of nuclear cathepsin L.
A: Synthesis and processing stages of cathepsin L depending on start-codon usage during protein translation. SC CTSL: single-chain cathepsin L, DC CTSL: double-chain cathepsin L.
B: Schematic representation of the proteolytic functions carried out by cathepsin L within the cell nucleus. EMT: epithelial-mesenchymal transition, cl. H2A: cleaved histone H2A, cl. H3: cleaved histone H3.

2.3. Epigenetic regulation

2.3.1. Principles of epigenetic regulation

The word epigenetics was coined by the British developmental biologist Conrad Waddington in 1956 (Waddington, 1956). At the time, there was a general disagreement on whether the phenotypes of adult organisms were pre-encoded in the zygote or created from sequential interactions between the cells of the embryo. Waddington reconciled these two hypotheses by understanding them as complementary processes, with the latter reflecting the dynamic nature of gene expression and the former representing the permanence of the gene (Van Speybroeck, 2002; Waddington, 1942). By combining both ideas, he came up with the term *epigenetics*, which he defined as the branch of science that addresses the interplay between genes and their products leading to the emergence of the phenotype (Waddington, 1956).

In the following decades, the discovery of some of the mechanisms by which genes interact with their products led to the diversification of the term epigenetics. Currently, molecular and cellular biologists define *epigenetics* as the study of changes in DNA function that do not arise from changes in DNA sequence (Deans & Maggert, 2015; Holliday, 1994). In turn, the *epigenome* is the genome-wide collection of all the epigenetic traits of a given cell in a given moment. Epigenetic modifications are usually preserved following cell division, granting cells with a source of memory that transcends the genetic code (Allis & Jenuwein, 2016). Therefore, some authors choose to define epigenetics as all forms of non-genetic biological inheritance (Deans & Maggert, 2015; Holliday, 1994).

Epigenetic mechanisms influence gene function by altering the structure of the chromatin, the repeating polymer formed by the wrapping of the DNA around histone proteins. The structural unit of the chromatin is the nucleosome, which is composed of 147 bp of DNA coiled approximately 1,65 times around a histone octamer. Histone octamers are in turn composed of two copies of each of the core histones, namely histone H2A, H2B, H3 and H4 (Allis *et al.*, 2007). Chromatin is further complexed with linker H1 and its variants, which associate directly with the DNA around the entry and exit point of nucleosome (Thoma *et al*, 1979). Nucleosomes have a dual impact on chromatin structure and function: On the one hand, they are highly-optimized DNA packaging devices, and thus offer a solution to the problem that represents fitting the nearly two meter-long eukaryotic genome into a volume of a few cubic micrometres (Allis *et al.*, 2007; Jorgensen *et al*, 2007). On the other hand, nucleosomal histones are heavily modified post-translationally, and therefore act as locus-specific scaffolds of epigenetic information. This critical feature endows eukaryotic cells with the ability to define, alter, maintain and inherit gene-specific activity profiles.

Apart from their role in DNA packaging and epigenetic regulation, histones have evolved to fulfil other cellular functions. For instance, H3-H4 tetramers bind oxidized copper and reduce it to Cu^{1+} in order to sustain intracellular copper bioavailability and copper-dependent mitochondrial respiration (Attar *et al*, 2020). Furthermore, the H2A-H2B dimer was recently identified as an integral part of the catalytic core of human telomerase, suggesting a crucial role for nucleosomal histones in telomere physiology (Ghanim *et al*, 2021).

Histone post-translational modifications (PTMs) are a defining trait of eukaryotic genomes (Brunk & Martin, 2019). They are mainly deposited on histone tails, which are flexible and highly basic amino acid stretches that protrude from the nucleosome. They are found at the N-terminus of all four nucleosomal histones as well as at the C-terminus of histone H2A. Histone PTMs can have a direct impact on inter-nucleosomal interactions, which in turn affect the overall structure of the chromatin. Besides, histone PTMs recruit specific factors and protein complexes that mediate further modifications to the local chromatin environment (Bannister & Kouzarides, 2011). The dynamic deposition, recognition and erasure of histone PTMs is carried out by a diverse collection of proteins known as "writers, readers and erasers", respectively. These enzymes are usually found in large multi-protein complexes that have evolved to interpret the multiple layers of information that constitute the "histone code". In 2018, researchers had identified approximately 100 "readers", 50 "writers" and over a dozen "erasers" (Stillman, 2018). However, several new modalities of histone modification have been recently identified (Lepack *et al*, 2020; Liu *et al*, 2019; Zhang *et al*, 2019), and therefore the number of epigenetic regulatory enzymes will probably continue to grow in the years to come.

Histone PTMs include acetylation, phosphorylation, lysine-methylation, arginine-methylation, deamination (also known as citrullination), β-N-acetylglucosamination, ADP-ribosylation, ubiquitination, sumoylation, proline-isomerization, butyrylation, succinylation, propionylation, malonylation, crotonylation, serotonylation, dopaminylation, lactylation, benzoylation and tail proteolysis (Huang *et al*, 2018; Ishiguro *et al*, 2018; Kebede *et al*, 2017; Lepack *et al.*, 2020; Liu *et al.*, 2019; Stillman, 2018; Tan *et al*, 2011; Wang *et al*, 2017; Zhang *et al.*, 2019). Beyond this extraordinary diversity, the same modification can have different downstream effects depending on amino acid position, genomic context and co-occurrence with other PTMs (Allis *et al.*, 2007).

2.3.2. Histone tail proteolysis

Conventional histone PTMs are reversible in nature. Histone tail proteolysis is special in that it permanently shortens the polypeptide backbone, and thus can only be removed by histone eviction or turnover (Zhou *et al*, 2014). Moreover, when histone tails are cleaved, critical PTMs might also be lost (Ferrari *et al.*, 2021; Paternoster *et al*, 2021b) (Fig. 3A). Histone tail cleavage is evolutionarily conserved in all eukaryotic organisms (Cheung *et al.*, 2021; Santos-Rosa *et al*, 2009). It is mediated by context-dependent proteases —including aspartic (Khalkhali-Ellis *et al*, 2014), cysteine (Duncan *et al.*, 2008), serine (Cheung *et al.*, 2021) and metalloproteases (Rice *et al*, 2021)— and it can affect all four nucleosomal histones as well as linker histone H1 (Dhaenens *et al*, 2015). Despite the increasingly evident role of this mechanism in regulating common biological processes, we are still lacking a comprehensive understanding of its significance and mechanistic basis (Kragesteen & Amit, 2021). Recently, the proteolytic modification of histone tails has caught the attention of the epigenetics community (Cheung *et al.*, 2021; Marruecos *et al*, 2021). The far-reaching implications of their preliminary findings suggest that it will shortly become an intense focus of scientific research (Dhaenens, 2021; Kragesteen & Amit, 2021).

2.3.2.1. Origins of the research field

The first report of proteolytic processing of histone proteins appeared in the 1964, ten years before the discovery of the nucleosome (Kornberg, 1974; Reid & Cole, 1964). While investigating histone

heterogeneity in the calf thymus, Professor David Cole from the University of California stumbled upon a histone variant that presented a complex band pattern upon gel electrophoresis. After excluding potential experimental pitfalls leading to sample contamination or degradation, they concluded that the bands corresponded to shorter histone species originated *in vivo*. In 1970 and 1974, two studies confirmed the existence of at least one chromatin-bound protease that sensitized histones to proteolytic processing in acid extracts from the rat liver and calf thymus, respectively (Bartley & Chalkley, 1970; Chong *et al*, 1974). Shortly after, the protease activity of the rat liver was shown to correspond to two different serine proteases that, to this day, remain unidentified (Tsurugi & Ogata, 1982).

Evidence that histone tail proteolysis is a physiologically regulated phenomenon arrived in 1980, when a group interested in the molecular mechanisms driving nuclear dimorphism in ciliates detected N-terminal truncation of histone H3 in association with the life cycle of *Tetrahymena*, a free-living protozoan (Allis *et al*, 1980). Using pulse-chase experiments with radioactive amino acids, they demonstrated that the heavier histone H3 was a precursor of the lighter one, which lacked six amino acids from its N-terminus. Importantly, the cleavage took place *in vivo*, following incorporation to the chromatin and in synchrony with the cell cycle (Allis *et al.*, 1980).

The list of chromatin-bound proteases increased rapidly in the following years. In 1986, a group in Japan identified three different proteolytic activities associated with the chromatin of *Saccharomyces cerevisiae* (Motizuki *et al*, 1986). One of them was an aspartyl protease, whereas the other two were serine proteases. The size and isoelectric point of the latter enzymes matched that of the serine proteases isolated from rat liver. Based on these observations, the authors argued that the proteolytic regulation of chromatin-bound proteins was probably present in all eukaryotic organisms.

2.3.2.2. Lessons from *in vitro* studies

The notion that histones, the packaging devices of the genome, are subject to proteolytic processing *in vivo* raised interest among structural biologists (Dhaenens *et al.*, 2015). This led to the publication of multiple studies addressing the structural implications of histone truncation *in vitro*. From this research, we learned that histone-tail cleavage has a direct impact on both nucleosome conformation and higher-order chromatin structure: Tail-less nucleosomes complexed with free DNA did not arrange into a 30 nanometer chromatin fiber, indicating that the positively charged histone tails contribute to DNA folding by neutralizing the negative charge of the DNA strand (Allan *et al*, 1982). More recently, the N-terminal domains of histones H2A and H2B were shown to be crucial mediators of intranucleosome stability, whereas those of histones H3 were mostly implicated in the establishment of interactions between nucleosomes (Bertin et al, 2007a; Bertin et al, 2007b). Furthermore, removing the N-terminal tail of histone H3 —but not that of histone H4— led to a more open nucleosome conformation with a wider range of motion during chromatin breathing (Nurse et al, 2013) (Fig. 3A). Nucleosomes devoid of the N-terminal tail of histone H3 displayed less and weaker nucleosome-DNA contacts, and therefore were significantly less stable than intact nucleosomes (Iwasaki et al, 2018) (Fig. 3A).

Mass spectrometry (MS) analyses of chromatin digested with common proteases including cathepsin B and cathepsin L showed that the N-terminus of all core histones is susceptible to proteolytic

cleavage *in vitro*, with that of histone H3 being the most sensitive to enzymatic digestion (Bohm *et al*, 1981; Dumuis-Kervabon *et al*, 1986; Harvima *et al*, 1988; Weintraub & Van Lente, 1974). This is probably due to the high exposure of this histone domain within the chromatin strand. The fact that histone tails are easily degraded *in vitro* should be acknowledged and properly addressed by scientists studying endogenous histone tail cleavage events (Dhaenens *et al.*, 2015). Close attention should be paid to histone proteolysis occurring during sample collection, storage and handling.

2.3.2.3. Cathepsin L-mediated histone tail proteolysis

In 2008, Duncan and colleagues described a histone tail cleavage event for the first time in a defined biological context (Duncan *et al.*, 2008). While investigating histone PTM dynamics of murine embryonic stem cells (mESC), they identified a lighter isoform of histone H3 that appeared specifically upon induction of differentiation and corresponded to canonical histone H3.1 and/or H3.2 truncated between alanine 21 and threonine 22 (histone H3 cleavage site 1, H3cs1). They also detected a number of secondary cleavage products that were mapped within the six amino acids distal to the main cleavage site. The truncated histones and N-terminal peptides were enriched in a specific set of PTMs characteristic of both active and repressive chromatin states. This observation suggested that signalling cross-talk exists between histone tail cleavage and other epigenetic regulatory pathways. Using chromatin-bound protein fractionation followed by MS, they detected peptides belonging to the cysteine protease cathepsin L specifically in fractions presenting histone H3 tail proteolysis. They also showed that cathepsin L generated H3cs1 *in vitro* and that the cleavage was abrogated following acute knockdown of cathepsin L. Three years later, another group confirmed Duncan's findings by solving the crystal structure of cathepsin L in complex with histone H3 (Adams-Cioaba *et al*, 2011). To date, cathepsin L remains the best characterized histone tail protease, insomuch that it is used as a tool to isolate the N-terminal tail of histone H3 *in vitro* for in mass spectrometry studies (Papanastasiou *et al*, 2019).

During mESC differentiation, cathepsin L also cleaves the N-terminus of a small fraction of histone H2A (Coradin *et al.*, 2021). In this case, the truncated histone lacks the first 23 amino acids and represents up to 1% of the total H2A pool, much less than that of cleaved histone H3 in the same biological context. Interestingly, the authors detected several cathepsin L-independent cleavage sites, suggesting that other enzymes participate in the proteolytic event. Suppressing H2A cleavage led to defective removal of H2A acetylation at pluripotency-related gene promoters, which in turn exhibited elevated transcription. In agreement with *in vitro* studies (Bertin *et al*, 2007; Iwasaki *et al*, 2013), nucleosomes containing cleaved H2A were more unstable than intact ones. Moreover, these nucleosomes contained histone H3 molecules enriched in activity-related PTMs and mostly devoid of repressive marks (Coradin *et al.*, 2021). Taken together, the authors concluded that H2A cleavage by cathepsin L serves as a mechanism to both repress pluripotency and promote cell-lineage commitment through the modulation of gene expression and nucleosome stability. Whether cleaved H2A co-exists with H3cs1 in the same nucleosome is not known.

Cathepsin L also cleaves the N-terminus of histone H3 during intestinal differentiation (Ferrari *et al.*, 2021). In this context, histone tail proteolysis affects other nucleosomal histones, namely H2B and H4, and is not only catalysed by cathepsin L but also by serine proteases of the trypsin family. Interestingly, the overall levels of common histone PTMs located proximal to the cleavage site are

severely reduced during intestinal differentiation. These include marks associated with both actively-transcribed and repressed chromatin domains such as H3K4me3 (histone H3 trimethylated at lysine 4) and H3K9me3 (histone H3 trimethylated at lysine 9), respectively. On the other hand, both the levels and distribution of other common PTMs located distal to the cleavage site remain largely preserved. Based on their observations, the authors interpret that H3 tail cleavage might help reduce the local concentration of certain H3 PTMs while preserving their cell type-specific distribution.

Besides cellular differentiation, cathepsin L facilitates histone tail cleavage in the context of cellular senescence (Duarte *et al.*, 2014). Senescent fibroblasts and melanocytes accumulate H3cs1, with its levels being much higher than those observed during mESC differentiation. Accordingly, senescent melanocytes of benign human nevi (moles) display elevated levels of H3cs1, confirming the relevance of this mechanism *in vivo*. In senescent cells, the preferentially cleaved form of histone H3 is H3.3, a replication-independent histone variant whose chromatin incorporation is mechanistically uncoupled from that of canonical H3.1 and H3.2. Suppressing H3.3cs1 through the inhibition of human cathepsin L (CTSL1) impairs the formation of senescence-associated heterochromatin foci (SAHF), whereas the ectopic expression of H3.3cs1 triggers a characteristic cellular senescence program. Overexpressing intact H3.3 was also sufficient to induce some of the phenotypes attributed to H3.3cs1, suggesting that the replication-independent incorporation of H3 is, on itself, a driver of cellular senescence. The authors did not uncover the genomic distribution of H3.3cs1, but provided evidence suggesting that H3 tail cleavage promotes cell cycle arrest during senescence by permanently removing H3K4me3 from E2F-target genes (Duarte *et al.*, 2014).

2.3.2.4. Histone H3 tail proteolysis

The limited proteolysis of the N-terminal tail of histone H3 (hereinafter referred to as H3 cleavage) has been reported more frequently and in greater detail than that of any other histone protein (Cheung *et al.*, 2021; Dhaenens *et al.*, 2015).

In 2009, Santos-Rosa and colleagues showed that yeast cells undergo H3 cleavage during state transitions, particularly upon induction of sporulation or following entry into a stationary growth phase (Santos-Rosa *et al.*, 2009). In accordance with the cleavage event described by Elisabeth Duncan one year before, the main cleavage site was mapped between alanine 21 and threonine 22 (Duncan *et al.*, 2008). However, in yeast cells H3cs1 is not catalysed by cathepsin L, but by a serine protease which is most likely cervesin (Prb1) (Xue *et al*, 2014). Santos-Rosa and colleagues were the first group to perform chromatin immunoprecipitation of tail-less histones, in this case using mutant yeast strains overexpressing a myc-tagged histone H3 (Santos-Rosa *et al.*, 2009). They showed that H3cs1 marks gene promoters of transcriptionally silent genes before their transition to an active state. Moreover, they found evidence strongly suggesting that H3 cleavage triggers nucleosome eviction, a key step of gene induction in yeasts. Accordingly, abrogating H3 cleavage led to deficient sporulation-related gene activation. The authors argue that H3 cleavage is an ancestral chromatin regulatory mechanism that probably serves as a converging point for multiple signalling pathways leading to gene expression reprogramming (Santos-Rosa *et al.*, 2009).

H3 cleavage has also been described in *Plasmodium falciparum*, a protozoan parasite responsible for the most severe form of human malaria (Herrera-Solorio *et al*, 2019). The cleavage is mediated by a

Cathepsin C-like cysteine protease, involves removal of the first 21 amino acid residues of histone H3 and is strictly associated with the replicative stages of the parasite´s life cycle. Ectopically expressed forms of the cleaved histone integrated into chromatin specifically at the promoter region of six key genes involved in DNA replication. This observation hints at the existence of specialized cellular machinery mediating chromatin incorporation of truncated histones (Herrera-Solorio *et al.*, 2019).

In mammalian organisms, H3 cleavage has diversified and specialized enormously to fit tissue-specific needs. This is reflected by the large repertoire of histone tail proteases, upstream signals and downstream effects identified in different mammalian cell types. For example, cathepsin D proteolytically processes histone H3 between lysine 23 and alanine 24 during post-lactational mammary gland involution (Khalkhali-Ellis *et al.*, 2014). Cathepsin D is an intracellular aspartyl protease that, in certain biological contexts, escapes its constitutive lysosome targeting and is secreted into the extracellular matrix (Vetvicka *et al*, 1993), where it is reuptaken by neighbouring cells (Laurent-Matha *et al*, 1998). Following endocytosis, cathepsin D disperses within the cytoplasm. This is true except if the source of cathepsin D is the involuting mammary gland. In this context, cathepsin D is tyrosine-nitrosylated before secretion, a modification that somehow allows it to translocate to the cell nucleus (Khalkhali-Ellis *et al.*, 2014). Mammary epithelial cells treated with nitrosylated cathepsin D show signs of entosis, a hallmark of tissue involution. Together, their findings support the view that cathepsin D-mediated H3 cleavage functions as a paracrine signalling mechanism that stimulates tissue remodelling in the mammary gland (Khalkhali-Ellis *et al.*, 2014).

The evidence indicating that H3 cleavage can function as a cell-to-cell signalling mechanism is not unique to the mammary gland. One of the most peculiar H3 cleavage events described to date is carried out by mast cells, an immune cell type that mediates inflammatory processes including allergy, host defence and wound healing. Mast cells interact with other cell types through the targeted secretion of specific molecules stored in their cytoplasmic granules. One such molecule is tryptase, a tetrameric serine protease. Tryptase is also found in the nucleus of mast cells, where it is known to process the N-terminus of histones H3 and H2B to maintain the cellular identity (Melo *et al*, 2014; Melo *et al*, 2017). However, when presented with a melanoma, mast cells release extracellular vesicles packed with tryptase (Rabelo Melo *et al*, 2019). Inside melanoma cells, tryptase re-localizes to the cell nucleus, where it cleaves the N-terminus of histone H3. It also degrades lamin B1 and ribonucleoprotein A2/B1, two crucial players in nuclear architecture and RNA stabilization, respectively. This event is followed by the transcriptional repression of oncogenes and oncogenic non-coding RNAs, subsequently leading to cell cycle arrest and melanoma cell inactivation.

To the best of this author's knowledge, only two studies in mammalian cells have reported the genomic distribution of a cleaved H3 product without relying on artificial expression of recombinant tagged histones (Cheung *et al.*, 2021; Kim *et al*, 2016).

In 2016, Kim and colleagues showed that MMP-9 proteolytically processes histone H3 during the last steps of osteoclast differentiation (Kim *et al.*, 2016). So far, metalloprotease-mediated H3 cleavage has been reported in four different biological contexts, namely osteoclastogenesis (Kim *et al.*, 2016; Kim *et al*, 2018), melanomagenesis (Shin *et al*, 2022), myogenesis (Rice *et al.*, 2021) and

ribosomal RNA transcription (Ali *et al*, 2021). The two former events are mediated by MMP-9 and are strictly dependent on H3K18 acetylation, whereas the two latter are catalysed by MMP-2 and seem to be mechanistically independent from one another. In all four cases, the main cleavage site is located between lysine 18 and glutamine 19. In the absence of a suitable antibody against this cleavage site, Kim and colleagues developed a ChIP-seq-based method to study the genome-wide localization of truncated H3 (Kim *et al.*, 2016). Briefly, fragmented chromatin is treated with acetic anhydride to induce complete acetylation of all lysine residues *in vitro*. Then, chromatin is probed with an antibody against H3K14ac, which detects all histone H3 molecules that preserve the lysine residue at position 14. Finally, ChIP-seq is performed in parallel using an antibody against the globular domain of histone H3. Subtraction of the H3K14ac output signal from that of total H3 exposes the areas where histone H3 has undergone proteolytic cleavage. In differentiating osteoclasts, truncated H3 species occupy gene promoters and/or coding regions of over 1200 genes. Approximately half of these genes exhibited altered expression levels in the absence of MMP-9. Out of these, approximately 80% were downregulated.

Recently, Kim and colleagues further showed that tetracycline analogues, a group of compounds that was previously shown to block bone reabsorption *in vivo* (Nagasawa *et al*, 2011), function as specific metalloprotease inhibitors (Kim *et al*, 2019). The authors argue that targeting the MMP-9/H3 cleavage osteoclast differentiation axis might be an effective therapeutic strategy for osteoporosis.

More recently, Cheung and colleagues showed that three serine proteases involved in pathogen digestion —neutrophil elastase (ELANE), cathepsin G and proteinase 3— proteolytically process the N-terminus of H3 in during monocyte to macrophage differentiation (Cheung *et al.*, 2021). The authors of this study performed ChIP-Seq to map the genome wide distribution of the main cleavage product (H3cs1) and the main histone protease (ELANE) of primary monocytes. They combined it with ATAC-Seq and RNA-Seq to obtain a readout of chromatin accessibility and gene expression, respectively. This remarkable effort allowed them to describe in unprecedented detail the biological significance of a histone cleavage event. They found that, while H3cs1 is enriched at specific DNA loci, it is also found at low levels across the genome. The widespread localization of the protease ELANE does not reflect the local concentration of H3cs1, suggesting that mechanisms other than protease-chromatin interaction dictate H3cs1 localization. The authors argue that the chromatin-associated serine protease inhibitor MNEI (Bird *et al*, 2001) could be a key factor regulating this process. H3cs1 is strongly enriched in genic over intergenic sequences, particularly in promoters and untranslated regions and at the transcription start site of actively transcribed genes. In almost 80% of the cases, H3cs1 peaks are surrounded by accessible chromatin. Upon H3cs1 depletion, these genes become either much more accessible or inaccessible, and undergo modest but consistent changes in gene expression. Based on these observations, the authors concluded that H3 cleavage probably primes the chromatin for transcriptional reprogramming during cellular identity transitions (Fig. 3B).

Importantly, the monocyte levels of H3cs1 are markedly reduced in patients of systemic juvenile idiopathic arthritis, an autoimmune disease involving abnormal macrophage activation (Cheung *et al.*, 2021). Reduced levels of H3cs1 are also detected in an epigenetically distinct cell population within paediatric pontine gliomas (Harpaz *et al*, 2022). Additionally, the transcriptional program driving melanoma cell growth is implemented via MMP-9-mediated H3 cleavage (Shin *et al.*, 2022).

Histone H3 in hepatocarcinoma cell lines exhibits a different cleavage signature than healthy hepatocytes, involving reduced variety of cleavage sites and preferential proteolysis of canonical histones over H3.3 (Tvardovskiy *et al*, 2015). Another study reported lower levels of cleaved histone H3 in cervical cancer cells than in healthy donor tissue (Sandoval-Basilio *et al*, 2016). Finally, H3 cleavage is upregulated in mice with loss-of-function mutations in *BRD1*, encoding a reader of acetylated histones linked with schizophrenia and bipolar disorder (Paternoster *et al.*, 2021b). Combined, these findings indicate that alterations in H3 cleavage are a common trait of a diverse collection of human disorders.

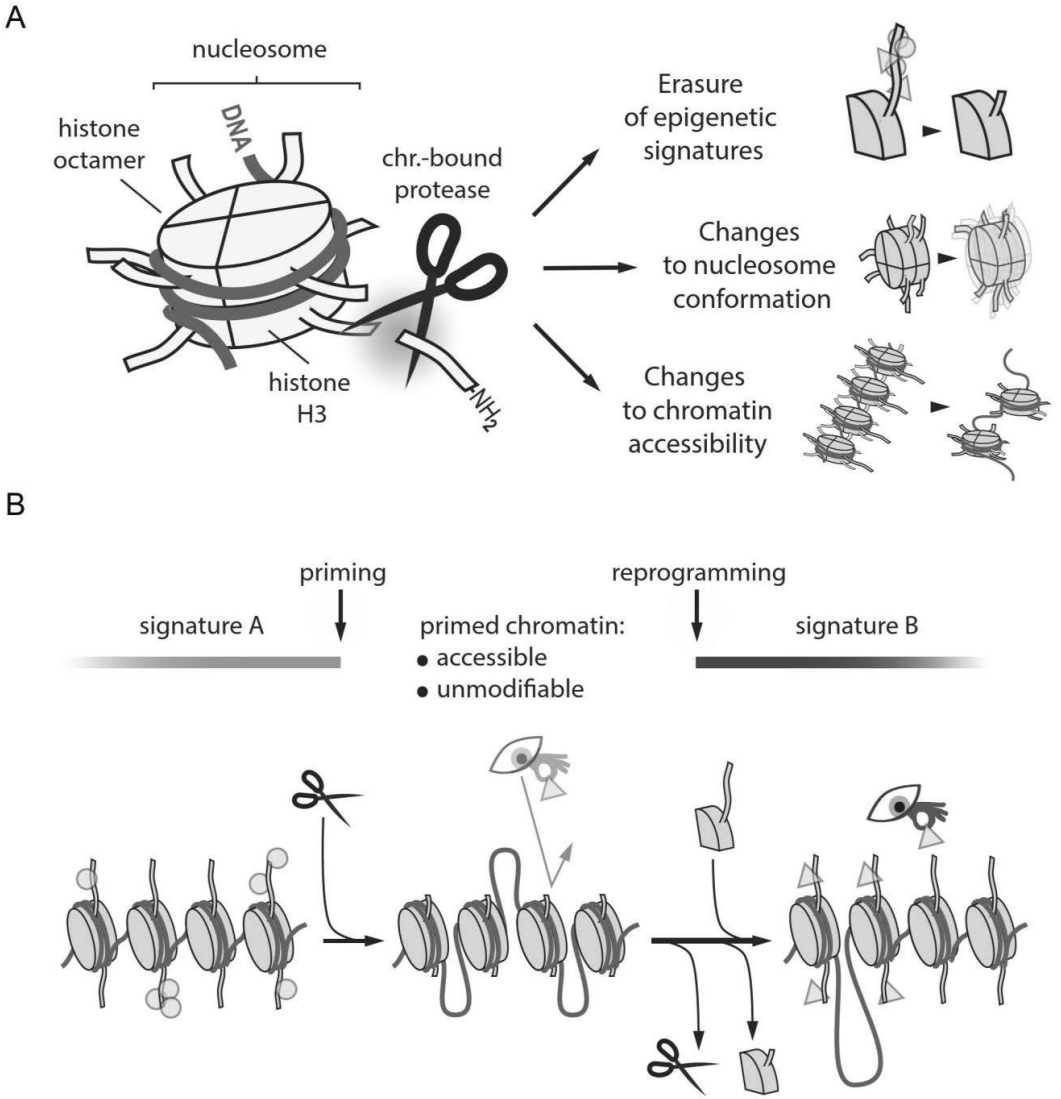


Figure 3. Biochemical and epigenetic regulatory effects of histone H3 N-terminal truncation.
A: The proteolytic modification of histone H3 results in erasure of epigenetic signatures deposited on the evicted N-terminal peptide. It also alters the conformation and stability of mononucleosomes, reducing nucleosome-DNA contacts and promoting an open chromatin conformation with a wider DNA breathing motion. Chr.: chromatin. **B:** Model depicting our current understanding of the role of histone H3 N-terminal proteolysis on gene expression reprogramming during cell state transitions. Briefly, the presence of H3-tail proteases creates a permissive chromatin state that neither contains nor allows *de novo* deposition of H3 PTMs. The repression of H3-tail proteases, probably followed by the substitution of cleaved histones by intact ones, promotes histone modification and chromatin reconfiguration.

2.3.2.5. Distinguishing histone tail cleavage from histone degradation

Histone tail cleavage and histone degradation are functionally distinct phenomena (Dhaenens *et al.*, 2015). However, they can be difficult to distinguish, as they usually share the same histone proteases (Duncan *et al.*, 2008; Morin *et al*, 2012) and can even occur simultaneously, as part of the same biological process (Duarte *et al.*, 2014; Ivanov *et al*, 2013).

Histone degradation occurs continuously as part of the protein turnover of cellular homeostasis (Dhaenens *et al.*, 2015). However, it is also upregulated in certain biological contexts. For instance, cathepsin L degrades protamines and histone remnants in the male pronucleus after egg fertilization (Imschenetzky *et al*, 1997; Morin *et al.*, 2012). Histone degradation also participates in chromosome segregation during mitosis (Hämälistö *et al*, 2020). The process involves the regulated leakage of a subset of lysosomes onto metaphasic chromosomes, which leads to the truncation of a small subset of histone H3 at its globular domain. The cleavage is mediated by cysteine cathepsins, and in particular cathepsin B. Interestingly, artificially blocking this process by introducing a recombinant histone H3 fused to CSTB led to severe nuclear defects including chromosome instability and aneuploidy. However, overexpressing CSTB in its native form did not abrogate histone cleavage, indicating that CSTB does not act as a physiological inhibitor of this process. The senescence program also involves lysosome-dependent histone degradation (Ivanov *et al.*, 2013; Lee *et al*, 2006). Cathepsin L is thought to play a central role in this process, and H3cs1 can be readily detected in DAPI-positive lysosomes during *in vitro* senescence (Ivanov *et al.*, 2013).

Histone degradation has also been linked to a multitude of cell-type specific chromatin decondensation events. Neutrophils, for instance, employ histone degradation as a mechanism to fight infections (Papayannopoulos *et al*, 2010; Tilley *et al*, 2022). Upon neutrophil activation, the serine protease ELANE translocates to the cell nucleus and cleaves all core histones. This mechanism participates in the formation of the neutrophil extracellular trap, a sticky DNA-based substance that engulfs and neutralizes pathogens (Li & Tablin, 2018). Pathogens also use histone cleavage as an offensive strategy; An FMD-virus protease cleaves the whole pool of histone H3 to shut down gene expression in the host (Falk *et al*, 1990), whereas mengovirus hijacks linker histone dynamics via selective H1-variant degradation (Traub & Traub, 1978).

2.4. Brain development

2.4.1 Differences in brain development between humans and mice

Mice are broadly used as a model organism for studying human development, physiology and disease. This is partly because they are easy to breed and manipulate, but more importantly because their genome is highly homologous to that of humans —even more so than that of other mammals such as dogs and elephants (Foley *et al*, 2016). Mice recapitulate the mechanisms of tissue morphogenesis and cell type diversification that govern human brain development. Accordingly, the basic architecture and circuitry of the adult cortex is common to both species (Defelipe, 2011; Mountcastle, 1997), and the vast majority of cell types that populate the human cortex (approximately 75) have an equivalent murine counterpart (Hodge *et al*, 2019). On the other hand, there are species-specific features that ought to be taken into account in translational studies.

First, several neurodevelopmental milestones occur prenatally in humans and postnatally in mice (Semple *et al*, 2013). For instance, in humans the blood brain barrier is established during gestational weeks 23 to 32 whereas in mice it develops at postnatal days one to three (Engelhardt, 2003). Likewise, the developing human brain reaches its maximum growth rate during the last four weeks *in utero*, whereas in mice this process does not occur until the second postnatal week (Dobbing & Sands, 1979).

Second, the human brain is larger than that of mice in terms of net weight (≈ 2500-fold), cortical surface (≈1000-fold) (Herculano-Houzel *et al*, 2006) and neuronal density (≈ four-fold) (Herculano-Houzel *et al*, 2007). Brain size is primarily influenced by the number of neural stem and progenitor cells generated during development (Florio & Huttner, 2014; Rakic, 2009). Therefore, the neural stem cell niche endures more rounds of division in humans than in mice, creating a window of enhanced vulnerability to both external and genetic insults (Semple *et al.*, 2013).

Third, the cellular makeup of the human cortex is somewhat more specialized that than of mice. For instance, human cortical astrocytes are larger, more diverse, and structurally more complex than rodent astrocytes (Oberheim *et al*, 2009), and rosehip neurons, a transcriptionally and electrophysiologically distinct type of GABAergic interneuron, is thought to be unique to the upper layer of the human cortex (Boldog *et al*, 2018). Finally, even if the majority of cortical cell types are found both in humans and in mice, they exhibit transcriptional signatures that are specific to their species (Hodge *et al.*, 2019). The greatest differences are observed in non-neuronal cell types, suggesting a that a larger degree of evolutionary divergence has occurred in glia than in neurons.

2.4.2 Neural stem cell differentiation

Bona fide neural stem cells originate in the neural plate, a columnar monolayer of primitive ectoderm that folds in upon itself to constitute the neural tube (Florio & Huttner, 2014; Grubb, 2006). The brain emerges from the subsequent enlargement, folding and patterning of the rostral end of the neural tube. Neural stem cells (neuroepithelial cells; NECs) ultimately give rise to all major cell types of the neural lineage, including neurons, astrocytes, oligodendrocytes, NG2-glia and ependymal cells. Initially, NECs self-amplify through a series of symmetric proliferative divisions (Rakic, 1995).

Mitoses take place near the ventricular surface of the neural tube (Fig. 4). During NEC proliferation, the vertical orientation of the division planes result in lateral expansion of the embryonic neocortex. As NECs progress through the cell cycle, their nuclei migrates back and forth along the apical-basal axis (Sauer & Walker, 1959). The process of interkinetic nuclear migration gives rise to the characteristic pseudostratified epithelium of the early neural tube.

Cortical neurogenesis begins at approximately day 12 post-fertilization in mice and at gestational week eight in humans (Grubb, 2006). By the time neurogenesis is initiated, the developing brain has already been colonized by microglia progenitors derived from the embryonic yolk sac (Nayak *et al*, 2014). At the onset of neurogenesis, NECs switch to an asymmetric division mode characterized by longer G1 phases (Huttner & Kosodo, 2005; Takahashi *et al*, 1995). Interestingly, arresting the cell cycle at G1 is sufficient to trigger ectopic initiation of the neurogenic phase (Misumi *et al*, 2008). These divisions give rise to other NECs and thus are self-renewing, but also to post-mitotic neurons and apical radial glia cells, a slightly more committed yet multipotent cell type that will ultimately prevail over NECs in the following developmental stages (Florio & Huttner, 2014) (Fig. 4). Both radial glia and NECs display apical-basal polarity, undergo interkinetic nuclear migrations and express the intermediate filament nestin. However, radial glial cells differ in that they also express the astrocyte marker GFAP and a distinctive set of transcription factors including Pax6.

At the peak of cortical neurogenesis, radial glial cells tend to differentiate into Tbr2-positive intermediate progenitors (IPs), a cell type responsible for the production of glutamatergic neurons (Hevner, 2019) (Fig. 4). Other stage-defining markers of the neuronal lineage include doublecortin (DCX), a microtubule associated protein that is expressed in late proliferative IPs and early post-mitotic neurons (Francis *et al*, 1999); βIII-tubulin (TuJ1), which marks neuronal processes from mid differentiation to adulthood; and NeuN, expressed inside and around the nuclei of the majority of terminally-differentiated neurons (Gusel'nikova & Korzhevskiy, 2015).

Neurons born at the dorsal ventricular side of the telencephalon use the apical-basal architecture of radial glia to migrate outwards and sequentially populate the layers of the developing cortex; from the inner-most to the outer-most (Grubb, 2006) (Fig. 4). This process is known as radial migration, and it is primarily used by excitatory neurons such as glutamatergic pyramidal cells. Conversely, inhibitory interneurons including GABAergic interneurons originate from neuronal progenitors located at the ventricular zone of the medial and caudal ganglionic eminences (GEs), a transitory structure located at both sides of the ventral telencephalon. New-born interneurons migrate tangentially, perpendicular to the radial axis of migration, until reaching their final destination in the neo-cortex (Fig. 4).

After neurogenesis and migration, neuronal development continues with the formation of connectivity or synaptogenesis (Semple *et al.*, 2013). In mice, the number of synapses reaches its absolute maximum during the second postnatal week. The brain then undergoes a transition from the creation of new synapses to the removal and re-organization of pre-existing ones, a process of connectivity refinement that is known as synaptic pruning and extends into early adulthood.

At embryonic day 17, radial glial cells in the developing mouse cortex cease to produce neurons and begin to generate astrocytes (Florio & Huttner, 2014) (Fig. 4). The majority of astrocytes are born during early postnatal development, but the process continues until adulthood. Cortical neurogenesis can also take place in the adult brain, but in mammals, this process is less frequent and regionally restricted than adult gliogenesis (Gage, 2019). Radial glia also differentiate into a functional, yet multi-potent cell type known as oligodendrocyte precursor cell or NG2-glia (Lin & Bergles, 2004; Sánchez-González *et al*, 2020). This cell type emerges in temporally- and spatially-defined waves during embryonic and early postnatal brain development, and constitutes up to five percent of all cells in the adult brain. NG2-glia typically differentiate into oligodendrocytes, the myelin-forming cells of the central nervous system. Nonetheless, NG2-glia can also generate astrocytes and neurons *in vivo*, both in the developing and in the adult brain (Aguirre & Gallo, 2004; Belachew *et al*, 2003). In mice, axon myelination begins around birth, peaks at approximately P20, and continues until puberty (Wiggins, 1986).

The degree of cell type specialization found in the mammalian brain is unmatched by any other biological system, and the process of neural stem cell differentiation is far more complex than conveyed above. It includes multiple intermediate neural progenitor cell types deriving —directly or indirectly— from radial glia (Götz & Huttner, 2005). The contribution of each neural progenitor to the adult brain will be determined by the developmental trajectory of its daughter cells. In turn, this will depend on the stepwise execution of elaborate transcriptional programs, beginning much earlier than actual fate commitment, in response to a complex milieu of internal and external stimuli (Faure *et al*, 2020).

2.4.2.1. Metabolic reprogramming during neural stem cell differentiation

Stem cells, including neural progenitors, have relatively low energy requirements but are particularly sensitive to the genotoxic effects of reactive oxygen species. Neurons, on the other hand, require staggering amounts of ATP only to maintain their basal requirements of energy consumption (Attwell & Laughlin, 2001). Therefore, the process of neurogenesis entails a radical transition between metabolic programs, in general involving downregulation of glycolysis and upregulating mitochondrial biogenesis and oxidative phosphorylation (Agostini *et al*, 2016; Zheng *et al*, 2016). Interestingly, the metabolic switch seems to play a crucial role in repressing neural stem cell renewal and promoting differentiation, and therefore is a driver, and not only a consequence, of neurogenesis (Iwata & Vanderhaeghen, 2021). Concretely, secondary metabolites produced in mitochondria influence neurogenesis by directly or indirectly altering histone PTM landscapes and subsequently modifying gene expression (Reid *et al*, 2017). In turn, it is becoming increasingly evident that epigenetic modifiers govern mitochondrial biogenesis prior to neurotrophic signalling (Uittenbogaard *et al*, 2018). As a proof of concept, neural stem cells treated with pan-histone-deacetylase inhibitors undergo upregulation of genes related to mitochondrial reprogramming and subsequently undergo neuronal differentiation.

2.4.2.2. Modelling neural stem cell self-renewal and differentiation with neurospheres

Studying neural stem cell differentiation *in vivo* is particularly challenging because of the high degree of complexity that characterizes the nervous system. This challenge can be overcome by using simplified *in vitro* models that recapitulate the key steps occurring *in vivo* (Azari & Reynolds, 2016). Neural stem and progenitor cells (hereafter referred to as NPCs) harvested from ventricle-proximal areas along the entire length of the central nervous system can be grown in a culture system known as the neurosphere assay (Ciccolini & Svendsen, 1998; Soares *et al*, 2021). In this setup, NPCs are selectively amplified using a serum-free culture media supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (FGF2). Under these conditions, NPCs can be propagated as free-floating colonies for at least ten passages with little or no change in their proliferative capacity and cell potency (Reynolds & Rietze, 2005). Mechanistically, FGF2-responsive NPCs divide asymmetrically and self-renew while producing a second cell population that also respond to EGF (Martens *et al*, 2000; Reynolds & Weiss, 1996). In turn, EGF-responsive NPCs increase in number via self-amplifying symmetric divisions. On the contrary, the growth of non-neural and differentiated cell types is not supported by this signalling milieu (Reynolds & Rietze, 2005).

Each neurosphere contains a unique collection of neuronal and glial progenitors in different states of differentiation (Suslov *et al*, 2002). The majority of clones in a neurosphere culture derive from relatively immature founder cells, evidenced by expression of radial glia markers including nestin and Pax6. Because the length of the cell cycle varies between different NPC populations (Florio & Huttner, 2014), the relative size of a neurosphere is thought to reflect the progenitor type from which it originated (Suslov *et al.*, 2002). Measuring the ability of a neurosphere population to form secondary neurospheres after dissociation and replating provides a readout of self-renewing capacity (Parmar *et al*, 2002).

Upon removal of growth factors and addition of serum to the culture media, intact or dissociated neurospheres differentiate into neurons and neuroglia (Helgason & Miller, 2005). Cell fate choice can be influenced by external cues such including growth factors and proteins of the extracellular matrix. For instance, supplementing the differentiation media with brain-derived neurotrophic factor (BDNF) promotes neurogenesis (Silva *et al*, 2009; Torrado *et al*, 2014), whereas increasing the concentration of serum favours the acquisition of an astrocyte fate (Brunet *et al*, 2004).

Like all technologies, the neurosphere culture system has its own limitations. Firstly, only a small fraction of neurospheres in any given culture derive from *bona fide* neural stem cells, whereas the vast majority originate from relatively more differentiated NPCs (Reynolds & Rietze, 2005). Consequently, the transition between neural stem cells and intermediate progenitors cannot be visualized in all neurospheres. Second, it is unclear whether neurosphere-derived progenitors preserve their regional identity, and whereas numerous reports indicate that these cells give rise to neuronal subtypes characteristic of the brain areas where they originated from (Klein *et al*, 2005; Ostenfeld *et al*, 2002; Parmar *et al.*, 2002), several other studies suggest otherwise (Hack *et al*, 2004; Santa-Olalla *et al*, 2003). Finally, neurosphere cultures are highly sensitive to environmental variations (Jensen & Parmar, 2006). Therefore, the results extracted from such experiments can be theoretically difficult to replicate unless the methodological procedures are properly standardized.

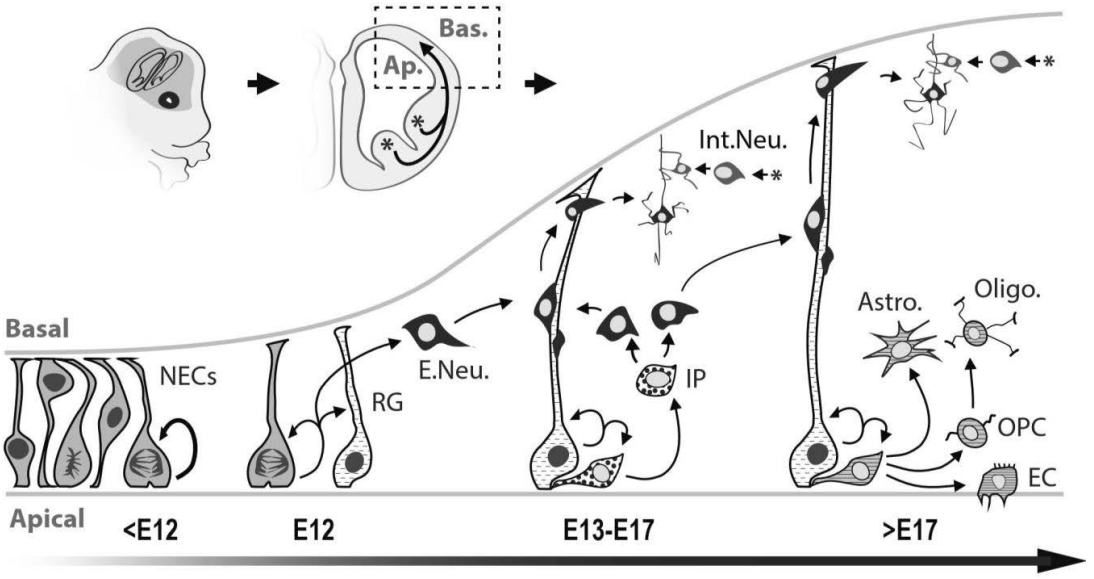


Figure 4. Hallmarks of cortical development in mice. Bas.: basal surface, Ap.: Apical surface, NECs: neuroepithelial cells, RG: radial glia, E.Neu.: excitatory neuron, Int.: interneuron, IP: intermediate progenitor, Astro.: astrocyte, Oligo.: oligodendrocyte, OPC: oligodendrocyte progenitor cell, EC: ependymal cell.

2.4.3. Postnatal development of the cerebellum

The cerebellum adjusts motor activity in response to sensory and cerebral inputs, participates in cognitive function and integrates neuronal networks related to visual spatial processing and affective control (Buckner, 2013). It is one of the earliest brain areas to undergo differentiation, but also one of the last to terminally differentiate and mature (Wang & Zoghbi, 2001). Indeed, neurogenesis in the human cerebral cortex occurs *in utero* (Grubb, 2006), whereas 85% of cerebellar granule neurons originate during the first year of life (Kiessling *et al*, 2014). The protracted development combined with a very fast growth in postnatal stages confers the cerebellum a particularly high vulnerability to genetic, epigenetic and environmental insults (Sathyanesan *et al*, 2019; Surchev *et al*, 2007). Accordingly, common neurodevelopmental diseases such as autism spectrum disorder and attention-deficit hyperactivity have been increasingly linked with anomalies in cerebellar development and cortico-cerebellar connectivity (Crippa *et al*, 2016; Mackie *et al*, 2007; Sathyanesan *et al.*, 2019).

The full spectrum of neuronal cell types that populate the cerebellum originate in a sequential manner from only two NPC populations (Leto *et al*, 2008). Glutamatergic lineages including granule cells arise from a distinct population of progenitors located in the rhombic lip of the developing metencephalon, whereas GABAergic lineages such as Purkinje neurons derive from another population of progenitors that proliferate near the ventricular zone. Granule cell progenitors migrate tangentially from the rhombic lip and form a transient germinal epithelium known as the external

granular layer (EGL) (Espinosa & Luo, 2008). In mice, proliferation in the EGL takes place from approximately E17.5 until P16 (Fig. 4). During this period, cell division rates are so high that granule cells end up becoming the most numerous type of neuron of the whole nervous system (Surchev *et al.*, 2007). Other hallmarks of cerebellar development concentrated during the first two postnatal weeks include the peak of synaptogenesis, the arrival of Purkinje cell axons to their target relay nuclei, the migration of new-born GABAergic interneurons to the cerebellar cortex, the proliferation of microglia and the birth of Bergmann glia, a specialized population of unipolar astrocytes with crucial functions in cerebellar morphogenesis (Das *et al*, 1974; Nikodemova *et al*, 2015; Sathyanesan *et al.*, 2019; Zhang & Goldman, 1996) (Fig. 4).

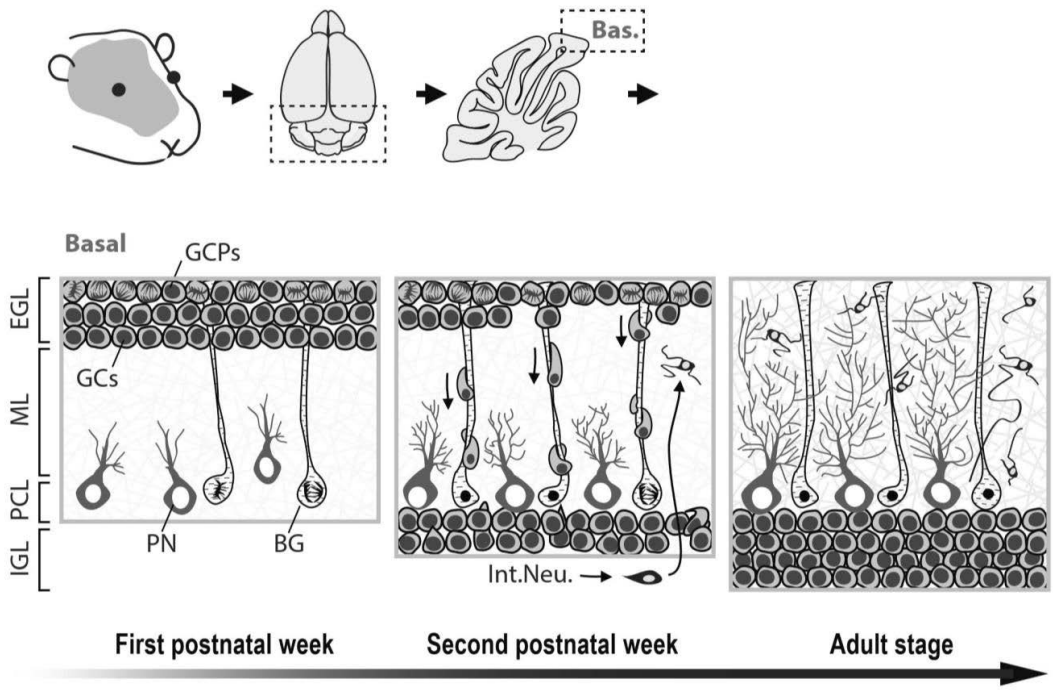


Figure 5. Hallmarks of postnatal cerebellar development in mice. Bas.: basal surface, EGL: external granular layer, ML: molecular layer, PCL: Purkinje cell later, IGL: internal granular layer, GCPs: granule cell progenitors, GCs: granule cells, PN: Purkinje neuron, BG: Bergmannn glia, Int.Neu.: interneuron.

From approximately P5 to P20, post-mitotic granule cells exit the EGL and migrate radially across the molecular layer following the processes of Bergmannn glia. Next, they break through the Purkinje cell layer and establish their final location at the bottom of the internal granular layer (Komuro &

Rakic, 1998) (Fig. 4). The main aspects of cerebellar growth and morphogenesis are orchestrated by the sonic hedgehog signalling pathway (Cheng *et al*, 2018; De Luca *et al*, 2016). On the other hand, the more delicate features of cerebellar maturation depend on spatiotemporally regulated expression of other molecules such as neurotrophins (Lindholm *et al*, 1997). Accordingly, suppressing BDNF signalling in cerebellar precursors results in apparently normal development with specific alterations in GABAergic markers and connectivity (Rico *et al*, 2002).

In humans, the maturation of the cerebellum is thought to continue until late adolescence. This phenomenon is evidenced by sex-specific fluctuations in cerebellar volume after reaching a growth peak at ages 12 in females and 16 in males (Tiemeier *et al*, 2010). A similar neuroimaging study reported progressive and regressive anatomical changes affecting multiple areas of the infantile and adolescent cerebellum (Sussman *et al*, 2016). Beyond these macroscopic features, the latter stages of cerebellar maturation remain largely uncharacterized (Sathyanesan *et al.*, 2019). Intriguingly, regionally-specified neurospheres can be derived from the adult mouse cerebellum (Klein *et al.*, 2005). The original stem cell population has never been properly identified, and even if adult neurogenesis occurs in the cerebellum of other mammals (Feliciano *et al*, 2015), it is unclear whether the same is true for humans and mice. Notably, Bergmann glia in the adult cerebellum express neural stem cell markers including Sox1, Sox2 and Sox9 (Sottile *et al*, 2006), raising the possibility that they might constitute an adult stem cell reservoir (Ahlfeld *et al*, 2017).

2.4.4 Epigenetic regulation in the developing brain

Epigenetic regulatory mechanisms enable diversification of gene expression programs during development. The mammalian brain, with an unparalleled degree of specialization in the cell types that integrate it, relies heavily on such mechanisms, and is especially sensitive to epigenetic alterations. Indeed, mutations in genes involved in epigenetic regulation often lead to neurodevelopmental disorders (Rajarajan *et al*, 2016; Urdinguio *et al*, 2009). For example, Rubinstein-Taybi syndrome is a multisystem disease characterized by mental impairment, skeletal abnormalities and high incidence of cancer that is caused by mutations in genes encoding a related pair of histone acetyltransferases (Van Gils *et al*, 2021). Coffin-Lowry syndrome, an X-linked form of mental retardation involving progressive motor dysfunction and skeletal anomalies, is caused by mutations in a kinase required for EGF-mediated histone H3 phosphorylation (Rogers & Abidi, 1993; Sassone-Corsi *et al*, 1999). Finally, Rett syndrome is another X-linked disorder characterized by neurodevelopmental arrest at approximately one year of age followed by progressive loss of intellectual and motor skills (Jawaid *et al*, 2017).

Rett syndrome is caused by mutations in *MECP2*, encoding a methyl- and hydroxymethyl-cytosine binding protein (Amir *et al*, 1999; Jawaid *et al.*, 2017). MeCP2 is preferentially expressed in neurons, where it is nearly as abundant as histone octamers (Skene *et al*, 2010). MeCP2 deficiency results in global rearrangement of neuronal chromatin, including two-fold increase in linker histone H1 occupancy. Conversely, the transcriptional signature of *Mecp2*-null mice cerebella is not too different from that of controls (Jordan *et al*, 2007). This is probably because MeCP2 is essentially a genome-wide repressor of transcriptional noise, mostly arising from heterochromatin elements such as satellite DNA and retrotransposons (Muotri *et al*, 2010; Skene *et al.*, 2010). An important unsolved question

is why neurons require MeCP2-mediated dampening of transcriptional noise whereas other somatic cell types do not.

2.4.4.1. Epigenetic regulation of neural stem cell differentiation: the example of bivalent chromatin

In stem cells, the promoters of many lineage-specific genes are simultaneously marked with active and repressive histone PTMs such as H3K4me3 and H3K27me3 (Bernstein *et al*, 2006). This is known as a bivalent epigenetic signature, and it is a chromatin priming mechanism that facilitates gene expression reprogramming upon lineage commitment. In neural stem cells, bivalent domains cooperate with other epigenetic mechanisms to preserve cell potency before the arrival of differentiation cues (Hatada *et al*, 2008; Liu *et al*, 2017b). For example, the bivalent signatures of astrocyte-specific genes tend to lose H3K27me3 already during embryonic to neural stem cell differentiation (Mikkelsen *et al*, 2007). However, they are kept transcriptionally silent through the repressive action of DNA methylation (Hatada *et al.*, 2008). If an astrocyte fate is chosen during neural stem cell differentiation, these genes will undergo activation via promoter demethylation. Alternatively, if a neuronal fate is chosen, astrocyte genes will remain repressed and bivalent genes related to the neuronal lineage will lose H3K27me3 and become activated (Burney *et al*, 2013; Liu *et al.*, 2017b).

2.4.4.2. Epigenetic regulation of brain maturation: the example of histone H3 turnover

In proliferative tissues, every round of division represents a new chance to re-organize the chromatin and modify its function (Moore, 2016). In post-replicative tissues, this opportunity does not exist. Therefore, epigenome plasticity becomes dependent on histone variants, whose deposition and eviction is uncoupled from DNA replication (Martire & Banaszynski, 2020; Stefanelli *et al*, 2018; Tvardovskiy *et al*, 2017). The brain, populated by long-lived and highly plastic cell types, is the paradigmatic example of such process. In neuronal chromatin, histone variant H3.3 accumulates to near-saturating levels by mid adolescence (Maze *et al*, 2015a). Consistently, canonical histones H3.1 and H3.2 become progressively downregulated from birth onward.

Age-dependent accumulation of H3.3 is also observed in other organs such as the liver, the kidney and the heart (Tvardovskiy *et al.*, 2017). However, the brain is unique in that the rate of histone turnover does not slow down in adult stages (Maze *et al.*, 2015a). H3.3-dependent nucleosomal turnover is essential for synapse formation and maintenance, mediates DNA responses to neuronal activity and promotes cell-type specific transcription throughout life. Moreover, it behaves similarly to MeCP2 in that it represses heterochromatin-derived transcriptional noise in neurons. Considering the vast influence of replication-independent histone dynamics on normal brain development and function, it is becoming increasingly evident that alterations in this process might be a common driver of brain ageing and disease (Wenderski & Maze, 2016).

3. Aims of the study

The overall aim of this study was to gain insight into the molecular mechanisms of EPM1 through the study of the nuclear role(s) of CSTB protein. To address this question, we utilized the murine model of EPM1 disease; the *Cstb*$^{-/-}$ mouse strain.

We hypothesized that CSTB modulates cysteine protease activities in the nucleus of brain cells, and that the pathogenesis associated with CSTB-deficiency originate from the dysregulation of nuclear proteolysis during brain development and/or maturation.
Specific aims were:

1. To determine whether nuclear cathepsin L and, in particular, histone H3 tail proteolysis participate in brain development and maturation.

2. To elucidate whether CSTB acts as a biologically-relevant inhibitor of nuclear cathepsin L in the brain.

3. To investigate the potential impact of CSTB-deficiency in the nucleus in the context of brain development and maturation.

4. Material and methods

Mouse model

The CSTB-deficient (*Cstb*$^{-/-}$) mouse strain used in this study is 129S2/SvHsd5-*Cstb*^{tm1Rm}, derived from the Jackson Laboratory strain 129-Cstbtm1Rm/J (stock number 003486) (Pennacchio *et al.*, 1998). Wild type mouse embryos of the same age and background (I) or wild type offspring generated through heterozygous matings (II) were used as controls. Genotypes were inferred by PCR-mediated detection of the *Cstb*^{tm1Rm} mutation using genomic DNA isolated from ear clippings and confirmed with the same strategy using DNA isolated from tail samples following euthanasia.

Table 1. Methods used in this study

Method	Used in
High-resolution respirometry	I
Immunocytochemistry	I, unpublished
Immunohistochemistry	II
In situ β-galactosidase activity assays	II
Microscopy	I, II
Neurosphere assay	I, unpublished
RNA sequencing	I, unpublished
RT-qPCR	I, II
Sub-cellular protein fractionation	II
Substrate-based protease activity assays	I, II
Transient transfection	I
Western blotting	I, II, unpublished

Table 2. Antibodies used in this study

Target	Host species	RRID	Clonality	Used in
53BP1	rabbit	AB_10003037	polyclonal	unpublished
Cathepsin B	goat	AB_2086949	polyclonal	II, unpublished
Cathepsin F	goat	AB_2576799	polyclonal	II
Cathepsin L	mouse	AB_305417	monoclonal	II
CUX1 (a.a.861)	rabbit	AB_2892622	polyclonal	unpublished
DCX	mouse	AB_10610966	monoclonal	I
GABA	rabbit	AB_477652	polyclonal	I
GAPDH	mouse	AB_2107448	monoclonal	II
GFAP	rabbit	AB_10013382	polyclonal	I
GFAP	rat	AB_2532994	monoclonal	I, II
H3cs1	rabbit	AB_2797961	monoclonal	I, II
H3K27me2	rabbit	AB_448222	polyclonal	I

H3K4me3	rabbit	AB_306649	polyclonal	I
histone H3	rabbit	AB_302613	polyclonal	I, II
IBA1	goat	AB_521594	polyclonal	II
IBA1	rabbit	AB_839504	polyclonal	II
Ki-67	rat	AB_10853185	monoclonal	I
Lamin B1	rabbit	AB_443298	polyclonal	II
LAMP1	rat	AB_2134500	monoclonal	II
Nestin	mouse	AB_94911	monoclonal	I
NeuN	mouse	AB_2298772	monoclonal	II
O4	mouse	AB_11213138	monoclonal	I
Olig2	goat	AB_2157554	polyclonal	II
$p21^{cip1}$	rabbit	AB_2734729	recombinant	II
VGAT	mouse	AB_887872	monoclonal	I
βIII-tubulin	mouse	AB_2210524	monoclonal	I
γH2AX	rabbit	AB_1640564	monoclonal	II

Table 3. RT-qPCR assays carried out in this study (TaqMan, TMO).

Target	**Description**	**Assay ID**	**Used in**
Atp5c1	Reference gene	Mm00662408_m1	I
Cstb	Gene of interest	Mm00432769_m1	I, II
Lmnb1	Gene of interest	Mm00521949_m1	II
Ndufs2	Gene of interest	Mm00467603_g1	I
Rpl13	Reference gene	Mm02526700_g1	II
Rpl19	Reference gene	Mm02601633_g1	I
Uqcrfs1	Gene of interest	Mm00481849_m1	I
Ywhaz	Reference gene	Mm03950126_s1	I, II

Table 4. Other key materials used in this study

Product	**Description**	**Used in**
CA-074	Protease inhibitor (CAS: 134448-10-5); Merck	I, II
Cathepsin B activity kit	Fluorometric enzymatic activity assay; Abcam	I
Cathepsin L activity kit	Fluorometric enzymatic activity assay; Abcam	I, II
CSTB-EGFP	Gene construct, *CSTB* in *pEGFP-N3* (GeneBank: U57609)	I
E-64	Protease inhibitor (CAS: 66701-25-5); Merck	I
EGF	Recombinant mouse protein; Merck	I
FGF2	Recombinant mouse protein; Peprotech	I
Histone extraction kit	Buffers for acid extraction of bulk histones; Abcam	I, II
Protein fractionation kit	Subcellular fractionation buffers; TMO	II
Z-FF-FMK	Protease inhibitor (CAS: 197855-65-5); Merck	I

Table 5. Statistical analyses carried out in this study

Statistical test	**Used in**
Student's *t*-test; paired	I (Fig. 7B)
Student's *t*-test; unpaired	I (Fig. 1D, Fig. 2D, Fig 3B, Fig. 4B-C, Sup. Fig. 9, Sup. Fig. 11)
Welch's *t*-test	I (Fig. 7B)
One-way ANOVA; Bonferroni or Šidák correction	I (Fig. 1C, Fig. 4A), II (Fig. 1A, Fig. 2D)
Two-way ANOVA; Greenhouse–Geisser or Tukey correction	I (Fig. 3A, Fig. 5C, Sup. Fig. 8), II (Fig. 2B, Fig. 2E, Fig. 3A, Fig. 3B, Fig. 3C, Fig. 3D, Fig. 3E, Sup. Fig. 3, Sup. Fig. 4), unpublished (Fig.8B)
Kruskal–Wallis test; Dunn's correction	II (Fig. 1A)
Linear mixed model; Wald's chi-squared test	I (Fig. 1F)
Wald test; Benjamini-Hochberg correction	I (Fig. 6B and Sup. Table 1)
Fisher's exact test; Benjamini-Hochberg correction	I (Fig. 6C and Sup. Table 2)

5. Results

5.1. Histone H3 tail proteolysis is a physiologically regulated phenomenon in the mouse brain (I and II)

5.1.1. Developmental regulation

The proteolytic removal of histone tails, particularly that of histone H3 , is usually reported during cell state transitions (Dhaenens, 2021). To investigate a potential role for H3 cleavage in the neural cell lineage, we set up an *in vitro* model of neural stem cell renewal and differentiation from the embryonic brain of wild type mice (Fig.1B in I). In brief, we cultured neural stem and progenitor cells (NPCs) from the ganglionic eminences of E13.5 mouse embryos as neurospheres (Ciccolini & Svendsen, 1998), induced their differentiation with a standard protocol to derive neurons and glia from NPCs within a 12-day period (Fig.1E, S.Fig. 2-4 in I) (Helgason & Miller, 2005) and probed the process at different stages.

Western blot analysis of histone extracts exposed two faster-migrating histone H3 species appearing specifically upon induction of differentiation (Fig.2B in I). The bands corresponded to N-terminal cleavage products, as evidenced by specific detection with antibodies against H3K27me2 but not H3K4me3 (Fig.2A-C, S.Fig.5 in I). The heavier product was recognized with an antibody against the new N-terminus generated by the proteolytical cleavage of histone H3 between amino acids A21 and T22 (H3cs1) (Fig.2A-C in I) (antibody validated in (Cheung *et al.*, 2021; Harpaz *et al.*, 2022)). H3cs1 was also present in the embryonic mouse brain and in primary neurosphere cultures (Fig.2D in I). However, it became undetectable upon neurosphere dissociation and passaging, probably reflecting the selective NPC amplification achieved with the neurosphere assay. The lighter cleavage species was detected in immunoblots against total histone H3, and based on its relative size and antibody recognition pattern, it corresponded to histone H3 truncated between amino acids K23 and R26 (Fig.2B in I, S.Fig.5 in I). K23-R26 cleavage was only observed in differentiating NPCs and not in primary neurospheres or in histone extracts from the E13.5 mouse brain (Fig.2D in I). Moreover, it could never be properly quantified due to its inconsistent expression among biological replicates.

Next, we asked whether H3 cleavage is switched off after brain development. To answer this question, we characterized the levels and distribution of H3cs1 in the postnatal mouse brain by performing immunohistochemical staining (IHC) of H3cs1 in tissue sections from the primary somatosensory cortex (S1), the striatum and the cerebellum of mice aged P7, P14, P21, P30 and P120. In all three regions, H3cs1 was readily detectable before P30, but not from P30 onwards (Fig.1A in II). In S1 and striatum, the levels of H3cs1 peaked at P14, a stage of neurodevelopment roughly corresponding to that of humans aged two (Bell, 2018). In contrast, the cerebellum displayed a steadily elevated H3cs1 content from P7 to P21. This is probably due to the relatively protracted development of the cerebellum in relation to the rest of the brain (Sathyanesan *et al.*, 2019). Western blot analysis of subcellular protein fractions prepared from P14 and P120 mouse brains confirmed that H3cs1 is abolished following brain maturation (Fig.1C in II). Notably, a band corresponding to H3cs1 was identified in chromatin-bound protein extracts, but not in lysosome-enriched cytoplasmic fractions (Fig.1C in II). Furthermore, IHC analysis of individual cell nuclei showed that H3cs1 does not overlap with DAPI-dense heterochromatin bodies (Fig. 1B), consistent with its previously reported

association with permissive chromatin (Cheung *et al.*, 2021; Kim *et al.*, 2016). Finally, a very faint band matching the exact size of H3cs1 could be distinguished in total histone extracts from the P14 mouse brain probed with an antibody against the H3 C-terminus (S.Fig.2 in II). This observation indicates that, in brain, H3cs1 represents a very small fraction of the total H3 pool, in contrast to analogous reports in other somatic tissues (e.g. (Ferrari *et al.*, 2021)). The H3 cleavage product mapped between K23 and R26 was not detected in postnatal brain tissue.

Together, our findings indicate that H3cs1 is confined to the process of brain development and maturation, in line with our current understanding of its physiological role in chromatin priming during cell state transitions (Cheung *et al.*, 2021; Santos-Rosa *et al.*, 2009). Of interest, H3cs1 is the most frequently reported cleavage product of histone H3, and, in our case, the only faster-migrating H3 species observed both in differentiating mouse NPCs and in mouse brain tissue.

5.1.2. Cell type specificity

Immuocytochemical (ICC) analysis during NPC differentiation revealed that H3cs1 was only present in a small fraction of cell nuclei, with intense signals detected in approximately 1 to 3% of the total cell count throughout the timeline of differentiation (Fig.3A in I). This observation suggested that H3cs1 displayed a cell-type specific enrichment. To test this hypothesis, we quantified the co-occurrence of H3cs1 with cellular identity markers. During differentiation, new-born astrocytes accumulate GFAP and downregulate nestin in an overlapping manner (Sergent-Tanguy *et al*, 2006) (Fig.1F in I). Triple ICC staining with antibodies against GFAP, nestin and H3cs1 showed that approximately 90% of H3cs1-positive cells expressed neither nestin or GFAP, hence they could not be astrocytes (S.Fig.6 in I). H3cs1 was detected in approximately 5% of cells expressing O4, a premyelinating oligodendrocyte antigen (S.Fig.8 in I). The O4-positive cell population was very scarce (0.75% of the total cell count, data not shown), and thus could not account for the main cell type undergoing H3 cleavage during NPC differentiation. Finally, we turned toward neurons. Nearly 20% of H3cs1-positive cells expressed the neuron-specific β-tubulin isoform III (TuJ1) (S.Fig.7 in I). TuJ1-positive cells represented only 3% of the total cell count by day 12 post-differentiation (Fig.1E in I), indicating that H3cs1 is enriched among neurons. Accordingly, approximately 88% of cells containing H3cs1 were immunopositive for DCX (Fig.5B in I), a neuronal lineage marker expressed in late-dividing progenitors and early post-mitotic neurons (Francis *et al.*, 1999). To assess the proliferation status of H3cs1-positive cells, we quantified the co-occurrence of H3cs1 with Ki-67, a cell cycle progression marker. Interestingly, 85% to 99% of H3cs1-positive cells from days 1 to 12 post-differentiation were devoid of Ki-67 immunoreactivity (Fig.5A in I). Together, the data indicate that H3 cleavage is mostly confined to the process of neurogenesis. To strengthen this conclusion, we sought to alter the neurogenic potential of NPCs and evaluate its impact on H3cs1. NPCs plated in media containing BDNF produced significantly more TuJ1-positive neurons than NPCs grown in high-serum conditions (S.Fig.9 in I). Consistent with our hypothesis, we obresrved an increased number of H3cs1-positive cells in BDNF-supplemented cultures (Fig.5C in I).

To assess whether the neuron enrichment reported during NPC differentiation was preserved in postnatal brain, we stained cortical, striatal and cerebellar tissue sections of P14 mice against H3cs1 and lineage markers NeuN, Olig2, GFAP and IBA1. Under the confocal microscope, H3cs1 could be

detected in the vast majority of cell nuclei regardless of the brain region analysed (S.Fig.1 in II). However, whereas practically all astrocytes and NeuN-positive neurons showed immunoreactivity for H3cs1, oligodendrocyte-lineage cells and microglia displayed an ON/OFF staining pattern reminiscent of that observed during NPC differentiation.

Together, the findings show that H3cs1 is subject to cell-type specific regulation in the mouse brain.

5.2. CSTB controls the neurodevelopmental profile of H3cs1 by inhibition of nuclear cathepsin L (I, II, unpublished)

5.2.1. CSTB functions as a negative regulator of H3cs1 in the neural cell lineage

To uncover the molecular role of CSTB during brain development, we utilized tissue samples from the *Cstb* knockout mouse strain (Cstb$^{-/-}$ mouse) described in section 2.1.3. We studied the impact of CSTB deficiency on H3 cleavage in the *in vitro* model of neurogenesis described above and explored the presence of H3cs1 in postnatal cerebellar tissue samples collected from mice aged P7 to P120.

CSTB mRNA was detected both in undifferentiated and in differentiating wt NPCs. Interestingly, the mRNA levels displayed a linear upregulation trend throughout the experimental timeframe (Fig. 1C in I). In wt cerebella, the levels of CSTB mRNA increased by approximately 50% from P14 to P30, and remained constant between P30 and P120 (Fig. 2D in II). The time-resolved changes in CSTB mRNA expression strongly suggest that *Cstb* is a developmentally-regulated gene. In turn, this observation supports the prediction that CSTB protein is involed in the process of brain development and maturation.

Motivated by a previous report indicating that CSTB interacts with histones and cathepsin L in the nucleus of T89G astrocytoma cells (Ceru *et al.*, 2010), we asked whether CSTB participates in the regulation of H3 cleavage in the neural cell lineage. Western blot analysis of histone extracts revealed that *Cstb*$^{-/-}$ NPCs present H3cs1 not only during differentiation, but also in the undifferentiated state (Fig. 2B in I). Additionally, the cleavage product H3cs1 was significantly upregulated in tissue samples and in primary neurospheres derived from CSTB-deficient mouse embryo brains (Fig. 2D in I). Finally, an ICC-based analysis of differentiating NPCs revealed a sharp increase in the proportion of *Cstb*$^{-/-}$ cells displaying H3cs1 immunoreactivity throughout the timeline of differentiation (Fig. 3A in I). Together, the findings indicate that CSTB-deficiency triggers ectopic histone tail cleavage both during self-renewal and during NPC differentiation.

To further investigate the role of CSTB as a regulator of H3 cleavage, we transfected *Cstb*$^{-/-}$ NPCs with a plasmid encoding CSTB fused to a fluorescent reporter (GFP) under a constitutive CMV promoter and assessed its ability to rescue the ectopic H3 cleavage phenotype. C-terminal tagging of CSTB was chosen over N-terminal tagging to avoid unwanted interactions with the protease inhibitory domain located near the N-terminus of the protein (Turk & Bode, 1991). Measurement of H3cs1 immunoreactivity among transfected cells 24 hours after induction of differentiation showed that CSTB overexpression is sufficient to abolish H3cs1 during differentiation (Fig. 3B in I). This observation supports a direct role for CSTB in modulating H3cs1 in the neural cell lineage.

We had previously shown that, during NPC differentiation, H3cs1 is mostly found in cells committed to a neuronal fate. To elucidate whether CSTB-deficiency leads to an imbalance in the cell type specificity of H3cs1, we examined the identity of H3cs1-positive cells during *Cstb*$^{-/-}$ NPCs differentiation. In accordance with the observations carried out in wt cells, we found that H3cs1 was largely confined to cells expressing neuronal markers; i.e. TuJ1 and, in particular, DCX (Fig. 3B, S.Fig. 6-7 in I). However, we also detected a sharp increase in the proportion of premyelinating oligodendrocytes (O4-positive cells) displaying H3cs1 immunoreactivity in the absence of CSTB (S.Fig. 8 in I). These observations suggest that CSTB is particularly important for the modulation of H3 cleavage during neurogenesis and oligodendrogenesis, and suggest that the cell type specificity of this chromatin event is defined by mechanisms other than CSTB expression.

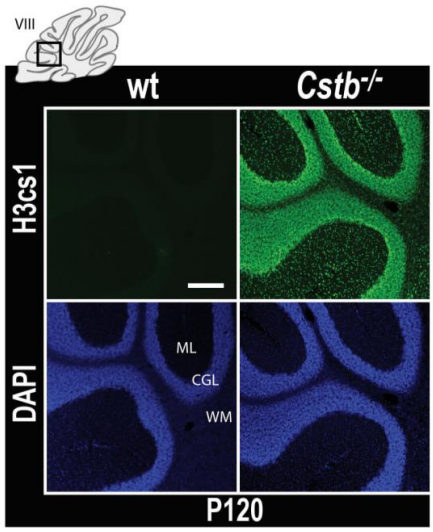


Figure 6: Representative set of epifluorescence microscopy images of H3cs1 immunohistochemical staining in the cerebellum of adult wt and Cstb$^{-/-}$ mice (P120). DAPI counterstaining is also shown. VIII = cerebellar lobule VIII, ML = molecular layer, CGL = cerebellar granular layer, WM = white matter. Scale bar = 100

Finally, we investigated a potential role for CSTB in modulating H3cs1 in the postnatal mouse brain. We chose the cerebellum as an experimental model due to its previously reported involvement in the pathophysiology of CSTB deficiency. First, we carried out IHC of H3cs1 in serial cerebellar sections from *Cstb*$^{-/-}$ and wt mice at P7, P14, P21, P30 and P120. In wt cerebella, H3cs1 immunoreactivity decayed from P14 onwards, with the steepest decrease detected between P21 and P30 (Fig. 1A in II). In *Cstb*$^{-/-}$ cerebella, H3cs1 displayed a marked decrease from P14 to P21, and a subsequent increase from P21 onwards (Fig. 2B in II). The greatest difference between genotypes was observed at P120 (Fig. 6), a time-point at which the levels of H3cs1 in the *Cstb*$^{-/-}$ cerebellum matched those of control mice at its peak of maximum abundance (P14) (Fig. 2B in II). To validate these observations, we carried out western blot analysis of whole cerebellar lysates at P14 and P30. At both of these time-points, H3cs1 was visibly more abundant in samples from *Cstb*$^{-/-}$ mice than in those from wt littermates (Fig. 2C in II). Moreover, in the absence of CSTB, H3cs1 increased by a factor of four from P14 to P30, whereas in control samples the levels of this histone species shrank by 50% during the same developmental window.

Together, the findings indicate that CSTB has a negative modulatory effect on H3 cleavage in the mouse brain. Specifically, CSTB activity is required to (i) prevent premature histone cleavage during NPC renewal, (ii) modulate the levels of H3cs1 throughout a developmental window spanning from NPC differentiation to brain maturation and (iii) mediate the permanent repression of H3cs1 after postnatal brain development.

5.2.2. Cystatin B represses nuclear cathepsin L signalling in the mouse brain

Next, we sought to identify the protease responsible for H3cs1 in the mammalian brain. Several proteolytic enzymes including serine and cysteine peptidases have been shown to catalyse H3cs1 *in vivo* (Cheung *et al.*, 2021; Duarte *et al.*, 2014; Duncan *et al.*, 2008). The role of CSTB in repressing H3cs1 suggested that, in the context of brain development, this PTM is mediated by one or several papain-like cysteine proteases. Cathepsin L, the enzyme responsible for H3cs1 during mouse stem cell differentiation (Duncan *et al.*, 2008), was the most obvious candidate. To test this hypothesis, we cultured undifferentiated *Cstb*$^{-/-}$ NPCs in the presence of pharmacological protease inhibitors with different specificities. Treatment with E-64, a wide-range inhibitor of papain-like cysteine proteases, abolished the ectopic production of H3cs1 associated with CSTB-deficiency (Fig.4A in I). H3cs1 was similarly sensitive to *Z*-FF-FMK, a specific inhibitor of cathepsin B and cathepsin L. To distinguish between these two proteases, we cultured cells with CA-074, a selective inhibitor of cathepsin B (Montaser *et al*, 2002). Interestingly, CA-074 caused an incomplete but significant reduction in the levels of H3cs1, suggesting that cathepsin B and cathepsin L are jointly responsible for ectopic H3 cleavage in *Cstb*$^{-/-}$ NPCs.

To validate this interpretation, we measured the enzymatic activities of both of these proteases in whole cell lysates of *Cstb*$^{-/-}$ and wt NPCs using commercial fluorometric enzymatic activity assays. In accordance with the expectations, both cathepsin B and cathepsin L were significantly more active in CSTB-deficient than in control NPCs (Fig. 4B in I). However, cathepsin L activity was only 0.2-fold greater, whereas that of cathepsin B was increased by sevenfold. In the light of the findings, we weighted the possibility that cathepsin B is solely responsible for H3cs1, and that the partial rescue effect observed in CA-074-treated cells resulted from an incomplete inhibition of the target protease. However, additional enzymatic activity assays confirmed that the CA-074 treatment used in our previous experiments was sufficient to completely suppresses cathepsin B activity (Fig. 4B in I). Taken together, our findings strongly suggest that CSTB modulates H3 cleavage in NPCs through the inhibition of cysteine cathepsins B and L.

Previous studies indicate that the proteases mediating H3 cleavage in cultured cells can be different from those carrying out the same function *in vivo* (Cheung *et al.*, 2021; Tvardovskiy *et al.*, 2015). To determine whether cathepsin B and cathepsin L are also responsible for H3cs1 during normal brain development, we investigated their sub-cellular localization in the postnatal and adult mouse brain. We also analyzed the distribution of cathepsin F, a cysteine protease with a previously reported chromatin association (Maubach *et al.*, 2008). Western blot analysis of sub-cellular protein fractions derived from P14 and P120 wt mouse brains showed that the active forms of both cathepsin B and cathepsin F localized exclusively in a lysosome-enriched cytoplasmic fraction (Fig.1C in II). On the contrary, cathepsin L was also detected in protein fractions eluted from chromatin pellets following MNase-mediated DNA digestion. In cytoplasmic fractions, we observed cathepsin L bands matching the sizes of murine pro-cathepsin L (39 kDa), single-chain cathepsin L (30 kDa) and double-chain cathepsin L (25 kDa) (Ishidoh *et al.*, 1998). In chromatin-bound protein fractions, we detected two cathepsin L signals corresponding to a slightly shorter form of pro-cathepsin L (37 kDa) and to the mature double-chain cathepsin L (25 kDa). The latter observation is consistent with a previous report indicating that the nuclear translocation of cathepsin L is accomplish through the production of

shorter pro-cathepsin L isoforms devoid of a lysosome import signal (Goulet *et al.*, 2004). Interestingly, nuclear pro-cathepsin L was predominantly expressed in the P14 mouse, whereas the lysosomal isoform was mostly expressed in the adult mouse brain (Fig.1C-D in II). These findings imply that that the subcellular targeting of *de novo*-synthetized cathepsin L shifts from nuclear to lysosomal after postnatal brain development. On the other hand, double-chain cathepsin L was equally present at P14 and at P120, indicating that mature forms of this protease can be found in the nucleus of brain cells until adulthood.

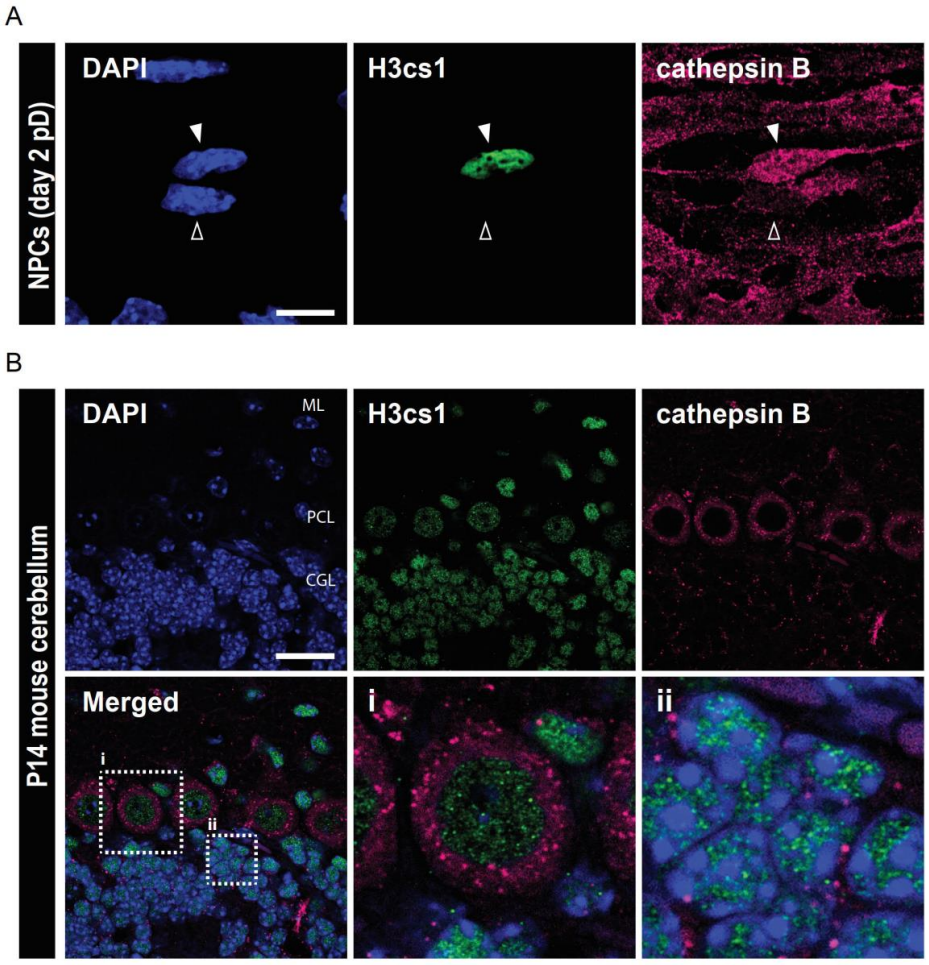


Figure 7: Subcellular localization of the cysteine protease cathepsin B in neurons. **A:** Representative confocal microscopy image of wt NPCs at day 2 after induction of differentiation stained against cathepsin B and H3cs1. DAPI counterstaining is also shown. The white arrowhead indicates a cell nucleus displaying immunoreactivity for both cathepsin B and H3cs1. The arrowhead indicates a cell nucleus where both markers are absent. Scale bar = 10 µm. **B:** Representative confocal microscopy image of cathepsin B and H3cs1 immunohistochemical staining in cerebellar tissue sections of wt mice at P14. DAPI counterstaining is also provided. Magnified details of Purkinje neuron and cerebellar granule neuron soma are shown in panels "i" and "ii", respectively. ML = molecular layer, PCL = Purkinje cell layer, CGL = cerebellar granular layer. Scale bar = 20 µm.

The observation that cathepsin B is excluded from the chromatin of brain cells at the peak of H3cs1 abundance contradicts with our previous findings indicating that cathepsin B contributes to the ectopic H3 cleavage observed in CSTB-deficient NPCs (Fig. 5 in I). To clarify the role of cathepsin B as an H3 protease in the neural lineage, we stained differentiating NPCs and cerebellar tissue sections from P14 mice against cathepsin B and H3cs1 and assessed their co-localization at the cellular level. In agreement with our previous findings, cathepsin B was readily detected in the nuclei of H3cs1-positive NPCs, but not in those of H3cs1-negative cells (Fig.7A). In the cerebellum, cathepsin B expression was detected in a variety of cell types including granule cells and Purkinje neurons (Fig.7B). However, it did not localize in the cell nucleus. These findings imply that cathepsin B is partially responsible for H3cs1 during *in vitro* NPC differentiation, and strengthen the findings suggesting the that it does not participate in H3cs1 during postnatal brain development.

Taken together, the results show that, of the three cysteine proteases with a previously reported function in the cell nucleus (Goulet *et al.*, 2004; Hämälistö *et al.*, 2020; Maubach *et al.*, 2008), cathepsin L is the only that interacts with the chromatin in the postnatal mouse brain. Considering that pro-cathepsin L is specifically targeted to the nucleus at the peak of histone cleavage, we find it justified to conclude that cathepsin L probably is responsible for H3cs1 in the postnatal mouse brain.

Finally, we sought to clarify whether CSTB regulates H3cs1 through its inhibitory effect on cathepsin L *in vivo*. We measured the enzymatic activity of cathepsin L in whole brain lysates and in chromatin-bound protein fractions prepared from *Cstb*$^{-/-}$ and wt mouse brains (Fig. 2E in II). We chose to carry out this experiment with mice aged P30 for they display the highest level of *Cstb* mRNA expression (Fig. 2D in II). In both genotypes, cathepsin L activity was higher within the chromatin-associated protein fractions than at the whole tissue levels (Fig. 2E in II), confirming that an enzymatically active form of cathepsin L exists in the nucleus of brain cells. In whole brain samples, cathepsin L activity did not differ between the genotypes. Conversely, chromatin samples derived from *Cstb*$^{-/-}$ mice displayed higher cathepsin L activity that those of wt mice. These findings strongly suggest that CSTB serves as a chromatin-specific inhibitor of cathepsin L in the mouse brain.

Prior studies in other cell types have shown that, apart from histone H3, cathepsin L can proteolytically process other nuclear proteins: the transcription factor CUX1 (Goulet *et al.*, 2004), 53BP1 (Gonzalez-Suarez *et al.*, 2011), histone H2A (Coradin *et al.*, 2021), lamin B1 and caspase 6 (Islam *et al.*, 2022). To elucidate whether the CSTB/cathepsin L axis affects other nuclear proteins, we performed western blot analysis of cerebellar lysates collected from *Cstb*$^{-/-}$ and wt mice at P14 and P30. We found that the cathepsin L-dependent isoform of CUX1 (CUX1 p110) was more abundant in *Cstb*$^{-/-}$ cerebella than in age-matched wt controls at P14 but not at P30 (Fig. 8). The levels of 53BP1 were similar in brains of both genotypes, suggesting that, in the mouse cerebellum, the stability of this protein is not regulated by CSTB. Similarly, we did not detect the characteristic 21-kDa band generated by the cleavage of lamin B1 by cathepsin L (S.Fig. 3A in II). The integrity of histone H2A and caspase 6 was not assessed.

Collectively, these results indicate that CSTB modulates a neural signalling axis involving cathepsin L-mediated proteolysis of at least two chromatin-interacting proteins—histone H3 and CUX1—in a developmentally regulated fashion (Fig.8).

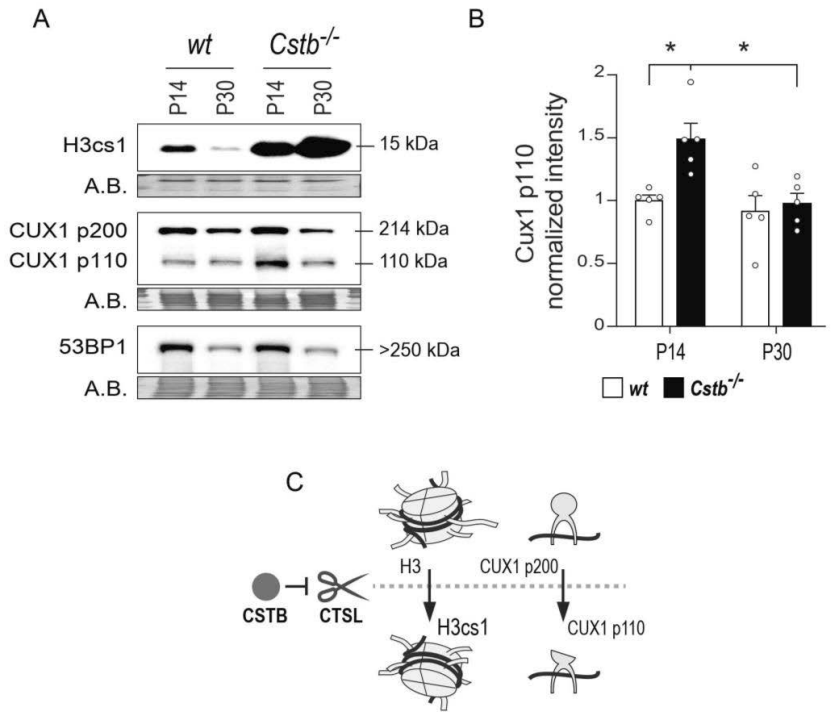


Figure 8: Nuclear cathepsin L targets affected by CSTB deficiency. **A:** Western blot detection of H3cs1, CUX1 and 53BP1 in whole cerebellar lysates of wt and *Cstb*$^{-/-}$ mice at P14 and P30. **B:** Bar plot depicting genotype-dependent changes in CUX1 p110 protein abundance. Normalized intensity values are plotted as means ± SEM (n = 5 mice / time-point and genotype). Confidence intervals: * p-value < 0.02. **D:** Schematic representation of nuclear cathepsin L activities regulated by CSTB in the mouse brain.

5.3. A role for CSTB in mouse brain development

5.3.1. CSTB-deficiency promotes neural stem cell differentiation but does not interfere with lineage commitment.

The lack of CSTB expression has been associated with neurodevelopmental defects both in humans and in mice (Di Matteo *et al.*, 2020; Mancini *et al.*, 2016). We sought to explore whether CSTB-deficiency impairs neural stem cell renewal and differentiation using neurosphere cultures derived form *Cstb*$^{-/-}$ mice. First, we grew single cells derived from dissociated neurospheres in separated culture wells and performed a clonal colony-forming assay. *Cstb*$^{-/-}$ NPCs gave rise to significantly less neurospheres than wt cells, indicating reduced self-renewing capacity (Fig. 1D in I). Next, we investigated the process of lineage specification taking place during NPC differentiation. CSTB-deficiency did not alter the neurogenic potential of NPCs, as judged by the equal proportion of *Cstb*$^{-/-}$ and wt cells expressing the stage-specific neuronal markers DCX and TuJ1 at early and late stages of

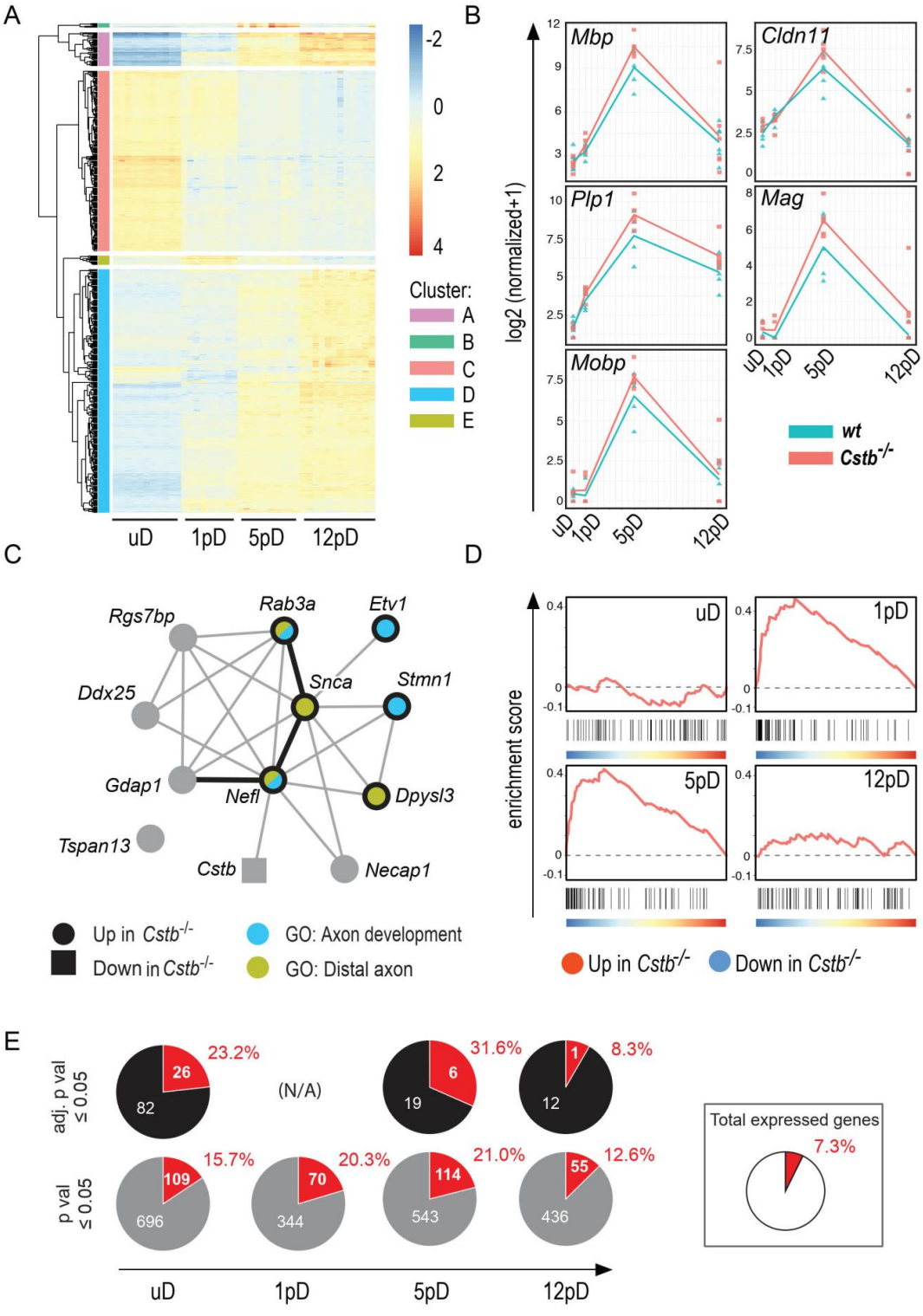
differentiation (Fig. 1E in I). Notably, neurons generated in the *in vitro* model of neurogenesis expressed GABAergic lineage markers VGAT and GABA, with no apparent differences between genotypes (S.Fig. 2 in I). Oligodendrocyte lineage cells could not be properly quantified due to their low and variable frequency. Finally, we investigated astroglia, the cell type that is more frequently produced upon differentiation of neurosphere-derived NPCs (Singec *et al*, 2006) (S.Fig. 4 in I), CSTB-deficient astrocytes adopted a maturity-related cytoskeletal phenotype —GFAP-positive nestin-negative (Sergent-Tanguy *et al.*, 2006)— significantly earlier than control cells. Accordingly, the proportion of nestin-positive GFAP-negative cells decayed earlier in the absence of CSTB than in wt cultures (Fig. 1F in I). Collectively, these findings strongly suggest that CSTB-deficiency does not affect NPC potency or obstruct cell fate specification, but has a stimulatory effect on NPC differentiation.

5.3.2. CSTB-deficiency induces transcriptional alterations during neural stem cell renewal and differentiation

Previous reports indicate that H3cs1 participates in gene expression reprogramming (Cheung *et al.*, 2021; Herrera-Solorio *et al.*, 2019; Santos-Rosa *et al.*, 2009). Based on these observations, we asked whether the ectopic presence of H3cs1 influences gene expression in *Cstb*$^{-/-}$ NPCs. We performed RNA sequencing (RNA-Seq) of total mRNA isolated from *Cstb*$^{-/-}$ and wt NPC colonies (44 in total) during self-renewal and at days 1, 5 and 12 after induction of differentiation, and determined gene expression changes in the absence of CSTB. To limit the variability arising from clonal heterogeneity within neurosphere cultures (Suslov *et al.*, 2002), we focused the study on neurospheres sized approximately 200 μm, containing approximately 400 cells.

Principal component analysis (PCA) of the RNA-Seq data revealed that approximately 93% of the variation between samples originated from cellular maturity rather than genotype (PC1-PC3; Fig. 6A and S.Fig. 10 in I). PC1 (77% of the variation) segregated the RNA samples chronologically, in a well-defined cluster for each of the time-point analysed (Fig. 6A in I). The samples derived from undifferentiated NPCs formed a tightest cluster, signifying that their transcriptional signatures where highly homologous. However, a PCA of this time-point alone consistently separated *wt* from *Cstb*$^{-/-}$ along PC1, in this case representing 32% of the phenotypic variation (Fig. 6A in I). This observation anticipated that the transcriptional effects of CSTB-deficiency manifest already before induction of differentiation, concomitant with the premature initiation of H3 cleavage.

A gene clustering analysis based on chronological expression profiles classified the top 500 genes with the highest degree of variation among samples into five groups (Fig. 9A). 97% of these genes were either monotonically upregulated (clusters A and D) or monotonically downregulated (cluster C) throughout differentiation, whereas the remaining 3% peaked at days 1 or 5 post-differentiation (clusters E and B, respectively). Interestingly, all five genes integrating cluster B encode for key structural components of the myelin sheath and displayed a non-significant upregulation trend in *Cstb*$^{-/-}$ samples (Fig. 9B).


A
-2
0
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4
Cluster:
A
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C
D
E
uD
1pD
5pD
12pD
B
log2 (normalized+1)
Mbp
Cldn11
Plp1
Mag
Mobp
wt
Cstb-/-
C
Rgs7bp
Rab3a
Etv1
Ddx25
Snca
Stmn1
Gdap1
Nefl
Dpysl3
Tspan13
Cstb
Necap1
Up in Cstb-/-
Down in Cstb-/-
GO: Axon development
GO: Distal axon
D
enrichment score
Up in Cstb-/-
Down in Cstb-/-
E
adj. p val ≤ 0.05
p val ≤ 0.05
26
82
23.2%
(N/A)
6
19
31.6%
1
12
8.3%
109
696
15.7%
70
344
20.3%
114
543
21.0%
55
436
12.6%
Total expressed genes
7.3%

Figure 9: Transcriptional changes associated with CSTB-deficiency during NPC renewal and differentiation. A: Heatmap and hierarchical clustering of the top 500 genes whose expression levels vary the most during NPC differentiation. The map is built on the transcriptomics data of all 42 samples analysed, independently of their genotype. Genes are distributed in five clusters (A-E) based on their time-resolved expression profiles. uD: undifferentiated NPCs; 1, 5 and 12 pD: NPCs at days 1, 5 and 12 post-differentiation. **B:** Transcriptional levels of the five genes whose expression peaks at day 5 after induction of differentiation (cluster B in panel A) in wt and *Cstb*$^{-/-}$ samples. **C:** Interaction network of proteins encoded by differentially expressed genes (DEGs) in *Cstb*$^{-/-}$ NPCs at day 12 post-differentiation. Nodes are depicted as circles or squares depending on whether they are upregulated or downregulated in CSTB-deficient cells. Interactions depicted with thick lines obtained high confidence scores (>0.7/1) in STRING database. Coloured nodes represent genes annotated under to gene ontology terms "axon development" (GO:0061564) and "distal axon" (GO:0030424). **D:** Gene set enrichment analysis (GSEA) of differentially expressed genes in *Cstb*$^{-/-}$ NPCs for the gene ontology term "electron transport chain" (GO:0022900) at the designated time-points. Black lines represent the position that each gene in the dataset occupies in the DEG ranking, from the most downregulated (left pole) to the most upregulated (right pole) in *Cstb*$^{-/-}$ samples. Statistically significant enrichment scores were reported at days 1 and 5 post-differentiation (q value = 0 in both cases). **E:** Pie charts depicting the proportion of DEGs in *Cstb*$^{-/-}$ NPCs encoding experimentally-verified components of the mitochondrial proteome, as annotated in MitoCarta2.0. For reference purposes, the frequency of mitochondrion-related genes among all expressed genes

Comparative transcriptome analysis of undifferentiated NPCs revealed a total of 112 differentially expressed genes (DEGs) between genotypes (Fig. 6B and S.Table 1 in I). The majority of these genes (97/112) were upregulated in CSTB-deficient cells. Next, we characterized functionally-related gene categories enriched within the 112-DEG dataset using Gene Ontology (GO) enrichment analyses (S.Table 2 in I). A GO analysis of biological processes and molecular functions showed over-representation of multiple gene categories related to mitochondrial and cytoplasmic protein translation, RNA metabolism and intracellular protein transport. The first group included several eukaryotic translation initiation factors (*Eif2s3y, Eif3a, Eif4a1, Eif4B,* and *Eif5A*) as well as genes encoding mitochondrial ribosomal proteins (*Mrpl54*, *Mrps10*, and *Mrps15*). We also noted a marked enrichment in key genes linked to mRNA processing and splicing including *Txnl4*a and *Hnrnpr*. GO cellular components identified a robust enrichment in mitochondrion-associated genes (Fig. 6C and S.Table 2 in I). These included *Ndufs3, Uqcrfs1,* and *Cox6a1*, which encode proteins belonging to mitochondrial respiratory complexes I, III and IV, respectively, *Ogdh* and *Dlst,* belonging to the oxoglutarate dehydrogenase complex, and *Samm50, Tomm20,* and *Tomm40,* encoding proteins of the outer mitochondrial membrane involved in cytosolic protein import and sorting.

We did not observe other DEGs than *Cstb* at post-differentiation day 1, and next investigated the 19 and 12 DEGs identified in *Cstb*$^{-/-}$ NPCs at days 5 and 12 following induction of differentiation, respectively (Fig. 6B in I). At day 5 post-differentiation, 14 out of the 19 DEGs were downregulated. Based on GO functional classifications, transcripts of electron transport chain and electron transfer

activity were significantly enriched in the dataset (S.Table 2 in I). Accordingly, GO cellular component analysis of DEGs in *Cstb*$^{-/-}$ mice (Fig. 5C) showed enrichment in transcripts of oxidative phosphorylation complexes I (*Ndufs2*), III (*Uqcrfs1*) and IV (*Cox6a1*, *Cox8a*). The 12 DEGs at day 12 post-differentiation were all upregulated in *Cstb*$^{-/-}$ samples (Fig. 6B in I). These genes formed a highly interconnected network mostly related to neuronal maturation and integrity, as judged by enrichment in neuron-related ontologies (Fig. 9C; S.Table 2 in I). Half of the genes encoded structural components of the distal axon and/or proteins involved in axon development (Fig. 9C). Our dataset also contained two out of three genes annotated under the biological process "regulation of thrombin-activated receptor signalling pathway" (*Stmn1* and *Snca*), a process involved in neuroinflammation, synaptic transmission and plasticity (Ben Shimon *et al*, 2015; Ebrahimi *et al*, 2017). Notably, mutations in five of the 12 DEGs have been individually linked to human disorders of the nervous system (Alsahli *et al*, 2018; Cuesta *et al*, 2002; Horga *et al*, 2017; Polymeropoulos *et al*, 1997; Yang *et al*, 2016).

5.3.3. CSTB-deficiency elicits mitochondrial dysfunction during neural stem cell differentiation

To further explore transcriptional regulation in the absence of CSTB, we ranked all genes measured by RNA-Seq from the most upregulated to most downregulated in *Cstb*$^{-/-}$ samples and performed a gene set enrichment analysis (GSEA) with phenotype permutation testing (1000 iterations). We found that the genes belonging to the GO term "electron transport chain" were significantly enriched at the bottom of the ranking, indicating reduced expression in *Cstb*$^{-/-}$ samples (Fig. 7A in I). The expression enrichment was present at days 1 and 5 post-differentiation, but not at day 12 or in undifferentiated NPCs (Fig. 9D). These findings could indicate that the transcriptional defects were specific for the initial stages of differentiation. However, contrasting the DEGs at other time-points with entries in MitoCarta2.0 (Pagliarini *et al*, 2008) showed that genes encoding components of the mitochondrial proteome were over-represented in undifferentiated NPCs DEG lists (Fig. 9E). In this case, 23 out of the 26 genes found in both datasets displayed upregulated expression with CSTB-deficiency. Overall, these findings show that the lack of CSTB induces a generalized dysregulation of nuclear encoded mitochondrial genes that changes from up to downregulation upon induction of differentiation.

Due to the modest changes in mRNA expression of individual genes, we could only validate the downregulation of electron transport chain genes *Ndufs2* and *Uqcrfs1* at post-differentiation day 5 using RT-qPCR on an independently generated set of mRNA samples (S.Fig. 11 in I).

To evaluate the functional consequences of these transcriptional alterations, we measured oxygen consumption in mitochondrial extracts from *Cstb*$^{-/-}$ and wt NPCs using high-resolution respirometry. In undifferentiated NPCs, oxygen consumption was relatively low and unchanged between genotypes. The induction of differentiation led to a sharp increase in mitochondrial respiration, reflecting the upregulation of OXPHOS taking place during neurogenesis (Agostini *et al.*, 2016; Zheng *et al.*, 2016). At day 5 post-differentiation, mitochondrial respiration was significantly reduced in *Cstb*$^{-/-}$ samples (Fig. 6C). Taken together, our findings strongly suggest that CSTB-deficiency impairs mitochondrial function upon NPC differentiation.

5.3.4. CSTB-deficiency induces hallmark phenotypes of cellular senescence in the developing cerebellum

Finally, we focused on the pathogenic changes predicted by sustained H3 cleavage in the *Cstb*$^{-/-}$ cerebellum. Both cathepsin L-dependent and ectopically overexpressed H3cs1 induce cellular senescence in fibroblasts and melanocytes (Duarte et al., 2014). We asked whether cellular senescence participates in the pathophysiology of CSTB-deficiency.

We first searched for signs of cellular senescence manifesting in the prodromal stages of the disease (P14 to P30), a period characterized by declining H3cs1 in wt mice and striking H3cs1 accumulation in the case of CSTB-deficiency (Fig. 2C in II).

The induction of senescence involves an irreversible cell cycle arrest that is mediated by one or several cyclin-dependent kinase inhibitors (CDKIs) (reviewed in (Martínez-Zamudio *et al*, 2017)). To test for the presence of arrested proliferation in the *Cstb*$^{-/-}$ mouse cerebellum, we stained tissue sections from *Cstb*$^{-/-}$ and wt mice for p16^{INK4a}, p27^{Kip1} and p21^{cip1}, the most frequently reported CDKIs. We identified a transient activation of p21^{cip1} in the Purkinje cell layer of the P14 cerebellum (Fig. 3D in II). IHC staining for GFAP showed that p21^{cip1} was exclusively expressed in Bergmann glia, a population of unipolar astrocytes with crucial functions during cerebellar development (Cheng *et al.*, 2018). This phenotype was consistently observed in mice of both genotypes. (Das *et al.*, 1974). However, the cerebella of *Cstb*$^{-/-}$ mice contained approximately six times more p21^{CIP1} positive cells than those of wt littermates (Fig. 3D). These data suggest that Bergmann glia undergo a developmentally programmed cell cycle arrest at P14, which is stimulated by CSTB-deficiency.

Cellular senescence is accompanied by changes in nuclear architecture that are typically associated with the enlargement of the cell nucleus (Mitsui & Schneider, 1976). To assess whether CSTB-deficiency induces neuronal phenotypes with nuclear swelling, we measured the area of NeuN-positive cell nuclei in the granule cell layer of *Cstb*$^{-/-}$ and wt mice (Fig. 3B in II). At all three time points analysed (P14, P21 and P30), the nucleus of *Cstb*$^{-/-}$ neurons was on average 25% larger than that of age-matched controls. We then asked whether this phenotype originated from changes in the expression of lamin B1, a critical component of the nuclear lamina that is typically downregulated in senescent cells (Freund *et al*, 2012). RT-qPCR and western blot analyses of cerebellar tissue samples showed that lamin B1 is similarly expressed between genotypes, both at the mRNA and at the protein levels (Fig. 3C in II). These findings show that while CSTB-deficiency is associated with nuclear swelling in cerebellar granule neurons, this phenotype does not derive from reduced expression of lamin B1.

Next, we sought to uncover whether *Cstb*$^{-/-}$ cerebella displayed abnormal or sustained DNA-damage responses (DDR), a characteristic feature of senescent cells (Collin et al, 2018; Sedelnikova et al, 2004). For this, we analysed the protein level and staining patterns of histone H2AX phosphorylated at serine 139 (γH2AX), a widely used DDR marker that targets sites of DNA double strand break (Kuo & Yang, 2008) (Fig. 3E and S.Fig 4 in II). In accordance with previous bibliography (Barral *et al*, 2014), the cerebellar levels of γH2AX declined significantly from P14 to P30 (S.Fig 4A in II). A similar profile was identified in cerebella of both genotypes, suggesting that DDR is not

fundamentally altered by CSTB-deficiency. Next, we used IHC to characterize DDR responses at the cellular level. We detected a small subset of astrocytes and oligodendrocytes displaying a bright γH2AX immunoreactivity (Fig. 3D and S.Fig 4B in II). The staining pattern was either pan-nuclear or annular (S.Fig 4C in II), indicating that these DNA damage responses are probably coupled to cell death by apoptosis (Ding *et al*, 2016; Solier & Pommier, 2014). In wt cerebella, this cell population vanished after P14, whereas in *Cstb*$^{-/-}$ mice it did not (Fig. 3E, S.Fig. 4B in II). These data expose the presence of persistent DDR in glial cells of the CSTB-deficient cerebellum, consistent with a cellular senescence phenotype.

Finally, we turned our attention toward microglia. A growing body of evidence indicates that the re-activation of microglial proliferation defines the prodromal stages of many chronic neurodegenerative diseases (Olmos-Alonso *et al*, 2016). In the long run, this pathologic behaviour elicits cellular senescence by proliferative exhaustion (Hu *et al*, 2021). To characterize the proliferation status of microglia in the pre-symptomatic *Cstb*$^{-/-}$ mouse brain, we quantified the proportion of IBA1 positive microglia expressing the cell cycle marker Ki-67 in cerebellar tissue sections from *Cstb*$^{-/-}$ and wt mice (Fig. 3F in II). In the cerebellum of control mice, microglial proliferation was abolished after the second postnatal week, in accordance with previous knowledge (Nikodemova *et al.*, 2015). On the contrary, microglia in the P14 *Cstb*$^{-/-}$ cerebellum the expression of Ki-67 was approximately twofold higher as that of age matched controls, and exhibited no significant decline by P30.

Collectively, we have exposed phenotypes associated with cellular senescence preceding the onset of neurodegeneration in *Cstb*$^{-/-}$ mice. These findings suggest that cellular senescence might play an important role in the neurodegeneration observed in *Cstb*$^{-/-}$ mice.

To explore this hypothesis further, we asked whether the accumulation of senescent cells is one of the progressive brain changes occurring during the course of the disease. For this we performed a colorimetric assay for the detection of acidic lysosomal beta galactosidase activity (SA-βGal), a widely reported senescence biomarker, in cerebellar tissue sections from *Cstb*$^{-/-}$ and wt mice. The experiments were carried out with cerebella from mice in the early and in the late stages of the disease (P30 and P180, respectively). In accordance with previous studies, SA-βGal was present in several regions of the wt brain including the Purkinje cell layer (Raffaele et al., 2020) (Fig. 3A in II and Fig. 12). In comparison to controls, *Cstb*$^{-/-}$ mice showed increased SA-βGal-positive cell densities both in the molecular and in the granule cell layers of the cerebellum (Fig. 3A in II). The phenotype was observed at both P30 and P180, with the SA-βGal-positive cell count being approximately four times greater in the older mice. The progressive accumulation of SA-βGal positive cells in the *Cstb*$^{-/-}$ mouse brain supports the notion that cellular senescence participates in the pathophysiology of CSTB-deficiency.

6. Discussion

In the present study, we show that the cysteine protease inhibitor CSTB delimits a temporal window for cathepsin L-mediated histone H3 tail proteolysis during mouse brain development. This chromatin regulatory mechanism is predicted to have an almost ubiquitous role in shaping cellular behaviour (Ali *et al.*, 2021; Dhaenens, 2021; Liu *et al*, 2017a). However, it remains fundamentally unexplored in the majority of mammalian tissues. Here we take the first steps in the characterization of the cleavage event in the neural cell lineage. Our findings have important implications to the molecular pathogenesis of CSTB-deficiency, revealing the origins of epigenetic regulation in the associated human diseases.

6.1. Role of H3cs1 in brain development

Cleavage of histone H3 between amino acids 21 and 22 (H3cs1) is an evolutionarily conserved histone modification previously linked to a multitude of cellular functions, including mESC and macrophage differentiation, yeast sporulation, malaria parasite infection and cellular senescence among others (Cheung *et al.*, 2021; Duarte *et al.*, 2014; Duncan *et al.*, 2008; Herrera-Solorio *et al.*, 2019; Santos-Rosa *et al.*, 2009). Here we find that it also participates in the development of the neural cell lineage, and provide evidence indicating that, even if this cleavage event has a relatively small scale in comparison to that of the above-mentioned examples, it must play an important role in brain physiology. This is partly evidenced by the heterogeneous distribution of H3cs1 among brain regions, developmental stages and cell types. In this biological context, H3cs1 was chromatin-bound and excluded from the lysosomal compartment, strongly suggesting that it is an epigenetically connoted histone modification and not an intermediate product of histone degradation (Dhaenens et al., 2015; Ivanov et al., 2013). Finally, the association between H3 cleavage and brain disease extends beyond this study. Indeed, the dysregulation of this process —up or down— has been recently reported in the context of infantile glioma and in a monogenic mouse model of neuropsychiatric disease (Harpaz *et al.*, 2022; Paternoster *et al*, 2021a).

The bulk of the existing knowledge, including our own findings, indicate that H3cs1 is strictly associated with cellular transitions (reviewed in (Dhaenens, 2021)). However, the molecular effects downstream of this H3cs1 remain poorly understood. In study I, we speculated that the sustained removal of histone tails resulting from CSTB-deficiency would likely interfere with the acquisition of maturity-related histone PTM profiles. In full agreement with this hypothesis, a recent study demonstrated that artificially abolishing H3cs1 triggers local epigenetic reprogramming (Cheung *et al.*, 2021). These findings implied that H3cs1 participates in chromatin priming, a group of transitional epigenetic activities required for the correct temporal regulation of gene expression (Bonifer & Cockerill, 2017). Therefore, cells probably use H3 cleavage as a way to protect genes from immediate reprogramming during temporally-protracted multi-step transitions such as those of development (depicted in Fig.3B). The observation that H3cs1 is essentially linked to the process of neurogenesis is concordant with the notion that neuron-specific epigenetic signatures are progressively shaped during NPC differentiation, whereas those of the astrocytic fate are present by default in uncommitted NPCs (Burney *et al.*, 2013; Mikkelsen *et al.*, 2007). In the postnatal mouse brain, the levels of H3cs1 are temporally correlated with the timeline of synaptogenesis, and the

endogenous repression of H3 cleavage occurs in a developmental period marked by the pruning of synapses and subsequent refinement of cellular behaviour (P14 to P30) (reviewed in (Semple *et al.*, 2013)). Taken together, our findings combined with the current understanding of epigenetic regulation suggest that the loss of H3cs1 facilitates gene expression reprogramming during the differentiation and maturation of the neural cell lineage.

6.2. Role of cysteine proteases in nuclear physiology and brain disease

To date, cathepsin L has been implicated in the proteolysis of six nuclear proteins —CUX1, histone H3, 53BP1, histone H2A, pro-caspase 6 and lamin B1— in several different biological contexts (Coradin *et al.*, 2021; Duncan *et al.*, 2008; Gonzalez-Suarez *et al.*, 2011; Goulet *et al.*, 2004; Islam *et al.*, 2022). Half of these interactions were published in the last two years, indicating that nuclear cathepsin L is an emergent player in molecular biology. The present study contributes to this research field by exposing the persistent yet developmentally regulated localization of cathepsin L in the chromatin of brain cells. Importantly, we identify histone H3 and the transcription factor CUX1 as downstream targets of cathepsin L during brain development.

On a more specific level, our findings identify nuclear cathepsin L as a key mediator of the molecular pathophysiology of CSTB-deficiency. The role of this protease in brain disease is further affirmed by recent findings showing that cathepsin L-mediated lamin B1 cleavage causes nuclear laminopathy in the context of Alzheimer's disease (Islam *et al.*, 2022). Of interest, *Cstb*$^{-/-}$ mouse cerebella did not contain the characteristic lamin B1 fragment generated by cathepsin L in a mouse model of AD, indicating that this protease is subject to multiple layers of regulation in the mammalian brain. Interstingly, our study further expands the link between nuclear cathepsin L and brain disease through the identification of histone H3 and CUX1 as proteolytic targets of cathepsin L in brain tissue; Recently, increased H3 cleavage by unknown mechanisms has been linked with schizophrenia-related mutations in transcription factor BRD1 (Paternoster *et al.*, 2021a). Moreover, the proteolytic truncation of CUX1, also by unknown mechanisms, has been reported to stimulate cancer progression in the nervous system by shifting gene expression toward EMT, glycolysis and cell cycle progression (Ceru *et al.*, 2010; Fei *et al.*, 2018; Li *et al.*, 2019). The data as a whole suggest that different forms of brain disease converge in the ectopic reactivation of nuclear cathepsin L signalling after brain development.

Interestingly, our data strongly suggest that cathepsin L and cathepsin B are jointly responsible for H3cs1 during NPC differentiation. The apparent redundancy in the molecular machinery driving H3 cleavage is not a unique feature of the neural cell lineage: trypsins and cathepsin L proteolytically process histone H3 during intestinal differentiation (Ferrari *et al.*, 2021), and a combination of three different serine proteases mediate H3cs1 during monocyte to macrophage differentiation (Cheung *et al.*, 2021). Overlapping roles for cathepsins B and L in the nervous system can be inferred from the ovserbation that genetically modified mice lacking either protease are viable, whereas double knockouts exhibit severe brain atrophy and die soon after birth (Felbor *et al*, 2002; Halangk *et al*, 2000; Nakagawa *et al*, 1998). The evidence suggesting that cathepsin B participates in chromatin regulation is not unique to our study: During mitosis, this protease stimulates chromosome

segregation by a mechanism involving proteolytic cleavage of histone H3 at its globular domain (Hämälistö *et al.*, 2020). Nonetheless, our data indicate that cathepsin B is excluded from the chromatin-bound proteome in the postnatal mouse brain. This observation suggests that cathepsin B-dependent H3cs1 is either restricted to embryionic brain development or artificially induced by culturing NPCs *in vitro*. In any case, the potential contribution of cathepsin B to histone tail cleavage should be clarified in future studies.

6.3. Cysteine protease inhibitors in epigenetic regulation

CSTB co-immunoprecipitated with cathepsin L and histones in the nucleus of astrocytoma cells (Ceru *et al.*, 2010). Moreover, the same study found that the presence of histones stimulates cathepsin L inhibition by CSTB *in vitro*. In agreement with these findings, we identify CSTB as an inhibitor of the fraction of cathepsin L that is associated with the chromatin of brain cells. Moreover, we provide evidence indicating that the effect of CSTB on cathepsin L activity is specific to this cellular compartment.

The participation of cysteine protease inhibitors in epigenetic regulation is not exclusive to CSTB. Heterochromatin-associated serpin B10 (MENT) induces changes to higher-order chromatin structure by inhibition of cathepsin L and possibly other cysteine proteases (Bulynko *et al*, 2006). Serpin B3 (SCCA1), another cross-class serpin, participates in gene regulation in the context of cervical cancer by inhibition of cathepsin L (Wang *et al*, 2022). Finally, cystatin D interacts with euchromatin and mediates gene expression reprogramming in colon adenocarcinoma cell lines (Ferrer-Mayorga *et al.*, 2015). Interestingly, none of these proteins is expressed in the mouse brain (Yao *et al*, 2021). Therefore, our data indicating that CSTB has an irreplaceable role in modulating nuclear cathepsin L in the neural cell lineage is consistent with previous findings, and provides a plausible explanation for the predominantly neural phenotype associated with CSTB-deficiency.

6.4. An epigenetic link to human brain disease via CSTB regulation of nuclear cathepsin L

The present study shows that loss of CSTB results in increased nuclear cathepsin L activity, with consequent alterations in downstream events mediated by this protease in the mouse brain. Our findings constitute the earliest molecular changes reported in *Cstb*$^{-/-}$ mice, suggesting their central role in initiating the pathogenesis downstream of CSTB-deficiency. Moreover, the notion that CSTB functions as a chromatin regulator throughout the timeline of brain development and maturation is consistent with the striking neurodevelopmental defects observed in human patients with biallelic *CSTB* null mutations (Mancini *et al.*, 2016; O'Brien *et al.*, 2017). Finally, the observation that H3cs1 is a low-abundance histone PTM has the potential to explain why residual levels of CSTB expression in EPM1 patients are sufficient to delay the onset of the symptoms from neonatal stages to childhood or adolescence (Joensuu *et al.*, 2007; Lalioti *et al.*, 1997b)

Cstb$^{-/-}$ NPCs undergo premature activation of H3 cleavage during self-renewal. In accordance with the role of H3cs1 in promoting cellular transitions (Dhaenens, 2021), we report slightly decreased progenitor self-renewing and faster fate specification with CSTB-deficiency. A recent study in

EPM1-patient-derived brain organoids also reported premature NPC differentiation, in this case evidenced by decreased proliferation and premature neurogenesis (Di Matteo *et al.*, 2020). On the contrary, we did not detect alterations in the neurogenic potential of murine *Cstb*$^{-/-}$ NPCs, but reported a striking H3cs1 enrichment in this cell type. Combined, the data of both studies suggest that the developing human brain is considerably more vulnerable to CSTB-deficiency than the murine counterpart. This is further evidenced by the observation that biallelic *CSTB* null mutations elicit neonatal-onset encephalopathy in humans, whereas *Cstb* knockout mice develop a much milder phenotype, with the associated symptomatology appearing later on in life (Joensuu *et al.*, 2014; Mancini *et al.*, 2016; Manninen *et al*, 2014a; Shannon *et al.*, 2002; Tegelberg *et al.*, 2012).

RNA sequencing of *Cstb*$^{-/-}$ NPCs unraveled gene expression changes in over a hundred genes. Of these, 86% were slightly upregulated in relation to wt cells, in agreement with reports in other cell types and with the current conception of H3 cleavage after amino acid 21 as a gene priming mechanism (Bonifer & Cockerill, 2017; Cheung *et al.*, 2021; Santos-Rosa *et al.*, 2009). During differentiation, the transcriptional alterations were in general so subtle that they could only be properly analysed using cutoff-independent methods such as GSEA. We reasoned that, if the changes are specific to neurons as expected from enrichment of H3cs1 in this cell type, these might be masked by the larger number of astrocytes present in our culture system. In agreement with this interpretation, we reported a generalized downregulation of electron transport chain genes, a functional category whose activation is intimately linked with the process of neurogenesis. Indeed, the upregulation of mitochondrial biogenesis and a metabolic transition from glycolysis to oxidative phosphorylation define the first stages of neuronal differentiation (Agostini *et al.*, 2016; Zheng *et al.*, 2016). *Cstb*$^{-/-}$ cells showed a delayed activation of OXPHOS-related genes at early and mid-stages of differentiation, with a concomitant decrease in mitochondrial respiratory capacity. An impairment in the temporal regulation of gene expression is consistent with the sustained activation of H3 cleavage during differentiation (Bonifer & Cockerill, 2017).

Recently, proteomics analysis of synaptosomes isolated from the cerebellum of pre-symptomatic *Cstb*$^{-/-}$ mice exposed alterations in the mitochondrial proteome as a central finding (Gorski *et al.*, 2020). This phenotype is temporally followed by a dramatic loss of mitochondrial respiratory capacity during the the initial stages of disease progression (Gorski, manuscript in preparation). Collectively, the data is consistent with our RNA-Seq findings, and indicate that mitochondrial dysfunction is an important contributor to the early pathophysiology of CSTB-deficiency. Of interest, the synaptosome proteomics and the NPCs transcriptomics data share other important similarities, stressing enrichment in ontologies related to intracellular transport and protein translation. The present data strongly suggest that these changes originate from alterations in the epigenetic regulation of gene expression initiated during brain development.

The latest developments in the field indicate that the repression of H3 cleavage opens a window of opportunity for gene expression reprogramming (Cheung *et al.*, 2021). Remarkably, the failure to repress H3cs1 observed in the *Cstb*$^{-/-}$ cerebellum is closely followed by the onset of myoclonus and neuronal death (Pennacchio *et al.*, 1998; Tegelberg *et al.*, 2012). In the light of the evidence, it is reasonable to speculate that the dysregulation H3cs1 plays a direct role in the pathophisology of CSTB-deficiency.

The pre-symptomatic *Cstb*$^{-/-}$ mouse brain is characterized by transcriptional and functional alterations in the establishment of GABAergic connectivity (Franceschetti *et al.*, 2007; Joensuu *et al.*, 2014). Interestingly, another gene defect —loss of transcription factor BRD1— causes strikingly similar alterations in GABAergic signalling, as well as increased histone H3 tail cleavage in the mouse brain (Paternoster *et al.*, 2021b; Qvist *et al*, 2017). These correlations suggest that over-activation of H3 cleavage might underlie the GABAergic connectivity defects downstream of both CSTB- and BRD1-deficiency. Moreover, *BRD1* is specifically upregulated following treatment with valproic acid (Dyrvig *et al*, 2017), currently the most efficient drug against the symptoms of EPM1 (Lehesjoki & Kälviäinen, 2020). The intriguing connection between BRD1 and CSTB should be weighed in future studies.

Our findings support the notion that CSTB-deficiency results in phenotypic alterations in all major cell types of the mammalian brain (Manninen *et al.*, 2014b; Pennacchio *et al.*, 1998; Tegelberg *et al.*, 2012). Besides the more obvious changes in developing neurons, we also detected glia-specific changes in pre-symptomatic stages of the disease. Astrocytes and oligodendrocytes in the *Cstb*$^{-/-}$ mouse cerebellum displayed apoptosis-associated DNA-damage responses before their generalized activation at P30. Bergmann glia, a population of astrocytes that preserves stem cell properties until adulthood (Ahlfeld *et al.*, 2017), showed signs of abnormal cell cycle arrest in the absence of CSTB. Oligodendrocytes represented less than 1% of cells produced upon *in vitro* NPC differentiation, yet the genes encoding key components of the myelin sheath showed a consistent upregulation trend in *Cstb*$^{-/-}$ samples. The presence of these early defects in gene regulation could explain the striking hypomyelination observed in brains of *CSTB*$^{-/-}$ patients (O'Brien *et al.*, 2017). Interestingly, cathepsin L and CSTB are expressed at consecutive stages of oligodendrocyte development, potentially creating a very narrow window for H3 cleavage (Marques *et al*, 2018; Marques *et al*, 2016). Accordingly, pre-myelinating oligodendrocytes lacking CSTB expression displayed an enrichment in H3cs1 unmatched by any other cell type. Collectively, these findings suggest that H3cs1 has a particularly important role in the establishment of the oligodendrocyte lineage, with potential implications for the pathophysiology of CSTB-deficiency. Finally, we found that microglia in the pre-symptomatic *Cstb*$^{-/-}$ cerebellum display sustained proliferation, a key feature that is currently understood as a converging point in the pathophysiology of many chronic and age-related neurodegenerative diseases (Hu *et al.*, 2021; Olmos-Alonso *et al.*, 2016). Considering that microglia does not originate from NPCs, the role of CSTB in this cell type remains to be determined.

Finally, we identified altered presentation of cellular senescence-associated markers in different cell types of the pre-symptomatic *Cstb*$^{-/-}$ cerebellum (Hu *et al.*, 2021; Martínez-Zamudio *et al.*, 2017). Many of the phenotypes were also present in the developing wt brain, in line with the notion that the mechanisms of cellular senescence also participate in organism development (Domínguez-Bautista *et al*, 2021; Muñoz-Espín *et al*, 2013; Storer *et al*, 2013). H3cs1 has been previously linked to the regulation of both development and senescence: During mESC differentiation, cathepsin L cleaves canonical histones H3.1 and H3.2 over the replication-independent histone variant H3.3 (Duncan *et al.*, 2008). On the other hand, the ectopic expression of cleaved H3.3 (H3.3cs1) specifically induced cellular senescence in fibroblasts and melanocytes (Duarte *et al.*, 2014). These data suggest that the signalling downstream of H3 cleavage depends on the molecular machinery mediating its

incorporation into the chromatin. In the mammalian brain, replication-independent histone dynamics gain a crucial after postnatal development, for they enable non-dividing or slowly diving cell populations to remain plastic throughout life (Maze *et al*, 2015b). Unfortunately, and due to methodological limitations, we could not visualize whether H3cs1 occurred preferentially on canonical histones or on H3.3. However, the findings combined support a model whereby H3cs1 is naturally repressed in the mature brain to prevent chromatin integration by replication-independent mechanisms, subsequently leading to cellular senescence (Duarte *et al.*, 2014).

Conclusions and future prospects

In this thesis, I have demonstrated that:

- A proteolytically modified form of histone H3 (H3cs1) marks immature post mitotic neurons during neural stem cell differentiation **(Aim 1)**.

- Histone H3 tail proteolysis is naturally repressed after postnatal brain development, being almost undetectable in the adult mouse brain **(Aim 1)**.

- Cathepsin L localizes in the chromatin of brain cells and is likely responsible for the truncation of histone H3 in the neural cell lineage **(Aim 1)**.

- Another cysteine protease, cathepsin B, might also participate in histone H3 tail cleavage during neural stem cell differentiation **(Aim 1)**.

- Cystatin B modulates the levels of H3cs1 by inhibition of nuclear cathepsin L **(Aim 2)**.

- Cystatin B-deficiency elicits ectopic histone H3 tail proteolysis before neural stem cell differentiation and in the adult mouse brain **(Aim 3)**.

- Cystatin B-deficiency results in defects in the expression of nuclear-encoded mitochondrial genes at early stages of brain development and premature brain ageing concomitant with disease progression **(Aim 3)**.

Collectively, this work identifies a layer of epigenetic regulation that couples CSTB function to healthy brain development. This study has also clarified some of the fundamental aspects of CSTB dysfunction in brain disease. However, the vast amount of new data has raised a number of questions that ought to be addressed in future studies: What is the genomic distribution of CSTB in the different cell types of the neural lineage? What is the genomic distribution of H3cs1, both in physiological conditions and in the absence of CSTB? What is the specific contribution of cysteine proteases cathepsin L and cathepsin B to the novel phenotypes associated with CSTB deficiency? What is the role of cellular senescence in the pathophysiology of CSTB-deficiency? Can we extrapolate the findings of this study to human EPM1 patients? Finally and most importantly: Can we target the molecular mechanisms reported here to develop an effective therapy for EPM1 patients?

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