Universidad Miguel Hernández - CSIC

Instituto de Neurociencias de Alicante

Departamento de Neurobiología Molecular

TESIS DOCTORAL

Role of lysine demethylase 5C in neurodevelopment and intellectual disability

Papel de la desmetilasa de lisinas 5C en desarrollo neural y discapacidad intelectual

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Programa de Doctorado en Neurociencias

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San Juan de Alicante, 20th of October 2017

DOCTORAL THESIS BY COMPENDIUM OF PUBLICATIONS

To whom it may concern:

The doctoral thesis developed by me, **Marilyn Scandaglia**, with title *"**Role of lysine demethylase 5C in neurodevelopment and intellectual disability**"*, is a compendium of publications and includes the following publications in which I am the first author.

- ***Loss of Kdm5c causes spurious transcription and prevents the fine-tuning of activity-regulated enhancers in neurons***

Scandaglia M, Lopez-Atalaya JP, Medrano-Fernandez A, Lopez-Cascales MT, Del Blanco B, Lipinski M, Benito E, Olivares R, Iwase S, Shi Y, Barco A.

Cell Rep. 2017 Oct 3;21(1):47-59. doi: 10.1016/j.celrep.2017.09.014.

- ***Fine-tuned SRF activity controls asymmetrical neuronal outgrowth: implications for cortical migration, neural tissue lamination and circuit assembly***

Scandaglia M, Benito E, Morenilla-Palao C, Fiorenza A, Del Blanco B, Coca Y, Herrera E, Barco A.

Sci Rep. 2015 Dec 7;5:17470. doi: 10.1038/srep17470.

I declare that these publications will not be used in any other thesis.

Yours sincerely,

Marilyn Scandaglia

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San Juan de Alicante, 24th of October 2017

DOCTORAL THESIS BY COMPENDIUM OF PUBLICATIONS

To whom it may concern:

The doctoral thesis developed by **Marilyn Scandaglia**, with title *"**Role of lysine demethylase 5C in neurodevelopment and intellectual disability**"*, includes a publication in *Cell Reports* (doi: 10.1016/j.celrep.2017.09.014), corresponding to the main topic of the dissertation, and a second article published in *Scientific Reports* (doi: 10.1038/srep17470) and presented as an Annex.

As the director of this Ph.D. Thesis and the corresponding author of both articles, I declare that Ms. Marilyn Scandaglia is the first author of the two articles and both articles were published in journals that belong to the first quartile (Q1) for the corresponding disciplines according to the last Journal Citation Reports (JCR).

- *Cell Reports*: Impact factor: 8.3 (2016: 26/190 Q1 Cell Biology)
- *Scientific Reports*: Impact factor: 4.3 (2016: 10/64 Q1 Multidisciplinary Sciences)

Yours sincerely,

Ángel Barco Guerrero

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Dr. Miguel Valdeolmillos
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INFORME DE LA COMISION ACADEMICA DEL PROGRAMA DE DOCTORADO EN NEUROCIENCIAS

Por la presente, la Comisión Académica del Programa de Doctorado en Neurociencias:

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Front cover: Mice lacking the lysine demethylase Kdm5c recapitulate many endophenotypes associated with Claes-Jensen type X-linked intellectual disability. Their neurons show spurious transcription from cryptic and germ line-specific promoters (depicted in the image by the leaky forefront Torricelli's column), which may importantly contribute to pathoetiology. In control mice (background column), the presence of Kdm5c (orange patches) prevents spurious transcription in adult neurons and is required during differentiation for the silencing by cytosine methylation of germ line genes (red patches) and the fine-tuning of activity-regulated enhancers (blue patches).

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... a Stefano, Silvana e Giusi

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"Quando muore il corpo sopravvive quello che hai fatto"

Rita Levi Montalcini

"Imperfection is beauty, madness is genius and it's better to be absolutely ridiculous than absolutely boring"

Marilyn Monroe

"Che mi arrendo se tu ti offendi lo sai, che con l'orgoglio non si puó amare mai"

Laura Pausini e Gianluca Grignani

"Me olvidé de poner en el suelo los pies y me siento mejor"

Extremoduro

"Everything faded into mist. The past was erased, the erasure was forgotten, the lie became truth"

George Orwell

"L'avenir a plusieurs noms. Pour les faibles, il se nomme l'impossible, pour le timides il se nomme l'inconnu, pour les penseurs et pour les vaillants, il se nomme l'idéal"

Victor Hugo

"Vita, si uti scias, longa est"

Seneca

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ABSTRACT

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Numerous neurodevelopmental syndromes are caused by mutations in chromatin-modifying enzymes. One of such enzymes, the lysine demethylase 5C (KDM5C) is mutated in Claes-Jensen X-linked intellectual disability (CJ-XLID), a neurodevelopmental disorder associated with intellectual problems and adaptive behaviour impairment. KDM5C demethylates histone H3 di- or trimethylated at lysine 4 (H3K4me2/3), two histone modifications associated with active transcription. In this thesis, I used murine and cellular models of the disease to gain deeper insight into the genomic actions of this protein and its differential role in the developing and adult brain. We compared the behaviour, transcriptome and epigenomic landscapes of *Kdm5c* null (KO) and forebrain-restricted inducible knockout (ifKO) mice. KOs showed strong neurological phenotypes mimicking patients' symptoms such as decreased learning and memory, epileptic seizure propensity and altered emotions. In contrast, ifKOs only presented some spatial learning deficits, suggesting a predominant developmental component in KDM5C-associated ID. Furthermore, consistent with the proposed general function for Kdm5c as a transcriptional repressor, both KO and ifKO mice showed excessive hippocampal transcription and histone H3K4 tri-methylation at promoters and enhancers. In particular, exclusively KO mice showed (i) neuronal expression of germ line genes that escaped developmental silencing by DNA methylation, and (ii) over-activation of activity-regulated enhancers involved in cognitive processes, such as those at the Npas4, Arc and Fos loci. Although the importance of these functions declines after neuronal maturation, Kdm5c still retains a genome surveillance role preventing the illegitimate activation of non-neuronal and cryptic promoters in adult neurons. Finally, to characterize how a disease-associated mutation affects early stages of human brain development, I produced the first cerebral organoids derived from induced pluripotent stem cells (iPSCs) of CJ-XLID patients.

Resumen (Español)

Numerosos síndromes que afectan el desarrollo del sistema nervioso están causados por mutaciones en enzimas modificadoras de la cromatina. El gen que codifica una de estas enzimas, la desmetilasa de lisinas 5C (KDM5C), está mutado en pacientes con discapacidad intelectual vinculada al cromosoma X de tipo Claes-Jensen (CJ-XLID), un trastorno asociado con problemas neurológicos, intelectuales y alteraciones del comportamiento adaptativo. KDM5C desmetila la histona H3 di- o trimetilada en la lisina 4 (H3K4me2/3), dos modificaciones de esta histona que están asociadas con la transcripción activa. En esta tesis, he utilizado modelos murinos y celulares de la enfermedad para determinar las acciones genómicas de esta proteína y su papel diferencial en el cerebro adulto y durante el desarrollo. Para ello, comparamos la conducta, el transcriptoma y los perfiles epigenómicos de ratones *knockout* para Kdm5c (KO) y ratones *knockout* inducibles en los que la perdida de Kdm5c se restringe al prosencéfalo adulto (ifKO). Mientras los ratones KO mostraron fuertes fenotipos neurológicos que reproducen síntomas observados en pacientes (como los deficits en aprendizaje y memoria, la propensión a ataques epilépticos y emociones alteradas), los ifKOs solo presentaron algunos déficits de aprendizaje espacial. Estos resultados sugieren un papel preponderante de Kdm5c durante el desarrollo en la etiología de los problemas cognitivos y neurológicos. Además, de acuerdo con la función general propuesta para Kdm5c como represor transcripcional, tanto los ratones KO como los ifKO mostraron una transcripción de hipocampo excesiva y un aumento de la tri-metilación de la histona H3K4 en promotores y secuencias reguladoras de tipo *enhancer*. Algunas de estas alteraciones son exclusivas de ratones KO, como (i) la expresión en neuronas de genes de línea germinal que escaparon al silenciamiento por metilación del ADN durante la diferenciación celular, y (ii) la sobreactivación de

secuencias reguladoras que modulan la transcripción de genes implicados en la respuesta neuronal a estímulos, como *Npas4*, *Arc* y *Fos*. Aunque la importancia de estas funciones disminuye en neuronas maduras, en estas Kdm5c todavía conserva un papel de vigilancia del genoma que evita la activación ilegítima de promotores no neuronales y crípticos. Finalmente, para caracterizar el impacto de una mutación asociada a CJ-XLID en las primeras etapas del desarrollo del cerebro humano, he producido los primeros organoides cerebrales derivados de células madres inducidas pluripotentes (iPSCs) procedentes de pacientes con CJ-XLID.

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TABLE OFABBREVIATIONS

5hmC	5-hydroximethylcytosine
5mC	5-methylcytosine
ADHD	Attention deficit hyperactivity disorder
AML	Acute myeloid leukemia
AMY	Amygdala
ARID	Autosomal Recessive ID
ARID/Bright	AT-rich interaction domain/B-cell-specific trans-activator of IgH transcription
ARX	Aristaless-related homeobox
ASD	Autism spectrum disorder
ATRX	Alpha thalassemia/mental retardation X-linked
BDNF	Brain derived neurotrophic factor
BWS	Beckwith-Wiedemann syndrome
CAS9	CRISPR associated system 9
ccRCC	Clear cell renal cell carcinoma
ChIP	Chromatin Immunoprecipitation
ChIP-Seq	Chromatin Immunoprecipitation sequencing
CNS	Central nervous system
CNV	Copy number variation
CO	Cerebral organoid
CoREST	REST corepressor
CPRF	Cleft palate, psychomotor retardation and distinctive facial features
CR	Chromatin remodelling
CRISPR	Clustered regularly interspaced short palindromic repeat
CtBP1	C-term binding protein 1
CUL4	Cullin 4
DD	Developmental delay
DDX3X	DEAD (Asp-Glu-Ala-Asp) Box Helicase 3, X-linked
DE	Differentially expressed
DER	Differentially expressed region
DNMT	DNA methyl transferase
DOT1L	DOT1 like histone lysine methyltransferase
EB	Embryoid body
EHMT	Euchromatic histone lysine methyltransferase
eRNA	enhancer RNA
ESC	Embryonic stem cell
EZH	Enhancer of zeste 1 homologue
FAD	Flavin adenosine dinucleotide
FXS	Fragile X syndrome
gDMR	Germline differential methylated regions
GABA	Gamma-aminobutyric acid

GO	Gene Ontology
GV	Germinal vescicle
HCC	Human hepatocellular carcinoma
HDAC	Histone deacetylase
HIF	Hypoxia-inducible factor
HP1	Heterochromatin protein 1
HPTM	Histone post-translational modification
ICF	Immunodeficiency centromeric instability and facial dysmorphism
ICR	Imprinting control region
ID	Intellectual disability
IDD	Intellectual disability disorder
IEG	Immediate early gene
IHC	immunohistochemistry
IQ	intelligence quotient
JARID1C	Jumonji/ARID domain-containing protein 1C
JmjC	jumonji C
JmjN	jumonji N
K	Lysine
KA	Kainic acid
KABUK1	Kabuki syndrome 1
KABUK2	Kabuki syndrome 2
KAT	Lysine acetyltransferase
KD	Knock-down
KDM	Lysine demethylase
KMT	Lysine methyltransferase
KO	Knock-out
KS	Kleefstra syndrome
KSS	Kleefstra syndrome spectrum
lincRNA	Large intergenic non-coding RNA
lncRNA	Long non-coding RNA
LoF	Loss of function
LSD1	Lysine-specific demethylase 1
MAX	Myc-associated factor X
MDS	Myelodysplastic syndrome
MeCP2	Methyl-CpG-binding protein 2
MEF	Mouse embryonic fibroblast
METH	Methanphetamine
MFH	Malignant fibrous histiocytoma
MII	meiosis II
miRNA	micro RNA
MLL	Mixed lineage leukemia
MR	Mental retardation
MWM	Morris Water Maze
Naa11	N(Alpha)-Acetyltransferase 11, NatA Catalytic Subunit

NAc	Nucleus Accumbens
ncRNA	non-coding RNA
NE	Novel environment
NPC	Neuroprogenitor cell
NRSE	Neuron-restrictive silencing element
NSD	Nuclear receptor-binding SET domain protein
NS-XLMR	Non syndromic X-linked mental retardation
PCGF6	Polycomb group ring finger 6
PFC	Prefrontal cortex
PGC	Primordial germ cell
PHD	Plant homeodomain
PHF8	PHD finger protein 8
piRNA	Piwi-interacting RNA
PNC	Primary neuronal culture
PRC	Polycomb Repressor Complex
PTM	Post-translational modification
PTZ	Pentylenetetrazol
PWS	Prader-Willi Syndrome
qPCR	quantitative PCR
R	Arginine
RA	Retinoic acid
Ras/MAPK	Rat sarcoma/Mitogen-activated protein kinase
RBBP5	Retinoblastoma-binding protein 5
RBP	Retinoblastoma-binding protein
RBP-J	Recombination signal-binding protein Jk
RBR	Retinoblastoma-binding protein related
REST	Repressor element-1 silencing transcription factor
RNA-Seq	RNA sequencing
RTT	Rett Syndrome
S	Serine
SAM	S-adenosyl-L-methionine
SCN2A	Sodium channel type 2A
SET	Su(var)3-9, Enhancer-of-zeste, Trithorax
SETD1A	SET domain containing 1A
shRNA	short hairpin RNA
siRNA	small interfering RNA
SLS	Sotos like syndrome
SMCX	Selected mouse cDNA on the X
SRF	Serum Response Factor
SS	Sotos syndrome
SYN1	Synapsin 1
TET	Ten-eleven translocation
TF	Transcription factor
TSS	Transcription start site

UTX	Ubiquitously transcribed tetratricopeptide repeat containing, X-linked
VHL	Von hippel Lindau
WDSTS	Wiedemann-Steiner syndrome
WHS	Wolf-Hirshhorn syndrome
WT	Wild-type
WVS	Weaver syndrome
XCI	X chromosome inactivation
XIST	X Inactive Specific Transcript
XLID	X-linked Intellectual disability
XLMR	X-linked mental retardation
Zf	Zinc finger
Zn	Zinc

GENERAL INTRODUCTION

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1.1. Intellectual disability

Intellectual disability (ID), which until recently was referred to as mental retardation (MR), is a prevalent global condition characterized by variable degrees of limitations of intellectual functioning, and social and behavioural adaptation problems in many daily practical skills. ID has an estimated prevalence of 2% to 3%, and about 0.3% to 0.5% of the population is severely handicapped.

The intellectual functioning or intelligence (the general mental capacity of learning, reasoning, problem solving and so on) is usually measured by an intelligence quotient (IQ) test. Generally, an IQ test score lower than 70 indicates a limitation in intellectual functioning. In turn, adaptive behaviour is the set of different skills that are learned and performed by people in their everyday lives. They can be subdivided into conceptual skills (language, time, and number concepts), social skills (interpersonal skills, self-esteem, naïveté, social problem solving), and practical skills (occupational skills, personal healthcare and safety). The limitations in adaptive behaviour can also be determined using standardized tests. Importantly, the community environment, the level of life and the linguistic and cultural diversity should be considered during assessment of ID because they influence the way people communicate and behave.

ID disorders (IDDs) represent a vast and heterogeneous group of neurodevelopmental disorders. They originate during the developmental period, before the age of 18 (pre-adult) (American Psychiatric Association 2000). Epidemiological studies often use a simplified classification, grouping their subjects into mild ID (IQ of 50-70) and severe ID (IQ < 50) (Ropers and Hamel 2005). Approximately 85% of all ID-affected patients can be classified as mild ID. IDDs are also often subdivided in syndromic and non-syndromic IDs (Kaufman, Ayub et al. 2010), depending on the presence or absence of other physical, neurological and/or psychiatric manifestations

apart from ID. However, the boundary between these two forms of ID is often arbitrary because of the difficulty in diagnosing syndromic features in the absence of careful comparative clinical examinations among several patients with a common etiology. The standard screening tests that take place during pregnancy or baseline genetics diagnostic tests conducted after birth do not identify most IDDs. Whole exome sequencing could be used to identify patients with ID but often medical professionals do not offer this option or the costs associated with it are not covered by insurance. As a result, patients are frequently given incorrect medical explanations and an unprecise broad diagnosis.

IDDs, in its more severe manifestations, lead to lifelong emotional and cognitive disability representing an important personal and healthcare burden worldwide. Together with other neuropsychiatric conditions, they constitute a leading socio-economic problem of health care costs in Western countries (Ropers 2010). Regarding the treatment and therapy, there is no specific cure or treatment for these syndromes. Children with ID may benefit from a range of supportive treatments such as physiotherapy, speech therapy, supplementary nutrition for poor feeding, and special educational support. Each individual with ID has a unique profile, based on his or her level of language functioning as well as cognitive level, speech production skills, and emotional status. Improvements often can be achieved by providing adequate personalized supports by medical team and professionals (Ropers 2010, Schalock and Luckasson 2015, Tasse, Luckasson et al. 2016).

1.1.1. Etiology of IDD

The causes of ID are diverse, including both environmental and genetic factors (van Bokhoven 2011) although for up to 60% of cases there is no identifiable cause (Rauch, Hoyer et al. 2006). Environmental exposure to certain teratogens, viruses or radiation

can cause ID, as can severe head trauma, injury leading to lack of oxygen in the brain, fetal alcohol exposure or prenatal disease of the mother. Despite its universal occurrence, there tends to be higher prevalence of ID in areas of lower socioeconomic status and developing countries, particularly for mild cases (Emerson 2007). While these factors explain some cases of ID, it is also important to consider that genetic causes of ID are responsible of 25–50% of cases. The greatest number of cases with a genetic cause are patients with Down syndrome (anomaly that occurs in 15 of every 10,000 births) and is caused by chromosome 21 trisomy or the translocation of chromosomes 21 and 15 (Rauch, Hoyer et al. 2006). Other much less frequent chromosomal abnormalities are those responsible of fragile X chromosome syndrome (FXS), Prader-Willi syndrome (PWS), Rett syndrome (RTT), neurofibromatosis, etc. In fact, a wide variety of genetic mutations, ranging from large cytogenetically chromatin abnormalities to single gene mutations, can cause ID (van Bokhoven, 2011). These genetic mutations can be either *de novo* or inherited and depending of their location can be also classified in X-linked (which account for 5-10% of male ID patients) (Tzschach, Grasshoff et al. 2015), or autosomal.

The X-chromosome has historically been the most thoroughly studied chromosome with regard to ID due to the high male to female ratio among the affected population (there is approximately 30% more male than female ID patients). IDDs originated by mutations into the sexual X chromosome are named X-linked ID (XLID) disorders and constitute >10% of all ID cases. Until now, more than 100 XLID genes have been identified (Ropers and Hamel 2005, Bassani, Zapata et al. 2013). Since males only have one copy of the X chromosome, they are naturally hemizygotes for all X-linked genes therefore a monoallelic mutation would cause loss of function. In contrast, females with one affected X and one normal allele would show no symptoms or milder

symptoms. Similarly to other IDDs, XLID can be classified as non-syndromic and syndromic (Gecz, Shoubridge et al. 2009, Raymond 2006, Lubs, Stevenson et al. 2012, Tzschach, Grasshoff et al. 2015) (**Figure 1**).

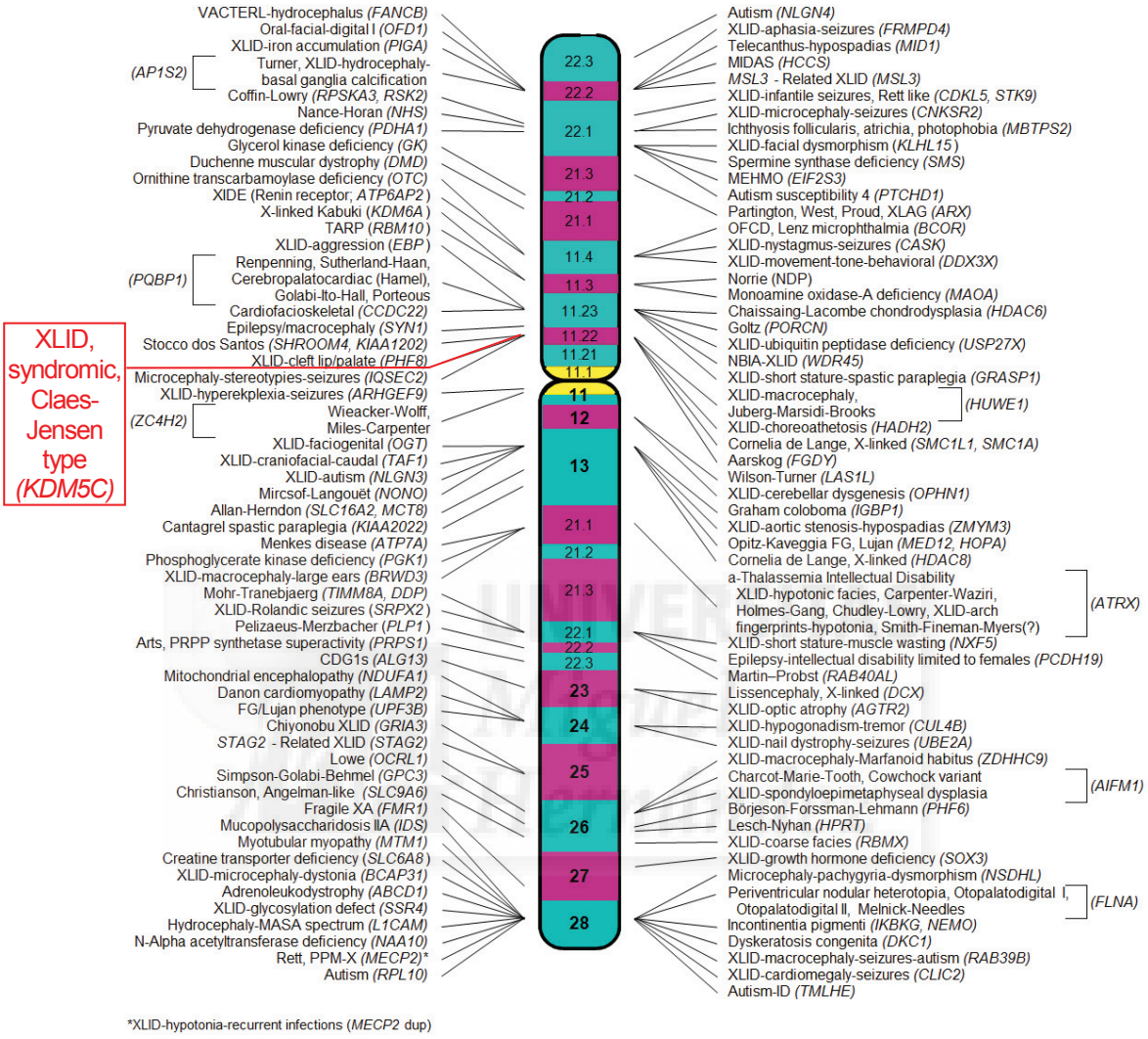

Figure 1. List of syndromic ID genes localized in the chromosome X. Alternate sub-bands are highlighted in violet and blue colours. The yellow band corresponds to the centromere. The location of *KDM5C*, main focus of this thesis, is labeled in red. From Greenwood Genetic Center, updated April 2017.

Studying the biological causes of ID might help us to decipher relevant pathways that are involved in neurodevelopment and human cognition and aid us in treating or relieving symptoms of IDD patients. IDD genes often belong to common cellular pathways and participate in related processes. This is the case, for example, of those

involved in Ras/MAPK signalling and components of the synaptic complex (Pavlowsky, Chelly et al. 2012, Bassani, Zapata et al. 2013). More important in the frame of this thesis are the genetic disorders linked to ID and caused by mutations in chromatin regulators, components of the epigenetic machinery (writers, erasers, readers, and remodelers), that are expected to have widespread downstream consequences (Kleefstra, Schenck et al. 2014, Bjornsson 2015) as I will explain more deeply in Section 1.2.6.

1.2. Principles of epigenetics

The development of multicellular organisms requires the specification of a plethora of different cell types and organs. This depends on the fine-tuned regulation of gene expression by the orchestrated action of transcription factors (TFs) and epigenetic mechanisms. The classical definition of *epigenetics* comes from Conrad Waddington in 1942 as ‘‘the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being’’ (Waddington 2012). Afterwards, Holliday added to that definition the concept of heritability of a phenotype passed on through either mitosis or meiosis (Holliday 1994). Along with the increase in the number of studies in the field and the discoveries of different epigenetic mechanisms, the word epigenetics has gained a definitional identity crisis (Deans and Maggert 2015). Nowadays, a working definition from Berger and colleagues (Berger, Kouzarides et al. 2009) contemplates a “stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence” and agrees with the definition present in the Oxford Dictionary of Biochemistry and Molecular Biology as ‘‘the study of factors that influence gene expression but do not alter the genotype, such as

chromatin methylation and acetylation involved in tissue specific patterns of gene expression, or the parental imprinting of genes''(Wood 2007).

Post-mitotic cells such as neurons contemplate a different meaning of epigenetics. Mature neurons are not dividing cells, therefore, chromatin modifications are not passed to daughter cells but these mechanisms have a pivotal role during embryonic stem cells (ESCs) differentiation and neurogenesis and the chromatin changes can be maintained until the neural lineage mature cells are formed (Yao, Christian et al. 2016, Atlasi and Stunnenberg 2017). In addition to these developmentally programed changes, the chromatin of a mature neuron can also be influenced by other factors including age, environmental conditions and disease states. For this reason, Day and Sweatt have proposed adopting the term *neuroepigenetic mechanisms* to distinguish chromatin changes in the adult brain from heritable epigenetic marks involved in development, cell-fate determination and cell division (Day and Sweatt 2010). It has been shown, indeed, that some chromatin marks change their profiles in neuronal cells after behavioural experiences (Sweatt 2009, Tognini, Napoli et al. 2015).

In the context of this thesis, I will refer to epigenetic mechanisms as the modifications of the DNA and its associated proteins that modify how genes are expressed without altering the underlying DNA sequence. This can be achieved, for instance, through the addition of chemical groups to histone tails or cytosine nucleotides in the DNA (**Figure 2**). Alterations to nucleosome positioning or composition, the action of regulatory non-coding RNAs (ncRNAs), irregular chromosome structure organization and histone replacements are also considered epigenetic modifications.

Understanding the mechanisms involved in the initiation, maintenance, and heritability of chromatin states is an important aspect of research in current biology. As chromatin changes contribute to define the distinct gene-expression profiles for each

individual cell type at specific developmental stages, it is evident that the disruption of such control can cause a multitude of diseases including disorders of tissue growth (i.e. cancer) and diverse neurological disorders. Furthermore, current research is focused on characterizing cell type or tissue-specific chromatin marks, in human health and disease, studying the interaction between them and the risks for common and adult onset disorders (Allis and Jenuwein 2016).

I will discuss more in detail the current conception of the main epigenetic mechanisms in the next section, focusing on their link with neuroscience.

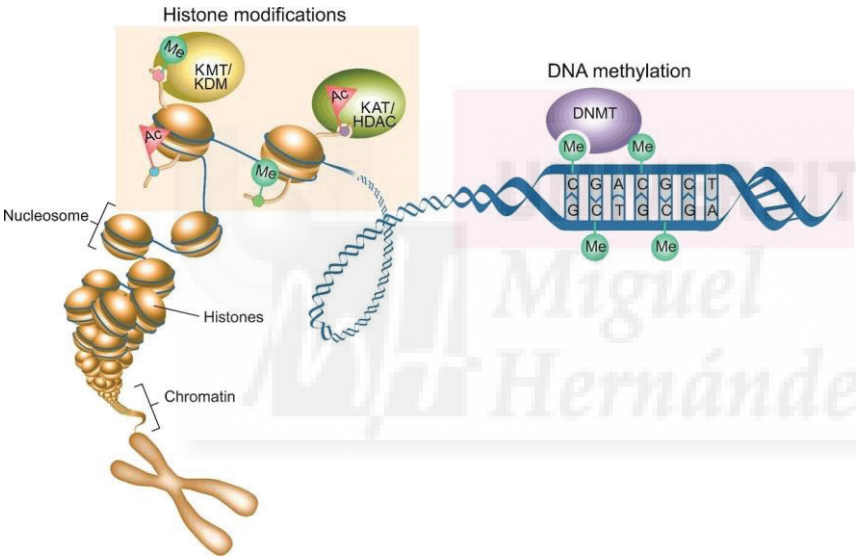

Figure 2. Main epigenetic mechanisms acting on the chromatin: histone modifications and DNA methylation. More details on their functioning are described in the next sections. Adapted from (Li and Casanueva 2016)

1.2.1. Chromatin organization

The nucleosome is the fundamental unit of chromatin structure. It is constituted by ~145-147 bp of double-helical DNA wrapped around the canonical histone octamer (two copies each of H2A, H2B, H3 and H4). Multiple levels of packaging establish chromatin conformations that can be either relaxed (euchromatin) or tightened (heterochromatin) to either enable or repress transcription in specific cells at critical times during development, respectively. Highly active genes locate within euchromatin and are associated with an open chromatin environment, often with a short nucleosome-

free segment of DNA near the transcriptional start site (TSS). In turn, heterochromatin exists in two forms; facultative and constitutive. Facultative heterochromatin is interspersed within chromosomes and mediates context-dependent, transient epigenetic silencing of genes in processes such as developmental programming, cell fate determination, mating-type gene silencing, and X-chromosome inactivation (XCI). Constitutive heterochromatin is present at sites marked by repetitive genetic elements, including telomeric and centromeric repetitive regions that cannot be transcribed due to the highly compact heterochromatin environment which limits the access of TFs and the RNA polymerase complex. Further regulation is accomplished by assembling promoter-enhancer complexes via long-range chromatin looping, a process that is regulated by specific DNA sequences called insulators, which often locate at the points of intra-chromosomal contacts from which the loops emanate (Chetverina, Fujioka et al. 2017).

1.2.2. DNA methylation

DNA methylation is considered one of the main chromatin modifications and it acts directly on the double helix. It involves the transfer of a methyl group to cytosine (5-methylcytosine or 5mC) in a CpG dinucleotide. Regions rich in CpG dinucleotides are called CpG islands and are critical regions for transcriptional regulation where several epigenetic modifications can occur.

In mammalian cells, DNA methylation occurs predominantly in CpG dinucleotides within repetitive elements and in some non-repetitive sequences in intergenic and intragenic regions (Smith and Meissner 2013, Shin, Ming et al. 2014). While CpG methylation is usually occurring on the promoters of genes that need to remain silent, it is excluded from active gene promoter regions. Typically CpG islands, not only in proximal promoters of expressed genes, are almost always unmethylated in

normal tissues, with the exception of imprinted genes, X-inactivated genes, retrotransposons, and a few genes with tissue-specific silencing (Costello and Plass 2001, Straussman, Nejman et al. 2009). DNA methylation at CpG island-associated promoters usually causes gene silencing, either by directly interacting with TFs or by recruiting methyl-binding proteins that in turn recruit histone-modifying enzymes responsible for establishing a repressive state (Portela and Esteller 2010).

Therefore, whereas DNA methylation is not necessary *per se* for transcriptional silencing, its presence is thought to represent a "locked" state that inactivates transcription. In particular, DNA methylation appears critical for the maintenance of mono-allelic silencing in the context of genomic imprinting and XCI (Bourc'his, Xu et al. 2001, Marzi, Meaburn et al. 2016), which is an essential mechanism to compensate for dosage imbalances between male and female embryos.

In mammals, DNA methylation is carried out by two general classes of enzymatic activities that either establish (*de novo* methylation) or maintain the patterns of methylation through cell division. This maintenance activity is necessary to preserve DNA methylation after every cellular DNA replication cycle. DNMT1 is the maintenance methyltransferase that recognizes hemi-methylated DNA and is responsible for copying DNA methylation patterns to the daughter strands during DNA replication, whereas, d*e novo* methylation is carried out by DNMT3A and DNMT3B that set up DNA methylation patterns early in development (Smith and Meissner 2013). Genetic engineering in mouse has informed that Dnmt1 and 3b are essential for embryonic development, while Dnmt3a KO mice survive for few weeks after birth (Li, Bestor et al. 1992, Okano, Bell et al. 1999).

DNA methylation patterns are largely erased and then re-established between generations. Almost all methylations from the parents need to be erased, first during

gametogenesis, and again in early embryogenesis, with demethylation and re-methylation occurring each time. Demethylation in early embryogenesis occurs in the pre-implantation period in two stages, first in the zygote, then during the first few embryonic replication cycles of morula and blastula. Subsequently, a wave of methylation takes place during the implantation stage of the embryo. In the post-implantation stage, methylation patterns are stage- and tissue-specific, with changes that would define each individual cell type lasting stably in post-mitotic cells throughout their lifetime (**Figure 3**) (Smallwood and Kelsey 2012, Messerschmidt, Knowles et al. 2014, Lucifero, La Salle et al. 2007, Borgel, Guibert et al. 2010, Hackett, Reddington et al. 2012).

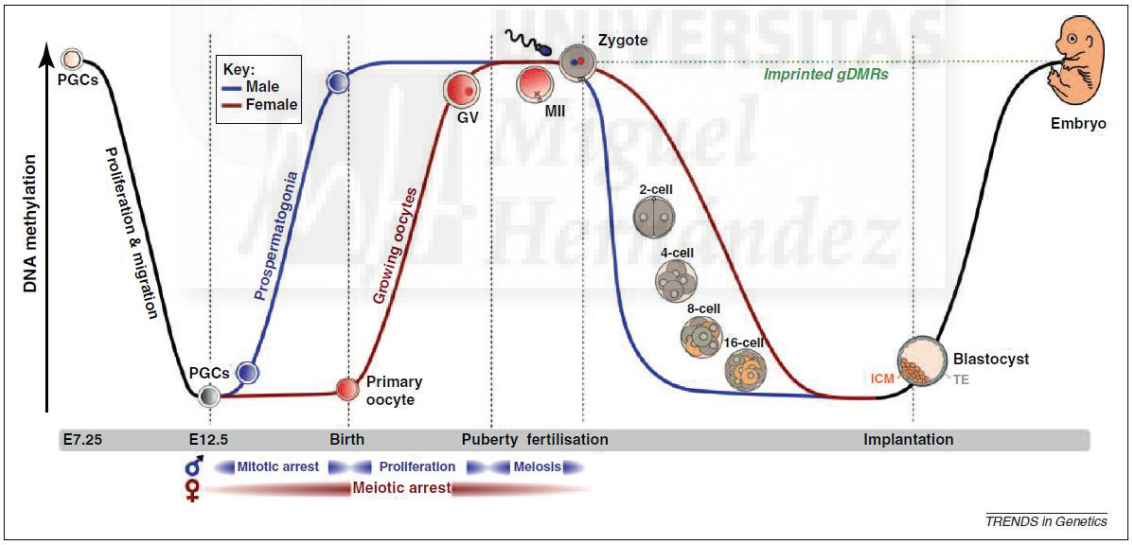

Figure 3. DNA methylation during developmental reprogramming. DNA methylation is erased while primordial germ cells (PGCs) proliferate and migrate and during the first stages of zygote mitoses. During female and male germ-cell precursors development and after the blastocyst stage, new DNA methylation patterns are differentially established. GV: germinal vesicle oocytes; MII: second meiotic division oocytes; gDMRs: germline differential methylated regions. From Smallwood and Kelsey 2012.

Importantly, the general protection of CpG islands from methylation can break down in pathological conditions, particularly in cancer cells, leading to aberrant gene

silencing (Jones and Baylin 2002, Delpu, Cordelier et al. 2013). Defects in the regulation or detection of 5mC cause severe disorders in humans including some IDDs such as immunodeficiency, centromeric instability, and facial dysmorphism (ICF), FXS and imprinting disorders (Xu, Bestor et al. 1999, Hagleitner, Lankester et al. 2008, Klein, Botuyan et al. 2011, Winkelmann, Lin et al. 2012, Weng, An et al. 2013, Al-Mahdawi, Virmouni et al. 2014, Xin, Cruz Marino et al. 2017).

In addition to 5mC, mammalian DNA contains a related modified base, 5-hydroxymethylcytosine (5hmC), that has been found in many cell types and tissues, with particularly high levels in the brain and in ESCs. Ten-eleven translocation (TET) proteins are dioxygenases that catalyze the conversion of the modified genomic base 5mC into 5hmC and are involved in further oxidative DNA demethylation process (Tahiliani, Koh et al. 2009, Wu and Zhang 2010).

1.2.3. Histone post-translational modifications

Chromatin is a very dynamic structure subjected to modulation in response to both internal and external signals. Cellular events, ranging from development to tumorigenesis, rely on gene regulation mechanisms that target the nucleosome but the function of the genome depends not only on the DNA sequence and the main histone octamer core but also on the presence of variant histones (i.e. H3.3, cenH3, H2A.X, H2A.Z) and the covalent post-translational modifications of histone amino acids (HPTMs) that modulate the action of effector molecules on transcription (Luger, Dechassa et al. 2012). As a result, chromatin plasticity depends on the activity of histone-modifying enzymes and on the transcriptional regulation that derives from these reversible covalent modifications (Strahl and Allis 2000, Zentner and Henikoff 2013).

HPTMs often occur at lysine (K), arginine (R) or serine (S) amino acid residues in the loosely structured N-terminal histone tails that project from the tightly structured nucleosome cores. They include acetylation, phosphorylation, methylation, mono- or poly-ADP ribosylation, ubiquitination, glycosylation and SUMOylation, but this list is likely to grow. HPTMs can have different effects on gene activity because they influence the recruitment of non-histone proteins such as TFs and repressors complexes to specific DNA sequences involved in transcriptional regulation. Histone tail marks or a combination of them constitute critical factors for the regulation of gene expression determining specific gene states spanning between active, silenced or poised. Certain histone modifications can establish, and potentially maintain, active or silent epigenetic states.

For example, histone acetylation is thought to be coupled to gene activation, while SUMOylation is often associated with gene repression. Histone methylation, on the other hand, is a more complex process and can be associated with both gene activation and repression depending on the particular amino acid residue modified and the number of methyl groups added. An example is given by the dynamic state of gene promoters during differentiation from stem cells to neural precursors and, finally, to differentiated neurons (Olynik and Rastegar 2012, Hardwick and Philpott 2014). Indeed, lineage-specific genes, that in stem/progenitor level are kept silenced but prompted to be activated in the following stages, present their promoters/regulative regions with both activating (e.g., H3K4me3, H3K9ac) and repressive (H3K27me3) marks. During fate transitions, specific enzymes can resolve these bivalent or poised states by removing the first or the latter, to keep the genes silenced (e.g., neuronal genes in astrocytes) or to turn them on (Mikkelsen, Ku et al. 2007, Mohn, Weber et al. 2008, Hirabayashi, Suzki et al. 2009).

Therefore, chromatin structure and function are largely regulated by the activities of histone-modifying enzymes. The enzymes dedicated to the deposition of the histone mark are usually called "writers", while the enzymes that catalyzed the opposite reaction, by removing the mark, are called "erasers". Other proteins can bind and "interpret" the mark and are so named "readers". The modifications catalysed by this large array of enzymes can be sequential and interdependent, mutually exclusive, or independent. Among the most studied cellular enzymes that catalyze histone modifications there are lysine acetyltransferases (KATs), histone deacetylases (HDACs), lysine methyltransferases (KMTs) and lysine demethylases (KDMs). Importantly, mutations in some of these chromatin modifiers can lead to human disease.

1.2.4. Regulatory non-coding RNAs

Another level of epigenetic regulation is achieved through the action of ncRNAs that have gained widespread interest in the past decade owing to their unexpected large number and function in a variety of biological processes. These include microRNAs (miRNAs), small interfering RNA (siRNA), Piwi-interacting RNAs (piRNAs), enhancer RNA (eRNAs) and long non-coding RNAs (lncRNAs). They all play important roles in the regulation of gene expression at several levels of transcription, mRNA degradation, splicing, transport and translation (Kaikkonen, Lam et al. 2011). Some of these species participate in epigenetic regulation through chromatin modifications.

The main function of miRNAs and siRNAs consists in regulating post-trascriptionally homologous mRNAs and may cause translational repression or degradation (Fabian, Sonenberg et al. 2010, Fiorenza and Barco 2016, Dana, Chalbatani et al. 2017). However, both types of small RNA molecules are also implicated in transcriptional gene regulation through modification of epigenetic marks. For instance,

the miRNA mir-320, one conserved miRNA encoded within the promoter region of the cell cycle gene *POLR3D* in the antisense orientation, silences transcriptionally POLR3D through recruitment of the H3K27 methyltransferase EZH2 (Kim, Saetrom et al. 2008).

PiRNAs show sequence complementarity to a variety of transposable and repetitive elements, thus the primary role of these small RNAs seems to be the suppression of transposon activity during germ line development (Aravin, Lagos-Quintana et al. 2003).

LncRNAs are found at various locations in relation to annotated protein-coding genes. They may be antisense, intronic, intergenic, promoter- or enhancer-associated and can regulate transcription both in *cis* and *trans* by a number of different mechanisms (Ponting, Oliver et al. 2009). A subgroup of lncRNAs, called large intergenic non-coding RNAs (lincRNAs) have been suggested to guide chromatin-modifying complexes to specific genomic loci and this way participate in the establishment of cell type specific epigenetic states (Guttman, Amit et al. 2009, Khalil, Guttman et al. 2009). The most famous is *XIST* lncRNA that is essential for initiating XCI (Zhao, Sun et al. 2008). HOTAIR is another example of non-imprinted lncRNA expressed from the *HOXC* gene. It recruits polycomb repressive complex 2 (PRC2) and histone demethylase LSD1 resulting in acquisition of silencing histone marks at genes within the HOXD gene cluster located on another chromosome (Tsai, Manor et al. 2010).

Lastly, some lncRNAs exert function as enhancers activating gene transcription by serving as cis-regulatory molecules. Recent studies have demonstrated that many enhancer elements can be transcribed and produce RNA molecules, which are named eRNAs. These eRNAs directly regulate enhancer activity in gene activation through

various mechanisms (Natoli and Andrau 2012, Chen, Du et al. 2017). Thus, mammalian genomes are populated with thousands of active enhancers that can be dynamically tuned to elicit alternative gene expression programs, which may underlie many sequential gene expression events in development, cell differentiation and disease progression (Wang, Garcia-Bassets et al. 2011). Interestingly for neuronal function it has been shown that the level of eRNA expression at neuronal enhancers positively correlates with the level of messenger RNA synthesis at nearby genes. The enhancers of immediate early genes (IEGs) have been particularly studied (Kim, Hemberg et al. 2010, Malik, Vierbuchen et al. 2014, Schaukowitch, Joo et al. 2014). eRNAs have also been identified in many non-neuronal cell types and their production is recognized as a reliable marker for active enhancers (Andersson, Gebhard et al. 2014).

1.2.5. Interaction between different epigenetic mechanisms

Similarly to the interplay between lncRNAs and histone modifiers commented above, DNA methylation and HPTMs crosstalk occurs through interactions of enzymes and other proteins that create and recognize these patterns (Lehnertz, Ueda et al. 2003, Epsztejn-Litman, Feldman et al. 2008, Cedar and Bergman 2009, Du, Johnson et al. 2015, Atlasi and Stunnenberg 2017). The relationship between these two central forms of chromatin modification is known to be bi-directional, with histone marks being more labile and DNA methylation more stable. In embryonic development, the modification of histone marks usually precedes and guides *de novo* DNA methylation, either by recruiting histone methyltransferase enzymes for repressive marks such as H3K9 methylation or by protecting DNA from *de novo* methylation through the addition of active marks like H3K4 trymethylation (Ooi, Qiu et al. 2007, Epsztejn-Litman, Feldman et al. 2008, Tachibana, Matsumura et al. 2008, Smallwood and Kelsey 2012). Thus,

DNA methylation can act to “lock-in” epigenetic states. However, regulating metastable states of gene expression is so crucial during development and tissue homeostasis that other mechanisms, in addition to histone modifications and DNA methylation, come into play to establish and maintain epigenetic states (Zhang, Cooper et al. 2015).

Developmental genes may be poised for activation during later stages of neuronal maturation and epigenetic patterns are essential for controlling gene expression in normal growth and development. Genes marked by bivalent histone marks (H3K4me3 and H3K9me2) are expressed at subthreshold levels during development but showed increased expression with postnatal neuronal maturation. This suggest that bivalent marking in post-mitotic neurons helps to maintain genes responsive to developmental cues, as it has been previously proposed for various stem cell types (Voigt, LeRoy et al. 2012, Yao, Christian et al. 2016, Podobinska, Szablowska-Gadomska et al. 2017).

Furthermore it is important to highlight that not only the main epigenetic modifications afore described can influence gene expression. In fact, modifications in nucleosome positioning and composition, as well as the nuclear architecture and chromatin folding, have been demonstrated to have important roles in transcriptional regulation both in brain physiological and pathological states (Takizawa and Meshorer 2008, Campos and Reinberg 2009, Spyropoulou, Piperi et al. 2013, Ito, Magalska et al. 2014, Shibayama, Fanucchi et al. 2014, Medrano-Fernandez and Barco 2016).

1.2.6. Epigenetics and ID

In the adult nervous system, both transient and stable changes in gene expression play very important roles regulating the neuronal response to the different environmental stimuli, such as those involved in acquired behaviours, learning and memory processes, neural plasticity, neurotoxicity and drug addiction. These changes rely on or are

associated with chromatin modifications (Takizawa and Meshorer 2008). For example, although for long time it was thought that there were no changes of the DNA methylation patterns acquired during neural differentiation, recent studies have shown that DNA methylation could alter the expression and splicing of genes involved in functional plasticity and synaptic wiring during memory formation. Changes in DNA methylation could induce a state change that alters responsivity to existing inputs and acts permissively to enable other long-term changes that are ultimately responsible for memory (Levenson, Roth et al. 2006, Borrelli, Nestler et al. 2008, Day and Sweatt 2010, Feng, Zhou et al. 2010, Miller, Gavin et al. 2010, Tognini, Napoli et al. 2015, Halder, Hennion et al. 2016). Similarly, histone H3K4me and H3K9me states are also usually established during development and specification of cellular types but other experiences can change, at least temporally, the pattern of these marks (Gupta, Kim et al. 2010, Benevento, Oomen et al. 2017).

Therefore, it has become clear that the dysfunction of epigenetic mechanisms can lead to various disorders including diseases with behavioural or neurologic manifestations such as cognitive impairment (Rudenko, Dawlaty et al. 2013). The etiology of ID is connected to epigenetics in two fundamentally different ways: first, through mutations that disrupt specific epigenetic regulators and second, through abnormal epigenetic regulation of ID-linked genes with other biological functions. In the first scenario, single gene mutations affecting epigenetic regulators can result in disruption of normal chromatin regulation in the brain. Currently, about 55 chromatin regulators involved in DNA methylation, nucleosome remodelling, or histone modification are implicated in monogenic forms of ID (Kleefstra, Schenck et al. 2014). In some cases, the disrupted genes encode proteins that have been involved in neuronal processes such as learning and memory or dendritic development and synaptogenesis.

However, in many cases, the neuronal function for these genes is not known yet (Fiala, Spacek et al. 2002, Kramer and van Bokhoven 2009, Champagne 2012). In the second scenario, for example, abnormal epigenetic silencing occurs in the nucleotide repeat disorder FXS (Tabolacci and Chiurazzi 2013) and in the imprinting disorders Angelman syndrome and Prader–Willi syndrome (Buiting 2010). In these disorders, underlying DNA changes result in an altered epigenetic state leading to the silencing of genes that would be otherwise transcribed. Such inappropriate gene silencing can be deleterious for normal brain function. Many of the diseases linked to mutations in epigenetic regulators show autosomal inheritance but a substantial proportion of them are X-linked, so they affect mainly males. Random XCI in female patients leads to somatic mosaicism for the mutation and disease pathology depends on which allele (wt or mutant) is silenced. This is the case, for example, of the methyl-CpG-binding protein (*MECP2)* gene in the Rett syndrome (Lyst and Bird 2015). Other X-linked mutations can skew X inactivation towards the X containing the mutant allele in carrier females, like in the case of the α-thalassemia/mental retardation syndrome, X-linked (*ATRX*) gene (Muers, Sharpe et al. 2007, Ratnakumar and Bernstein 2013).

Gaining a more complete understanding of the essential components and underlying mechanisms involved in epigenetic regulation could lead to novel treatments for a number of neurological and psychiatric diseases (Millan 2013).

1.3. Focus on histone lysine methylation

Lysine methylation occurs in a specific and well-regulated manner, and plays key roles in controlling important biological processes such as transcription, DNA damage and cell cycle. Histone methylation has been shown to occur on 5 lysine residues of histone H3 (K4, K9, K27, K36 and K79) and on four arginine residues (R2, R8, R17 and R26).

Other histones can also be methylated, like the histone H4 on the lysine 20 (H4K20) or on the arginine 3 (H4R3) and the histone H2B on the lysine 37 (Black, Van Rechem et al. 2012, Gardner, Zhou et al. 2011). These residues can be unmodified, mono-, di- or trimethylated, and different degrees of methylation on the same residue have been suggested to differentially affect chromatin structure and transcription (Black, Van Rechem et al. 2012). The fact that distinct modification states occur on a single residue can modulate the accessibility of that particular residue to modifying enzymes (Blackledge and Klose 2010).

Importantly, the methylation states do not affect the positive charge of the residue, and so the effect of methylation on nucleosome dynamics is thought to be less direct than for lysine acetylation. In fact, while acetylation is mostly associated with transcriptional activation, histone H3 lysine methylations can be associated with both activation (e.g., H3K4, H3K36 and H3K79 methylations) and repression (e.g., H3K9 and H3K27 methylations). For instance, elevated levels of H3K27 methylation correlate with gene repression (Boyer, Plath et al. 2006); in contrast, high levels of histone H3K4 methylation are detected in promoter regions of active genes along with different acetylation marks (Bernstein, Kamal et al. 2005).

1.3.1. KMTs and KDMs

In 1964 two studies demonstrated that transcription can be modulated by methylation at the ε–amino group of lysine (K) of histone proteins (Allfrey and Mirsky 1964, Murray 1964). Afterwards Paik and Kim demonstrated that this modification could occur also in the guanidine group of arginine (R) (Paik and Kim 1967) and was catalysed by enzymes that use the S-adenosyl-L-methionine (SAM) as the methyl

group donor (Paik and Kim 1971). However, it was only in 2000, when Jenuwein and colleagues discovered the first protein catalysing one of these reactions, the histone methyltransferase SUV39H1 (KMT1A) highly conserved from yeast to human (Rea, Eisenhaber et al. 2000). Since then many other KMTs were identified containing the catalytic SET domain (Dillon, Zhang et al. 2005). Proteins containing the 130 aa SET-domain constitute the bigger of the two classes of KMTs. KMT4 (known as Dot1p in yeast and Dot1L in human) is the only enzyme belonging to the second class that lacks a SET domain (Okada, Feng et al. 2005). KMTs have a high degree of catalytic specificity for the lysine residue within the histone substrate and its degree of methylation. However, their interacting partners can alter this specificity and modulate their catalytic activity.

As other HPTMs such as phosphorylation and acetylation, methylation is reversible. Yang Shi and colleagues demonstrated in 2003 the existence of the first lysine demethylase (KDM), LSD1/KDM1A (Shi, Sawada et al. 2003). The protein is part of the C-term binding protein 1 (CtBP1) corepressor complex (Shi, Sawada et al. 2003, Shi, Lan et al. 2004). KDM1A contains a flavin adenine nucleotide (FAD)-dependent amine oxidase domain that demethylates H3K4me2 and H3K4me1 (Shi, Lan et al. 2004). An additional class of histone demethylases was discovered afterwards (Tsukada, Fang et al. 2006). This second class is characterized by the jumonji C (JmjC) domain that catalyses demethylation through oxidation of methyl groups. It uses α-ketoglutarate, molecular oxygen, and Fe(II) as co-factors for demethylation (Shi and Whetstine 2007). Six different classes of JmjC-domain-containing proteins have been characterized: from KDM2 to KDM7 families. All the KMTs and KDMs currently known are presented in **Figure 4**.

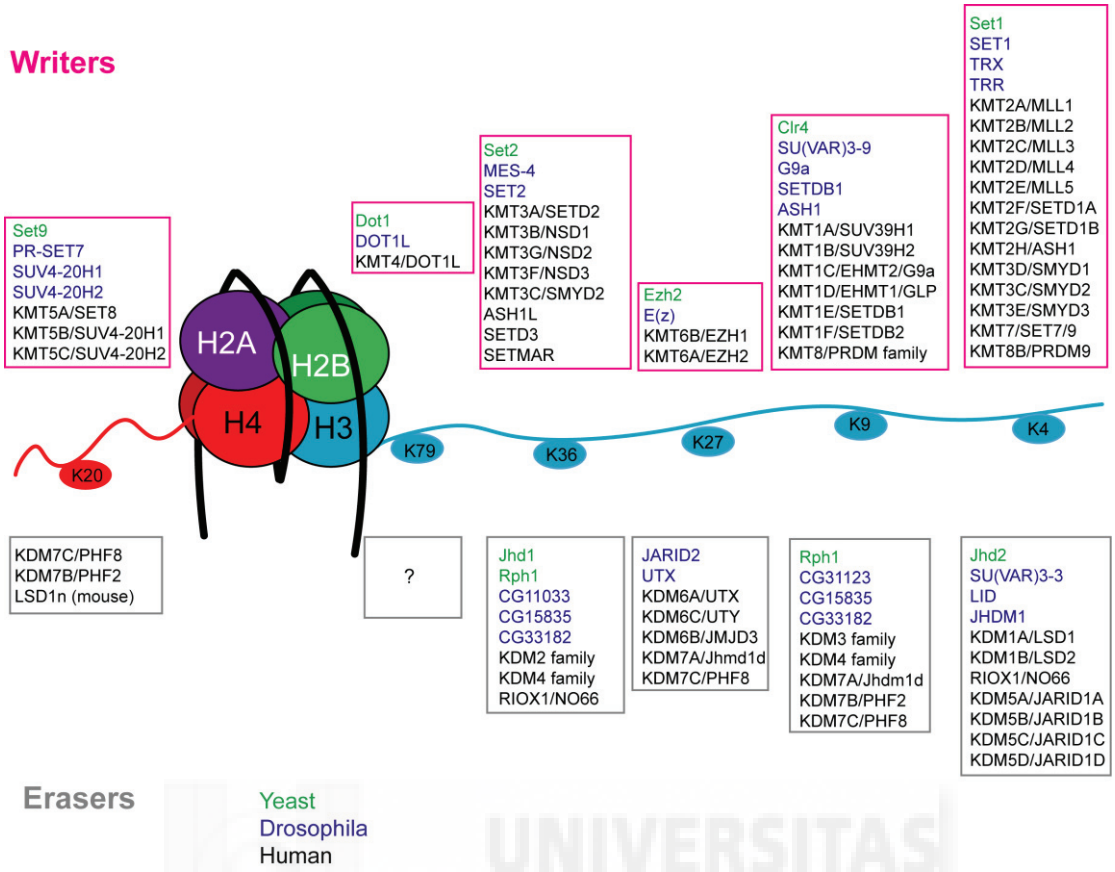

Figure 4. Scheme depicting writers and erasers of H3 and H4 histone tail lysine methylation. Erasers of H3K79 methylation marks have not been identified yet. Adapted from (Hyun, Jeon et al. 2017).

The identification of all these KMTs and KDMs has uncovered a dynamic methylation balance that importantly contributes to modulate appropriate levels of gene expression (Dimitrova, Turberfield et al. 2015). Gene expression levels of various KDMs, as I will later explain, show distinct patterns of embryonic and adult expression and respond to different environmental cues, suggesting that KDMs levels must be tightly regulated for normal development.

1.3.2. Histone H3 methylation as a major hub for IDDs

Histone methylation regulates a variety of processes including maintenance of genome integrity, cell proliferation and tumour progression, and is now believed to

function in neurons of the CNS to mediate the process of memory formation and other changes in behaviour (Gupta, Kim et al. 2010, Zovkic, Guzman-Karlsson et al. 2013). Moreover, the role of histone methylation in ID, addiction, schizophrenia, autism, depression, and neurodegeneration is currently being intensively investigated. Indeed, several IDDs have been associated with mutations in genes codifying enzymes that alter the balance of histone H3 methylation marks (Kim, Lee et al. 2017). Although some of them have been shown to regulate intellectual development in humans, the specific roles of these enzymes in neurodevelopment, learning and memory in the adult brain remain obscure (Parkel, Lopez-Atalaya et al. 2013). The biological basis for the involvement of these genes in ID is unclear; however, abnormalities of neuronal morphology, and in particular of dendritic development, have been reported in the brains of patients with these diseases or in the animal models developed (Shi 2007).

Here, I will summarize the main IDDs associated with altered histone H3 methylation, to have a general view of the strong link between this epigenetic modification and mental retardation (**Figure 5**).

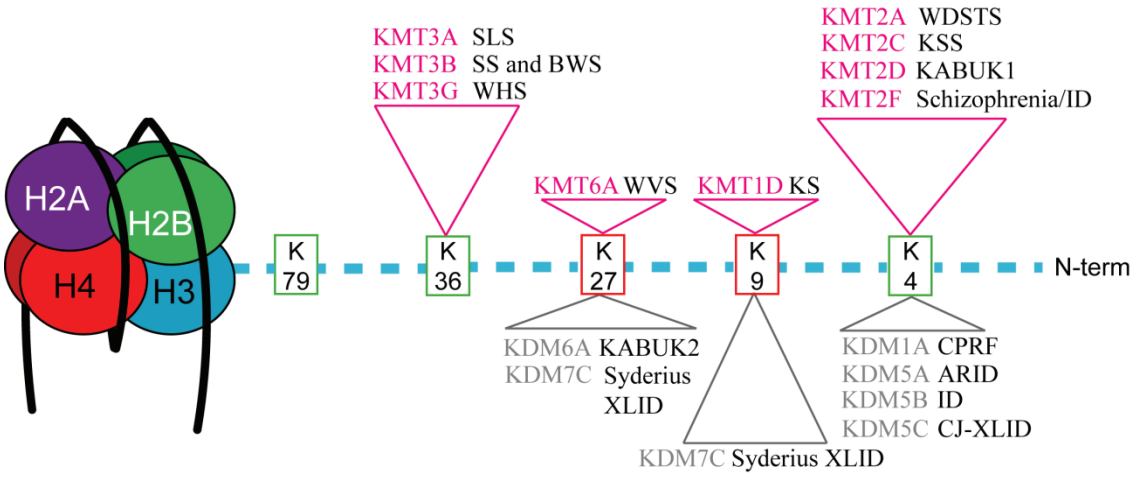

Figure 5. IDDs linked with H3 KMTs or KDMs. Disease names are in black, while lysine methyltransferases and demethylases are in pink or grey, respectively. Lysine boxes indicate residues associated to gene transcription (green) or to gene silencing (red).

IDDs related to KMTs

KMT2A and Wiedemann-Steiner syndrome (WDSTS). This syndrome (OMIM #605130) is a rare autosomic disorder resulting from mutations in the *Mixed-lineage leukemia 1 (MLL1)*/*KMT2A* gene encoding a KMT for H3K4. Pathogenic variants in KMT2A lead to global changes in gene expression throughout development (Milne, Briggs et al. 2002). Interestingly, *Kmt2a* heterozygous mutant mice recapitulate abnormal brain functions, displaying profound deficits in long-term contextual fear memory (Gupta, Kim et al. 2010). Recently, the related KMT Kmt2b has also been found to regulate memory function in mice (Kerimoglu, Sakib et al. 2017).

KMT3B/NSD1, Sotos syndrome (SS) and Beckwith-Wiedemann syndrome (BWS). Mutations or deletions of the gene *KMT3B/NSD1* encoding a KMT for H3K36 and H4K20 (Kudithipudi, Lungu et al. 2014) cause Sotos syndrome (SS, OMIM #117550), an overgrowth condition associated with behavioural problems including ADHD, macrocephaly, facial dysmorphology and learning difficulties (Kurotaki et al, 2002). In addition, recent results demonstrated genome-wide aberrant DNA methylation (Choufani, Cytrynbaum et al. 2015). Another overgrowth condition similar to SS where NSD1 is involved in establishing or maintaining 11p15 region imprinting (Mussa, Chiesa et al. 2010) is the Beckwith-Wiedemann syndrome (BWS, OMIM #130650). Mouse models based on the most common mutation reported in BWS patients, LoF of the cell cycle inhibitor CDKN1C, causes somatic overgrowth in mice, modelling this key aspect of BWS (Tunster, Van de Pette et al. 2011).

KMT6A/EZH2 and Weaver syndrome (WVS). EZH2 is known to interact with EED to form PRC2, an H3K27me3 methyltransferase complex (Cao, Wang et al. 2002). Haploinsufficiency of *EZH2* lead to another overgrowth condition called Weaver syndrome (OMIM # 277590), an autosomal dominant disorder characterized by skeletal

abnormalities, macrocephaly, mild ID, poor coordination and balance (Tatton-Brown, Hanks et al. 2011, Gibson, Hood et al. 2012, Tatton-Brown and Rahman 2013). Animal models deficient for Ezh2 display abnormal neurogenesis in the cerebral cortex (Pereira, Sansom et al. 2010), cerebellum (Feng, Juan et al. 2016), and neural tube (Akizu, Garcia et al. 2016) during embryonic development. Moreover, Ezh2 is also implicated in adult hippocampal neurogenesis and memory (Zhang, Ji et al. 2014).

KMT1D/EHMT1 and Kleefstra syndrome (KS). Heterozygous mutations of euchromatic histone-lysine N-methyltransferase 1 *(EHMT1)* (aka *KMT1D* or *GLP*) gene cause the 9q subtelomeric deletion syndrome, also called Kleefstra syndrome (KS, OMIM #610253) characterized by developmental delay, moderate to severe ID, limited or absent speech, facial dysmorphology and hypotonia. *EHMT1* encodes a KMT catalyzing mono- and dimethylation of H3K9 (Tachibana, Ueda et al. 2005). Animal models have been described in mice and flies (Balemans, Huibers et al. 2010, Kramer, Kochinke et al. 2011, Balemans, Kasri et al. 2013). Ehmt1 forms a heteromeric complex with another H3K9 methyltransferase, Ehmt2 or G9a, and KOs of either of these genes lead to very similar phenotypes in mice, including embryonic lethality. Finally, impaired homeostatic synaptic scaling was also recently described in Ehmt1$^{+/-}$ neurons (Benevento, Iacono et al. 2016) along with decreased hippocampal cell proliferation (Benevento, Oomen et al. 2017).

KMT2C/MLL3 and Kleefstra syndrome spectrum (KSS). Among 4 of 9 EHMT1 mutation-negative patients with core phenotypic features of KS but with phenotypic heterogeneity, referred to as Kleefstra syndrome spectrum (KSS, OMIM #610253), Kleefstra and colleagues identified *de novo* mutations in 4 genes encoding epigenetic regulators: *MBD5*, *MLL3*, *SMARCB1*, and *NR1I3*. Using Drosophila, they demonstrated that MBD5, MLL3, and NR1I3, cooperate with EHMT1 while SMARCB1 was known

to interact directly with MLL3 (Kleefstra, Kramer et al. 2012). KO mice for MLL3 (a KMT for H3K4) exhibit delayed growth but need still to be investigated under a behavioural point of view (Lee, Saha et al. 2008).

KMT3G/NSD2 and Wolf-Hirschhorn syndrome (WHS). Wolf–Hirschhorn syndrome candidate 1 (*WHSC1*) encodes NSD2, a H3K36-specific methyltransferase that is frequently deleted in WHS (OMIM #194190), a malformation syndrome which key features include severe growth and ID, microcephaly and seizures (Battaglia, Carey et al. 1999). As for NSD1, NSD2 deficiency causes defects in H3K36 methylation, although WHS is not an overgrowth syndrome. Mice with Nsd2 gene deletions are growth-retarded, show midline, craniofacial, and ocular anomalies (Nimura, Ura et al. 2009), although they do not show any learning deficits (Naf, Wilson et al. 2001).

KMT3A/SETD2 and Sotos-like syndrome (SLS). Mutations in *SETD2*, codifying for the only known enzyme able to directly catalyze H3K36 tri-methylation, produce a variegated phenotype ranging from cancer (deletions/missense mutations) to SLS and autism spectrum disorders (ASD), likely through its haploinsufficiency (Wagner and Carpenter 2012, Luscan, Laurendeau et al. 2014, Lumish, Wynn et al. 2015).

KMT2F/SETD1A and Schizophrenia/ID. Rare loss-of-function variants found in *SETD1A* codifying for an H3K4 methyltransferase have been recently associated with schizophrenia and developmental disorders (Singh, Kurki et al. 2016).

KMT2D and Kabuki syndrome (KS). Heterozygous mutations in the *KMT2D* (previously called also *MLL2*) cause KS. Patients display ID, autism, microcephaly, postnatal dwarfism and a distinctive face with long palpebral fissures (reminiscent of the make-up of actors Kabuki, traditional Japanese music-drama) (Makrythanasis, van Bon et al. 2013). Mutations in *KMT2D* are found in more than 50% of patients with Kabuki syndrome 1 or KABUK1 (OMIM #147920). It codifies for KMT involved in

the apposition of H3K4me1/2/3. Interestingly, in mice Kmt2d has been shown to be crucial for the epigenetic re-programming that takes place before fertilization in oocytes by trimethylation of H3K4, with deficiency of Kmt2d resulting in anovulation (Bjornsson 2015).

IDDs related to KDMs

KDM6A/UTX and Kabuki syndrome (KS). Mutations in *KDM6A* are reported to contribute to less than 10% of KS cases (Bogershausen et al, 2013). These cases are referred to as Kabuki syndrome 2 (KABUK2; OMIM #300867) (Lederer et al, 2012; Yang et al, 2016). KDM6A is responsible for the removal of H3K27me3. *Kdm6a* mutations have been involved in mouse embryonic development (Shpargel et al, 2012).

KDM7C/PHF8 and Syderius XLID syndrome. Siderius mental retardation syndrome (OMIM #300263) is an XLID condition; patients display mental retardation, a long face and broad nasal tip, and cleft lip and palate (Siderius, Hamel et al. 1999). The disease has been associated with mutations in PHD finger protein 8 (*PHF8*, aka *KDM7C*) (Laumonnier, Holbert et al. 2005, Abidi, Miano et al. 2007). PHF8 has a demethylase activity towards two different methylated lysines, H3K9me1/2 and H4K20me1, and also functions as a trimethylated H3K4 reader (Feng, Yonezawa et al. 2010, Qi, Sarkissian et al. 2010). Injection of zebrafish PHF8 morpholino caused brain and craniofacial development defects (Qi, Sarkissian et al. 2010). Furthermore, Salcini's lab recently found that loss of a PHF8 homolog (JMJD-1.2) in *C. elegans* resulted in axon guidance defects (Riveiro, Mariani et al. 2017). However, a recent study showed that Phf8-deficient mice had no obvious developmental defects and cognitive impairment, although they are resilient to stress-induced anxiety- and depression-like behaviour. Additionally, Phf8-deficient primary cells had reduced proliferative potential (Walsh, Shen et al. 2017).

KDM1A/LSD1 and Cleft palate, psychomotor retardation and distinctive facial features (CPRF). Recently, the first-discovered KDM LSD1 (aka KDM1A) has been linked to an autosomal dominant form of neurodevelopmental disorder (not fully characterized yet) featuring distinctive traits of facial dysmorphisms and ID (Pilotto, Speranzini et al. 2016, Rauch, Wieczorek et al. 2012, Tunovic, Barkovich et al. 2014). KDM1A erases mono- and di-methyl groups from histone H3K4 (Shi, Lan et al. 2004) and H3K20me1/2 (Wang, Telese et al. 2015). It is considered an epigenetic transcriptional corepressor, interacting with CoREST and histone deacetylases HDAC1/2 (Shi et al, 2005). Mutations in humans have a possible dominant negative function (Wang, Scully et al. 2007). A neuronal specific splicing isoform for Lsd1 (neuroLsd1 o Lsd1n) seems to be able to facilitate neuronal maturation (Zibetti, Adamo et al. 2010, Laurent, Ruitu et al. 2015). Selective genetic ablation of LSD1n leads to deficits in spatial learning and memory, revealing the functional importance of LSD1n in neuronal activity–regulated transcription necessary for long term memory formation. While Lsd1 KO mice are lethal, Rosenfeld and colleagues reported that neuroLSD1^{KO} display significant memory impairment (Wang, Telese et al. 2015). Consistently, Battaglioli's group showed that neuroLSD1 haploinsufficiency provokes an aberrant acquisition of stress-evoked plasticity, leading to decreased anxiety-like behavior in neuroLSD1^{HET} (Rusconi, Grillo et al. 2016). Finally, a recent study showed that LSD1 is required to maintain terminally differentiated neurons and prevents the activation of neurodegenerative pathways, by continuously repressing the re-activation of stem cell TFs (Christopher, Myrick et al. 2017).

KDM5C and Claes-Jensen XLID (CJ-XLID). Clinical features in males with *KDM5C* mutations include mild to severe ID, epilepsy, short stature, hyperreflexia, aggressive behaviors, and microcephaly (Goncalves, Goncalves et al. 2014). Different studies have

been published on Kdm5c function and relationship with CJ syndrome, which will be outlined in more details in section 1.4.3.

1.4. Histone H3K4 methylation, KDM5C and ID

1.4.1. H3K4 methylation and its regulation during development and disease

Trimethyl-H3K4 (H3K4me3) is perhaps the best-studied chromatin modification. It is enriched at promoters and enhancers of actively transcribed genes throughout the genome. Looking at the genomic distribution of H3K4me3, its presence at promoters is very tightly linked to active transcription, and 91% of all the RNA polymerase II binding sites on DNA correlate with H3K4me3 enrichment (Barski, Cuddapah et al. 2007). However, whether this mark has an instructive role in transcription is still debated because of missing conserved mechanism among species (Pinskaya and Morillon 2009, Howe, Fischl et al. 2017). Another *in vitro* study suggested that H3K4 trimethylation may also facilitate pre-mRNA maturation via bridging of the spliceosomal components and CHD1 to actively transcribed genes (Sims, Millhouse et al. 2007).

The first H3K4 methylase complex was COMPASS, identified in the yeast *S. cerevisiae* (Miller, Krogan et al. 2001, Eissenberg and Shilatifard 2010). It consists of Set1/KMT2 and seven other polypeptides. Its mammalian homologs, the MLL proteins, are found in COMPASS-like complexes able of methylating H3K4 (Tenney and Shilatifard 2005, Shilatifard 2012). Although mammals have many more KMTs affecting this specific residue than yeasts, these different KMTs do not seem to be functionally redundant because, for example, the deletion of *MLL1* and *MLL2* are both embryonically lethal (Yu, Hess et al. 1995, Lubitz, Glaser et al. 2007). However, it is not surprising that the regulation of the plethora of distinct epigenomes present in a

multicellular organism requires more KMTs; the situation is not comparable with the epigenetic regulation of a single-cell eukaryote (Sims, Millhouse et al. 2007).

During development, the genes of important regulatory proteins in ESCs carry both the activating H3K4me3 and the repressive H3K27me3 mark and are referred to as "bivalently marked" (Bernstein, Mikkelsen et al. 2006). This mark keeps the developmental genes poised for later activation during neuronal lineage commitment (Mikkelsen, Ku et al. 2007). After differentiation, the promoters of neuronal genes retain the H3K4me3 label and lose the repressive mark, resulting in transcriptional activation (Bernstein, Mikkelsen et al. 2006, Podobinska, Szablowska-Gadomska et al. 2017).

H3K4 methylation is usually gene- and cell-type specific and the removal of specific KMTs or KDMs more likely changes methylation at the level of specific loci instead of at the global level. In fact, when any single MLL family member is ablated *in vivo*, global levels of H3K4 methylation are only slightly reduced (Lubitz, Glaser et al. 2007, Wang, Lin et al. 2009). By contrast, H3K4 methylation levels are significantly decreased when any of the core subunits of the complex (RbBP5, Ash2L and WDR5) is ablated (Dou, Milne et al. 2006). Interestingly, depletion of RBBP5 in mouse ESCs reduces H3K4 methylation and leads to defects in specification of the neural lineage, whereas self-renewal is not affected (Jiang, Shukla et al. 2011). Mll2 H3K4 methyltransferase seems to be essential during development because *Mll2*$^{-/-}$ embryos are lethal while conditional KOs present only spermatogenesis problems with consequent infertility (Glaser, Lubitz et al. 2009).

Several neurodevelopmental disorders such as schizophrenia, bipolar disorders, ID and ASDs are characterized by disruption of modifying enzymes for this epigenetic mark and have been extensively revised (Wynder, Stalker et al. 2010, Cruceanu, Alda et

al. 2013, Shen, Shulha et al. 2014, Vallianatos and Iwase 2015). Moreover, the high importance of this epigenetic mark in the regulation of gene expression highlights the possibility of its targeting in the treatment of neurodevelopmental disorders (Shilatifard 2008, Shen, Shulha et al. 2014, Ricq, Hooker et al. 2016).

1.4.2. The KDM5 family of lysine demethylases

The lysine demethylase 5 (KDM5) family acts specifically on the H3K4 and includes KDM5A, KDM5B, KDM5C, and KDM5D proteins. These are also referred to as JARID1A (Jumonji, AT rich interactive domain 1A)/RBP2 (retinoblastoma-binding protein 2), JARID1B/PLU-1, JARID1C/SMCX, and JARID1D/SMCY, respectively. All these enzymes act demethylating di- and trimethylated H3K4 (Agger, Christensen et al. 2008, Zhang, Wen et al. 2012). All KDM5 family proteins from *C.elegans* to mammals have several conserved functional domains, including one JmjN domain, one ARID/Bright domain responsible for DNA binding (Wilsker, Patsialou et al. 2002), one catalytic JmjC domain, one Zinc-finger (Zf) C5HC2-type DNA binding domain and two or more PHD domains, responsible for histone binding (**Figure 6**) (Baker, Allis et al. 2008, Huang, Chandrasekharan et al. 2010, Horton, Engstrom et al. 2016).

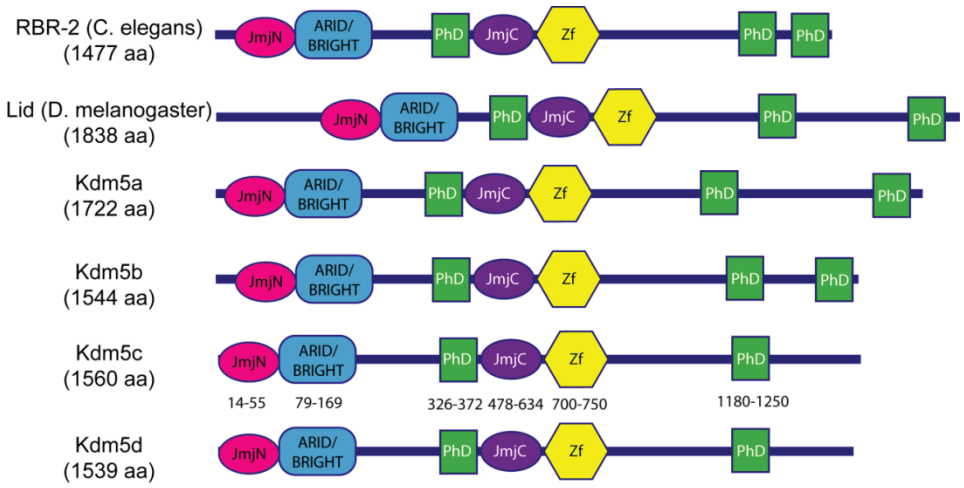

Figure 6. Domain organization in KDM5 family.

KDM5 family appears to play key developmental functions as demonstrated in *Drosophila* and *C. elegans*. In *Drosophila*, homozygous mutations in the dKDM5C/Lid (little imaginal discs), cause larval lethality and several developmental defects in surviving animals (Gildea, Lopez et al. 2000) while in *C. elegans*, deletion of the JmjC domain of RBR-2 (retinoblastoma binding protein related 2) leads to defects in vulva formation (Christensen, Agger et al. 2007). Salcini's group recently demonstrated that RBR-2 acts cell-autonomously and in a catalytic-dependent manner to control vulva precursor cells fate acquisition. RBR-2 reduces the H3K4me3 level at TSSs and in enhancer regions upstream of the TSSs, and acts both as a transcriptional repressor and activator (Lussi, Mariani et al. 2016).

More interesting in the frame of this thesis, KDM5 family has been involved also in neural development (Roidl and Hacker 2014, Fueyo, Garcia et al. 2015). It has been shown how RBR-2 ensures correct axon guidance by controlling the expression of the actin regulator wsp-1 by interacting with NURF-1, an H3K4me3-binding protein and member of the chromatin-remodelling complex NURF (Mariani, Lussi et al. 2016). Moreover, Liu and Secombe found that Lid activates genes critical for mitochondrial function and metabolism through recognition via its PHD domain (Liu and Secombe 2015).

Hereafter, I will describe the mammalian homologues of these proteins.

KDM5A: Mammalian KDM5A as well as *Drosophila* Lid have several interaction partners, including RBP-J (recombination signal-binding protein-Jk), Sin3, c-Myc (Secombe, Li et al. 2007, Liefke, Oswald et al. 2010, Gajan, Barnes et al. 2016), and PcG proteins of the PRC2 complex, which harbours H3K27 methyltransferase activity (Pasini, Hansen et al. 2008). Interestingly, KDM5B, KDM5C and KDM5D seem also to

interact with PcG proteins, which are involved in transcriptional repression (Lee, Norman et al. 2007, Zhou, Chen et al. 2009).

Surprisingly, contrary to the studies in worms and flies, Kdm5a KO mice display only subtle phenotypes, such as haematological abnormalities that correlate with derepression of cytokine genes (Klose, Yan et al. 2007), maybe because of functional compensation by other KDM5 family members. In cell culture systems, functional studies have linked KDM5A to the regulation of differentiation, cell cycle progression and mitochondrial function (Benevolenskaya, Murray et al. 2005, Lopez-Bigas, Kisiel et al. 2008). Recently, a *de novo* mutation in *KDM5A* has been found in a patient with autosomal recessive ID (ARID) (Najmabadi, Hu et al. 2011).

KDM5B: Kdm5b binds to neural master regulators such as Pax6 and Otx2 to maintain appropriate H3K4me3 levels in mouse ESCs. When it is missing, mouse embryos present high neonatal lethality and several neural defects due to impaired differentiation and failure to silence pluripotency genes, because of elevated H3K4me3 levels (Albert, Schmitz et al. 2013). ESCs need Kdm5b for proper differentiation to NSCs by maintaining a low level of H3K4me3 marks (Schmitz, Albert et al. 2011) and overexpression of this demethylase decreases the expression of other regulators of cell fate such as Egr1, p27 and BMI1 resulting in reduced numbers of differentiated cells and increased numbers of proliferating progenitors. This suggests that Kdm5b plays a developmental role maintaining uncommitted progenitors (Dey, Stalker et al. 2008). Similarly to *KDM5A*, a recent study highlighted a mutation in this gene potentially associated with ID (Athanasakis, Licastro et al. 2014). Furthermore, human *KDM5B* displays high expression levels in breast and prostate cancers (Lu, Sundquist et al. 1999), supporting a potential role in malignant transformation.

KDM5C: The lysine-specific demethylase 5C in humans is encoded by the *KDM5C* gene, also known as *SMCX* (selected mouse cDNA on the X) or *JARID1C*, a member of the SMCY homolog family. KDM5C can bind to the repressive histone mark H3K9me3 through its PHD domain and, like all the proteins belonging to KDM5 family, can remove the active epigenetic marks H3K4me3/2, thus establishing a repressive chromatin state (Christensen, Agger et al. 2007, Iwase, Lan et al. 2007). *KDM5C* is located on the proximal Xp (Xp11.1-Xp11.2) region of the sexual X chromosome and is ubiquitously expressed in almost all human tissues including white blood cells, with the highest levels of expression found in the brain and in skeletal muscle (Jensen, Amende et al. 2005, Jensen, Bartenschlager et al. 2010). Its role has been linked to brain development as I will explain in the following section.

KDM5D: KDM5D/JARID1D/SMCY interacts directly with the polycomb protein Ring6a/MBLR and leads to enhanced demethylation activity. Kdm5d is recruited to the Engrailed2 gene (a gene thought to be relevant in neurodevelopmental diseases) to regulate its transcriptional output by demethylating trimethyl H3K4 in its TSS (Benayed, Gharani et al. 2005, Titomanlio, Pierri et al. 2005, Lee, Norman et al. 2007).

The characterization of mouse strains deficient for the four Kdm5 proteins should clarify the specific and overlapping functions for the different family members in development, cancer and neuronal disorders (Pedersen and Helin 2010, Rasmussen and Staller 2014).

1.4.3. KDM5C as a chromatin modifier linked to ID

Shi and colleagues first described the family of H3K4me3 demethylases to which KDM5C belongs. In *Danio rerio*, depletion of the KDM5C homologue caused brain-

patterning defects and neuronal cell death, while KDM5C knockdown (KD) in primary rat cerebellar granule neurons impaired dendritic morphogenesis and this was dependent on its catalytic activity (Iwase, Lan et al. 2007). Shortly afterwards, KDM5C was isolated as part of a transcriptionally repressive complex containing HDAC1/2, EHMT2 (G9a), and REST (Tahiliani, Mei et al. 2007). Chromatin immunoprecipitation (ChIP) experiments in HeLa cells showed that KDM5C co-localizes with REST, a transcriptional repressor in the neuron-restrictive silencing elements (NRSE), in the promoters of a subset of REST target genes, including the brain-derived neurotrophic factor (*BDNF)*, the sodium channel type 2A (*SCN2A)* and synapsin 1 *(SYN1)*, suggesting that the loss of KDM5C activity impairs REST-mediated neuronal gene regulation in non-neuronal cells (Tahiliani, Mei et al. 2007). Genes like *Scn2A* and *Syn1* are themselves implicated in epilepsy, autism, and schizophrenia. Thus, it is possible that mutations in *KDM5C* lead to alterations in KDM5C function, causing disregulation of target genes whose products are directly involved in regulating neuronal activity. Although the specific molecular mechanism by which loss of function (LoF) of KDM5C causes impairment in neuronal development is not understood, epigenetic deregulation is presumed to play an important role.

The KDM5C protein is likely to play a role not only in ID but also in sex-specific differences in brain function. As *KDM5C* has a widely expressed homologue on the Y chromosome (*KDM5D*), it appeared a good candidate for genes that escape X-inactivation. Indeed, this was the case (Agulnik, Mitchell et al. 1994, Carrel and Willard 2005) because, not surprisingly, Kdm5c shows higher expression levels in XX females compared to XY males in mouse adult brain (Xu, Burgoyne et al. 2002). This difference has been shown to be associated with sex chromosome complement (XX vs. XY), rather than gonadal sex of the animals (Xu, Burgoyne et al. 2002). The Y-linked functional

homologue Kdm5d has been shown to be expressed at lower levels than Kdm5c in murine neurons and is not able to compensate for Kdm5c differences between females and males (Xu, Deng et al. 2008).

More recent reports have demonstrated a prominent role of KDM5C in modulating H3K4me3 levels throughout the genome. ChIP-sequencing of a set of chromatin remodelling (CR) proteins in the leukemia cell line K562 had shown that specific combinations of CRs co-localize in characteristic patterns at distinct chromatin environments, at genes of coherent functions, and at distal regulatory elements. When comparing between cell types, CRs redistribute to different loci but maintain their modular and combinatorial associations. KDM5C along with other transcriptional repressors binds to a wide range of promoters, including those that are active, competent, and repressed (Ram, Goren et al. 2011). In mouse ESCs and in NPCs, Kdm5c is recruited on regulatory regions such as enhancers and promoters. Intriguingly, a recent study proposed that Kdm5c restrains transcription, by reducing H3K4me3, at promoters while it stimulates their activity at enhancers (**Figure 7**) (Outchkourov, Muino et al. 2013). However, an ulterior study demonstrated that the interaction between the chromatin reader RACK7 and KDM5C induces the repression of enhancer and super enhancers. Loss of these two proteins led to overactivation of enhancers and their respective eRNAs and nearby genes because of the increase in H3K4 trimethylation and H3K27 acetylation at their regulatory sites. Therefore, according to this second study RACK7/KDM5C complex would function as an enhancer ‘‘brake’’ to ensure appropriate enhancer activity (Shen, Xu et al. 2016).

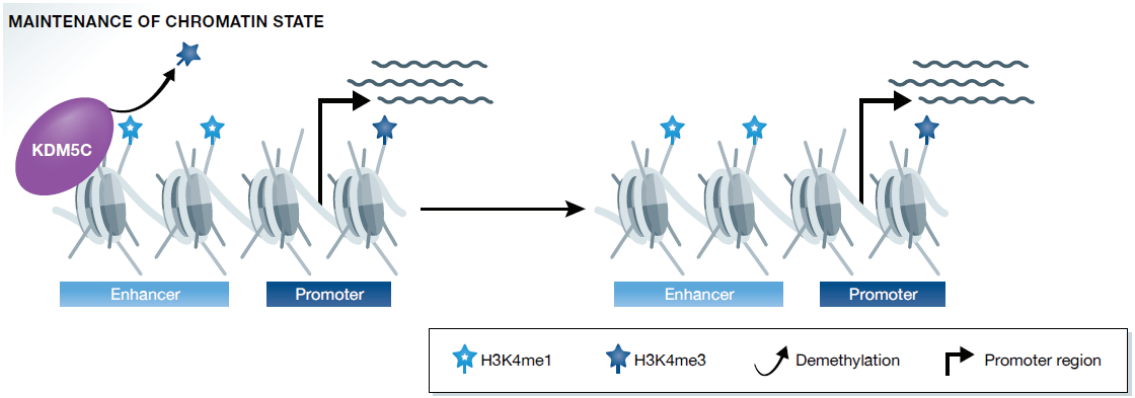

Figure 7. KDM5C and maintenance of chromatin state. KDM5C plays an important role in the maintenance of enhancer identity by preserving H3K4 monomethylation vs trimethylation ratios. From (Dimitrova, Turberfield et al. 2015).

Additionally, it was recently identified a non-transcriptional role for Kdm5c in DNA replication (Rondinelli, Schwerer et al. 2015) and DNA damage response (Hendriks, Treffers et al. 2015). During early S phase, KDM5C functions to actively remove H3K4me3 from replication origins, promoting the formation of the pre-initiation complex; if KDM5C is absent H3K4me3 persists at these sites and early origin replication sites fail to efficiently initiate replication leading to cell cycle arrest (Rondinelli, Schwerer et al. 2015). In response to DNA damage, KDM5C is SUMOylated causing an increase in its chromatin occupancy leading to transcriptional inhibition by removal of H3K4me3 prior to DNA repair (Hendriks, Treffers et al. 2015).

Another recent study from Tonon and colleagues demonstrated that KDM5C broadly binds to H3K9me3 heterochromatic chromatin domains and is required for heterochromatin replication in a protein complex that includes SUV39H1 and HP1α, as well as proteins not previously associated with heterochromatin assembly such as the cullin 4 (CUL4) complex adaptor protein DDB1. Transcription on heterochromatin is tightly suppressed to safeguard the genome (Rondinelli, Rosano et al. 2015).

A recent study in *C. elegans* showed that the absence of RBR-2, orthologue of the human *KDM5C*, led to developmental delay, reproductive defects and decreased

survival. Interestingly, mutant worms also displayed behavioural deficits such as reduced motor coordination, abnormal sensory chemotactic response and an increased sensitivity to Pentylenetetrazol (PTZ, a GABA antagonist). Furthermore neuronal process positioning and migration were also affected suggesting a contribution of this demethylase to GABA-dependent neuronal functions (Rodrigues, Bessa et al. 2015).

1.4.4. KDM5C and Claes-Jensen syndrome

As previously indicated, KDM5C is involved in XLID (Jensen, Amende et al. 2005, Santos, Rodriguez-Revenga et al. 2006, Tzschach, Lenzner et al. 2006, Abidi, Holloway et al. 2008, Abidi, Holloway et al. 2009, Rujirabanjerd, Nelson et al. 2010, Santos-Reboucas, Fintelman-Rodrigues et al. 2011). Claes and colleagues first described in 2000 a family with a syndromic form of X-linked mental retardation (XLMR) in which four males in two generations presented severe mental retardation, slowly progressive spastic paraplegia, facial hypotonia, and maxillary hypoplasia (Claes, Devriendt et al. 2000). Then, Jensen and colleagues identified families with non-syndromic XLMR, in which more than 30% of mutations clustered on proximal Xp. In particular, they detected seven different mutations in *KDM5C,* including one frameshift mutation and two nonsense mutations that introduced premature stop codons, as well as four missense mutations affecting evolutionarily conserved amino acids. This study first suggested that *KDM5C* might play an important role in human brain function (Jensen, Amende et al. 2005).

Indeed, *KDM5C* has been later implicated not only in CJ-XLID but also in autism susceptibility (Adegbola, Gao et al. 2008) and in the pathology of ARX-related ID (Poeta, Fusco et al. 2013). A nondysmorphic patient with developmental delay and ASD presented a novel missense mutation in the JmjC domain (exon 16) of the gene that

results in an arginine to tryptophan substitution at amino acid 766 (R766W). The affected amino acid was conserved in the homologous proteins of mouse and zebrafish (Adegbola, Gao et al. 2008). Moreover, Miano and colleagues discovered that *KDM5C* is directly regulated by aristaless-related homeobox ARX through the binding in a conserved non-coding element. Interestingly, Polyalanine (polyA)-expansion-encoding mutations of ARX cause a spectrum of XLID diseases and chronic epilepsy (Poeta, Fusco et al. 2013).

Mutations leading to XLID have been found in most of the functional domains of this protein (Jensen, Bartenschlager et al. 2010). Point mutations, found in patients with diverse phenotypes, which includes syndromic and non-syndromic forms of ID, can suppress demethylase activity and/or H3K9me3 binding in vitro, depending on the location of the mutation (Iwase, Lan et al. 2007, Brookes, Laurent et al. 2015). Notably, a number of missense mutations have been demonstrated to reduce or abolish the cellular demethylase activity (Tzschach, Lenzner et al. 2006, Goncalves, Goncalves et al. 2014). One such mutation is a non-sense mutation in the interaction domain that results in the generation of a premature termination codon, which led to reduced levels of Kdm5c transcript (probably due to nonsense-mediated mRNA decay) and therefore to reduced activity (Santos-Reboucas, Fintelman-Rodrigues et al. 2011, Brookes, Laurent et al. 2015) (**Table 1**). Some of these mutations, such as p.Y751C and p.F642L have been shown to downregulate genes as *Ntng2*, encoding for Netrin G2, and to induce shorter neurites in Neuro2a cells (Wei, Deng et al. 2016).

Table 1. Main patient mutations in *KDM5C* gene reported to date.

Mutation	***Type of mutation***	***Domain***	***Degree of intellectual disability***	***References***
p.M1_E165del	Missense	N-terminus	Severe	(Ounap, Puusepp-Benazzouz et al. 2012)

p.R68fs*7	Frameshift	—	Severe	(Jensen, Amende et al. 2005)
p.A77T	Missense	ARID/Bright	Severe	(Abidi, Holloway et al. 2008)
p.D87G	Missense	ARID/Bright	Mild-moderate	(Jensen, Amende et al. 2005)
p.R332Ter	Nonsense	PHD	Severe	(Tzschach, Lenzner et al. 2006)
p.A388P	Missense	PHD	Mild	(Jensen, Amende et al. 2005)
p.D402Y	Missense	—	Severe	(Jensen, Amende et al. 2005)
p.S451R	Missense	—	Severe	(Santos, Rodriguez-Revenga et al. 2006)
p.E468fs*2	Frameshift	JmjC	Severe	(Abidi, Holloway et al. 2008)
p.P480L	Missense	JmjC	Mild-moderate	(Grafodatskaya, Chung et al. 2013)
p.V504M	Missense	JmjC	Mild-moderate	(Abidi, Holloway et al. 2008)
p.P544T	Missense	JmjC	Moderate	(Rujirabanjerd, Nelson et al. 2010)
p.F642L	Missense	JmjC-C5HC2	Severe	(Tzschach, Lenzner et al. 2006)
p.A683Pfs*81	Frameshift	—	Segregation with ID in the family	(Tzschach, Grasshoff et al. 2015)
p.R694Ter	Nonsense	—	Severe	(Jensen, Amende et al. 2005)
p.E698K	Missense	JmjC-C5HC2	Severe	(Jensen, Amende et al. 2005)
p.C724Ter	Nonsense	C5HC2	Severe	(Santos-Reboucas, Fintelman-Rodrigues et al. 2011)
p.L731F	Missense	JmjC-C5HC2	Severe	(Jensen, Amende et al. 2005)
p.R750W	Missense	C5HC2	Severe	(Tzschach, Lenzner et al. 2006)
p.Y751C	Missense	C5HC2	Moderate	(Tzschach, Lenzner et al. 2006)
p.R766W	Missense	—	ID with autistic feature	(Adegbola, Gao et al. 2008)
p.C1028*	Nonsense	—	Segregation with ID in the family	(Tzschach, Grasshoff et al. 2015)
p.V1075Yfs*2	Nonsense	—	Severe	(Brookes, Laurent et al. 2015)
p.K1087fs*43	Frameshift	—	Severe	(Rujirabanjerd, Nelson et al. 2010)
p.W1288Ter	Nonsense	C-terminus	Severe	(Jensen, Amende et al. 2005)
p.R1481fs*9	Frameshift	—	Severe	(Abidi, Holloway et al. 2008)

The presence of KDM5D, the Y homolog of KDM5C, may be critical for male survival given that homozygous females are not described. However, studies in females carrying the mutation in heterozygosis display short term memory deficits (Simensen, Rogers et al. 2012). Furthermore, as previously said, the region containing Kdm5c displays intrinsic escape from XCI (Wu, Ellison et al. 1994, Li and Carrel 2008) and interestingly, females patients of other IDDs also seem to show skewed X-inactivation pattern (Fieremans, Van Esch et al. 2016).

Sub-microscopic duplications at Xp11.2 encompassing *KDM5C* and other two known ID genes (*TSPYL2* and *IQSEC2*) were also described. These provoke copy number variations (CNVs) in four males presenting with ID and significant deficits of speech development. Some patients also manifested behavioural disturbances such as hyperactivity and ADHD (Moey, Hinze et al. 2016).

Using a genome-wide approach, Weksberg and colleagues identified genes with significant loss of DNA methylation in blood of males with ID and mutations in the *KDM5C* gene. Loss of DNA methylation was detected at the promoters of the three top candidate genes *FBXL5*, *SCMH1*, *CACYBP* and parallel sex-specific DNA methylation profiles were observed in brain samples from control males and females at *FBXL5* and *CACYBP*. They supported the functional interdependency of DNA methylation and H3K4 methylation and provided new insights into the molecular pathogenesis of ID suggesting that some DNA methylation marks identified in blood can serve as biomarkers of epigenetic status in the brain (Grafodatskaya, Chung et al. 2013). Interestingly, the transcription factor ZNF711 has been also related to ID and it seems to bind together with another ID-linked KDM (PHF8/KDM7C) the promoter of *KDM5C* regulating its expression (Kleine-Kohlbrecher, Christensen et al. 2010, van der Werf, Van Dijck et al. 2017).

A recent study in 2016 described the first mouse model for this disease. KO male mice recapitulate many of the cognitive and adaptive impairments described in patients including long-term memory deficits and aggression. Molecular and cellular correlates of neurological dysfunction, such as altered dendritic growth and reduction in the number of spines in structure as the amygdala (AMY) and prefrontal cortex (PFC), were also shown. The study also reported the dysregulation of genes related to neuronal structure (**Figure 8**) (Iwase, Brookes et al. 2016). In the study presented in Chapter I of Results and recently published in *Cell Reports*, we extend the behavioural, transcriptomic and epigenomic characterization of those mice and compare them with inducible forebrain specific KO mice (**Table 2**) (Scandaglia, Lopez-Atalaya et al. 2017).

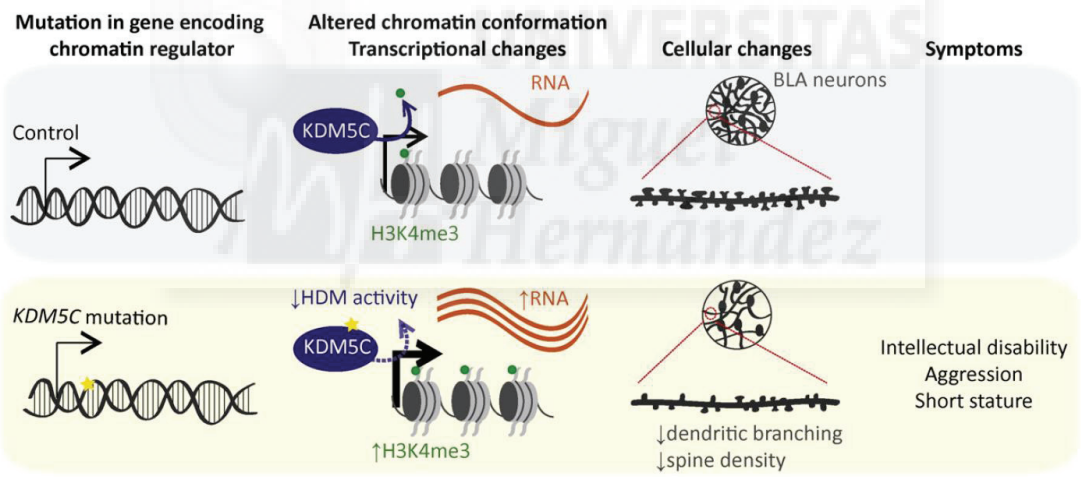

Figure 8. Scheme depicting how mutations in KDM5C cause disease. General view of the discoveries described in (Iwase, Brookes et al. 2016). From (Brookes 2016)

Table 2. Main findings from studies on Kdm5c function

Study	*System*	*Methodology*	*Main findings*
(Iwase, Lan et al. 2007)	Zebrafish, primary rat neurons	Morpholinos, shRNA transfection	Kdm5c is important for neuronal survival and dendritic development
(Tahiliani, Mei et al. 2007)	Nuclear extracts of HeLa cells stably expressing doubly tagged (with Flag and haemagglutinin, HA) human SMCX	OE-cell line; RNAi	KDM5C interacts with E2F, DP1, Max and Mga; NCOR1, REST, HDAC1, HDAC2, G9a, HP1γ, RING1, RING2, H-l(3)mbt-like
		HeLa cells stably expressing SMCX-directed or control	KDM5C regulated neuronal genes co-occupying their promoters with REST
		shRNA	Its demethylase activity is not important to determine the severity of the disease
(Outchkourov, Muino et al. 2013)	Mouse ESCs and NPCs	KD of Kdm5c, ChIPseq	Kdm5c acts as a promoter repressor and an enhancer activator
(Brookes, Laurent et al. 2015)	Human fibroblasts from patients	WB	Human mutations reduce protein stability and demethylase activity
(Iwase, Brookes et al. 2016)	Mouse (PNCs, adult AMY and PFC)	ChIPseq and RNAseq	First model of CJ-XLID, Kdm5c is important for dendritic growth and spine density
(Shen, Xu et al. 2016)	Cancer cells	ChIPseq and RNAseq	KDM5C cooperates with RACK7 to repress super-enhancers
(Scandaglia, Lopez-Atalaya et al. 2017)	Mouse (PNCs, adult HP)	ChIPseq and RNAseq	Kdm5c regulates different genes along life (germ line genes, eRNAs and spurious transcripts)

ESCs= embryonic stem cells; NPCs= neuroprogenitors; PNCs= primary neuronal culture, HP= hippocampus, AMY= amygdala, PFC= prefrontal cortex; shRNA= short hairpin RNA.

1.4.5. KDM5C and other diseases

In addition to ID and autisms, KDM5C has also been involved in other neuropsychiatric conditions, such as Huntington's disease (Vashishtha, Ng et al. 2013) and drug addiction (Aguilar-Valles, Vaissiere et al. 2014). Thus, Thompson and colleagues found that reducing the levels of this KDM in primary neurons reversed down-regulation of key neuronal genes caused by mutant huntingtin expression, suggesting a link between the transcriptional alterations caused by alteration of the levels of either protein (Vashishtha, Ng et al. 2013). In mice, Miller and colleagues

demonstrated that knocking-down Mll1 reduced H3K4me3 at target genes disrupting methamphetamine (METH)-associated memory in the nucleus accumbens (NAc). On the other hand, knocking-down Kdm5c resulted in hypermethylation of H3K4 and prevented the expression of METH-associated memory (Aguilar-Valles, Vaissiere et al. 2014). However, the main disease associated with *KDM5C* mutations and changes in KDM5C levels is cancer (Ricketts and Linehan 2015), although its role in this pathology is not well understood yet.

Clear cell renal cell carcinoma (ccRCC) is the most common form of adult kidney cancer. It is characterized in most cases by the presence of inactivating mutations in the VHL (Von Hippel-Lindau) Tumor Suppressor gene (Rini, Campbell et al. 2009). Inactivating mutations were found in different chromatin modifying enzymes such as SETD2, KDM6A and KDM5C (Dalgliesh, Furge et al. 2010). *KDM5C* is mutated in about 3% of ccRCCs. Moreover, like other KDMs, including KDM1A and KDM2B, KDM5C is induced by hypoxia-inducible factors (HIF) and hence potentially deregulated in pVHL-defective tumor cells. Increased expression of KDM5C in pVHL-defective tumor cells decreases H3K4 methylation and decreases cell proliferation *in vitro* and *in vivo*. Knock-down (KD) of KDM5C seems to greatly enhance tumor growth, suggesting that KDM5C is tumor suppressive in kidney cancer (Niu, Zhang et al. 2012). In ccRCC cells, KDM5C inactivation led to the unrestrained expression of heterochromatic ncRNAs which, in turn, triggers genomic instability. Thus, the inactivation of KDM5C in renal cancer led to heterochromatin disruption, genomic rearrangement, and aggressive ccRCC (Rondinelli, Rosano et al. 2015).

Another study highlighted that nuclear KDM5C expression is an independent prognostic parameter of prostate cancer (Stein, Majores et al. 2014). Indeed, KDM5C is functionally involved in proliferation control of prostate cancer cells, its KD induced

regulation of several proliferation associated genes. Thus, it can be used as a candidate marker for therapy failure in patients after prostatectomy (Kim, Shin et al. 2008, Stein, Majores et al. 2014).

KDM5C has been reported to have a role also in invasive human hepatocellular carcinoma (HCC) cells where it is found abundantly expressed. KD of KDM5C inhibited HCC cell migration, invasion and epithelial-mesenchymal transition *in vitro*, and markedly decreased the metastasis capacity of invasive HCC cells in the liver and lung. On the other hand, ectopic expression of KDM5C in HCC cells promoted cell migration, invasion and epithelial-mesenchymal transition (Ji, Jin et al. 2015).

KDM5C mutations have also been detected in malignant fibrous histiocytoma (MFH) (Li, Liu et al. 2017) and atypical meningioma (Lee, Lee et al. 2015). Moreover, loss of RACK7 or KDM5C leads to de-repression of S100A oncogenes and various cancer-related phenotypes in human breast cancer cells. Thus RACK7/KDM5C complex, when compromised, could have a tumorigenic potential (Shen, Xu et al. 2016). Further supporting a link between KDM5C and cancer, recent studies demonstrated that KDM5C is overexpressed in breast cancer cells (Denis, Van Grembergen et al. 2016) and that its expression level positively correlated with metastasis (Wang, Wei et al. 2015).

OBJECTIVES

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1. To extend the characterization of the first mouse model for Claes-Jensen type XLID, including the investigation of both Kdm5c-KO males and carrier females.
2. To dissect the developmental and adult component of the syndrome by producing forebrain inducible conditional Kdm5c-KO (ifKO) mice and comparing this new strain with conventional KOs.
3. To describe the changes of H3K4 methylation resulting from the elimination of Kdm5c in hippocampal cells.
4. To determine how these changes affect the hippocampal transcriptome with the aim of drawing causal links between epigenetic and transcriptional changes in both strains.
5. To delineate the different genetic programs and genomic actions regulated by Kdm5c during development and in the adult brain.
6. To produce the first brain organoids derived from CJ-XLID patients to investigate early developmental deficits in a human cellular context.

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GENERAL MATERIALS AND METHODS

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The work presented in this thesis is highly interdisciplinary. The materials and methods include the following techniques and methodologies:

- Molecular biology techniques: gene expression and epigenetic analyses *in vivo*, including western-blot, immunocytochemistry, immunohistochemistry, MRI analysis, quantitative RT-PCR, ChIP-qPCR and DNA methylation assays.
- Cell biology techniques: *in utero* electroporation, murine neuronal cultures, hiPSCs and cerebral organoids cultures, DNA transfection and viral transduction, luciferase and cell death assays.
- Genomic analyses: microarray analysis, RNAseq and ChIPseq. Bioinformatics and functional genomics using R/Bioconductor packages and online tools (e.g., Samtools, STAR, HTSeq2, DESeq2, Hisat2, MACS2, etc).
- Mouse genetics: the projects require different genetically modified mouse strains (transgenic mice and conditional knockouts).
- Behavioural studies: we have established a battery of behavioural tasks to evaluate basic behaviour and cognitive abilities in mice. This battery includes fear conditioning, spatial navigation in a water maze, novelty exposure, marble burying, open field, elevated plus maze, etc.

More details can be found in the following sections: Results, Chapter I in **Experimental Procedures** and **Supplementary Experimental Procedures** sections, Results, Chapter II, in the **Experimental methods** section, Annex I chapter in the **Materials and Methods** section.

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RESULTS:

CHAPTER I

Loss of Kdm5c causes spurious transcription and prevents the fine-tuning of activity-regulated enhancers in neurons

Marilyn Scandaglia, Jose P. Lopez-Atalaya, Alejandro Medrano-Fernandez, Maria T. Lopez-Cascales, Beatriz del Blanco, Michal Lipinski, Eva Benito, Roman Olivares, Shigeki Iwase, Yang Shi, and Angel Barco

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Some of the experiments presented in this article were performed in collaboration with members of my laboratory and with researchers from other institutions that are co-authors of the article.

Dr. Shigeki Iwase and Dr. Yang Shi provided the *Kdm5c* floxed mouse line used in this study. Dr. Eva Benito conducted seminal experiments involving Kdm5c cDNA. Roman Olivares genotyped all the mouse lines. Alejandro Medrano-Fernandez collaborated in the behavioural characterization of mouse strains. Dr. Beatriz del Blanco helped me in specific PNC experiments. Dr. José López-Atalaya processed and analysed ChIPseq data. Maria Teresa López Cascales processed and analysed RNA-seq data. Michal Lipinski generated the H3K27Ac ChIPseq profile used in some bioinformatical analyses. I designed and performed most of the behavioural, cellular, and molecular biology experiments presented in this thesis, collaborated and conducted specific bioinformatical analyses, performed statistical tests and other analyses, interpreted the data, and wrote the article in collaboration with my supervisor Prof. Angel Barco.

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Loss of Kdm5c causes spurious transcription and prevents the fine-tuning of activity-regulated enhancers in neurons

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Summary

During development, chromatin-modifying enzymes regulate both the timely establishment of cell type-specific gene programs and the coordinated repression of alternative cell fates. To dissect the role of one such enzyme - the intellectual disability-linked lysine demethylase 5C gene (Kdm5c) – in the developing and adult brain, we conducted parallel behavioral, transcriptomic and epigenomic studies in *Kdm5c* null and forebrain-restricted inducible knockout mice. Together, genomic analyses and functional assays demonstrate that Kdm5c plays a critical role as a repressor responsible for the developmental silencing of germ line genes during cellular differentiation, and in fine-tuning activity-regulated enhancers during neuronal maturation. Although the importance of these functions declines after birth, Kdm5c retains an important genome surveillance role preventing the incorrect activation of non-neuronal and cryptic promoters in adult neurons.

eTOC Blurb

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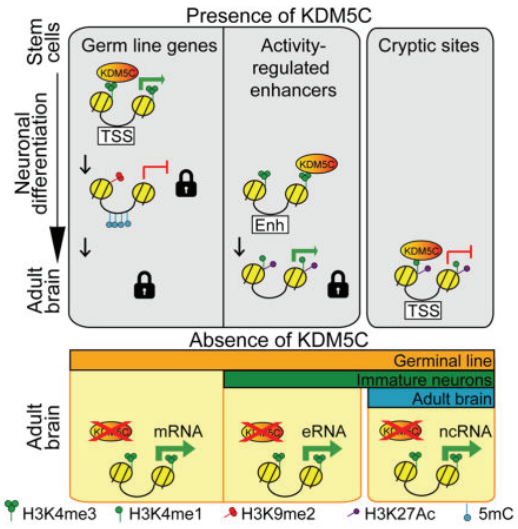
Authors' contributions: M.S. performed and analyzed cellular and molecular biology experiments with the collaboration of A.M.F., B.B., M.L. and R.O. in specific assays. A.M. and M.S. collected and analyzed behavioral data. J.P.L.A. conducted bioinformatics analyses. M.T.L.C., M.S. and A.B collaborated in bioinformatics analyses. S.I. and Y.S. generated the *Kdm5c* null and floxed strains. E.B. conducted seminal experiments related to KDM5C. A.B. conceived the study and secured funding. A.B. and M.S. designed the experiments and wrote the manuscript. All authors read and commented on the manuscript.



Scandaglia et al. show that Kdm5c plays critical roles constraining transcription during neuronal differentiation and maturation. Although Kdm5c contribution to neuronal transcription regulation later declines, it retains a genome surveillance role precluding spurious transcription in adult neurons. These functions likely contribute to the pathoetiology of Claes-Jensen type X-linked intellectual disability.

Introduction

The development of the nervous system is a highly organized process that requires precise spatial and temporal regulation of gene programs involved in differentiation, maturation and survival of neurons, but also the repression of alternative cell fates and restriction of cell type-specific gene expression (Lilja et al., 2013). Such dynamic expression patterns are sustained by extensive changes in the epigenome, and consequently, mutations of genes encoding chromatin-modifying enzymes can lead to severe neurodevelopmental disorders (Bjornsson, 2015; Kleefstra et al., 2014). In particular, mutations in the X-linked gene encoding for the lysine-specific demethylase 5C (*KDM5C*, also known as *JARID1C* or *SMCX*) can cause Claes-Jensen type X-linked intellectual disability (CJ-XLID) (Claes et al., 2000; Jensen et al., 2005). This rare syndrome accounts for approximately 1–3% of all XLID cases, and besides producing severe intellectual disability, is characterized by autistic behavior, short stature, hyperreflexia, emotional outbursts, spastic paraplegia, and epileptic seizures (Adegbola et al., 2008; Goncalves et al., 2014).

KDM5C mediates the demethylation of tri- and di-methylated lysines in position four of histone H3 (H3K4me3 and H3K4me2, respectively), functioning in non-neuronal cells as a transcriptional co-repressor of the RE1-silencing transcription factor (REST) complex (Iwase et al., 2007; Tahiliani et al., 2007) and as an enhancer modulator (Outchkourov et al., 2013; Shen et al., 2016). Notably, several other intellectual disability disorders (IDDs) also originate from mutations in enzymes that either modify or interact with the different species

of K4-methylated histone H3 (Parkel et al., 2013; Vallianatos and Iwase, 2015). Furthermore, changes in H3K4 methylation are correlated with memory acquisition (Graff et al., 2011; Gupta et al., 2010; Kerimoglu et al., 2013), but whether this histone posttranslational modification (HPTM) has a functional role in the process is still unknown.

Although KDM5C is mainly expressed in brain and skeletal muscle in adult human tissues (Jensen et al., 2005), the mechanisms that link transcriptional deregulation with impaired Kdm5c function in the developing and adult brain remain largely unexplored. A recent and seminal study, upon developing the first animal model of CJ-XLID, demonstrated that the germinal loss of Kdm5c in mice causes dendritic and spine anomalies, alterations of the neural transcriptome, and behavioral deficits that resembled clinical symptoms (Iwase et al., 2016), but did not determine the specific genomic actions of Kdm5c in neurons throughout life. Shortly afterwards, Shen et al. demonstrated that loss of Kdm5c results in the activation of a set of enhancers in human breast cancer cells (Shen et al., 2016), but whether enhancer over-activation also occurs in neurons and contributes to ID remains unexplored. To address these questions, we conducted parallel behavioral and genomics screens in mice exhibiting either germinal or adult forebrain ablation of *Kdm5c*. Our comprehensive analyses, together with loss- and gain-of-function experiments in neuronal cultures, demonstrate the dose- and time-dependent control of cognitive development by Kdm5c and its distinct functions in developing and mature neurons.

Results

Temporal dissection of CJ-XLID endophenotypes in mice

To determine the specific role of Kdm5c in the adult brain and its contribution to cognitive processes, we generated *CaMKIIα-creERT2::Kdm5C*$^{f/f}$ mice (from now on referred to as ifKO and their control littermates as ifWT) in which gene ablation was spatially restricted to principal neurons of the forebrain and temporarily regulated by tamoxifen administration in the adult stage (Fig. 1A–B). In order to distinguish between the adult and developmental effects of *Kdm5c* ablation, we conducted parallel neurological and behavioral assessments of ifKOs and *Kdm5c* null mice (KOs).

Kdm5c$^{-/y}$ males had smaller bodies and brains (Fig. 1C and S1A–B), but normal brain histology and anatomy (Fig. S1C–G), while ifKO males displayed normal weight and brain size (Fig. 1C and S1H–J). Exhaustive behavioral testing confirmed the cognitive impairments of *Kdm5c* KOs (Iwase et al., 2016) (Fig. 1D–E) and revealed traits related to CJ-XLID endophenotypes (Claes et al., 2000; Fujita et al., 2016; Goncalves et al., 2014) that had not been described before. These included hyperreflexia (Fig. 1F), increased impulsivity and emotional responses (Fig. 1G–I), impaired motor coordination (Fig. 1J), and epileptic seizure propensity (Fig. 1K; see also Fig. S2A–L and Table S1). In contrast, ifKOs were indistinguishable from control littermates in our battery of behavioral tasks with the exception of a significant learning delay in the Morris water maze (MWM), both in the hidden platform and reversal phases (Fig. 1D–J and 1L, S2M–O and Table S2).

Clinical investigations indicate that females carrying *KDM5C* mutations suffer from mild cognitive impairments (Rujirabanjerd et al., 2010; Simensen et al., 2012). To model this

aspect of *KDM5C*-related pathology, we evaluated *Kdm5c*$^{+/-}$ females and found that they were slightly smaller than their control littermates (Fig. 2A), showed hind-paw clasping (although to a lesser extent than males, Fig. 2B and S2A), exhibited memory deficits in the fear conditioning task (Fig. 2C), and presented a learning delay in the cued phase of the MWM task (Fig. 2D–E). Other behavioral abnormalities, however, were corrected for by the presence of the wild-type allele (Table S1 and S2). We also searched for behavioral impairments in ifKO females, but did not observe any significant differences in basal conditions or after stimulating the animals through environmental enrichment (Fig. S2P–R).

Overall, these experiments validate *Kdm5c*–KO mice as a suitable model to investigate CJ-XLID, including the milder impairments seen in female carriers. Furthermore, the comparison of conventional and conditional KOs indicate that (i) Kdm5c plays a more prominent role during development than in the adult brain, and (ii) Kdm5c still retains some function in mature principal neurons responsible for the spatial navigation defects observed in ifKO males. We cannot, however, discard that Kdm5c ablation in other cells or brain regions could cause stronger phenotypes than those reported here for ifKOs.

Kdm5c restrains H3K4me3 content at specific promoters and enhancers in adult neurons

To identify the molecular causes of these phenotypes, we next conducted parallel genomic screens in KOs and ifKOs. We reasoned that the analysis of KOs would clarify CJ-XLID pathoetiology and Kdm5c roles during development, whereas the investigation of ifKOs could provide novel insights into the specific genomic actions of Kdm5c in mature neurons. We focused on epigenetic and transcriptional alterations in the hippocampus because this brain region is highly relevant for cognitive processes (including those affected in both KOs and ifKOs) and has a high level of Kdm5c expression (Xu et al., 2002).

The occupancy profile of Kdm5c in hippocampal chromatin confirmed the loss of Kdm5c binding in KOs (Fig 3A). No global difference was observed in bulk H3K4me3 levels for KOs and ifKOs (Fig. S3A). However, chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) revealed a prominent increase (~12%) in the number of H3K4me3-enriched regions in KOs (Fig. S3B–C), as well as local increases in H3K4me3 levels in both strains (Fig. 3B and S3D). Differential profiling analysis identified 1,423 and 540 differentially methylated H3K4me3 peaks (DHMPs) in KOs and ifKOs, respectively (Fig. 3C and Table S3). These changes largely corresponded to increases in H3K4 methylation, with each strain having a negligible number of regions showing reduced methylation. The overlap between these epigenomic changes in KOs and ifKOs was very high. For instance, 85% of the changes found in ifKOs were also observed in KOs, and about one third of the changes detected in KOs were observed in ifKOs, albeit with a smaller magnitude (Fig. 3D).

In addition to H3K4me1 and H3K4me3 profiles, we also determined H3K27ac enrichment in hippocampal chromatin from wild type mice to identify putative enhancers across the genome. H3K4me3 peaks preferentially locate at transcription start sites (TSSs) and putative enhancers (i.e., at intra- and intergenic regions that show a concomitant enrichment for H3K27ac and H3K4me1; Fig. S3E). However, DHMPs were overrepresented at intra- and intergenic regions, particularly for *de novo* peaks (Fig. 3E and S3F), suggesting that enhancers are particularly sensitive to the absence of Kdm5c. The impact of Kdm5c loss on

H3K4me3 at enhancers followed an inverted-U function, in which H3K27ac-rich enhancers with a moderate H3K4me3 content were the most affected (Fig. 3F and S3G). The same pattern was also observed in ifKOs (Fig. S3H). This indicates that regions depleted of H3K4me3 or with very high H3K4me3 content are both resilient to Kdm5c absence. Consistent with this view, the regions showing stronger Kdm5c binding in wild type mice exhibited reduced H3K4me1 signal in KOs, while the increase was not evident in the case of H3K4me3 (Fig. S3I). Therefore, Kdm5c seems to be required in regions in which it is necessary to maintain a given tri-methylation to mono-methylation balance.

Kdm5c modulates the expression of plasticity-related genes and fine-tunes activity-dependent enhancers during neuronal maturation

RNA-seq-based differential expression (DE) screens revealed that transcriptional changes were both larger and more numerous in KOs than in ifKOs (248 and 107 differentially expressed genes (DEGs), respectively) (Fig. 4A–B, S4A and Table S4). In fact, most of the DEGs in ifKOs were also altered in KOs with larger fold changes (Fig. S4B). In both strains, the changes consisted predominantly of upregulations, thereby supporting Kdm5c role in transcriptional repression. The correlation between H3K4me3 gain at TSSs and transcript levels was remarkably high for both mutants (Fig. 4C), indicating that the increment of H3K4me3 resulting from the loss of Kdm5c is highly predictive of transcriptional upregulation. In fact, in both KOs and ifKOs, 97% of the upregulated genes associated with H3K4me3 peaks showed an increase of H3K4me3 signal.

To link genomic and phenotypic alterations in Kdm5c-deficient mice, we first focused on genes exclusively altered in KOs (Fig. 4D) because we reasoned that these changes should be accountable for the severe phenotypes observed in those mice. This DEG set included a number of genes linked to neuronal terms according to Gene Ontology (GO) enrichment analysis. These genes showed appreciable hippocampal expression, and relatively modest changes in transcription and H3K4 methylation (light green dots in Fig. 4E). Downregulations affected genes important for synaptic transmission and neurodevelopment (e.g., *Grik3*, *Nrp2*, and *Syt2*), while upregulated genes were involved in neuronal signal transduction (e.g., *Hexim1*, *Hexim2*, *Dusp5*, and *Dusp18*). Consistent with our results, transcriptome analyses in the amygdala and frontal cortex of Kdm5c-KO mice had also revealed misexpression of neuronal genes, but whether these changes were directly linked to altered H3K4 methylation was not explored (Iwase et al., 2016). Our parallel epigenome and transcriptome screens enabled the direct comparison of both effects *in vivo*. The correlation between differential H3K4me3 methylation at TSSs and changes of transcript levels for DE neuronal genes was modest, suggesting that the impact of Kdm5c loss in some of these loci might rely on changes at more distant regulatory sequences.

Given the role of Kdm5c in regulating enhancer function, as well as the potential causal link between KO phenotypes (such as learning impairment and epilepsy propensity) and altered activity-driven transcription, we next investigated the contribution of Kdm5c to gene induction upon exploration of a novel environment (NE). This experience is known to trigger hippocampal transcription of numerous immediate early genes (IEGs) involved in synaptic plasticity and memory consolidation (Fig. 5A, S5A and Table S5) (Flavell and

Greenberg, 2008). Strikingly, NE-induced changes in KOs represented about one third of those observed in WTs (66 *versus* 186 genes). This was because a significant proportion of the NE transcriptional program was moderately overexpressed in KOs in naïve situation (homecage: HC) (Fig. 5A–B), suggesting a role for Kdm5c fine-tuning activity-driven transcription. Independent RT-qPCR for the IEGs *Fos* and *Arc* confirmed this observation (Fig. 5C).

We next investigated the impact of NE on H3K4me3 profiles and its relationship with Kdm5c loss. Our ChIP-seq experiment revealed that NE caused a 5% increase in the number of detected peaks in hippocampal chromatin (Fig. 5D), demonstrating that the levels of H3K4me3, like the transcriptome, are susceptible to modulation by this experience. Notably, NE-induced changes in H3K4me3 profiles were partially occluded in KOs (Fig. 5D–E), which is consistent with Kdm5c playing a role in the regulation of activity-dependent enhancers. H3K4me3 levels at proximal enhancers of important IEGs involved in neuroplasticity were significantly elevated in naïve *Kdm5c*-KO mice, even when the TSS was not affected. A clear example of this is the IEG *Npas4*, which encodes a transcription factor that regulates cognition-related transcription and GABAergic synapse formation (Ramamoorthi et al., 2011). H3K4me3 levels at the enhancer region 10 Kb upstream of the *Npas4* TSS were higher in KOs than in WTs, and this increase was associated with augmented transcription of the enhancer RNA (eRNA) at the basal state (Fig. 5F–G). A similar scenario was also observed at enhancers 2 and 5 of *Fos* (Fig. S5B–C) and at the *Arc* enhancer (Fig. S5D–E), although for the latter, H3K4me3 levels at the promoter were also higher in KOs' chromatin.

In contrast to KOs, basal expression and induction of IEGs and H3K4me3 profiles at these activity-regulated enhancers were normal in ifKOs (Fig. S5F–H). This suggests that Kdm5c plays a specific role in establishing the ground activity of IEGs during neuronal maturation. To tackle this model, we investigated the fine-tuning of activity-regulated enhancers using primary neuronal cultures (PNCs), and found that *Kdm5c* ablation in PNCs from *Kdm5c*$^{f/f}$ embryos using a Cre recombinase-expressing lentivirus (LV) enhanced basal expression of IEGs such as *Fos*, *Arc* and *Npas4* (Fig. 5H–I). Moreover, normal basal IEG expression was observed upon co-infection with a second LV driving the expression of wild type human KDM5C (hKDM5C) (Fig. 5H–I). These experiments show that these loci are still susceptible to epigenetic tuning at this stage, and demonstrate that Kdm5c is both necessary and sufficient for proper modulation of activity-regulated enhancers during neuronal maturation.

Germinal loss of Kdm5c precludes the silencing of germ line genes

The exploration of DEG exclusive of KOs also revealed a subset comprised of genes related to reproduction and gametogenesis, such as *D1Pas1*, *Naa11*, *Ddx4* and *Ccnb1iP1,* that had not been reported in previous screens. These genes had very low or undetectable expression in the hippocampus of control mice, and presented large, highly correlated changes in transcript and H3K4me3 levels in the hippocampus of *Kdm5c*-KO mice (pink and purple dots in Fig. 4E). In fact, most of the genes presenting the largest differences between KO and wild type littermates belonged to this category (Fig. 4D). Interestingly, some of these

genes (pink dots) showed robust Kdm5c binding at their promoters in the chromatin of embryonic stem cells (ESCs), although this occupancy decreased over time, from neuroprogenitor cells (NPCs) to mature neurons (MNs) (Fig. 6A–B). Kdm5c disappearance coincides with the strong DNA methylation of these loci in wild-type mature neurons, according to available MeDIP data (Halder et al., 2016) (Fig. 6B and S6A, pink track). To confirm this model, we next examined DNA methylation levels in the hippocampi of KO mice and control littermates. Reduced CpG methylation at the promoter of the germ line genes *D1Pas1* and *Naa11* (Fig. 6C) was concomitant with their enhanced transcription (Fig. 6D). Further supporting our hypothesis, PNCs from *Kdm5c*$^{-/y}$ embryos also showed de-repression of *D1Pas1* and *Naa11* (Fig. 6E–F), whereas PNCs from *Kdm5c*$^{f/f}$ embryos transduced with Cre recombinase showed normal germ line silencing despite of a rapid and strong reduction in the levels of Kdm5c (Fig. S6B). These results indicate that Kdm5c is necessary for germ line gene silencing during early development but becomes dispensable at later developmental stages once a repressed status has been established through other mechanisms such as DNA methylation (Fig. S6C).

To examine whether the spurious expression of germ line genes in neurons can be rescued by restoring Kdm5c activity, PNCs from *Kdm5c*$^{-/y}$ embryos were transduced with a hKDM5C-expressing lentivirus (Fig. 6D). Interestingly, hKDM5C expression did not restore the silent status of germ line genes (Fig. 6E), which suggests that the mechanism of Kdm5c repression requires of cofactors that are absent in postmitotic neurons. Together these experiments show that Kdm5c is necessary to assure the silencing of germ line genes during early development. Consistent with this view and underscoring the clinical relevance of our finding, blood cells of CJ-XLID patients show multilocus loss of DNA methylation (Grafodatskaya et al., 2013).

Kdm5c retains a role preventing transcription from cryptic and non-neuronal promoters and enhancers in adult neurons

We next explored the transcriptional changes occurring in both strains (referred to as common DEGs in Fig. 4D). These genes are likely to be direct and permanent targets of Kdm5c and could thus provide insight into the somatic actions of this enzyme. Moreover, although these genes may not play a primary role in the most severe behavioral deficits observed in KOs, their deregulation was associated with impaired spatial navigation in ifKOs.

The integration of DHM and DE data revealed that common DEGs behaved remarkably similar in both strains. Prominently upregulated transcripts of this set (FC > 2) showed significant H3K4me3 increases at their TSSs, and characteristically low hippocampal expression in control mice (< 10 transcripts per million, TPM) (Fig. 7A). Their transcriptional and H3K4me3 profiles were thereby similar to those observed at de-repressed germ line genes. According to GO enrichment analyses, the common DEGs were associated with biological processes that are not related to neuron function, such as *Muscle contraction* and *Extracellular matrix assembly* (Fig. 7B). The set was comprised of both protein-coding genes and a number of non-coding RNAs (ncRNAs) (25%, including both miRNAs and lincRNAs). Although some of these strongly upregulated transcripts corresponded to full-

length messenger RNAs (mRNAs) (e.g., *Tnxb*, Fig. 7C), intriguingly, others were truncated mRNAs or partially overlapping ncRNAs. For example, upregulation of the muscle genes *Dnah1* (Fig. 7C) and *Acta1* (Fig. S7A) was restricted to their terminal exons and started at an internal DHMP. These truncated transcripts did not coincide with shorter isoforms of assigned biological function, but instead resembled a recently described class of eRNAs referred to as multiexonic eRNAs (meRNAs) that act as alternate promoters and utilize splicing and poly-adenylation signals from their protein-coding hosts (Kowalczyk et al., 2012).

To further explore this phenomenon in an unbiased manner, we extended our DE screen to non-annotated transcripts using *de novo* transcriptome assembly. This approach allowed us to identify several dozen additional Kdm5c-dependent transcripts, including both novel isoforms transcribed from internal TSSs, and non-annotated intergenic transcripts (Table S4 and Fig. S7B). Like for annotated genes, there was a large overlap between the expression changes in KOs and ifKOs (100% of ifKO changes were observed in KOs) and a high correlation with H3K4me3 levels (Fig. 7D, see examples in Fig. 7E and S7C). Indeed, examination of RNA-seq data around DHMPs located at intergenic regions revealed a robust and consistent association between elevated H3K4me3 and the production of transcripts (Fig. 7F and S7D). These DHMPs showed H3K27ac and Kdm5c occupancy in the chromatin of control mice, suggesting that these regions may have a regulatory or structural role in wild type mice (Fig. 7G and S7E). Re-analysis of publicly available transcriptomic data (Iwase et al., 2016) confirmed that the spurious transcription described here also occurs in other brain regions of *Kdm5c*-KOs (Fig. S7F). Notably, the spurious activation of these loci was also observed in PNCs from *Kdm5c*$^{f/f}$ embryos few days after triggering gene ablation (Fig. 7H), which demonstrate that, contrary to the situation described above for germ line genes, Kdm5c is constantly required to prevent the transcription from these cryptic promoters/regulatory elements.

The overexpression of some of these ncRNAs can directly alter the expression of protein-coding genes. For example, the downregulation of *Zfhx2*, which encodes a homeobox transcription factor highly expressed in the developing brain and whose germinal loss causes behavioral abnormalities (Komine et al., 2012), was not associated with H3K4me3 changes at its promoter, but rather with the upregulation of an anti-sense ncRNA (partially overlapping with the previously described *Zfhx2os* (Komine et al., 2006)) transcribed from a downstream DHMP (Fig. 7I). A similar type of interference, in which the absence of Kdm5c results in the production of an anti-sense ncRNA, may also occur at other gene loci (e.g., *Ssh1* and *Depdc5*). It is also possible that some of these ncRNAs are *trans*-acting, which could explain the downregulation of neuronal genes with no apparent H3K4me3 changes at their promoters and proximal regulatory regions.

Discussion

Dose- and time-dependent control of cognitive development by Kdm5c

Our comprehensive characterization and exhaustive analyses of conventional and inducible forebrain-restricted knockout mice demonstrate that this chromatin-modifying enzyme exerts a tight dose- and time-dependent control of cognitive development. The correlation in

the magnitude of epigenetic, transcriptional and cognitive dysfunctions across the two mouse strains, and the result of our loss- and gain-of-function experiments in neuronal cultures strongly supports a role for epigenetic dysregulation in the etiology of the syndrome. More specifically, our results suggest that the inability of Kdm5c-deficient neurons to silence germ line genes, to fine-tune the neuronal epigenome, and to preclude spurious transcription are probable causes of neurological symptoms. This insight opens up new avenues for therapy and pinpoints specific genes as potential biomarkers for this disorder. Moreover, since Kdm5c directly interacts with other ID-linked epigenetic regulators, our findings go beyond CJ-XLID and can also benefit other chromatin-related IDDs that target the same processes and gene networks.

Kdm5c is essential for the silencing of germ line genes in early development

During the development of multicellular organisms gene silencing is achieved through the implementation of precise DNA methylation patterns by DNA methyltransferases (DNMTs). The recruitment of these DNMTs requires the previous action of lysine demethylases (KDM) that remove methyl group from specific lysine residues associated with active transcription (Ooi et al., 2007), including (as we showed here) Kdm5c. Particularly interesting is our discovery that Kdm5c plays an essential role in the repression of germ line genes. Intriguingly, some of these genes are also upregulated in mouse embryos deficient for enzymes that introduce repressive marks in the chromatin, such as the methyltransferase of H3K9 Ehmt2 and the DNA methyltransferase Dnmt3b (Auclair et al., 2016), underscoring the interplay between DNA methylation, and H3K9 and H3K4 methylation (Du et al., 2015). Dnmt3b silences this class of genes by recruiting the transcriptional repressor E2F6 (Velasco et al., 2010). The ablation of other transcriptional repressors, such as Myc-associated Factor X (Max) and polycomb group ring finger 6 (Pcgf6), also leads to robust de-repression of germ cell-related genes affecting cell growth and viability (Endoh et al., 2017; Maeda et al., 2013). Most of these proteins (MAX, PCGF6, E2F6, EHMT2) seem to interact with KDM5C (Endoh et al., 2017; Outchkourov et al., 2013; Tahiliani et al., 2007), indicating that a large multimeric repressive complex is responsible for the silencing of germ line in somatic cells. This, together with our results, suggests that two mechanisms are necessary for promoting the silencing of these germ line genes: the removal of H3K4me3 by Kdm5c and the addition of H3K9me2 by Ehmt2. If either one of these processes is disrupted, these loci will not be inactivated by CpG methylation and will consequently remain transcriptionally active. Although the specific impact that the spurious expression of germ line genes has on neuronal differentiation and maturation remains unknown, it is striking that several chromatin-modifying enzymes linked to neurodevelopmental defects target the same or similar loci (Auclair et al., 2016; Hansen et al., 1999; Katz et al., 2009; Schaefer et al., 2009), which suggests that their defective silencing may play a role in the etiology of intellectual disability.

Kdm5c fine-tunes activity-regulated enhancers during neuronal maturation

Our study also clarifies conflicting *in vitro* results regarding the function of Kdm5c at enhancers (Outchkourov et al., 2013; Shen et al., 2016). Recent studies have blurred the functional boundary between enhancers and promoters by demonstrating that enhancers are often major sites of extragenic noncoding transcription (Andersson, 2015; Li et al., 2016;

Paralkar et al., 2016). The lack of Kdm5c would tip the balance between H3K4me3 (a HPTM strongly associated with active promoters) and H3K4me1 (which is enriched at enhancers), thereby favoring eRNA transcription from Kdm5c-regulated enhancers. Among the enhancers fine-tuned by Kdm5c, we identified regulatory regions that influence the transcription of genes of singular importance in the context of neuronal plasticity, such as *Fos*, *Arc* and *Npas4*. Kdm5c modulation of the ground activity of activity-regulated enhancers seems to take place during neuronal maturation because the phenomenon was reproduced in floxed PNCs but not in ifKO brains, which indicates that these regions become resilient to the H3K4 methylation imbalance caused by Kdm5c loss in the adult brain. Tuning down activity-driven genes is essential for the maturation of dendritic arbors and the control of neural circuit responses to upcoming signals (Yang et al., 2016). It is thereby tempting to speculate that some of the phenotypes observed in KOs that recapitulate clinical symptoms, such as epilepsy propensity and learning disability, could be caused by the mistuning of activity-regulated enhancers. This opens the possibility that compounds that tune down neuronal activity could have therapeutic value in the treatment of CJ-XLID.

A role for Kdm5c in neuronal genome surveillance

The comparison of genomic effects after gene ablation in the germinal line, developing neurons and mature neurons, presented here indicates that the biological relevance of Kdm5c in neurons declines over time, probably in parallel with the progressive locking of the cell type-specific epigenetic status (Smith and Meissner, 2013) that narrows the possibility of spurious transcription. Nonetheless, Kdm5c seems to retain a life-long surveillance role in mature neurons preventing extemporaneous transcription from numerous intragenic and intergenic regulatory sites with transcriptional potential. Even though spurious transcription likely occurs in all tissues, the clinical picture of CJ-XLID indicates that this form of deregulation particularly affects the central nervous system. The function of most of these Kdm5c-sensitive loci and associated lncRNAs is unknown. However, their weak transcription in control animals and apparent tolerance to transcriptional deregulation *in vivo* would suggest that many of these transcripts do not have a critical function, and that the evolutionary pressure for establishing a locked state is lower than in protein coding genes. However, some of these Kdm5c-regulated ncRNAs may play key regulatory roles in specific cell types or developmental stages (e.g., the consequences of altering the *Zfhx2*/*Zfhx2os* expression balance are likely more severe during development than in mature neurons). Furthermore, the consequences of the spurious activation of intergenic sites may be more severe in humans than mice since a recent study linked the expression of thousands of unannotated, non-exonic differentially expressed regions (DERs) in the human prefrontal cortex to a higher risk of developing brain disorders and variations in neuronal phenotype (Jaffe et al., 2015). It is also possible that more prominent phenotypes could emerge in *Kdm5c*-ifKOs upon exposure to specific situations or challenges. For instance, Kdm5c ablation in breast cancer cells was not deleterious by itself, but did favor the emergence of a tumorigenic phenotype (Shen et al., 2016).

In the context of cancer research, KDM5C-inactivating mutations have been associated with genome instability and poor prognosis (Rondinelli et al., 2015). In agreement with our results in neurons, these cancer cells overexpressed lincRNAs that are normally silent.

Recent studies have underscored the relevance of genome instability in brain pathology, particularly in the context of aging and age-related neurodegenerative disorders (Madabhushi et al., 2014; McKinnon, 2013). Neurons are very long-living cells that have to withstand challenge from numerous stressors over their lifetime, which makes critical to safeguard their DNA integrity (Pan et al., 2014). The lifelong role of Kdm5c in genome surveillance described here can not only explain its retained expression in adult neurons and the deficits observed in ifKOs, but also pinpoints new possible mechanisms of pathoetiology.

Experimental Procedures

For details, refer to Supplemental Experimental Procedures.

Animals and neuronal cultures

The generation of *Kdm5c*$^{-/y}$, *Kdm5c*$^{f/f}$ (Iwase et al., 2016) and CaMKIIα-creERT2 (Erdmann et al., 2007) mice have been previously described. Experiments were conducted in 2–6 months old animals, except when it is otherwise indicated. Primary cortical and hippocampal neurons were obtained from E17.5–E18.5 embryos, and were infected with lentiviral pseudovirions at the times indicated in the text. Mice were maintained and bred under standard conditions, consistent with Spanish and European regulations and approved by the Institutional Animal Care and Use Committee.

Behavioral testing

Independent cohorts of adult *Kdm5c*-KO males, *Kdm5c*$^{-/+}$ females and *Kdm5c*-ifKOs from both sexes were tested in series of behavioral tasks. The different tasks are described in detail in Supplemental Experimental Procedures.

Histology

Nissl staining and immunostainings were performed as described (Ito et al., 2014). The primary antibodies used in this study are α-NeuN (Chemicon, MAB377), α-MAP2 (Sigma, M9942), α-GFAP (Sigma, G9269), α-DCX (Abcam, ab18723), α-Parvalbumin (Swant, PV235), α-Cre (gift from Schütz's lab), α-H3K4me3 (Millipore 07-473), and α-GFP (Aves Labs, GFP-1020, valid also for recognizing EYFP). Nuclei were counterstained with a 1 nM DAPI solution (Invitrogen) before mounting. For magnetic resonance imaging (MRI), 4-month old *Kdm5c*-KO mice and wild-type littermates were examined.

Western blots, DNA isolation and pyrosequencing

Western blot analyses were carried out using Western Lightning ECL kit (Perkin-Elmer, Boston, MA), and α-H3K4me3 (Millipore 07-473) and α-actin (Sigma F5441) antibodies. DNA was extracted from adult hippocampi with QIAamp DNA Mini Kit (Qiagen). Bisulfite conversion of genomic DNA was performed with the EpiTect Fast bisulfite kit (Qiagen). Primer sequences for PCR amplification and pyrosequencing are provided in Supplemental Experimental Procedures.

RT-qPCR and RNA-seq

Total RNA from hippocampal cultures and tissue was extracted with TRI reagent (Sigma-Aldrich) and reverse transcribed to cDNA. Each independent sample was assayed in duplicate and normalized using GAPDH levels. RNA preparation for sequencing was performed as described in (Fiorenza et al., 2016). Information on library preparation and size, mapping to reference genome and differential expression and functional genomics analyses can be found in Supplemental Experimental Procedures.

ChIP assays and ChIP-seq

Chromatin immunoprecipitation (ChIP) experiments were conducted as described (Lopez-Atalaya et al., 2013) using anti-H3K4me3 (Millipore 07-473) anti-H3K4me1 (Abcam ab8895) and anti-Kdm5c (Iwase et al., 2016). See Supplemental Experimental Procedures for additional details.

Statistical analyses

All statistical analyses were two-tailed. P-values were considered to be significant when $\alpha < 0.05$. Mean ± s.e.m. and percentages are represented in bar graphs. For additional details see Supplemental Experimental Procedures.

Accession numbers

RNA-seq (GSE85874) and ChIP-seq (GSE85873) data can be accessed at the GEO repository.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Dose- and time-dependent control of cognitive development by Kdm5c
- Kdm5c is required for germ line gene silencing during early development
- Kdm5c functions as a fine-tuner of enhancers in maturing neurons
- Kdm5c retains a genome surveillance role in adult neurons

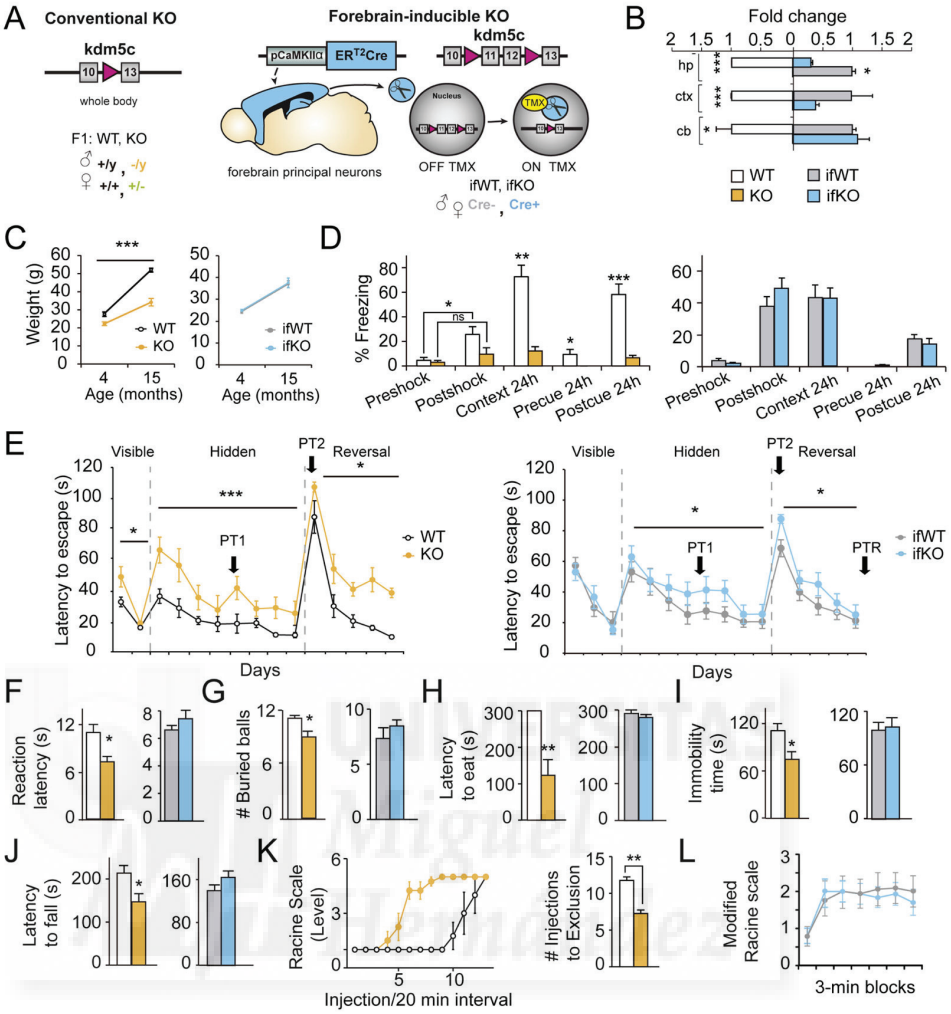

Figure 1. *Kdm5c*-KOs recapitulate CJ-XLID clinical symptoms and exhibit stronger phenotypes than ifKOs

A. Schematics of the genetic strategy used to obtain *Kdm5c*-KOs and ifKOs. **B.** Kdm5c transcript level in hippocampus (hp), cortex (ctx), and cerebellum (cb). RT-qPCR assays were conducted using primers specific for the deleted exons (hp: KO t_2=5.93, $p < 0.0001$; ifKO t_2=10.23, $p = 0.009$; ctx: KO t_2=82.27, $p = 0.0001$; ifKO t_8=2.21, $p = 0.06$; cb KO t_2=7.43, $p = 0.02$; ifKO t_2=1.63, $p = 0.2$). **C.** Average weight at different ages (KOs: $F_{(1,23)}$ = 67.79, $p < 0.0001$, post-hoc 4 months p<0.05, post-hoc 15 months p<0.0001; ifKOs: $F_{(1,33)}$ = 0.13, $p = 0.7$). **D.** Fear conditioning (FC) task in KOs (left: pre/post shock comparison, WT: U = 4.00, $p = 0.01$; KO: U =18.50, $p = 0.3$; context 24h: U = 0.00, $p = 0.0006$; pre-cue 24h: U = 8.00, $p = 0.02$; post-cue: t_{12}=5.93, $p < 0.0001$) and ifKOs (right: pre-shock, U = 74.00, $p = 0.3$; post-shock, t_{26}= 0.99, $p = 0.3$; context: t_{27}= 0.38, $p = 0.7$; pre-cue, U = 85.00, $p = 0.5$; post-cue U = 84.50, $p = 0.5$). **E.** MWM escape latency graph. Left: KOs showed deficits in the visible platform ($F_{(1,12)}$ = 4.913, $p = 0.05$), hidden platform ($F_{(1,108)}$ = 31.43, $p < 0.0001$), and reversal ($F_{(1,66)}$ = 5.60, $p = 0.02$) tests, as well as in the different probe

trials (PT, see Fig. S2). Right: ifKOs showed milder impairments in the hidden ($F_{(1,216)}$ = 6.26, p = 0.01) and reversal ($F_{(1,135)}$ = 7.22, p = 0.008) tests. **F.** Reaction latency in the hot plate (KOs: t_{12}= 3.05, p = 0.01; ifKOs: t_{21} = −1.26, p = 0.2). **G.** Marble burying test (KOs: t_{12} = 2.68, p = 0.02; ifKOs: t_{21} = −0.97, p = 0.3). **H.** Novelty suppressed feeding test (KOs: U = 49, p = 0.001; ifKOs: U = 92.00, p = 0.5). **I.** Mobility in the tail suspension test (KOs: t_{26} = 2.51, p = 0.01; ifKOs: t_{27} = 0.33, p = 0.7). **J.** Latency to fall in the accelerating rotarod task (KOs: t_{12} = 2.66, p = 0.02; ifKOs: t_{27} = 1.45, p = 0.2). **K.** Kainate-induced seizures measured in the Racine scale (left), and number of injections needed for exclusion (right: t_6 = 6.65, p = 0.0006). **L.** Sensitivity to pentylenetetrazol (PTZ)-induced seizures. Data are expressed as mean ± s.e.m. ns: non significant; * = p < 0.05; ** = p < 0.005; *** = p < 0.0005 in Mann-Whitney, Student's T-test or 2-way ANOVA.

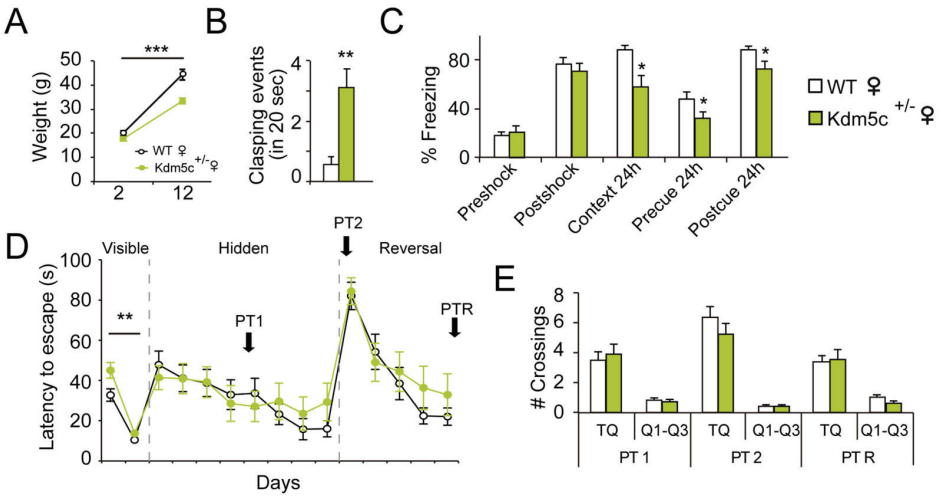

Figure 2. Cognitive impairments are *Kdm5c* dose-dependent

A. *Kdm5c*$^{+/-}$ females are smaller than their WT female littermates ($F_{(1,54)} = 17.86$, $p < 0.0001$; post-hoc 12-m $p < 0.0001$). **B.** Hemizygous females show hindpaw-clasping ($U = 31.50$, $p = 0.001$), although less severe than KO males (see Fig. S2A–B). **C.** Fear conditioning task: *Kdm5c*$^{+/-}$ females exhibit fear memory deficits (pre-shock, $t_{27} = 0.59$, $p = 0.6$; post-shock, $t_{27} = 0.7$, $p = 0.5$; context 24h, $U = 57.00$, $p = 0.04$; pre-cue 24h, $t_{27} = 2.10$, $p = 0.05$; post-cue 24h, $t_{27} = 2.49$, $p = 0.02$). **D–E.** Morris water maze: The escape latency curve (D) revealed mild learning difficulties during the first day of the task ($F_{(1,54)} = 8.91$, $p = 0.004$; post-hoc day 1 $p < 0.01$), but no difference in performance during the PTs (E). Data are expressed as mean ± s.e.m. * = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.0005$ in the Mann-Whitney test, Student's T-test and two-way ANOVA.

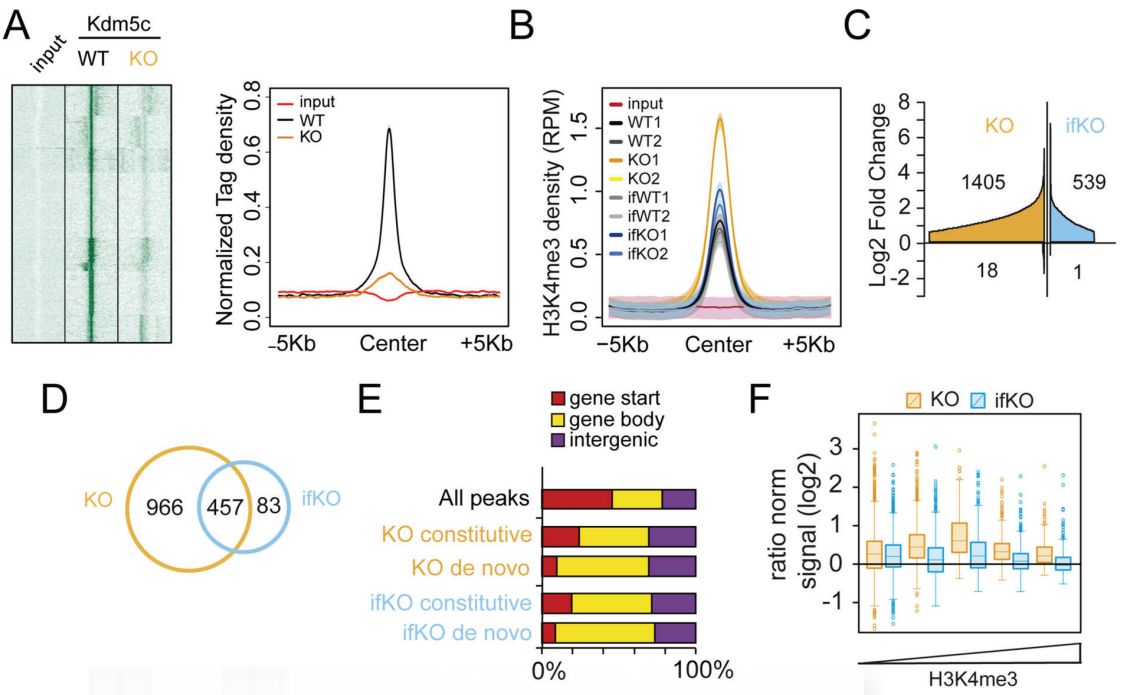

Figure 3. Lack of Kdm5c increases H3K4me3 levels throughout the neuronal epigenome

A. Heatmap (right) and line plot (left) showing Kdm5c density at Kdm5c-enriched regions (as detected in hippocampal chromatin of wild type mice) in KO and control littermates. Color intensity in the heatmap is proportional to read density. **B.** Density plot showing the average H3K4me3 signal (expressed as reads per million, RPM) of DHMPs for WT, KO, ifWT and ifKO replicates. Input values are also shown. Shaded areas represent the 95% confidence interval (CI). **C.** Cumulative graph showing the number of DHMPs (BH p adj. < 0.05, LFC > 0.58) and their fold change with respect to the relative control (i.e., KO *vs* WT; ifKO *vs* ifWT). DHMPs were ordered by Log2FC and plotted as separate entities on the x-axis. **D.** Venn diagram depicting the overlap between the DHMPs of KO (*vs* WT) and ifKO (*vs* ifWT) mice. **E.** Distribution of DHMPs in function of gene feature, in *Kdm5c*-KOs and ifKOs. "Gene start" refers to peaks located between ±1 Kb of the first TSS; "Gene body" denotes internal peaks (including alternative TSSs); and "Intergenic" refers to peaks that are farther than −1 Kb from the TSS of any Refseq annotated gene. **F.** H3K4me3 changes at putative enhancers (intra- and intergenic H3K27ac-rich regions not overlapping with a TSS) in KOs and ifKOs. Enhancers were classified according to their H3K4me3 content (see Fig. S3G–H for additional detail).

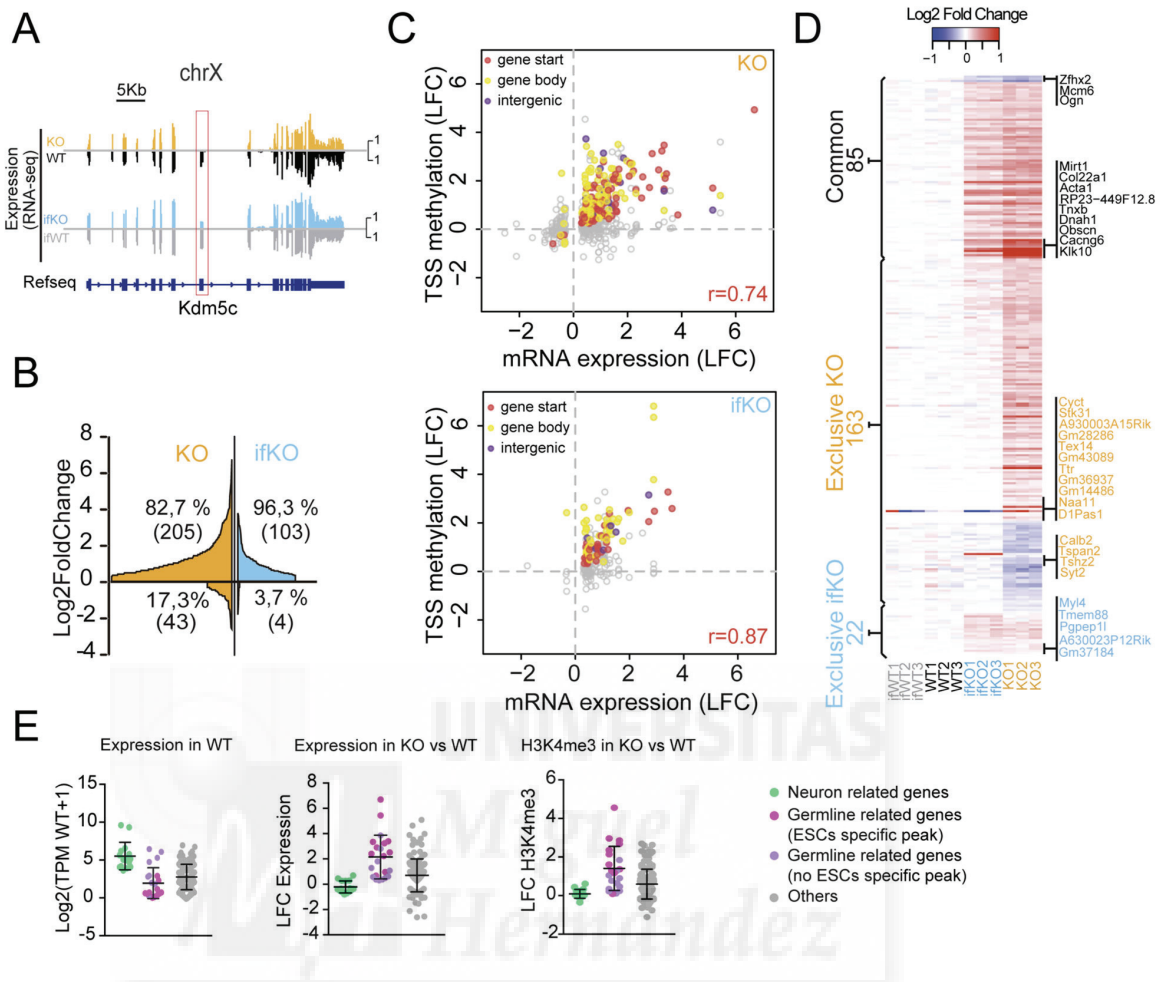

Figure 4. Transcriptional deregulation in Kdm5c-deficient mice correlates with altered H3K4me3 levels

A. RNA-seq profile of the *Kdm5c* gene in the hippocampus of KOs and ifKOs confirms the absence of exons 11 and 12 (red box). Note that only neuronal cells are affected in the hippocampus of ifKOs (~70% reduction). **B.** Gene expression changes in KOs and ifKOs (BH p adj. < 0.05, absolute Log2FC > 0.3). Genes were ordered by Log2FC and plotted as separate entities on the x-axis. **C.** Scatter plots for KOs (upper panel) and ifKOs (lower panel) showing the correlation between H3K4me3 levels at peaks located in close proximity to annotated DEGs and the transcript changes. Filled dots represent DHMPs (p adj. < 0.1) and colors indicate the location of the peak with respect to the gene feature. **D.** Heatmap showing the DEGs common to both KOs and ifKOs (common), as well as the DEGs altered exclusively in either strain. The number of genes in each category is indicated. **E.** Box plot showing Log2(TPM_{WT}+1), Log2FC of transcript level, and Log2FC of H3K4me3 at TSS for KO-exclusive DEGs. Light green dots correspond to genes with significant hippocampal expression and related to neuronal function. Pink dots: germ line-related genes associated with a Kdm5c-peak observed in embryonic stem cells (ESCs) but not in neural precursor cells (NPCs). Purple dots: germ line-related genes that are not associated with an ESC-

specific Kdm5c-peak. Grey dots: DEGs not falling in previous categories. Note that the genes with the largest transcript changes (germ line-related genes) have very low TPM values. Bars represent means ± SD.

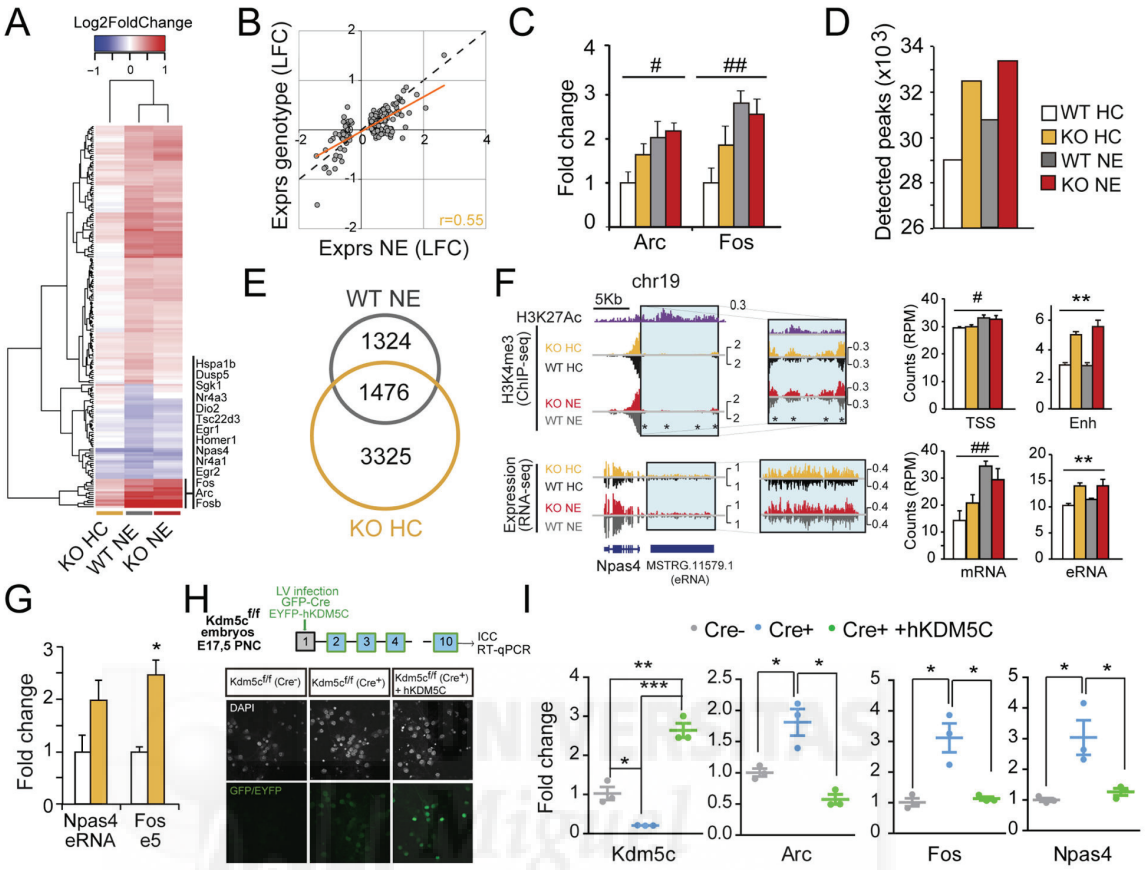

Figure 5. Kdm5c modulates neuronal activity-dependent enhancers

A. Heatmap of DEGs upon novelty exposure (NE) in WT mice together with their values for naïve (HC) and NE KOs. **B.** Scatter plot comparing NE effect with genotype effect in KOs for the set of NE-induced genes. **C.** RT-qPCR assays for *Arc* ($F_{(1,20)condition} = 8.73$, $p = 0.008$; $F_{(1,20)genotype} = 2.22$, $p = 0.2$; post-hoc WT $p < 0.05$) and *Fos* ($F_{(1,20)\ condition} = 12.67$, $p = 0.002$; $F_{(1,20)\ genotype} = 0.72$, $p = 0.4$; post hoc WT $p < 0.01$) using hippocampal RNA. **D.** Number of H3K4me3 peaks detected in WT-HC, WT-NE, KO-HC and KO-NE replicates. **E.** Venn diagram depicting the overlap between the DHMPs exclusive of WT-NE (*vs.* WT-HC) and KO-HC (*vs.* WT-HC). **F.** Left: Genomic profiles for *Npas4*. Right: Bar graphs representing the read density for H3K4me3 at the TSS ($F_{(1,4)condition} = 11.56$, $p = 0.03$; $F_{(1,4)genotype} = 0.0073$, $p = 0.9$; post-hoc ns) and upstream enhancer ($F_{(1,4)condition} = 1.31$, $p = 0.3$; $F_{(1,4)genotype} = 100.9$, $p = 0.0006$; post-hoc HC and NE $p<0.01$) of *Npas4*; and for the transcripts corresponding to *Npas4* mRNA ($F_{(1,8)condition} = 19.33$, $p = 0.002$; $F_{(1,8)genotype} = 0.05$, $p = 0.8$; post-hoc WT $p < 0.01$, post-hoc KO $p > 0.05$) and its putative eRNA ($F_{(1,8)condition} = 0.70$, $p = 0.4$; $F_{(1,8)genotype} = 20.03$, $p = 0.002$, post-hoc ns). **G**. RT-qPCR assays of IEG-associated eRNAs. *Npas4*-enhancer ($t_4 = 1.93$, $p = 0.13$); *Fos*-enhancer 5 ($t_4 = 4,74$, $p = 0.009$). **H**. Up: Scheme of PNC experiment; Down: Immunostaining of *Kdm5c*$^{f/f}$ PNC co-infected with LVs expressing Cre and hKDM5C. **I.** RT-qPCR assays confirmed the loss of the endogenous Kdm5c, the expression of hKDM5C and their

modulation of IEG expression (*Kdm5c*: Cre^{-} *vs* Cre^{+}, $t_4 = 4.92$, $p = 0.008$; Cre^{-} *vs* Cre^{+}+hKdm5c, $t_4 = 6.54$, $p = 0.003$; Cre^{+} *vs* Cre^{+}+hKdm5c, $t_4 = 13.74$, $p = 0.0002$; *Arc:* Cre^{-} *vs* Cre^{+}, $t_4 = 3.62$, $p = 0.02$; Cre^{+} *vs* Cre^{+}+hKdm5c, $t_4 = 5.43$, $p = 0.006$. *Fos:* Cre^{-} *vs* Cre^{+}, $t_4 = 4.28$, $p = 0.01$; Cre^{+} *vs* Cre^{+}+hKdm5c, $t_4 = 4.17$, $p = 0.01$. *Npas4*: Cre^{-} *vs* Cre^{+}, $t_4 = 3.58$, $p = 0.02$; Cre^{+} *vs* Cre^{+}+hKdm5c, $t_4 = 3.07$, $p = 0.04$). Data are expressed as means + s.e.m. * = $p < 0.05$, ** = $p < 0.005$; *** = $p < 0.0005$ (genotype effect); # = $p < 0.05$, ## = $p < 0.005$; ### = $p < 0.0005$ (condition effect) in 2-way ANOVA (C and F) and Student t-test (G and I).

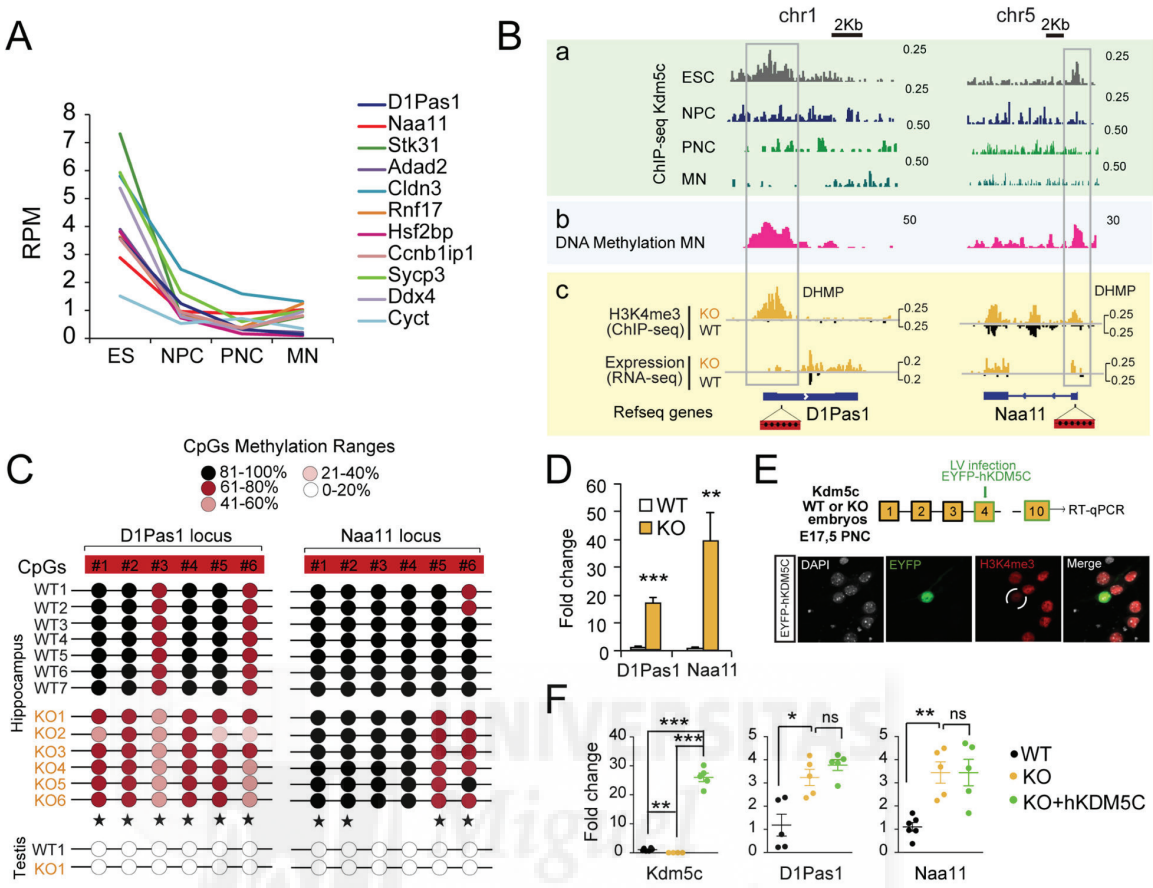

Figure 6. Germinal loss of Kdm5c prevents the silencing of germ line genes

A. Developmental loss of Kdm5c occupancy at the TSS of germ line genes (pink dots in Fig 4E). RPM: reads per million. **B.** Genomic profiles for representative germ line genes. From top to bottom, we show (a) Kdm5c-ChIP-seq profiles in ESC, NPC (Outchkourov et al., 2013), PNC (Iwase et al., 2016), and adult brain (MN, this study); (b) MeDIP profiles in adult brain of wild type mice (Halder et al., 2016), and (c) RNAseq and H3K4me3-ChIPseq experiments in adult hippocampus of WT and KO mice (this study). **C**. DNA methylation at specific CpGs (red boxes in Fig. 6B) at the promoter of *D1Pas1* and *Naa11* in hippocampal chromatin of WT and KO littermates (star symbols label significant DNA methylation reduction in KOs). Testis DNA was used as control for un-methylated DNA. **D**. RT-qPCR assays confirm the expression of germ line genes in the hippocampi of KOs (*D1Pas1*: $t_{10} = 8.53$, $p < 0.0001$; *Naa11*: $t_{10} = 3.90$, $p = 0.003$). **E.** Scheme of the experiment (top). Functionality of the hKDM5C construct was confirmed by transient transfection and examination of H3K4me3 levels in EYFP-expressing neurons (bottom). **F**. RT-qPCR assay confirmed the overexpression of germ line genes in PNCs from *Kdm5c*$^{-/y}$ embryos, infected or not with a hKDM5C-expressing lentivirus (*Kdm5c*, WT *vs* KO: $t_8 = 4.64$, $p = 0.002$, KO *vs* KO+hKDM5C: $t_7 = 15.57$, $p < 0.0001$, WT *vs* KO+hKDM5C: $t_9 = 18.54$, $p < 0.0001$; *D1Pas1*, WT *vs* KO: $t_8 = 3.49$, $p = 0.008$; KO *vs* KO+hKDM5C: $t_8 = 1.27$, $p = 0.2$; *Naa11*,

WT *vs* KO: $t_9 = 5.05$, $p = 0.0007$; KO *vs* KO+hKDM5C: $t_8 = 0.0007$, $p = 1.0$). Data are expressed as mean + s.e.m. ns: non-significant; * = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.0005$ in Student's t test.

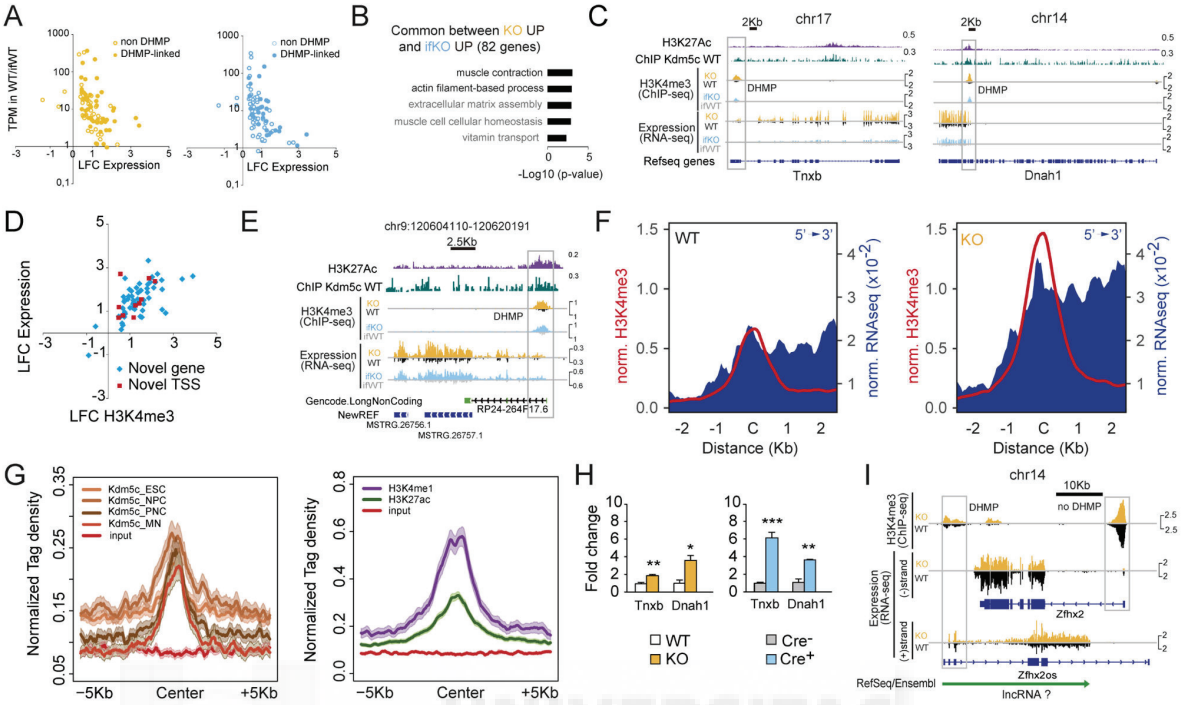

Figure 7. Kdm5c loss causes the activation of cryptic and non-neuronal regulatory sequences in adult neurons

A. Representation of Log2FC for common DEGs respect to the transcript levels in WT counterparts, for both KO (left) and ifKO (right) strains. TPM: transcripts per million; DHMP: differentially methylated H3K4me3 peak. **B.** GO enrichment for biological processes (GOBP) for common DEGs. Terms in grey correspond to an overlap of less than 5 genes. **C.** Genomic profiles for *Tnxb* and *Dnah1*, two representative examples of non-neuronal genes exhibiting spurious transcription and H3K4 methylation in *Kdm5c*-deficient strains. In the case of *Dnah1*, upregulation was restricted to the terminal exons and might correspond to the production of a meRNA. **D.** Scatter plots showing H3K4me3 level in KOs at peaks overlapping the TSS of non-annotated DE transcripts. **E.** Genomic profiles for one of the non-annotated ncRNAs identified in our *de novo* transcriptome assembly screen. **F.** Transcript upregulation in intergenic DHMPs in KOs. Reads for the plus and minus strand were independently scored 2 Kb either side of the peak center. **G.** Graphs show the presence of enhancer marks (H3K27ac and H3K4me1; right) and Kdm5c occupancy (left) at the same regions. ESC: embryonic stem cells; NPC: neuroprogenitor cells; PNC: primary neuronal cultures; MN: mature neurons. **H**. RT-qPCR assays confirmed spurious transcription of non-neuronal genes in *Kdm5c*$^{-/y}$ PNC (left, *Tnxb*: $t_9 = 4.16$, $p = 0.003$; *Dnah1*: $t_3 = 6.00$, $p = 0.009$) and in *Kdm5c*$^{f/f}$ PNC infected with Cre-expressing LV (right, *Tnxb*: $t_4 = 13.62$, $p = 0.0002$; *Dnah1*: $t_3 = 8.75$, $p = 0.003$). **I.** *Zfhx2* downregulation in both strains coincides with upregulation of an antisense lncRNA that overlaps with *Zfhx2os*. Data are expressed as mean + s.e.m. ns: non-significant; * = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.0005$ in Student's t test.

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Supplemental Information

Loss of Kdm5c Causes Spurious Transcription and Prevents the Fine-Tuning of Activity-Regulated Enhancers in Neurons

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Supplemental Experimental Procedures

Animals and treatments

The generation of *Kdm5c*$^{-/y}$, *Kdm5c*$^{f/f}$ (Iwase et al., 2016) and CaMKIIα-creERT2 (Erdmann et al., 2007) mice have been previously described. ifKOs where maintained in a pure C57BL/6J background, while KOs were produced in a mixed genetic background (F1: C57/129) because their viability was severely reduced in a isogenic background (**Table S6**), which suggests that multiple loci interact with *Kdm5c* during early development. Experiments were conducted in 2-6 months old animals, except when it is otherwise indicated. Mice were maintained and bred under standard conditions, consistent with Spanish and European regulations and approved by the Institutional Animal Care and Use Committee. For the induction of seizures by kainic acid (KA), we used the protocol described by (Umpierre et al., 2016) starting with a 5 mg/kg and injecting 2.5 mg/kg with 20 min intervals until the animal reached twice the level 4 in the Racine scale or level 5 once. For pentylenetetrazol (PTZ)-induced seizures, mice were injected with a 35 mg/kg dose and evaluated during 15 minutes by employing a modified Racine Scale. Data were averaged in bins of three minutes. Mice were treated with tamoxifen as described in (Fiorenza et al., 2016) when they were ~2 month-old. Environmental enrichment was produced as described in (Lopez-Atalaya et al., 2011). Exposure to novelty consisted on placing individual animal in a white Plexiglas square box containing plastic tubing and small toys for 1 h.

Neuronal cultures and lentiviral production

Primary cortical and hippocampal neurons were obtained from E17.5–E18.5 embryos. Hippocampi and cortices were dissected and processed as described (Benito et al., 2011). In the case of Kdm5c$^{f/f}$ mice all embryos were pooled together before plating, while *Kdm5c*$^{-/y}$ embryos were processed separately and only WT and KO male embryos-derived neurons were used in the analyses. The day of plating was considered day in vitro 1 (DIV1). Primary neurons were infected at the times indicated by adding the necessary volume of the concentrated viral preparation to achieve an effective multiplicity of infection of 1 to 10, which provided a percentage of neuronal infection close to 100%. In co-infection experiments, equal volumes of each virus were mixed. Recombination of endogenous Kdm5c gene was checked by RT-qPCR. The Cre recombinase expressing construct was obtained from Addgene (#20781); hKDM5C cDNA was obtained from Shi's lab (Iwase et al., 2007), cloned into a synapsin promoter-bearing lentiviral vector derived from LenLox 3.7 (Gascon et al., 2008) and used in the production of lentiviral pseudovirions as described (Benito et al., 2011). H3K4 demethylase activity was assessed by transfecting neurons using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Viral stocks were tittered by RT-qPCR and concentrated by ultracentrifugation.

Behavioral testing

Two independent cohorts of adult (3-6 months old) *Kdm5c*-KO and control littermate males were tested in series of behavioral tasks. Cohort 1 (n = 7 per genotype) was evaluated in Open field, Rotarod, Tail Suspension Test, Hot Plate Test, Morris Water Maze, Novel Suppressed-feeding test, Marble Burying and Fear Conditioning (contextual and cued test); Cohort 2 (n = 14 per genotype) was examined in Open field, Elevated Plus Maze, matching-to-place Y water maze and prepulse inhibition task. Behavioral assessment of *Kdm5c*-ifKO mice started 3 months after the first tamoxifen injection. The behavioral evaluation of *Kdm5c*-ifKO mice was conducted using a large cohort of male mice (n = 11 and 18 for ifWT and ifKO, respectively, except for the EPM and PTZ experiments in which n = 11 for both genotypes). The environmental enrichment experiment was performed with females because, contrary to males, they do not fight when housed in an enriched environment (EE). *Kdm5c*$^{f/f}$ females, negative (ifWT, n=13) and positive (ifKO, n=13) for the Cre recombinase transgene were treated with tamoxifen, and 3 months later split into two groups (EE and SC). After 30 days in the different housing conditions, behavioral testing was conducted. Experimenters were blind to genotype prior to behavioral tests. Sample size was chosen based on previous literature. Inclusion/exclusion criteria were not pre-established and no randomization was employed.

The open field (OF), elevated plus maze (EPM) and fear conditioning (FC) task were performed as previously described (Viosca et al., 2009). To evaluate motor coordination and learning, mice were trained on a Rotarod (Ugo Basile) at a constant speed (4 rpm) three times in one day. 24 h later, the Rotarod was set to increase its speed 1 rpm every 4s starting at 4 rpm up to 40 rpm, and the latency to fall off was measured. Hot plate was performed as previously described (Ito et al., 2014). The tail suspension test (TST) was performed with Med. associates Inc. equipment; mice were tail suspended during 6 min and their activity recorded. To evaluate physiological mild stress using the marble burying (MB) task, 12 opaque marbles were placed in a 6x2 arrangement (3 cm separation between marbles) on top of fresh sawdust; then, the number of marbles with more than 2/3 of their surface covered with sawdust (buried) during the 15 min test session was scored. Pre-pulse Inhibition (PPI) was carried as previously described

(Viosca et al., 2009) but using different intensities for the prepulse (PP): 20-ms PP trials of 83 dB, 86 dB and 92 dB. The acoustic startle reflex was tested using a Panlab S.L. startle testing system. Mice were placed in a Plexiglas testing cylinder 15 cm from a speaker mounted on a piezoelectric accelerometer. Background white noise was delivered through the entire experiment. Startle amplitude was assessed as the maximal response in 100 ms after the startle stimulus. For the habituation of the acoustic startle response, mice were left undisturbed inside the testing cylinder for 5 minutes and later presented with 80 startle pulses of 120 dB each (15 s inter-stimulus interval, ISI). After a 5-min acclimation period, mice were presented with a total of 99 trials (15 s ISI). Trials delivered either no stimulus or 40 ms noise bursts with randomly varied stimuli from 75 to 120 dB. PPI stimuli were selected on the basis that they would not elicit significantly different responses among groups. Following a 5-min acclimation period, mice were presented with 20 habituation trials (120 dB, ISI 15 s). In the prepulse inhibition phase, mice were presented with a total of 90 trials. Three prepulse intensities were tested: 75, 78 and 83 dB. Prepulse stimuli were 20 ms in duration and delivered either alone, prepulse + startle stimulus, and startle stimulus alone. Stimuli were pseudorandomly delivered.

Spatial learning in the Morris water maze (MWM) was assessed in a circular tank (170 cm diameter) filled with opaque white water as described previously (Viosca et al., 2009). Mice were monitored throughout the training and testing sessions with the video-tracking software SMART (Panlab S.L.). A platform of 10 cm diameter was submerged 1 cm below the water surface in the center of the target quadrant. The task was divided in three phases: visible platform (2 or 3 days), hidden platform (5 or 8 days) and reversal (5 days) in which the hidden platform was relocated to the opposite quadrant. Training each day consisted on four trials with a 45 min inter-trial interval. If the mice did not find the platform after 120 s, they were gently guided to it. Mice were transferred to their cages only after remaining for at least 10 s on the platform. Memory retention probe trials (PT) of 60 s were performed at different time points during the task. In the novel suppressed feeding test, mice were deprived from food for 24 h and then put in a novel open and bright arena with a piece of food in the center for 5 min and the time to get the food was measured. In the matching-to-place water Y-maze experiment, mice were placed in an 8-armed radial water maze in which only three arms were open shaping a Y-maze. Water was kept at about 20ºC and stained with non-toxic white paint to hide the platform under the surface. In the swimming phase, mice were placed and left to swim for 1 minute in the arm in which they were released and the arm that will not contain the platform in the sample phase. The sample phase started after 1 min inter-trial interval (ITI); mice were released from the same arm and the 3 Y arms were open. Once mice located the platform, they were left on it for 15 sec. After another ITI, the matching phase was conducted: mice were released from the starting arm and expected to find the platform in the same arm than before. The number of errors was counted. Errors were defined as entering without culminating in platform allocation. The experiment was finished once half of the mice located the platform with a single error.

Histology and MRI analyses

Nissl staining was performed as described in (Ito et al., 2014). For immunohistochemistry, mice were perfused with 4% paraformaldehyde in PBS. Coronal or sagittal vibratome sections (50 µm) were obtained, washed in PBS and PBS−0.1% Triton X-100 (PBT) and incubated for 0.5 h at room temperature with 3% NCS-PBT. After that, slices were incubated O/N at 4ºC with the primary antibodies diluted in 2% NCS-PBT. Primary neuronal cultures grown on glass coverslips were fixed and stained in similar conditions. The primary antibodies used in this study are α -NeuN (Chemicon, MAB377), α -MAP2 (Sigma, M9942), α-GFAP (Sigma, G9269), α-DCX (Abcam, ab18723), α-Parvalbumin (Swant, PV235), α-Cre (gift from Schütz's lab), α-H3K4me3 (Millipore 07-473), and α-GFP (Aves Labs, GFP-1020, valid also for recognizing EYFP). Nuclei were counterstained with a 1 nM DAPI solution (Invitrogen) before mounting. Images were taken with Olympus Confocal Inverted Microscope or a Leica epifluorescence Microscope. Four-month old *Kdm5c*-KO mice and wild-type mice were analyzed using magnetic resonance imaging (MRI). Experiments were carried out in a horizontal 7 Tesla scanner with a 30 cm diameter bore (Biospec 70/30v, Bruker Medical, Ettlingen, Germany). The system had a 675 mT/m actively shielded gradient coil (Bruker, BGA 12-S) of 11.4 cm inner diameter. Phantoms with perfused mice heads in agarose were placed in a custom-made phantom holder and positioned fixed in the magnet isocenter. T_2 weighted anatomical images were collected using a rapid acquisition relaxation enhanced sequence (RARE), applying the following parameters: field of view (FOV) 10 x 10mm, 15 slices, slice thickness 0.8 mm, matrix 128 x 128, effective echo time (TE_{eff}) 56 ms, repetition time (TR) 2 s and a RARE factor of 8. The B0 field distribution in a large voxel (30 x 30 x 30 mm^3) containing the whole head was acquired (FieldMap). Briefly, the brain was localized with T2-weighted RARE sequence, and first- and second-order shims adjusted with MAPSHIM application in a sufficiently large voxel containing the whole brain. 3D data were acquired using a RARE 3D sequence with TR 1500 ms, TE_{eff} 48.8 ms, RARE factor 16 and 18 averages. The matrix size was 316 x 220 x 154 and FOV was 1.65 x

1.15 x 0.80 cm^3, which yielded an isotropic resolution of 52 µm in 20 h acquisition time. Data were acquired and processed with a Hewlett-Packard console running Paravision 5.1 software (Bruker Medical GmbH, Ettlingen, Germany) operating on a Linux platform. Orthogonal planes were generated using Jive 7.9.0 application running under Paravisión 5.1 software. Briefly, after rotating the image to obtain the desired orientations, new 2D images were generated. The resolution of these newly generated images was doubled digitally by linear interpolation, rendering a final isotropic resolution of 26 µm.

Western blots

For Western blot, extraction buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, and 1%NP-40) containing protein inhibitor mix (Roche) was added to the hippocampi for homogenization, 50% 2x SDS buffer and 1% 2-mercaptoethanol were added to the samples and samples were heated 5 min at 95ºC. Equal volumes were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analyses were carried out using Western Lightning ECL kit (Perkin-Elmer, Boston, MA). α-H3K4me3 (Millipore 07-473) and α-actin (Sigma F5441) antibodies were used.

RNA extraction, RT-qPCR and RNA-seq

Total RNA from tissue and hippocampal cultures was extracted with TRI reagent (Sigma-Aldrich) and reverse transcribed to cDNA using the RevertAid First-Strand cDNA Synthesis kit (Fermentas). qPCR was performed in an Applied Biosystems 7300 real-time PCR unit using Eva Green qPCR reagent mix. The primer sequences used in RT-qPCR assays are:

Kdm5c (ex12-13) Fw: TCGTCACCCTCATGAATCCTAA, Rv: GTGATGACAAATTCTCCTGCACA;
Endogenous Kdm5c Fw: GGCTGACATCCATTCCAAAG, Rv: AGCATACTCCTCCTCCTCTG;
Fos Fw: GCTTCCCAGAGGAGATGTCTGT, Rv: GCAGACCTCCAGTCAAATCCA;
Arc Fw: GCAGGAGAACTGCCTGAACAG, Rv: AAGACTGATATTGCTGAGCCTCAA;
Npas4 Fw: CTGGCCCAAGCTTCTTCTCA, Rv: TCCATGCTTGGCTTGAAGTCT;
Gapdh Fw: CTTCACCACCATGGAGAAGGC, Rv:CATGGACTGTGGTCATGAGCC;
Npas4 eRNA Fw: CTCTGCGGTCAAATAACAAGACTG, Rv: GTCAGAGATGTCTAGGCCCAATAG
Fos e5 Fw: CAACCCTGTCATCTATTTAGC, Rv: TAAGAACTGCGGGGGTCTTC;
D1Pas1 Fw: CTTGGCTCCGACCAGAGAAT, Rv: GACGAACTCGAGAGCGGTAT;
Naa11 Fw: GGACAGCTCAGAGGACTTGG, Rv: TGTGGCTGAGACATGAAGGC;
Dnah1 (last exons) Fw: AATCCTCTTCTATGGGCGGC, Rv: ATTCAATAGGCCCGGGAACC;
Tnxb Fw: GTGGATGCCTATGAAATCCAGT, Rv: CGAGCTGTGAATGGTGGACT.

Each independent sample was assayed in duplicate and normalized using GAPDH levels. RNA preparation for sequencing was performed as described in (Fiorenza et al., 2016). Three independent samples were sequenced according to manufacturer instructions in a HiSeq2500 sequencer (Illumina, Inc). In each sample, the hippocampi of 2 (KOs) or 3 (ifKOs) mice were pooled together. Information on library preparation procedure, library size, and mapping to reference genome can be found at the end of this section. Briefly, RNA-seq reads were mapped to the mouse genome (Mus_musculus.GRCm.38.83) using STAR (v2.5.0c) (Dobin et al., 2013). Aligned reads were counted to gene transcripts using HTSeq (v0.6.1) (Anders et al., 2015). Differential expression analysis was performed using DESeq2 (v1.10.0) (Love et al., 2014) of the bioconductor suite (Huber et al., 2015) in the R (v3.2.2) statistical computing platform. Genes were considered differentially expressed at Benjamini-Hochberg (BH) adjusted p-value < 0.05 and absolute log2 fold change > 0.3. RNA-seq tracks were visualized using IGV (v2.3.72) (Thorvaldsdottir et al., 2013). GO analysis was performed using the platform *Enrichr* (Chen et al., 2013) and redundant categories were removed from the top five enriched terms. To discover novel transcripts RNA-seq reads were first aligned to reference genome Ensembl GRCm38 (genome_snp) using Hisat2 (v2.0.4) (Kim et al., 2015) and further processed using samtools (v1.3.1) (Li et al., 2009). Novel transcripts were identified in individual samples using StringTie (v1.3.0) (Pertea et al., 2015) with annotation reference provided (Mus_musculus.GRCm38.86.gtf). Transcripts from all samples were subsequently merged and compared with the reference annotation (Pertea et al., 2016). RNA-seq data can be accessed at the GEO repository (GSE85874).

Sample	*Type of sequencing*	*Nº of reads*	*Library type*
wt_HC_1	Single end	21543257	RNAseq
wt_HC_2	Single end	29090246	RNAseq
wt_HC_3	Single end	31582062	RNAseq
KO_HC_1	Single end	61519199	RNAseq
KO_HC_2	Single end	29799874	RNAseq
KO_HC_3	Single end	39961350	RNAseq
wt_NE_1	Single end	36998229	RNAseq

wt_NE_2	Single end	43005046	RNAseq
wt_NE_3	Single end	28477492	RNAseq
KO_NE_1	Single end	29543951	RNAseq
KO_NE_2	Single end	22371404	RNAseq
KO_NE_3	Single end	26459792	RNAseq
ifWT_1	Paired end	50876528	RNAseq
ifWT_2	Paired end	49467749	RNAseq
ifWT_3	Paired end	57959090	RNAseq
ifKO_1	Paired end	56764225	RNAseq
ifKO_2	Paired end	51001355	RNAseq
ifKO_3	Paired end	47717366	RNAseq

ChIP assays and ChIP-seq

Chromatin immunoprecipitation (ChIP) experiments were conducted as previously described (Lopez-Atalaya et al., 2013) using anti-H3K4me3 (Millipore 07-473) anti-H3K4me1 (Abcam ab8895) and anti-Kdm5c (Iwase et al., 2016). Hippocampi derived from two different animals were pooled together for each sample. In the case of H3K4me3, we examined two independent biological replicates. ChIPped DNAs were sequenced according to manufacturer instructions in a HiSeq2500 apparatus (Illumina, Inc). Information on library preparation method, size of the libraries, and mapping to reference genome can be found at the end of this section. ChIP-seq reads were aligned to the mouse genome (Mus_musculus.GRCm.38.83) using bowtie2 (v2.2.9) (Langmead and Salzberg, 2012) and further processed using samtools (v1.3.1) (Li et al., 2009) and bedtools (v2.25.0) (Quinlan and Hall, 2010). Peak calling for H3K4me3 ChIP-seq datasets was performed using MACS2 (v2.1.0) (Zhang et al., 2008) with default parameters. Peak calling for Kdm5c ChIP-seq in wild-type mice was run using MACS2 (v2.1.0) with the following parameters: -q 0.01 --nomodel --extsize 131 --broad --broad-cutoff 0.1. Read counts on aligned bam files were performed using featureCounts (Rsubread v1.22.3) (Liao et al., 2014). Differential peak methylation analysis was performed using DESeq2 (v1.10.0) (Love et al., 2014) of the bioconductor suite (Huber et al., 2015) in the R (v3.3) statistical computing platform. Peaks were considered differentially methylated at Benjamini-Hochberg (BH) adjusted p-value < 0.05 and absolute log2 fold change > 0.58. To identify potential enhancers in mouse hippocampal chromatin, we performed ChIP-seq experiments using antibodies against H3K27ac (Abcam ab4729) on chromatin samples from wild type mice. This experiment was performed in biological duplicates using pools of hippocampal tissue from three mice. We next retrieved genomic regions enriched for H3K27ac using MACS2 and selected peaks whose location was outside ±1 Kb of Ensembl annotated TSSs (Mus_musculus.GRCm.38.83). These criteria retrieved 11772 putative enhancers. Read densities from H3K4me3 datasets for wild-type and *Kdm5c*-KOs were collected in a window of 10 Kb around enhancers and the collected values were subjected to k-means clustering (using linear normalization method) using seqMiner (Ye et al., 2011). Five mayor groups of enhancers could be distinguished according to H3K4me3 levels. We used previously published ChIP-seq data from mouse cortex to evaluate enrichment for H3K4me1 (GSM722632), another chromatin mark of regulatory elements associated with enhancers and other distal elements (Shen et al., 2012), at these genomic locations. This dataset was also used to investigate presence of H3K4me1 at differentially methylated H3K4me3 peaks located at intergenic genomic locations. Kdm5c enrichment at enhancers and at differentially methylated intergenic H3K4me3 peaks, was evaluated using previously released datasets for primary cultures of cortical neurons (GSE75866) (Iwase et al., 2016) and mouse embryonic stem cells and derived neuroprogenitors (GSE34975) (Outchkourov et al., 2013). External data (i.e. data not generated for this study) were obtained in its primary form (e.g. fastq) and aligned to reference (Mus_musculus.GRCm.38.83) for use in the current study. Heatmaps of ChIP-seq read density and aggregate density plots were performed using SeqMiner (v1.3) (Ye et al., 2011) and R custom scripts. A 95% confidence interval was included into the aggregate density plots to estimate sample variance and significance. ChIP-seq peaks were annotated using ChIPpeakAnno (Zhu et al., 2010). ChIP-seq tracks were visualized using IGV (v2.3.72) (Thorvaldsdottir et al., 2013); each profile is the combination of 3 (RNA-seq) or 2 (H3K4me3 ChIP-seq) replicates. ChIP-seq data can be accessed at the GEO repository (GSE85873).

Sample	*Type of sequencing*	*N° of reads*	*Library type*	*Target*	*Antibody*
wtHC_1	Single end	32211897	ChIPseq	H3K4me3	Millipore (07-473)
wtHC_2	Single end	34834734	ChIPseq	H3K4me3	Millipore (07-473)
KOHC_1	Single end	35160470	ChIPseq	H3K4me3	Millipore (07-473)
KOHC_2	Single end	37001550	ChIPseq	H3K4me3	Millipore (07-473)

wtNE_1	Single end	33664646	ChIPseq	H3K4me3	Millipore (07-473)
wtNE_2	Single end	42890879	ChIPseq	H3K4me3	Millipore (07-473)
KONE_1	Single end	44699254	ChIPseq	H3K4me3	Millipore (07-473)
KONE_2	Single end	35485093	ChIPseq	H3K4me3	Millipore (07-473)
ifWT_1	Single end	36816774	ChIPseq	H3K4me3	Millipore (07-473)
ifWT_2	Single end	44487531	ChIPseq	H3K4me3	Millipore (07-473)
ifKO_1	Single end	44333838	ChIPseq	H3K4me3	Millipore (07-473)
ifKO_2	Single end	32672737	ChIPseq	H3K4me3	Millipore (07-473)
wtHC	Single end	48833425	ChIPseq	Kdm5C	Iwase et al, 2016
KOHC	Single end	45929814	ChIPseq	Kdm5C	Iwase et al, 2016
wtHC	Single end	46966588	ChIPseq	H3K4me1	Abcam (ab8895)
KOHC	Single end	48924123	ChIPseq	H3K4me1	Abcam (ab8895)

DNA isolation and pyrosequencing

DNA was extracted from adult hippocampi with QIAamp DNA Mini Kit. Bisulfite conversion of genomic DNA was performed with the EpiTect Fast bisulfite kit (Qiagen). PCR amplification of converted DNA followed by sequencing was conducted with the PyroMark Q24 apparatus using PyroMark CpG Assays (Qiagen): Mm_D1Pas1_01_PM PyroMark CpG assay (PM00212422) and Mm_Naa11_01_PM PyroMark CpG assay (PM00372379).

Statistical analyses

All statistical analyses were two-tailed. For pairwise comparison of averages, data were tested for normality using Shapiro's test. If any of the two samples were significantly non-normal, a non-parametric Mann-Whitney U/ Wilcoxon rank-sum test was executed instead. If the data from the two samples met the normality criterion, an F test was used for equality of variances. When variances between the groups were different, a Welch's t-test was employed; otherwise a standard two-sample t-test was used. A two-way ANOVA was used in MWM, NE and EE experiments. Bonferroni-corrected pairwise tests were used where appropriate post-hoc to correct for multiple comparisons. P values were considered to be significant when $\alpha < 0.05$. Mean ± s.e.m. and percentages are represented in bar graphs.

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Scandaglia et al_ FigS1. Related to Fig1

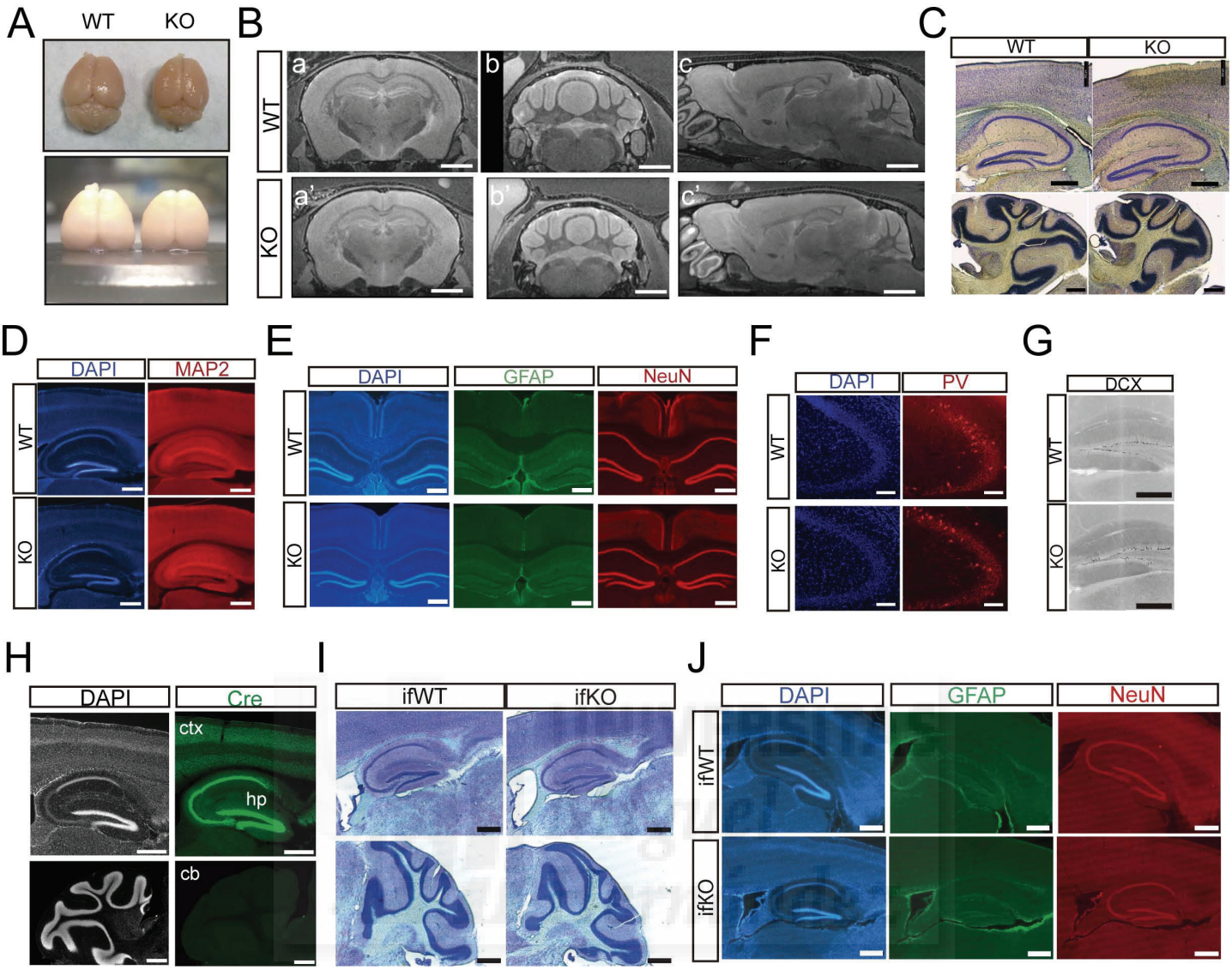

Figure S1. Related to Figure 1. Neuroanatomical and histological characterization of *Kdm5c*-KOs and -ifKOs. A. *Kdm5c*-KOs have smaller brains than their control littermates. **B.** Coronal (a, a': forebrain; b, b': cerebellum) and sagittal (c, c') MRI brain images of 4-month old mice. **C-G.** Nissl staining (C) and immuno-staining for different cell type markers (D-G) did not reveal gross anomalies between genotypes: Map2 (D) and GFAP and NeuN (E) in cortex and hippocampus; parvalbuminin in the CA3 region (F); doublecortin in dentate gyrus (G). **H.** Expression pattern of Cre recombinase in ifKOs. Brain sagittal slices were counterstained with DAPI. Hp: hippocampus; ctx: cortex; and cb: cerebellum. **I-J.** Nissl staining (I), and GFAP and NeuN immunostainings (J) did not reveal anomalies in ifKO brain slices. Scale bars: 2 mm in B; 500 µm in C-E and G-J; 100 µm in F.

Scandaglia et al_FigS2. Related to Figure 1

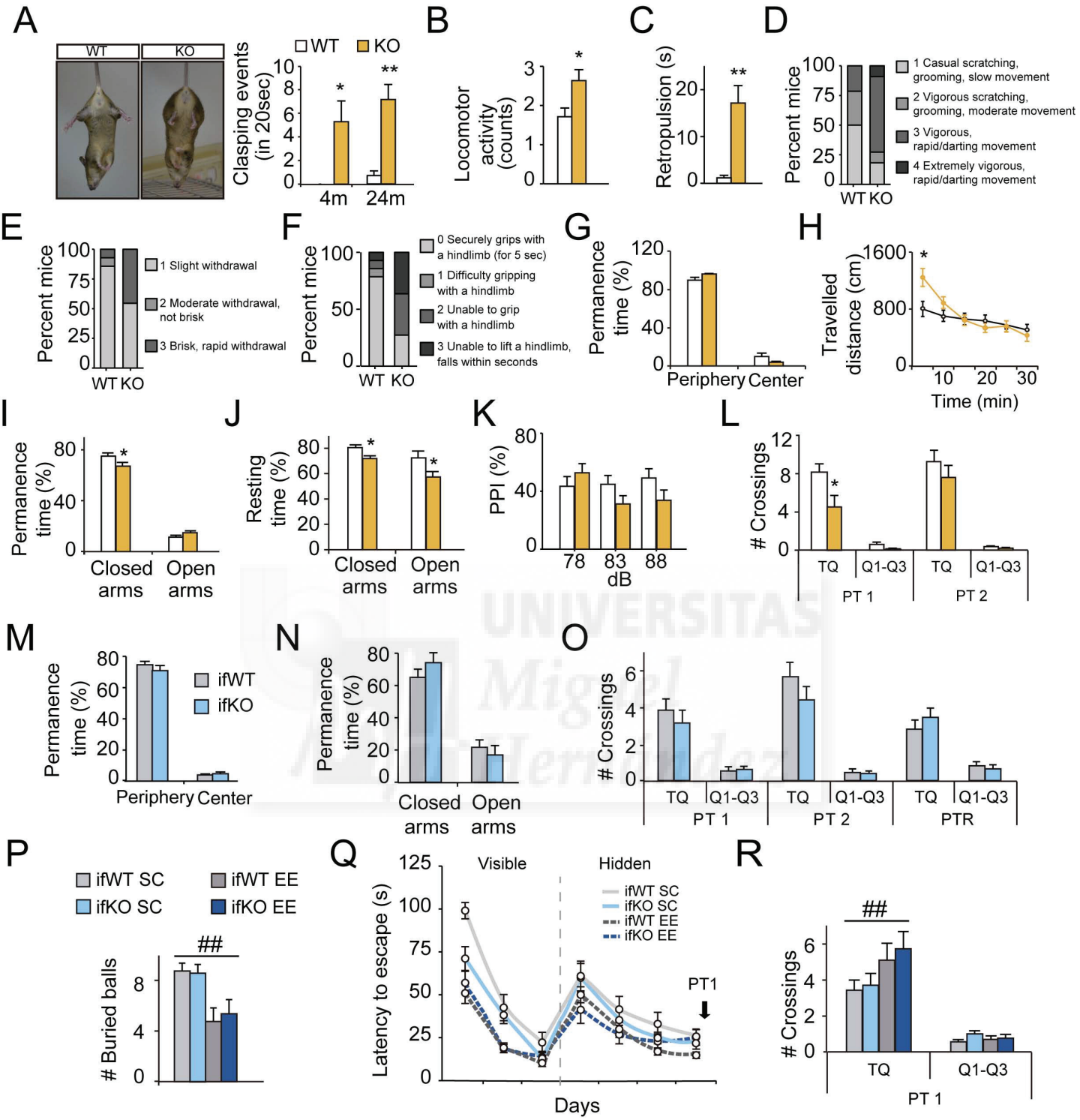

Figure S2. Related to Figure 1. Expanded behavioral characterization of KOs and ifKOs. A. Left: Photos showing hind-paw clasping in a 4-month old KO compared to a littermate control. Right: Quantification of clasping events in adult and aged mice (U = 4.00, $p = 0.005$ at 4 months, U =0.00, $p = 0.003$ at 24 months). **B-F.** SHIRPA testing: KO mice show enhanced locomotor activity (B), retropulsion (C), more vigorous spontaneous behaviors (D, U = 0.00, $p = 0.002$) and reaction to a toe pinch (E, $p = 0.07$), and difficulty for gripping on a wire (F). **G-H.** Open field: No difference in total time spent in the periphery or the center of the arena (G: U = 191, $p = 0.5$)**,** but hyperactivity during the first 5 min of testing (H: $F_{(1,156)} = 5.30$, $p = 0.02$, post-hoc 5-min $p < 0.01$). **I-J.** Reduced anxiety (I: closed arms, $t_{26} = 2.42$, $p = 0.02$; open arms: $t_{26} = 1.33$, $p = 0.2$) and hyperactivity (J: closed arms, $t_{26} = 2.70$, $p = 0.01$; open arms: $t_{26} = 2.14$, $p = 0.04$) in the elevated plus maze. **K.** Pre-pulse inhibition: No differences in PPI were found between genotypes (78 db, $t_{26} = -1.01$, $p = 0.3$; 83 db, $t_{26} = 1.64$, $p = 0.1$; 88 db, $t_{26} = 1.62$, $p = 0.1$). **L.** Number of platform crossings in the MWM (PT1, $t_{12} = 2.52$, $p = 0.03$). TQ: target quadrant, Q1-Q3: other quadrants. **M.** Evaluation of ifKOs in the OF (time in periphery, $t_{28} = 0.15$, $p = 0.9$; time in center, U = 96.00, $p = 0.6$). **N.** Evaluation of ifKOs in the EPM (closed arms, $t_{21} = -1.16$, $p = 0.3$; open arms: U = 86.00, $p = 0.2$). **O.** Number of platform crossings in the MWM (PT1, U = 89.00, $p = 0.7$; PT2, $t_{27} = 1.01$, $p = 0.3$; PTR, $t_{27} = 1.10$, $p = 0.3$). **P-R.** EE had a similar impact in ifKOs and control littermates, both in the MB task (P: $F_{(1,48)housing} = 14.08$, $p = 0.0005$; $F_{(1,48)genotype} = 0.0055$, $p = 0.9$; post-hoc ifWT and ifKO $p < 0.05$), learning curve in the MWM (Q: visible, $F_{(1,144)housing} = 44.85$, $p < 0.0001$, $F_{(1,144)genotype} = 3.52$, $p = 0.06$; hidden, $F_{(1,192)housing} = 11.84$, $p = 0.0007$; $F_{(1,192)genotype} = 0.43$, $p = 0.5$), and number of platform crossings during the PT (R: $F_{(1,47)housing} = 5.13$, $p = 0.003$; $F_{(1,47)genotype} = 0.31$, $p = 0.82$; post-hoc no significant). Two-way ANOVA revealed significant differences between housing conditions but not between genotypes. Data are expressed as mean ± s.e.m. * or # = p <0.05; ** or ## = p <0.005; *** = p <0.0005 in Mann-Whitney test, Student's T-test, and two-way ANOVA.

Scandaglia et al_FigS3. Related to Figure 3

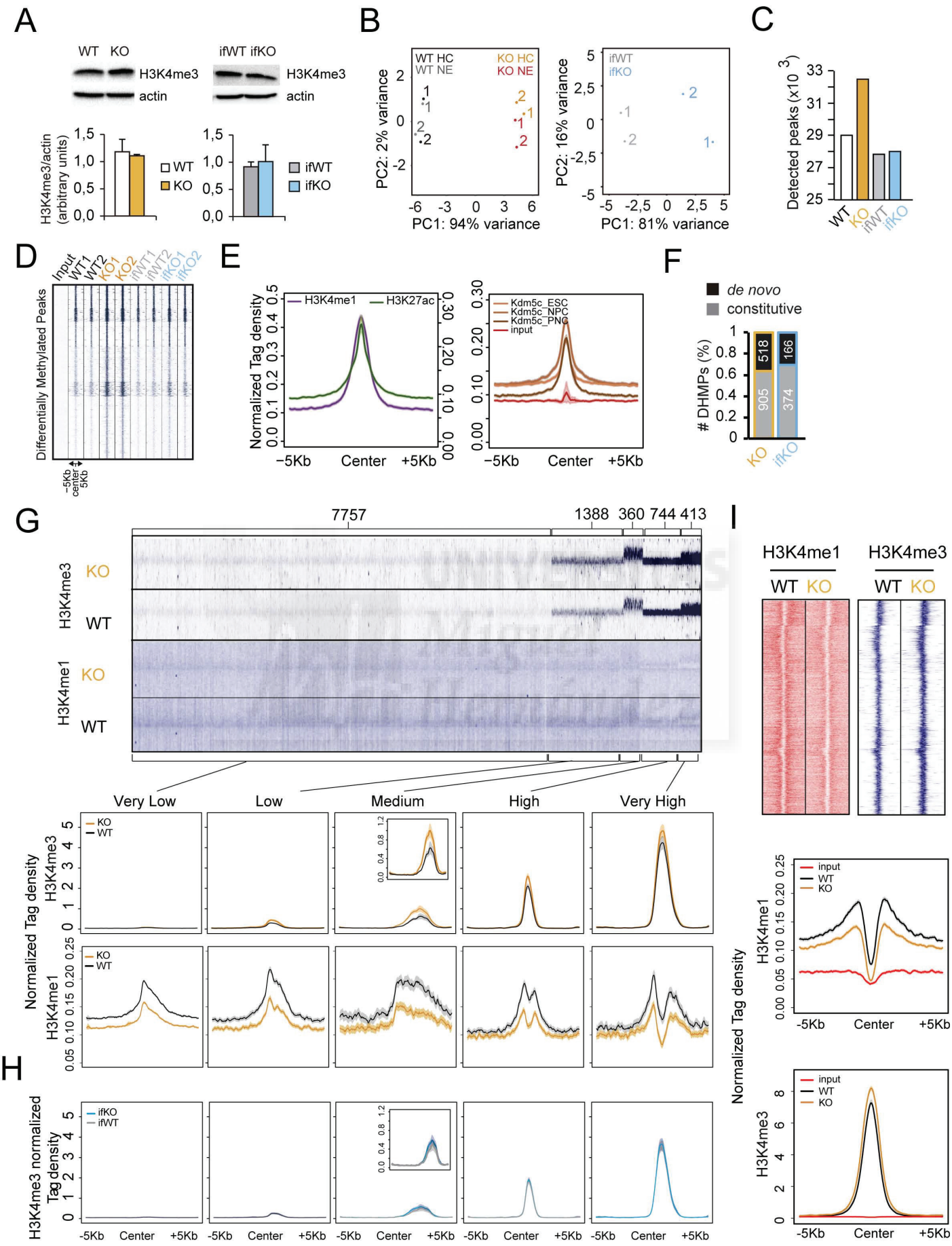

Figure S3. Related to Figure 3. H3K4me3 profiling of hippocampal chromatin in Kdm5c-deficient strains. A. Immunoblot of hippocampal protein extracts revealed no difference in H3K4me3 in bulk chromatin of KOs and ifKOs. **B.** PCA plots show that most of the variability across replicates in ChIP-seq samples is due to genotype. **C.** Bar graph showing the number of detected H3K4me3 peaks in the hippocampal chromatin of KOs, ifKOs and their respective control littermates. **D.** Heatmap shows H3K4me3 density across DHMPs in input, WT, KO, ifWT, and ifKO replicates. Color intensity is proportional to read density. **E.** Line plot showing H3K27ac, H3K4me1 and Kdm5c signal (normalized read density) at intergenic H3K4me3 peaks. The enrichment of H3K27ac (this study) and H3K4me1 (Shen et al., 2012) suggest that these regions are enhancers; Kdm5c profiles demonstrate the presence of this enzyme in wild type chromatin at these loci (Outchkourov et al., 2013; Iwase et al., 2016). **F.** Bar graph shows proportion and number of DHMPs in KOs and ifKOs that are detected as peaks in WT chromatin (constitutive peaks) or found exclusively in mutants (*de novo* peaks). **G.** Up: Heatmap showing H3K4me3 and H3K4me1 density at putative enhancers. Color intensity is proportional to read density. Down: Line plot showing H3K4me3 and H3K4me1 signal (normalized read density) for WT and KO animals. Peaks were subdivided in very low, low, medium, high, very high according to H3K4me3 content. Enhancers with delocalized (not overlapping with the center of the H3K27ac peak) and strongly asymmetrical H3K4me3 signal were discarded. The number of peaks in each category is indicated. **H.** H3K4me3 signal for ifWT and ifKO animals in the same enhancer groups shown in panel G. **I.** Heatmaps (top) and line plots (bottom) showing H3K4me1 and H3K4me3 density at Kdm5c-enriched regions (see **Fig. 3A**) in KO and control littermates. Color intensity is proportional to read density.

Scandaglia et al_FigS4. Related to Figure 4

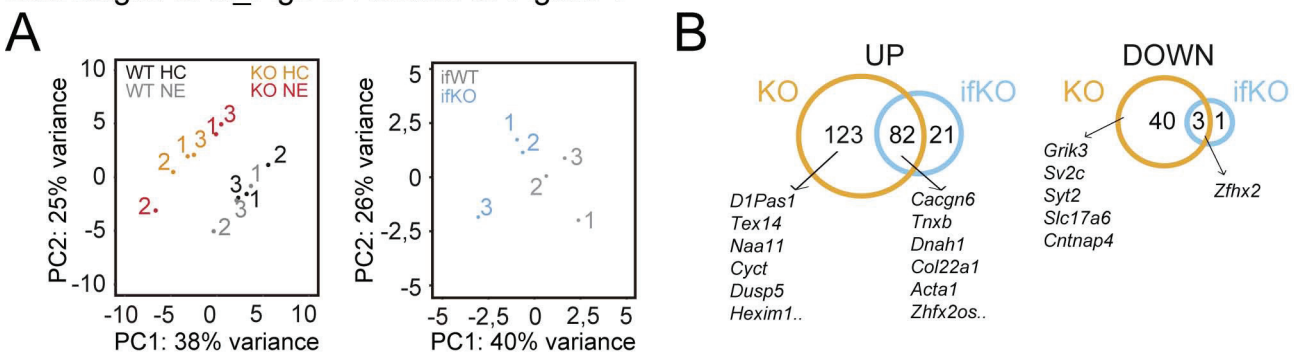

Figure S4. Related to Figure 4. Transcriptome analysis in Kdm5c-deficient strains. A. PCA plot indicates that most of the variability across RNA-seq replicates is due to genotype. **B.** Venn diagram showing overlap between upregulated and downregulated genes in KOs and ifKOs.

Scandaglia et al_FigS5. Related to Fig 5

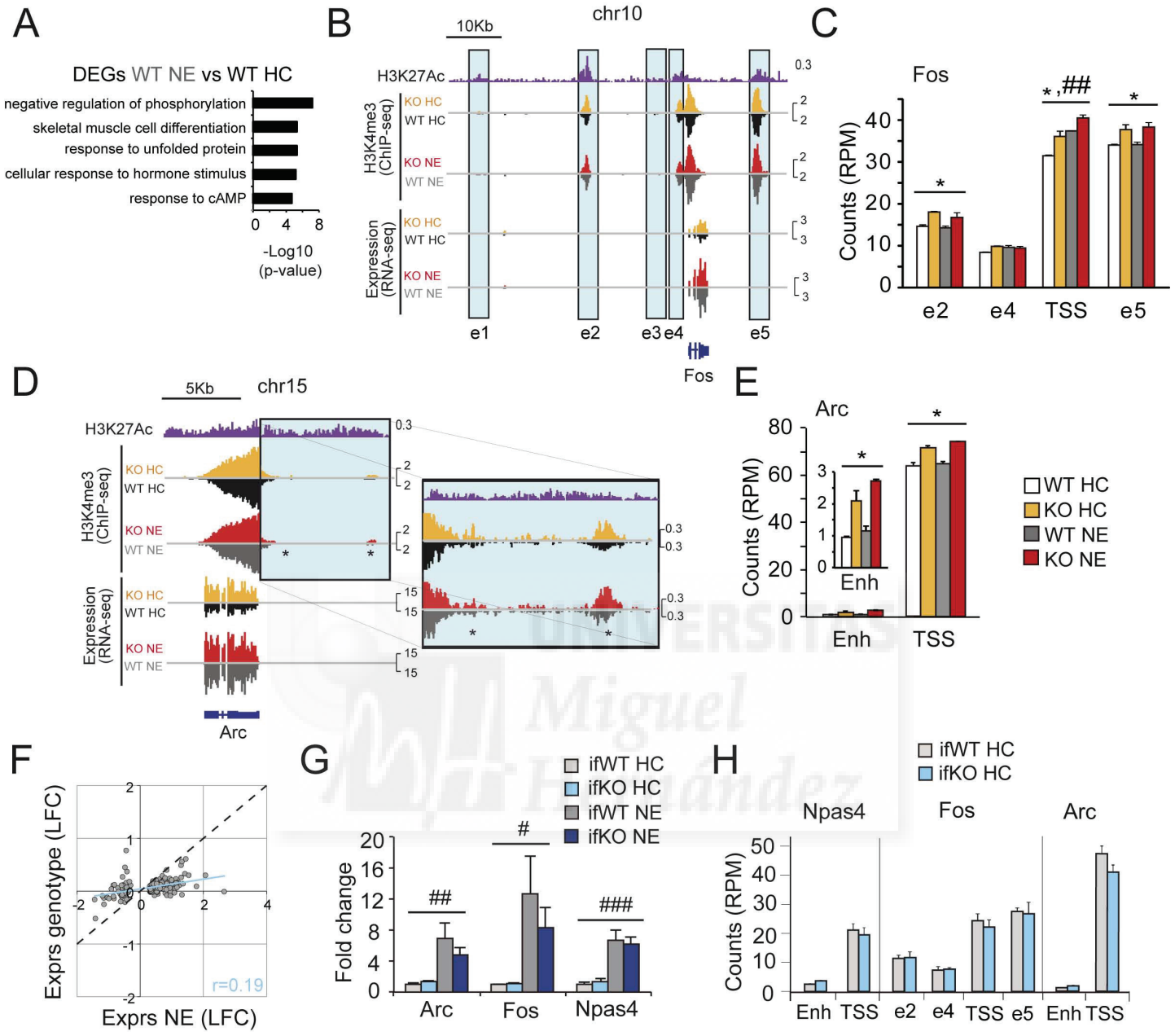

Figure S5. Related to Figure 5. Derepression of IEGs in KOs is associated with increased H3K4me3 at enhancers. A. GO for biological processes (GOBP) analysis of DEGs upon NE in WT mice. **B-E.** Snapshots of KO RNA-seq and H3K4me3 profiles at the *Fos* (B) and *Arc* (D) loci, both at basal state and after NE. Rectangular insets label and/or zoom at surrounding enhancers. Bar graph shows read density at regulatory elements and TSS of *Fos* (C; e2: $F_{(1,4)condition} = 1.62$, $p = 0.3$; $F_{(1,4)genotype} = 21.64$, $p = 0.01$, post-hoc HC $p<0.05$; e4: $F_{(1,4)condition} = 1.72$, $p = 0.3$; $F_{(1,4)genotype} = 4.48$, $p = 0.1$; TSS: $F_{(1,4)condition} = 49.40$, $p = 0.002$; $F_{(1,4)genotype} = 27.42$, $p = 0.006$, post-hoc HC < 0.05; e5: $F_{(1,4)condition} = 0.19$, $p = 0.7$; $F_{(1,4)genotype} = 21.63$, $p = 0.01$, post-hoc HC > 0.05, post-hoc NE $p<0.05$) and *Arc* (E; Enh: $F_{(1,4)condition} = 5.21$, $p = 0.08$; $F_{(1,4)genotype} = 56.51$, $p = 0.002$, post-hoc HC $p < 0.05$, post-hoc NE $p < 0.01$; TSS: $F_{(1,4)condition} = 3.77$, $p = 0.1$; $F_{(1,4)genotype} = 76.86$, $p = 0.0009$, post-hoc HC and NE $p < 0.01$). **F.** Scatter plot showing that NE and ifKO genotype effects are not positively correlated. **G.** RT-qPCR assays in hippocampal RNA of ifKOs show NE induction of *Arc* ($F_{(1,11)condition} = 15.11$, $p = 0.002$; $F_{(1,11)genotype} = 0.51$, $p = 0.5$, post-hoc ifWT $p < 0.05$), *Fos* ($F_{(1,11)condition} = 9.88$, $p = 0.0009$; $F_{(1,11)genotype} = 0.50$, $p = 0.5$, post-hoc ifWT $p<0.05$) and *Npas4* ($F_{(1,11)condition} = 35.04$, $p = 0.0001$; $F_{(1,11)genotype} = 0.007$, $p = 0.9$, post-hoc ifWT and ifKO $p < 0.01$), but no genotype effect. **H.** H3K4 trimethylation profiles at *Fos*, *Npas4* and *Arc* enhancers were not altered in ifKOs. Data are expressed as mean + s.e.m. * = $p < 0.05$ (genotype effect); # = $p < 0.05$, ## = $p < 0.005$; ### = $p < 0.0005$ (condition effect) in 2-way ANOVA.

Scandaglia et al_FigS6. Related to Fig 6

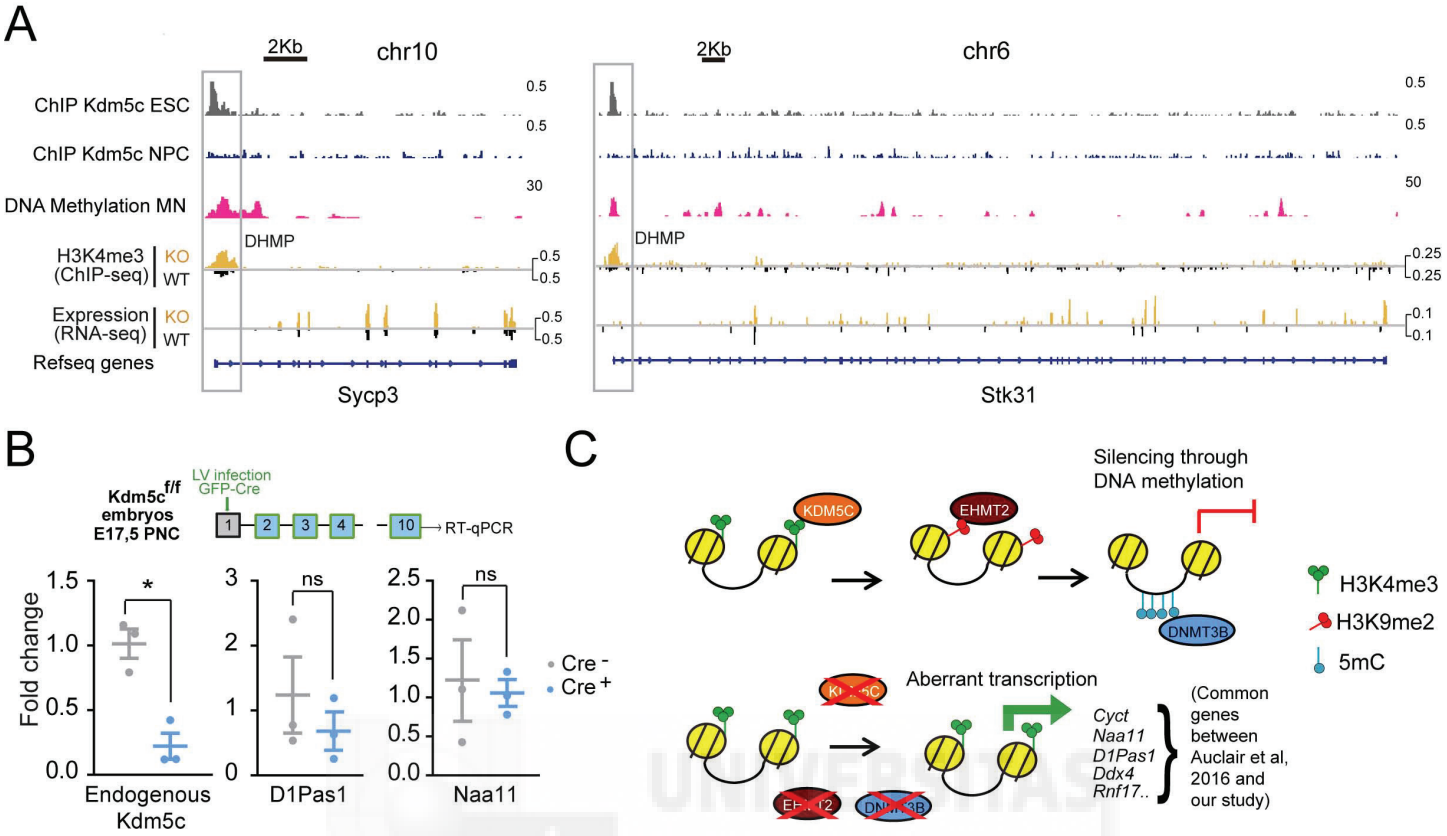

Figure S6. Related to Figure 6. Expression of hKdm5c precludes germ line gene silencing. A. Additional genomic profiles of germ line genes. From top to bottom, we show Kdm5c-ChIP-seq profiles in ESCs and NPCs (Outchkourov et al., 2013), MeDIP profiles in mature neurons (MN) (Halder et al., 2016), and H3K4me3-ChIPseq and RNAseq experiments in adult hippocampus (this study) for the *Sycp3* and *Stk31* genes. **B.** *Kdm5c*$^{f/f}$ PNCs infected with Cre-expressing LV do not show spurious expression of germ line genes (*endogenous Kdm5c*: $t_4 = 5.24$, $p = 0.006$; *D1Pas1*: $t_4 = 0.85$, $p = 0.5$; *Naa11*: $t_4 = 0.33$, $p = 0.8$). Data are expressed as mean ± s.e.m. * = p <0.05; ns = no significant, in Student's T-test. **C.** Model of germ line gene silencing through Kdm5c, Ehmt2 and Dnmt3b actions. It combines the evidence presented in this study and that of Auclair *et al.* (2016).

Scandaglia et al_FigS7. Related to Figure 7

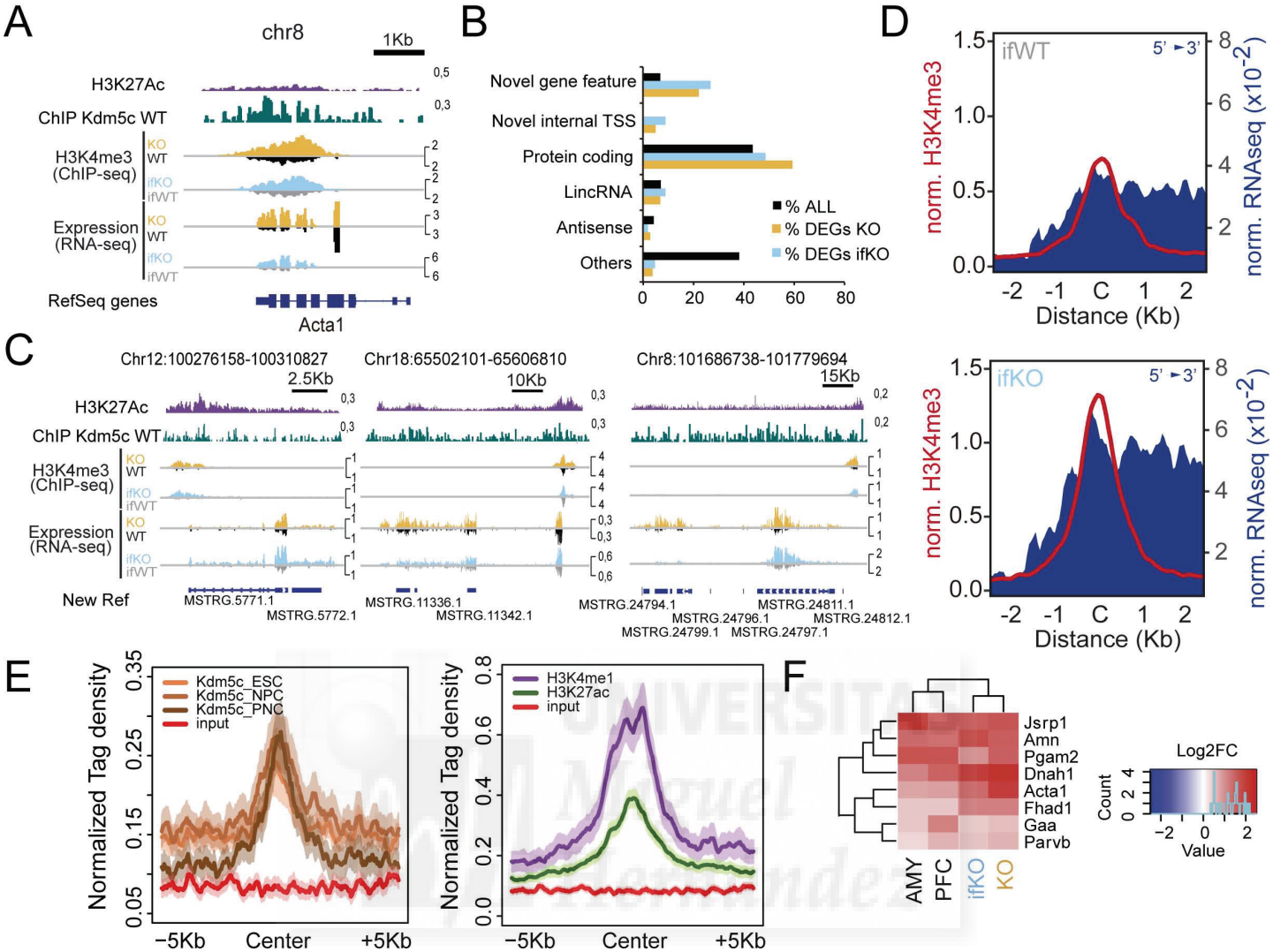

Figure S7. Related to Figure 7. H3K4 hypermethylation at intergenic elements is associated with spurious transcription. A. Genomic profiles for *Acta1*. Like *Dnah1* (see **Fig. 7C**), transcript upregulation in *Kdm5c*-KO neurons was restricted to the terminal exons and might correspond to the production of a meRNA. **B.** Detection of non-annotated transcripts is more abundant in Kdm5c-deficient mice than in their control littermates. **C.** Additional representative examples of RNA-seq and H3K4me3 profiles around top ranked intragenic DHMPs. **D.** Graphs show upregulation of transcripts from intergenic DHMPs in ifKOs. As for KOs, reads for the minus (-) and plus (+) strand +/-2 Kb from the peak center were independently scored. **E.** Graphs show the presence of Kdm5c occupancy and enhancer marks (H3K27ac and H3K4me1) at the same regions. **F.** Spurious transcription can be also detected in the amygdala (AMY) and prefrontral cortex (PFC) (Iwase et al., 2016).

Table S1. Related to Figure 1 and 2. SHIRPA evaluation of *Kdm5c*-KO males and heterozygous females.

Parameter	Kdm5c$^{+/y}$	Kdm5c $^{-/y}$	p	Kdm5c $^{+/+}$	Kdm5c $^{+/-}$	p
Weight (g)	34.4 ± 7.2	28.6 ± 6.1	0.01	30.2 ± 6.2	25.6 ± 3.9	0.05
Body length (mm)	9.47 ± 0.9	9.04 ± 0.54	0.04	9.02 ± 0.6	8.68 ± 0.80	0.13
Spontaneous activity	1.5 (1-2)	3 (2.5- 3)	0.02	1 (1-1)	1 (1-2)	0.09
Respiration rate	2 (2-2)	2 (2-2)	1	2 (2-2)	2 (2-2)	1
Tremor	2 (2-2)	2 (2-2)	1	0 ± 0	0 ± 0	1
Body position	0 (0-0)	0 (0-0)	0.29	3 (3-4)	4 (3 4	0.24
Barbering	4 (4-4)	4 (4-.4)	0.83	1 (1-1)	1 (1-2)	0.09
Urination	1 (0-1.75)	1 (0.5-1.5)	0.84	0 (0-0)	0 (0-0)	1
Defecation	0 (0-0)	0 (0-0)	0.29	0 (0-0.25)	0 (0-1)	0.67
Transfer arousal	0.5 (0-1)	0 (0-1)	0.52	1 (1-1)	1 (1-1)	0.06
Fear	0 (0-0)	0 (0-0)	0.73	0 (0-0)	0 (0-0)	0.73
Locomotor activity	1.93 ± 4	1.82 ± 8.5	0.03	6.31 ± 4.25	5.85 ± 5	0.72
Palpebral closure	0 (0-0)	0 (0-0)	1	0 (0-0)	0 (0-0)	1
Piloerection	1 (1-1)	1 (1-1)	1	0 (0-0)	0 (0-0)	1
Gait	0 (0-0)	0 (0-0)	1	0 (0-0)	0 (0-0)	1
Pelvic elevation	2 (0-2)	2 (0-2)	0.92	2 (2-2)	2 (2-2)	0.12
Tail elevation	2 (0-2)	2 (0-2)	0.92	1 (1-1)	1 (1-1)	1
Touch scape	1 (1-1)	1 (1-1)	0.87	1 (1-2)	1 (1-2)	0.46
Positional passivity	2 (1-2)	2 (1.5-2)	0.32	0 (0-1)	0 (0-0)	0.13
Trunk curl	0 (0-1)	0 (0-0)	0.07	0 (0-0)	0 (0-0)	1
Visual placing	2 (2-2)	2 (1.5-2)	0.50	2 (2-2)	2 (2-2)	1
Negative geotaxis	0 (0-0)	0 (0-0)	1	0 (0-0)	0 (0-0)	1
Grip strength	3 (3-3)	3 (3-3)	1	3 (3-3)	3 (3-3)	0.30
Pinna reflex	1 (1-1)	1 (1-1)	1	1 (1-2)	1 (1-2)	0.30
Corneal reflex	1 (1-1)	1 (1-1)	1	1 (1-2)	1 (1-2)	0.30
Toe pinch	1 (1-1)	1 (1-3)	0.07	2.5 (1-3)	3 (3-3) 0.124	0.12
Wire maneuver	0 (0-0)	2 (1-3)	0.01	0 (0-0)	0 (0-0)	0.05
Righting reflex	0 (0-1)	0 (0-0)	1	0 (0-0)	0 (0-0)	0.12
Irritability	0 (0-1)	0 (0-0)	0.44	1 (0-1)	1 (0-1)	0.98
Skin color	1 (1-1)	1 (1-1)	1	1 (1-1)	1 (1-1)	1
Limb tone	0 (0-1)	0 (0-0)	0.91	1 (1-1)	1 (1-1)	0.12
Abdominal tone	0 (0-0)	0 (0-0)	0.73	0 (0-0)	0 (0-0)	0.12
Lacrimation	0 (0-0)	0 (0-0)	1	0 (0-0)	0 (0-0)	1
Provoked biting	0 (0-1)	0 (0-0)	0.08	0 (0-0.25)	0 (0-1)	0.46
Aggression	0 (0-0)	0 (0-0)	1	0 (0-0)	0 (0-0)	1
Body tone	0 (0-0)	0 (0-0)	0.73	1 (1-1)	1 (1-1)	1
Vocalizations	1 (0-1)	1 (1-1)	0.36	1 (0-1)	1 (0-1)	0.98

Table S2. Related to Figure 1 and 2. Comparison of behavioral deficits in Kdm5c$^{-/y}$, Kdm5c$^{-/+}$ and Kdm5c-ifKO mice

	Kdm5c$^{-/y}$	**Kdm5c$^{-/+}$**	**Kdm5c-ifKO**
Spontaneous behavior			
Back circling	yes	non tested	no
Self-grooming	no difference	non tested	no difference
Rearing	no difference	non tested	no difference
Behavioral tests			
Open field	no difference	no difference	no difference
Elevated Plus Maze	no difference	no difference	no difference
Marble burying	↓ burying	non tested	no difference
Tail suspension	↓ immobility	non tested	no difference
Passive avoidance	no difference	non tested	non tested
Fear conditioning	↓↓ post-shock freezing ↓↓↓ context freezing ↓↓↓ cued freezing	↓ context freezing ↓ cued freezing	no difference
Morris water maze	↓↓↓ learning & memory	↓ learning	♂↓ & ♀ normal learning
Y-maze	↓ learning	non tested	non tested
Novelty-Suppressed feeding	↓ latency	non tested	no difference
Porsolt forced swimming	↓ immobility	non tested	no difference
Vocalization under stress	yes	non tested	no
Grip strength	no difference	no difference	non tested
Hotplate	↑ sensitivity	no difference	no difference
Rotarod	↓ latency	no difference	no difference
Other features			
Hindpaw clasping	yes	yes	no
Shaking after ketamine/xilacine injection	yes	non tested	no

Table S6. Related to Methods. Viability of Kdm5c$^{-/y}$ mice in a pure C57BL/6J genetic background

	Wild-type	*Kdm5c$^{-/y}$ or Kdm5c$^{-/+}$*	*P*
Males	42 (100%)	0 (0%)	< 0.0001
Females	39 (59,1%)	27 (40,9%)	0.30

For C57BL/6J genetic background, number and percentage of individuals obtained from wild-type males × heterozygote Kdm5c$^{+/-}$ females crosses are shown. The percentage of mutant mice was compared with same-sized samples following a mendelian distribution using Fisher's exact test.

Table S3, Table S4 and Table S5 are attached as EXCEL files and available at the following link: http://www.cell.com/cell-reports/fulltext/S2211-1247(17)31275-5

- **Table S3. Related to Figure 3. DHMPs in *Kdm5c*-KO and ifKO mice (**Excel file with two spreadsheets).
- **Table S4. Related to Figure 4. DEGs in *Kdm5c*-KO and ifKO mice** (Excel file with three spreadsheets).
- **Table S5. Related to Figure 5. DEGs upon NE** (Excel file with two spreadsheets).

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RESULTS:

CHAPTER II

Production of cerebral organoids derived from CJ-XLID patients for modelling intellectual disability

The unpublished data presented in this second Chapter of Results was produced during a 4-month stay at the Shi´s lab (Boston Children's Hospital – Harvard Medical School) where I set up the protocol for obtaining cerebral organoids from iPSCs with the collaboration and supervision of Dr. Violetta Karwacki-Nelsius.

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Introduction

In 2006, Yamanaka's lab introduced the technology for producing induced pluripotent stem cells (iPSCs), i.e., stem cells generated starting from adult differentiated cells. They discovered that this conversion was induced by the transduction of four TFs (Oct4/Pou5f1, Sox2, cMyc, and Klf4), later on referred to as the Yamanaka factors (Takahashi and Yamanaka 2006). Human iPSCs constitute a great advance in regenerative medicine because of their similarity to ESCs in terms of pluripotency. They can give rise to every kind of cells, and, importantly, they bypass the controversy associated to the usage of human ESCs derived from pre-implantation embryos.

Human fibroblasts or other cell types can be collected from healthy people or patients and reprogrammed to pluripotent cells using different delivery systems for the TFs' cocktail (Lim, Yang et al. 2015). The generated patient-specific iPSC lines can be then expanded and differentiated though several different protocols into the cell types in which the disease manifests. For instance, treatment with retinoic acid (a neuroectoderm inducer) or pharmacological inhibition of transforming growth factor-b and bone morphogenic protein pathways (dual SMAD inhibition) are usually employed to elicit neural commitment (Shi, Kirwan et al. 2012). Nowadays, there are shorter transformation protocols to produce iPSCs that imply the forced expression of a single TF (e.g. Neurogenin-2) through lentiviral expression (Zhang, Pak et al. 2013).

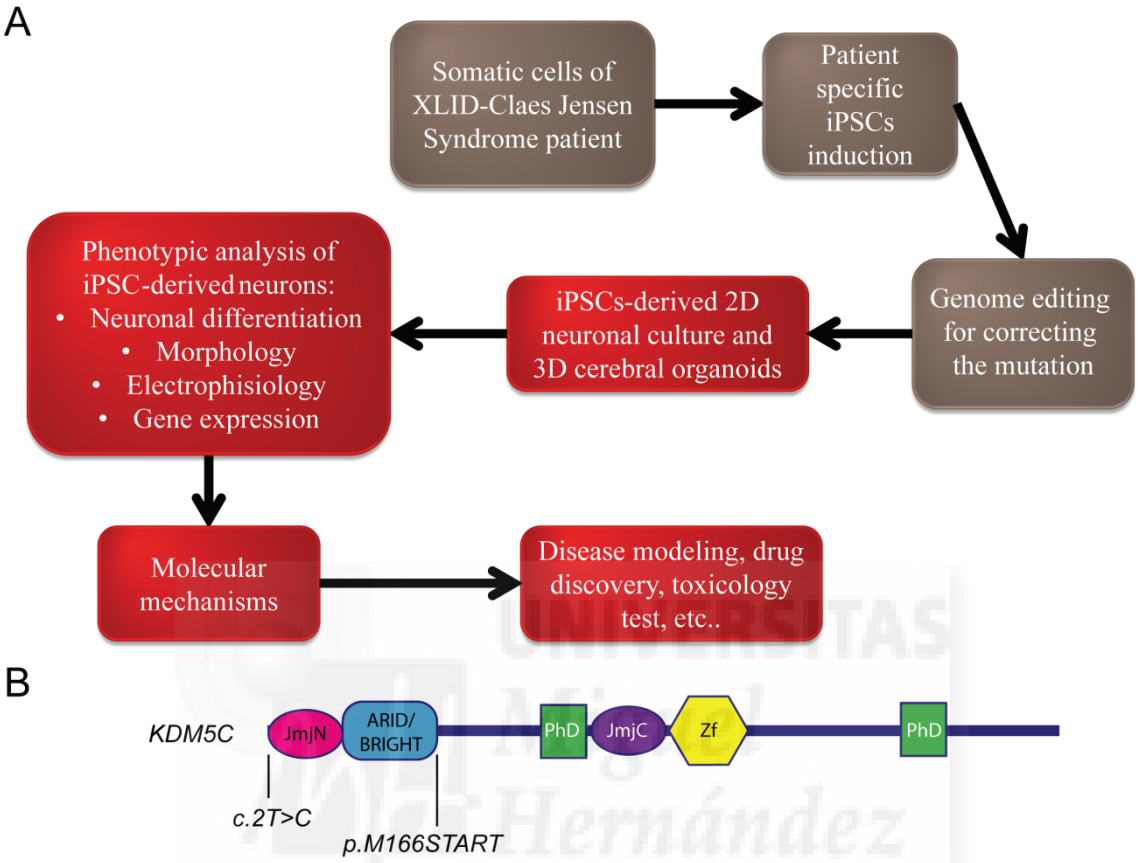
Heretofore, iPSCs-derived neurons from patients of several different neurological diseases, from autism to Hungtington's disease, have been generated and used in various studies (Vitale, Wolvetang et al. 2011, Sandoe and Eggan 2013, Telias and Ben-Yosef 2014, Velasco, Salazar et al. 2014, Avior, Sagi et al. 2016, Fu, Xu et al. 2016, Golas and Sander 2016, Habela, Song et al. 2016, Kang, Tang et al. 2016, Smith, He et al. 2016, Xiao, Ng et al. 2016). Human iPSCs-derived neurons have the advantage of

being species-specific for human disease modelling and are used for studying important aspects including electrophysiological properties, gene expression changes, molecular and morphological characteristics. Genome and epigenome editing techniques such as clustered regularly interspaced short palindromic repeat/CAS9 RNA-guided nucleases (CRISPR/CAS9), in combination or not with chromatin effectors, provide a novel system to correct the intended mutation or the epigenetic alterations for further patient-specific cell replacement or therapy approaches (Robinton and Daley 2012, Li, Suzuki et al. 2014, Zentner and Henikoff 2015, Thakore, Black et al. 2016, Brocken, Tark-Dame et al. 2017).

In the last few years, several labs have developed methods to produce *in vitro* complex structures, known as organoids, which resemble whole organs. These methods have revolutionized the field of stem cell research and developmental biology. Organoids can be generated either from resident progenitors in adult organs or from pluripotent stem cells (ESC and iPSC). These cells are grown in a 3D environment with specific factors that drive them to recapitulate the mechanisms of organogenesis that occur *in vivo*. They are able to differentiate and acquire tissue-specific patterning, for example, by developing into endoderm, mesoderm and ectoderm-derived tissues (Huch and Koo 2015).

Cerebral organoids (COs) are nowadays a cutting-edge technology able to unravel the mechanisms of mammalian stem cell differentiation and unveil the complexity of human brain development and evolution (Giandomenico and Lancaster 2017). In addition, the generation of iPSC-derived human COs is becoming an outstanding tool to model early neurodevelopmental disorders (Kelava and Lancaster 2016). The production of COs bearing the very same disease-associated mutation linked to neurodevelopmental disorders can reproduce early defects in stem cells proliferation,

differentiation, maturation and growth. These approaches should contribute to the definition of the molecular mechanisms impaired in patients respect to healthy people and to the design of possible drugs and therapies (**Chapter II, Figure 1A**).



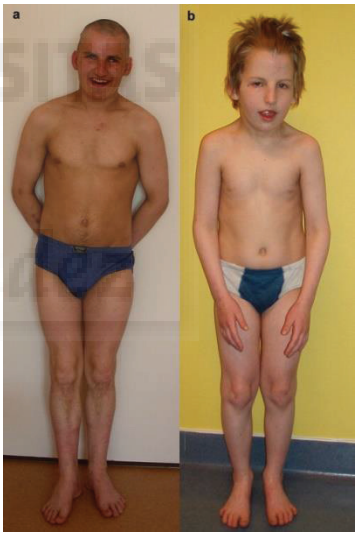
Chapter II, Figure 1. Project workflow. A. Scheme of the project. I specifically collaborated in the first trial of preparation of COs derived from CJ-XLID patients. **B.** Mutation c.2T>C induces a start codon shift resulting in a truncated protein were the N-terminal part until codon 166 is missing (Ounap, Puusepp-Benazzouz et al. 2012) (Brookes, Laurent et al. 2015).

To use this technique in the context of CJ-XLID research for the first time, we started to work with fibroblasts carrying the c.2T>C mutation (**Chapter II, Figure 1B**). This mutation was found in a large family where 2 males and 5 females were affected (Ounap, Puusepp-Benazzouz et al. 2012). The two CJ-XLID-affected brothers exhibited similar phenotypes and are referred to as AK and KK, respectively. Both presented developmental delay (DD), profound speech impairment, short stature, spasticity,

dysmorphic features and over-friendliness. The older brother AK also displayed epilepsy, aggressiveness and strabismus while the younger brother KK showed microcephaly (Ounap, Puusepp-Benazzouz et al. 2012) (**Chapter II, Table 1** and **Chapter II, Figure 2**).

Chapter II, Table 1. List of patients under study.

Patient	Age of biopsy	DNA mutation	Protein mutation	KDM activity	ID	Clinical notes
AK	23 years	c.2T>C	M166	Reduced	Severe	DD, strabismus, ataxia, short stature, aggression, epilepsy
KK	13 years	c.2T>C	M166	Reduced	Severe	DD, ataxia, short stature, microcephaly



Chapter II, Figure 2. Pictures of the AK and KK brothers. The older brother AK (left) and the younger KK (right). From (Ounap, Puusepp-Benazzouz et al. 2012).

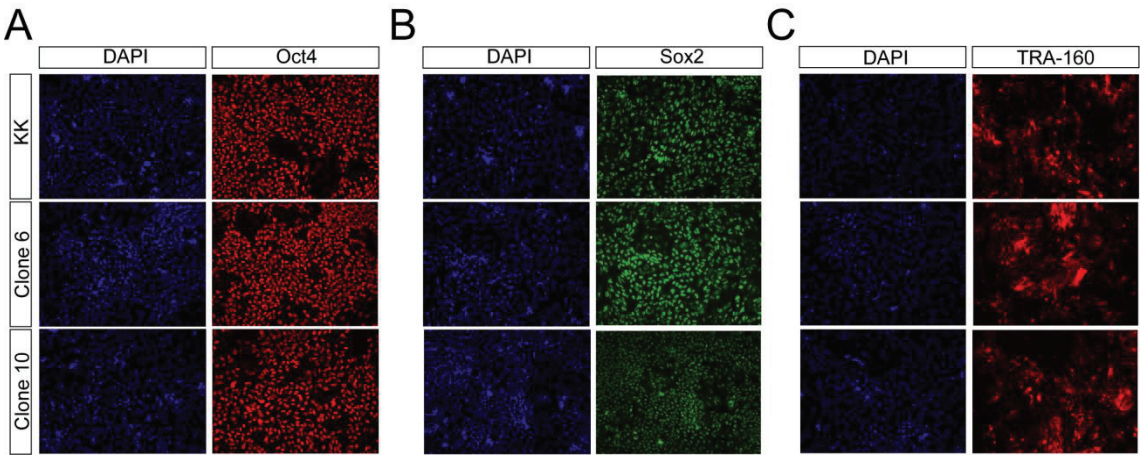
The mutation c.2T>C leads to the production of an N-terminally truncated protein (20 kDa smaller than the wild-type) that starts at codon 166 (p.M166START or p.M1_E165del) (**Chapter II, Figure 1B**). This protein is unstable (half-life of 2 h instead of 12 h), lacks the highly conserved JmjN and ARID domains and, although it still binds chromatin, lacks detectable KDM activity. Fibroblasts derived from the two brothers did not show global changes in histone methylation but presented specific changes in gene expression (Brookes, Laurent et al. 2015).

Shi and colleagues obtained fibroblasts of these two CJ-XLID patients, and reprogrammed them into iPSCs using non-integrating episomal plasmid vectors (Okita, Matsumura et al. 2011). They also corrected the mutation in specific clones using a genome editing approach that combined CRISPR-Cas9 (to generate double strand breaks at the mutation site) and *piggyBac* technology to seamlessly remove a drug selection cassette and correct the mutation, as described in (Yusa 2013). By doing this, they obtained control isogenic iPSC lines in which the IDD-causing mutation was corrected in an unaltered genetic background. The comparison of mutation bearing and corrected iPSCs represent a perfect system to identify and describe mutation-originated phenotypes.

Results

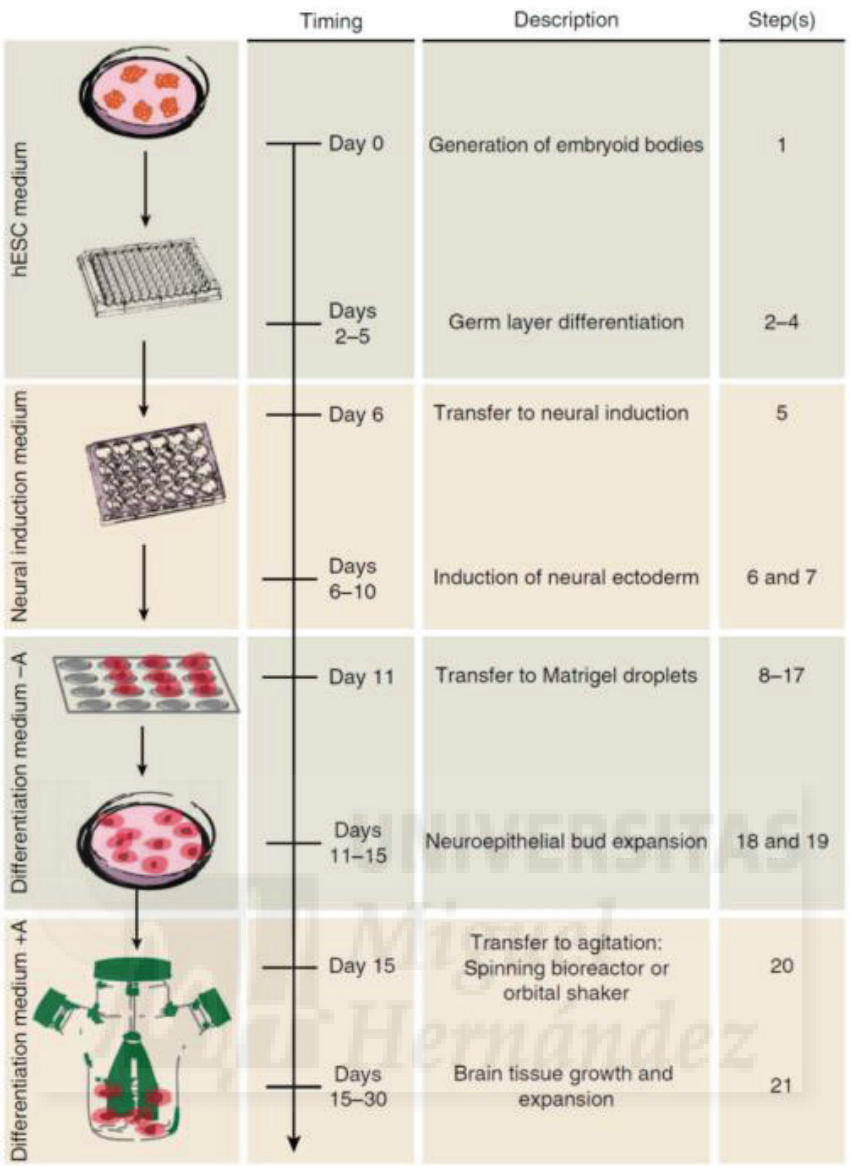
Production of cerebral organoids

We started our trial with KK brother-derived iPS cells and with clones in which the KDM5C mutation was corrected (Clone 6 and Clone 10). All iPSC lines were tested previously in Shi laboratory for lack of reprogramming plasmid genomic integration (quantified by EBNA qPCR), morphology assessment, expression of pluripotency markers by qPCR and immunoflourescence and a normal 46, XY karyotype. Genome editing in corrected lines was verified by genome sequencing. The aim of my work was to establish a cerebral organoid culture system in collaboration with the Shi laboratory to study defects in the KDM5C patient lines. We decided to focus on the protocol described by (Lancaster and Knoblich 2014). Before each differentiation attempt KDM5C mutant line and corrected clones were stained for pluripotency markers. No evident anomalies were detected in the mutant clone when compared to the corrected clones. All the clones expressed three main markers of pluripotency: Oct4, Sox2 and TRA-160 (**Chapter II, Figure 3**).



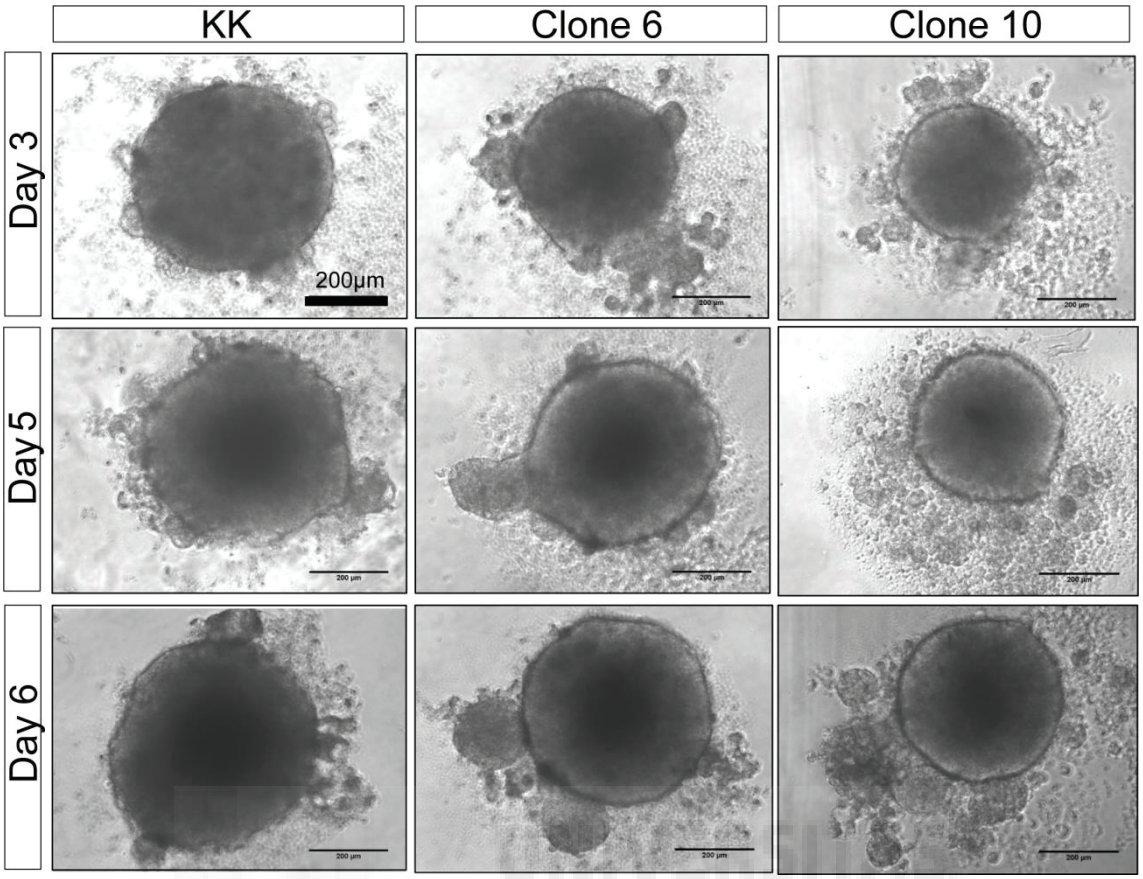
Chapter II, Figure 3. Pluripotency of mutant and corrected cells. A-C. Pluripotency of iPSCs was assayed by using three pluripotency markers: Oct4 (A), Sox2 (B) and TRA-160 (C). No gross differences were noticed among the three conditions.

After this quality assessment, we started the protocol of COs formation. First, iPSCs clones were cleaned by removing the differentiated and/or dead cells around the stem cells colonies. The day after cleaning, iPSCs colonies were lifted, taking care not to aspirate also the surrounding mouse embryonic fibroblasts (MEFs), and separated to single cells. These were plated at a given concentration (more details in *Experimental procedures* and **Chapter II, Figure 4**) to form embryoid bodies (EBs).



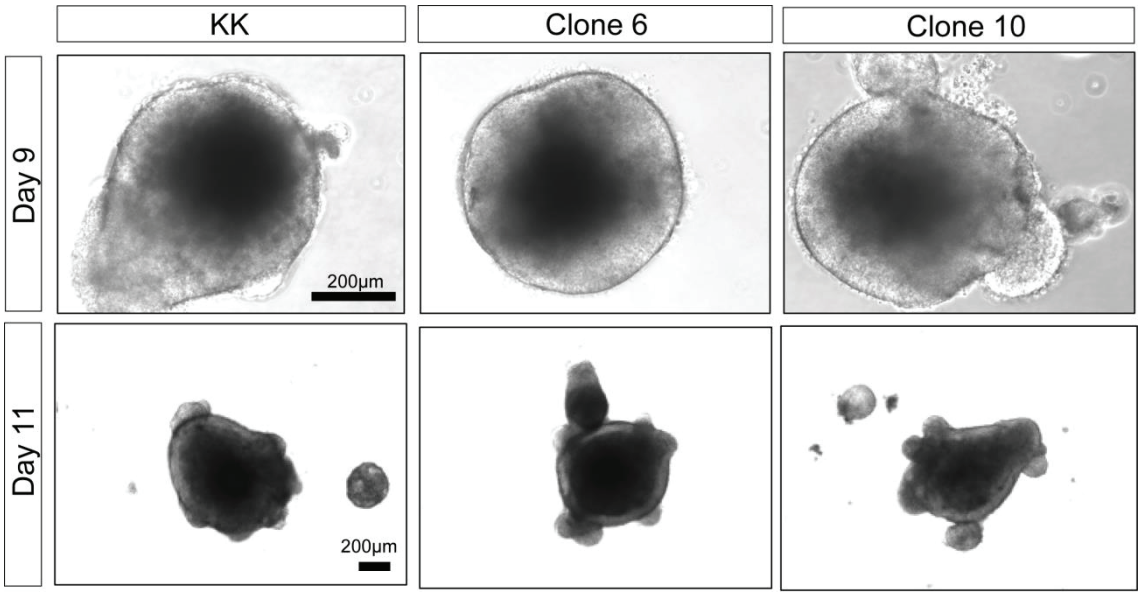
Chapter II, Figure 4. Protocol for COs production. The protocol followed in this trial was taken from (Lancaster and Knoblich 2014).

24h after plating the cells, EBs were clearly seen at the bottom of the well. These EB continued growing for 6 days. We observed that KK-derived EBs were bigger than those derived from the corrected clones because the cells attached more rapidly to the central body respect to the Clone 6 and 10, where a higher number of single cells remained un-attached (**Chapter II, Figure 5**).



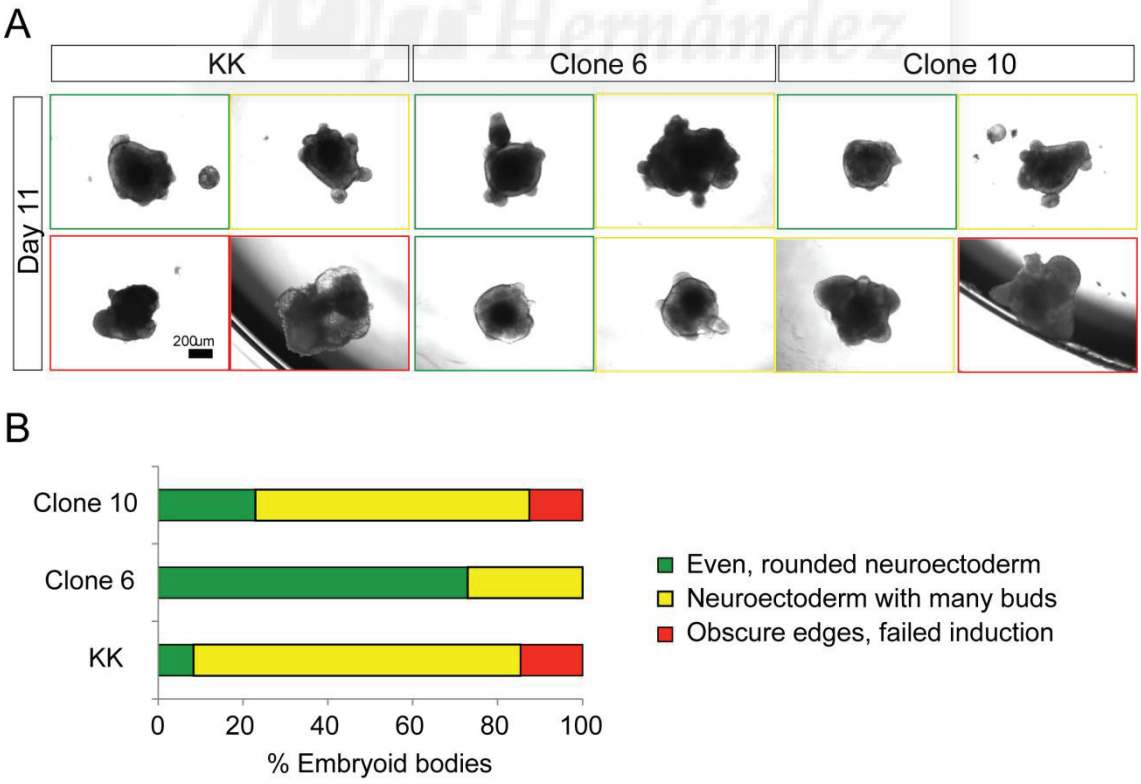
Chapter II, Figure 5. Embryoid bodies formation. Pictures of the EBs at day 3, 5 and 6, when EB formation occurs. KK-derived EBs look bigger than corrected clones.

At day 6, EBs were moved to ultra-low attachment 24-well plates (1 per well) and ESC media was substituted with neural induction media, a minimal media that does not support the growth of endoderm and mesoderm, allowing only the neuroectoderm to develop. Neuroectoderm *in vitro* spontaneously acquires a radial organization establishing the apico-basal polarity reminiscent of the neuroepithelium (**Chapter II, Figure 6**).



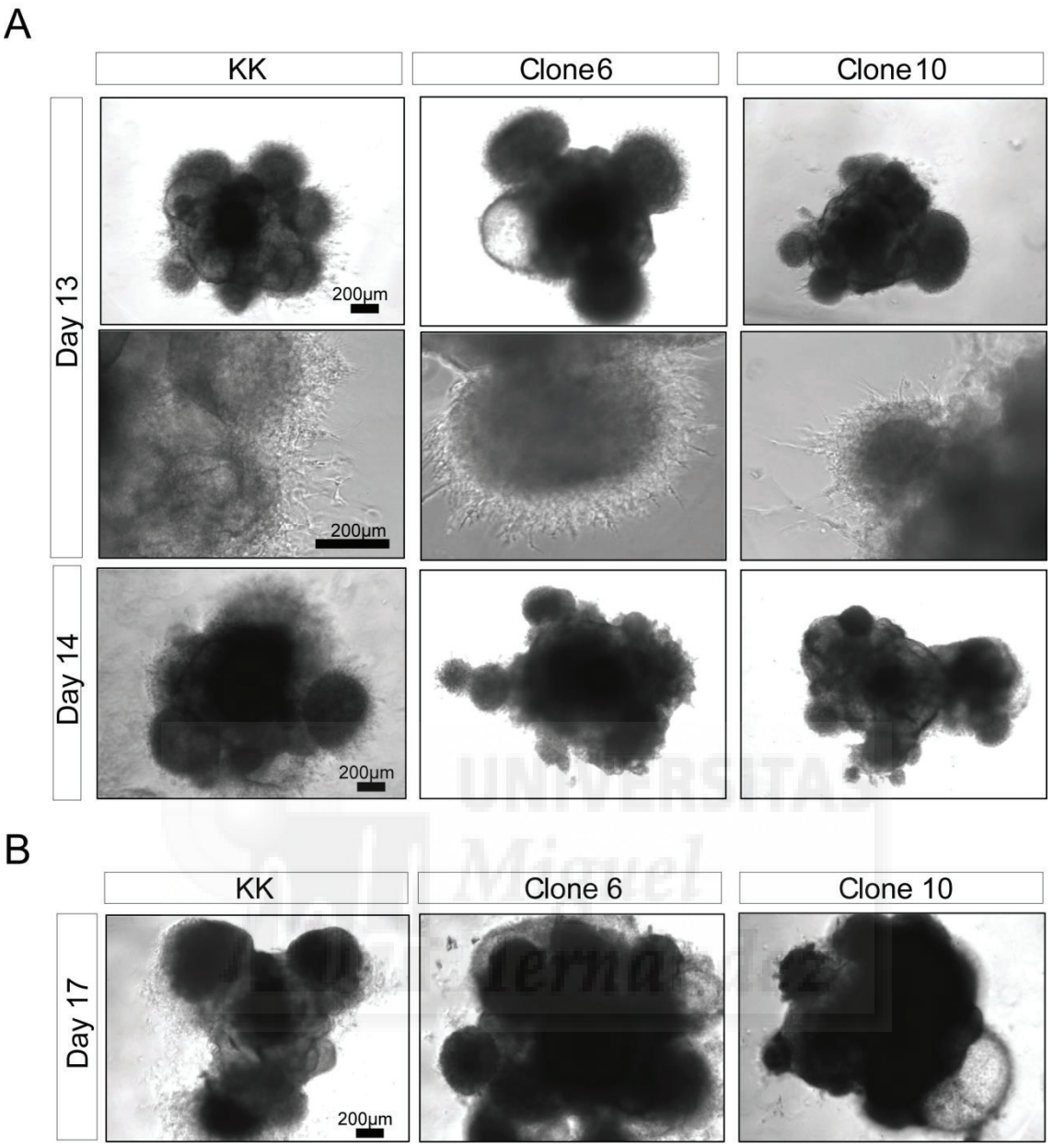
Chapter II, Figure 6. Neural induction. A. Pictures of the EBs at day 9 and 11 after neural induction. Brilliant edges indicated a good neural induction.

Neural induction was not always successful. Failure of the induction was detected in some of the EBs from KK and Clone 10 while Clone 6 had the best neural ectoderm induction (**Chapter II, Figure 7A-B**).



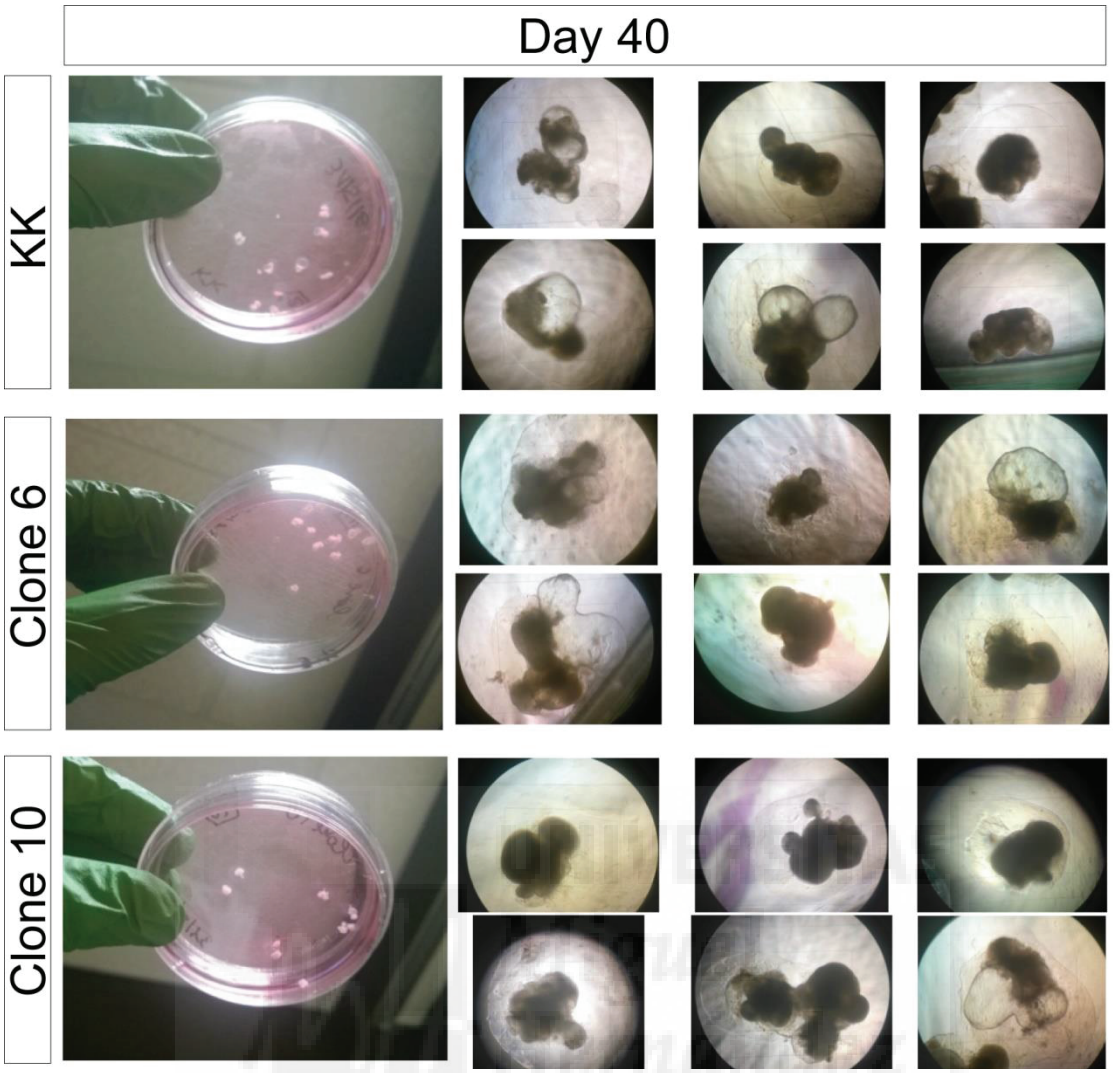
Chapter II, Figure 7. Variability in the induction success. A. Pictures of the EBs at day 11 showing the first neuroectoderm formation around the central body in the three conditions. Border colours indicate the quality and aspect of the neural induction quantified in panel B. **B.** Quantification of the percentage of EBs with a precise aspect categorized as *good* (green) when an even and rounded neuroectoderm was formed; *normal* (yellow), when the neuroectoderm contained many buds; and *bad* (red), when obscure edges and cysts were present, symptoms of a failed neural induction. Interestingly, the mutation bearing clone (KK) presented the lowest percentage of *good* EBs.

At day 11, neuroepithelial tissues were embedded in Matrigel (**Chapter II, Figure 8**). Matrigel droplets promote outgrowth of large buds of continuous neuroepithelium that expand and contain fluid filled lumens reminiscent of brain ventricles, indicative of proper apicobasal orientation. The first stage of the COs expansion is stationary and does not require vitamin A (retinoic acid) containing media whereas, at day 15, COs are transferred to a differentiation media containing vitamin A and they are put in an orbital shaker at 37ºC to induce rotational movement of the media. Retinoic acid (RA) has been reported to be secreted from the brain meninges and to promote neural differentiation (Siegenthaler, Ashique et al. 2009). However, it is also known to act as a potent caudalizing factor *in vivo* (Petros, Tyson et al. 2011). Therefore, in order to limit the presence of exogenous patterning factors, RA was not included at early stages and it was only added to the differentiation media at later stages in the form of vitamin A provided in the B27 supplement. Moreover, movements promote nutrient and oxygen exchange to allow more extensive growth and further development. COs can reach the maximum size at 2 months but they can be maintained up to 1 year (Lancaster and Knoblich 2014).



Chapter II, Figure 8. Cerebral organoids development in Matrigel. A. Pictures of the COs at day 13 and 14 showing the filamentous structures going out from the central body into the Matrigel in the three conditions. **B.** Pictures at day 17 showing the presence of multiple buds, rounded sectors.

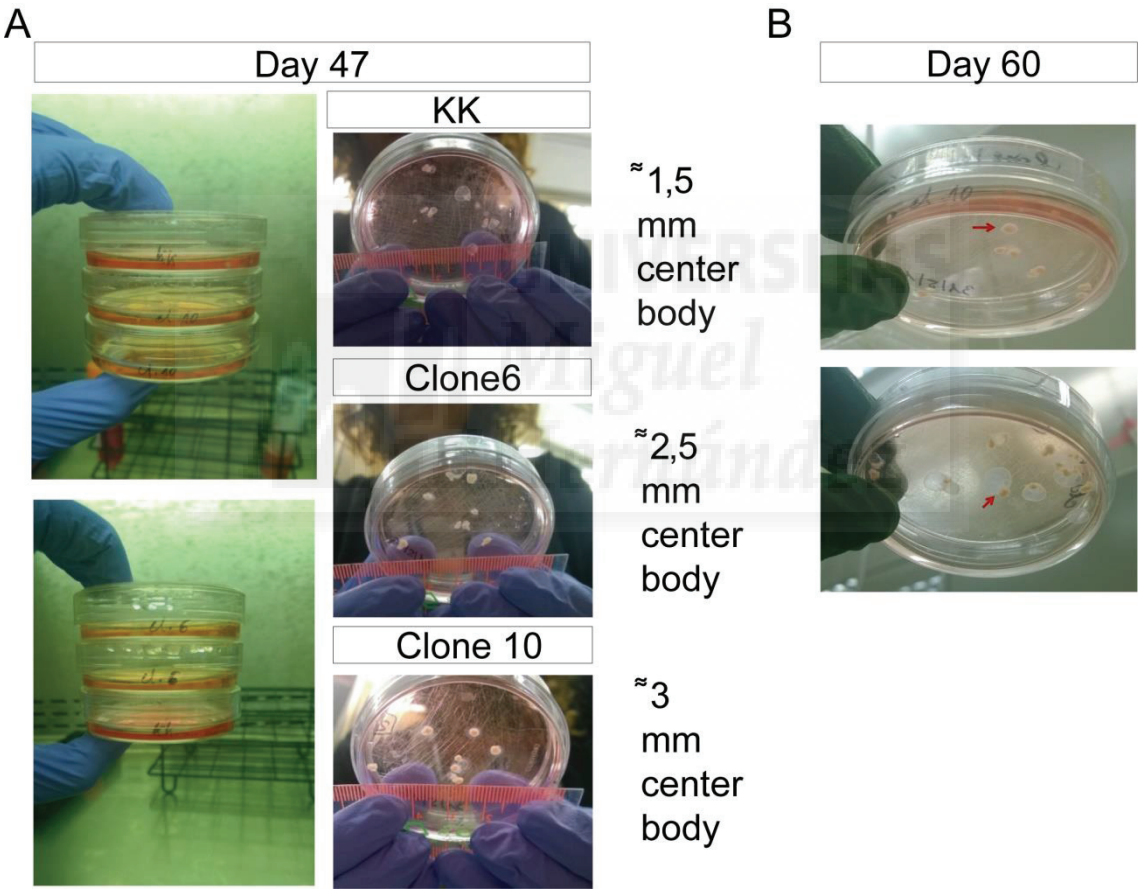
Following the protocol, media was changed every 5 days, and at day 40 the variability in shape and growth among COs of the same condition was evident (**Chapter II, Figure 9**).



Chapter II, Figure 9. Heterogeneity in shapes. External aspect of COs at day 40. Pictures of the plate and photographs in the bright field microscope of the developing structures in the three conditions. Images show the variability in the shape and size of each single COs.

Characterization of cerebral organoids

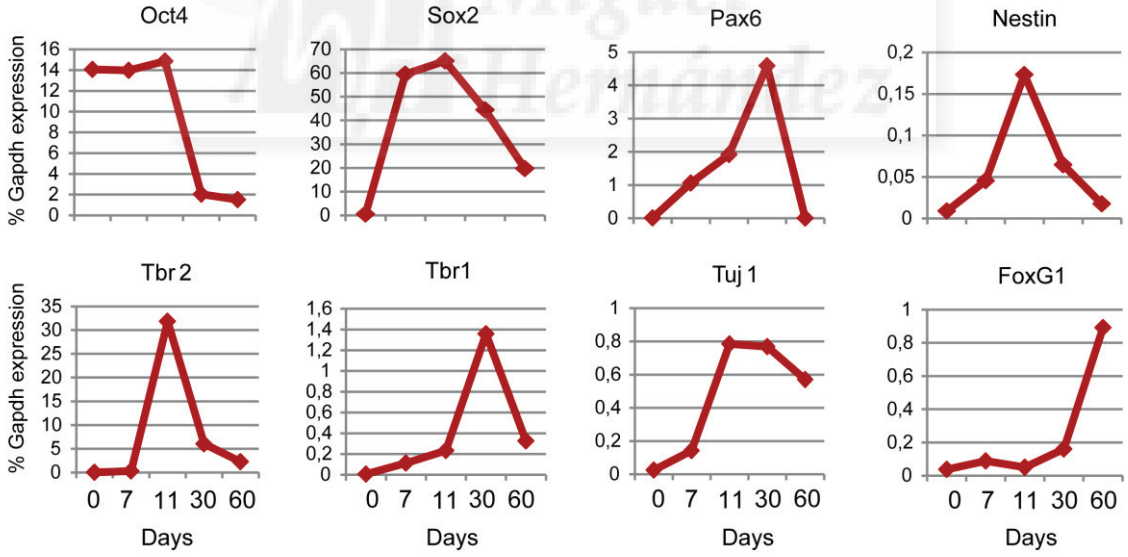
At day 47, we observed clear differences between KK-derived COs and those derived from the corrected clones. KK-derived COs seemed to be smaller and grew less. In fact, both the colour of the media and measurement of the center body size suggested this (**Chapter II, Figure 10A**). After 2 months, Clone 6 and 10 were enriched with healthy looking COs (large, up to 4 mm, and with a rounded shape), while the KK-derived COs often presented large fluid-filled cysts. I should however note that all three conditionss displayed COs of heterogeneous shape and size (**Chapter II, Figure 10B**).



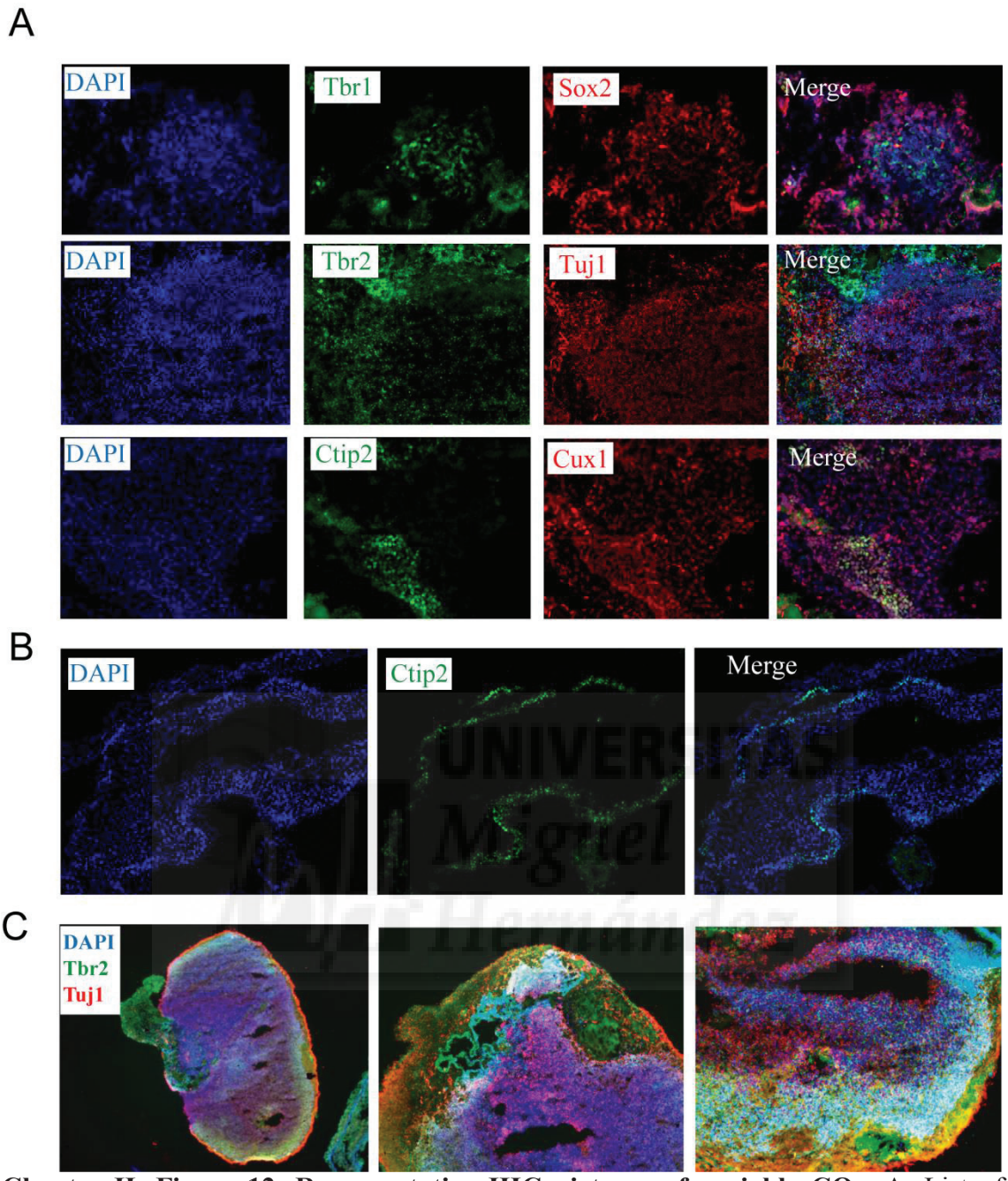
Chapter II, Figure 10. KK COs grow less. A. External aspect of COs at day 47. The colour of the media can be used as an indicator of the growth rate of the organoids. KK plates medium is less yellowish than the one of the corrected clones highlighting reduced growth. KK COs were in general smaller with about 1.5 mm of center body compared to the biggest one produced form clone 10 with 3 mm-thick center body. **B.** At day 60, the best COs obtained in this trial were rounded (top). Fluid filled cysts were observed in many COs after 2 months (bottom). Red arrows show representative COs.

To have a general idea of how pluripotency and neuronal markers were changing during embryoid development, I performed some RT-qPCRs experiments demonstrating the global decrease of pluripotency and the increase of neuronal specific genes along time (**Chapter II, Figure 11**). Note that in these preliminary experiments I could not distinguish between clones because of the small sample size (n) available.

Finally, in the last experiment of this first trial, I tried to corroborate the expression of some of these markers by immunohistochemistry (IHC). COs were cut in the cryostat and stained for different markers (**Chapter II, Figure 12**). These immune-stainings, like the expression analysis experiments reported above, aimed to test the specificity and efficiency of the primers and antibodies available and the suitability of the protocol for further investigation. Therefore, we did not directly compare the three different samples.



Chapter II, Figure 11. Gene expression dynamics during COs differentiation. RT-qPCRs of different markers in EBs and COs along their development showing a decrease in the expression of pluripotency markers (Oct4, Sox2, Pax6) and an increase in the expression of progenitors markers (Nestin and Tbr2) and neuronal markers (Tbr1, Tuj1 and FoxG1).



Chapter II, Figure 12. Representative IHC pictures of variable COs. A. List of antibodies tested: Tbr1: Pre-plate/layer 1 and deep layer neurons; Sox2: Radial glia/NSCs; Tbr2: Intermediate progenitors; Tuj1: neurons; Ctip2: deep layers neurons; Cux1: progenitor cells and upper layer neurons. Pictures in the first row show the coexistence of Sox2+ cells representing neural stem cells and Tbr1+ deep layer neuronal cells in the same CO. As expected, these markers do not co-localize. In the second row, intermediate progenitors and neurons are stained with Tbr2 and Tuj1 respectively, while in the third row, Ctip2 and Cux1 are used to distinguish between deeper and upper layers of the forming cortex. **B.** Ctip2 staining nicely marks all the cortical plate of the CO indicating the expansion of the deep layer neurons. **C.** Images of 3 COs with irregular morphology, including the presence of internal holes that resemble ventricle structures. DAPI was used in nuclear counterstaining.

Lastly, I tried to plate again KK-derived iPSCs (to repeat the experiment). The only observation, I could make during the first days after plating was again the initial larger appearance of KK-derived EBs, according to the conclusion achieved during the first trial. Because my stay in the Shi laboratory was restricted up to 4 months and organoids differentiation takes several months I could not perform a detailed analysis of the lines. However I was able to establish the culture system in the Shi laboratory which will enable future detailed investigation of the organoid cultures from ID patients.

Discussion

Although the data presented in this Chapter are very preliminary, the system has the potential to provide new and interesting insights into CJ-XLID. One phenotype that became apparent in the course of those experiments was the initial bigger size and the slower growth of EBs produced from patient KK. This suggests that KDM5C-deficient iPSCs rapidly aggregate at the beginning of EB formation but then show a slower growing rate. The smaller size could be caused, for example, by impaired proliferation of neuroprogenitor cells compared to controls. In addition, a higher percentage of failed COs in KK samples could indicate differentiation problems as well. We do not know yet if these cells are more prompt to differentiate to some other non-induced germ layers, such as endoderm or mesoderm, compromising the correct neuroectoderm induction. Additional immunostainings during EBs maturation using antibodies against markers for those layers might address this question. Another hypothesis would be that both processes (NPCs proliferation and differentiation) are affected in patient iPSCs at the same time: (i) the proliferation rate is low resulting in a smaller center body and (ii) only a small proportion of progenitor cells undergoes the correct neuronal induction, triggering a less efficient differentiation and a lower number of mature neurons.

Interestingly, the findings from Winder and colleagues, studying the function of Kdm5b in early development, revealed that day 9-Kdm5b-overexpressing EBs displayed increased proliferation through increased expression of cyclins, suggesting that they retained highly proliferative cells, refractory to differentiation. Conversely, $Kdm5b^{+/-}$ EBs have problems in the initial aggregation of iPSC cells (contrary to our *KDM5C* mutant cells) and seem to have decreased levels of pluripotency markers and increased levels of Egr1, an immediate early gene that is upregulated during differentiation (Dey, Stalker et al. 2008). However, although Kdm5b plays a role in both the proliferation of stem cells and in the repression of cell lineage genes, allowing cells to remain uncommitted, it is not said that Kdm5c has the same role at early developmental stages, so more investigations are needed to clarify their similarity and differences.

Some of the future experiments should also include the improvement of this protocol trying to integrate the new achievements in the field with the aim of reducing the variability between organoids and the differences in environmental conditions (Kelava and Lancaster 2016, Lancaster, Corsini et al. 2017). In fact, getting homogeneous COs will allow defining epigenomic and transcriptomic profiles in different developmental stages and to compare those with findings in the mouse model KO for Kdm5c, as well as to perform morphological and electrophysiological studies which can relate to the patients' phenotype.

Experimental methods

Cerebral organoid cultures

We followed the *Nature protocol* published in 2014 by Lancaster and Knoblich (Lancaster and Knoblich 2014) Briefly, hiPSCs colonies from KK and corrected clones were dissociated from mouse embryonic fibroblasts (MEFs) by dispase treatment followed by trypsinization to generate single cells. About 9000 cells were plated in each well of an ultra-low binding 96-well plate (Corning) in human ES media with low concentration basic fibroblast growth factor (bFGF) (4 ng/ml) and 50 mM Rho-associated protein kinase (ROCK) inhibitor (Calbiochem). Embryoid bodies were fed every other day for 6 days and then transferred to low-adhesion 24-well plates (Corning) in neural induction media containing Dulbecco's modified eagle medium (DMEM)/F12, 1:100 N2 supplement (Invitrogen), Glutamax (Invitrogen), minimum essential media-nonessential amino acids (MEM-NEAA) and 1 µg/ml heparin (Sigma). The neuroepithelial tissues were fed every other day for 5 days. On day 11, tissues were transferred to droplets of Matrigel (BD Biosciences) by pipetting into cold Matrigel on a sheet of Parafilm with small dimples. These droplets were allowed to gel at 37ºC for 30 min and then they were removed from the Parafilm and let grow in differentiation media containing a 1:1 mixture of DMEM/F12 and Neurobasal containing 1:200 N2 supplement (Invitrogen), 1:100 B27 supplement without vitamin A (Invitrogen), 3.5 µl/l 2-mercaptoethanol, 1:4000 insulin (Sigma), 1:100 Glutamax (Invitrogen), 1:200 MEM-NEAA. After 4 days of stationary growth, the droplets in 60 mm small dishes were transferred to an orbital shaker. The media from this moment till the end of the protocol contained differentiation media as above, except that B27 supplement with vitamin A (Invitrogen) was used.

RNA isolation, RT and qPCR

RNA isolation from 1 or more EBs or COs was performed with TriReagent following manufacturer's instructions. PrimeScript First Strand cDNA Synthesis Kit (Takara) was used for cDNA synthesis and LightCycler® 480 Instrument II (Roche) to run qPCR assays. Primers used were specific for human transcripts.

Preparation of organoids for cryosectioning and immunostaining

Organoids were transferred at different time points to a standard 24-well plate by gently pipetting with a cut 200-µl pipette tip for organoids not yet embedded in Matrigel, or a cut 1-ml pipette tip for organoids embedded in Matrigel droplets. Media was removed and organoids washed with PBS. 4% PFA was added and let stand at 4°C for 15 min before washing again with PBS for 3 times, 10 min each. PBS was replaced by 1 ml of 30% (wt/vol) sucrose solution and the plate was placed at 4°C overnight to allow tissues to sink into sucrose solution. The next day, gelatin/sucrose solution was warmed at 37°C for 20–30 min and a small amount was poured in a medium-sized weighing dish to just cover the bottom of the dish. The dishes were put at 4°C to allow the gelatin to polymerize. The sucrose solution on organoids was replaced by 1 ml of warmed gelatin/sucrose solution and the plate was placed at 37°C for 15 min to equilibrate the tissues. Then, organoids were carefully transferred from the 24-well plate to the polymerized gelatin/sucrose layer in the weighing dish (we use a cut 200-µl pipette tip for small tissues or a cut 1-ml pipette tip for large organoids). We allowed the small amount of gelatin/sucrose that was transferred with the organoids to solidify at room temperature for 2–3 min. Then warm gelatin/sucrose solution was poured in the weighing dish to completely cover the tissues and place it at 4°C allowing it to polymerize for 15–20 min. Next, we first removed the entire polymerized gelatin from

the weigh boat by pushing the sides, and then we cut out using a scalpel blade a small block containing the organoids. A freezing bath was prepared by dropping several small pieces of dry ice into a bath of isopentane until a low-temperature thermometer reads between −50 and −30°C. The entire blocks of gelatin containing organoids were immersed into the cold bath allowing them to freeze for 1–2 min. Carefully the block were taken out of the bath using a spatula and store at −80°C until ready to section. 20-μm sections were cut using a standard cryostat, and collected on Ultra Plus slides for immunostaining.

Immunohistochemistry

Immunohistochemistry (IHC) was performed following a standard protocol for tissue cryosections. During this first trial several antibodies were tested but images shown in the Results are derived from the following stainings: anti-Eomes (Oct4) (H-109) sc-98555, anti-Sox2 (E-4) sc-365829, anti-TRA160 (e-Bioscience), anti-Tbr1 ab31940, anti-Tbr2, anti-Tuj1 Biolegend 801202, anti-Cux1 sc-13024, anti-Ctip2 ab18465. Pictures were taken with a classical microscope or with a mobile phone camara.

GENERAL DISCUSSION

UNIVERSITAS
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The research article presented in Chapter I of Results includes a brief discussion of the main findings achieved in this thesis. Here, I will discuss in further detail some aspects of my research that were not covered in that manuscript due to space limitations. I will also discuss our main results in a broader context and relate our conclusions with some recent articles in the field that were published during or after the publication of our article. Finally, I will comment on the preliminary results obtained during my stay at the laboratory of Dr. Shi in the Boston Children's Hospital (Boston, MA, USA).

5.1. Modelling Claes-Jensen type X-linked intellectual disability

The etiology and pathogenesis of IDDs are still poorly understood. Although tremendous progress has been made, pharmacological intervention and therapeutic strategies remain limited. Appropriate animal and cellular models are necessary to explain basic mechanisms of these conditions and scientific discoveries must be translated into practical applications to finally improve human health. Thus, progress in understanding and treating each form of ID and trying to unveil the common molecular pathways affected is of great relevance. In the frame of this thesis, I used mouse lines and human COs to model CJ-XLID.

5.1.1. Modelling CJ-XLID in the mouse

Until the last decade animals (in particular mice) constituted the most-studied and best-known way to model a disease, including IDD, and big progresses have been done thanks to them. Here, I expanded the description of phenotypical traits of Kdm5c-KO mice that resemble clinical features of CJ-XLID patients. This includes behavioural impairments as hyperreflexia, impulsivity, altered emotional responses, motor defects, and increased epilepsy susceptibility. My experiments do not only confirm the

suitability of Kdm5c-KOs as a model of CJ-XLID, first outlined by Shi and colleagues (Iwase, Brookes et al. 2016), but they also show that the complete loss of Kdm5c in full body KOs in a pure murine background is embryonically lethal, underscoring the key role of Kdm5c in early development and its interaction with other genetic factors. Additionally, we showed that Kdm5c heterozygous females exhibit mild motor and cognitive defects that resemble those recently described for human females carrier of KDM5C mutations (Simensen, Rogers et al. 2012), indicating that a full dose of KDM5C is required for the normal development of neuronal circuits underlying motor coordination and cognitive functions.

On the other hand, our characterization of ifKO revealed comparatively milder impairments than conventional KO, namely deficits in spatial learning and memory in the MWM, which indicates that Kdm5c plays a prominent role during development of neuronal circuits and that the range of its genomic actions is reduced in adult neurons. This is reflected both in the number of genes affected and the degree of change. The highly specific cognitive defects found in these mice would suggest a specific role for Kdm5c in hippocampal dependent spatial navigation memory although surprisingly this deficit was not reproduced in homozygous conditional female mice. We should also note that tests evaluating other behaviors and abilities or conducted in different conditions, such as after stressful stimuli or other insults, may reveal in the future other Kdm5c-dependent phenotypes.

Although we do not know yet the precise mechanisms by which the mutations of human *KDM5C* cause CJ-XLID, our study provide some likely candidate processes. These novel insights resulted from the comprehensive analyses aimed to identify the genomic effects and targets of murine Kdm5c during development and in the adult forebrain. We achieved this by comparing H3K4me3, Kdm5c and mRNA profiles in

Kdm5c-deficient mice and control littermates at different developmental stages. In the way, we have also provided the scientific community with genomic profiles of adult hippocampal principal neurons that will be useful for further research on neurogenomics.

5.1.2. Human iPSCs and cerebral organoids as tools for IDD modelling

Despite genome-wide transcriptomic and epigenetic studies have been conducted in cell lines and model organisms bearing IDD-linked mutations providing very interesting results and important cues concerning the role of IDD-linked enzymes, it is still far from clear to what extent the genomic loci and biological pathways identified in those studies are also affected in nervous tissue from human patients. Therefore, studies with neuronal cultures and COs derived from human iPSCs are of a great value nowadays.

In particular, COs, because of their complex structures integrating different cellular types, provide a unique opportunity to model human brain early stages in a system remarkably similar to *in vivo* development. Although they cannot reproduce the behavioural outcome typical of a complex organism, they allow researchers to study cellular and molecular etiology of neurodevelopmental diseases in a specific human background (Lancaster and Knoblich 2014, Quadrato, Brown et al. 2016).

In the Chapter II of my results, I showed how COs derived from a CJ-XLID patient can reproduce phenotypical aspects detected previously in mice such as microcephaly. However, more investigations are needed to characterize and compare the organoids bearing the ID-causing mutation and those in which the mutation was eliminated after genomic editing.

5.2. Epigenetic etiology of IDDs

Our studies highlight the importance of Kdm5c in three specific processes that are likely to contribute to the etiology of CJ-XLID: the silencing of germ line genes, the fine-tuning of activity-regulated enhancers and the prevention of cryptic transcription in adult neurons. As I will discuss in the next sections, deregulation of neither one of these processes is an exclusive feature of CJ-XLID. In fact, we believe that these events may also underlie other forms of ID and participate in the pathoetiology of other neurological conditions.

5.2.1. Germ line genes silencing and ID

ESCs have identical genotypes and phenotypes. Later, external signals trigger developmental events that lead to the differentiation of these cells into different types, a process that requires the activation of cell type-specific transcriptional programs together with the simultaneous repression of alternative cell fates. The latter is achieved through the integration of specific DNA methylation patterns by DNMTs, which, in turn, requires the action of other epigenetic factors (Ooi, Qiu et al. 2007).

Although mature differentiated cells remain genotypically identical, they become phenotypically distinct upon differentiation. The differences in gene expression persist in the face of numerous cell divisions, which indicates that they are self-sustaining. As discussed in the Introduction, these developmentally induced changes in gene expression are mediated by epigenetic mechanisms. How does Kdm5c contribute to triggering the persistence of an epigenetic mark? In our study, we concluded that Kdm5c plays a critical role in the silencing of specific germ line genes that are very lowly expressed in the adult WT brain. Specifically, we found that among the most upregulated DEGs in the hippocampus of adult Kdm5c-KOs mice there are at least

twenty germline related genes. The upregulation of these genes correlated with a strong increase of H3K4me3 at their promoters. Recently, another study has demonstrated the upregulation of germline related genes in KO embryos for *Ehmt2* and *Dnmt3b* (Auclair, Borgel et al. 2016). Interestingly, some of these target genes coincided with DEGs in Kdm5c-KOs (e.g., *D1Pas1, Naa11, Cyct*). Looking deeper at the genomic profiles of these genes we noticed that in most of them, Kdm5c had a specific occupancy during ESC stage that was lost already in NPCs (Outchkourov, Muino et al. 2013). That suggests that Kdm5c is specifically required at this early time point to demethylate H3K4 and trigger or enable the recruitment of the H3K9 methyltransferase Ehmt2 and the *de novo* DNA methyltransferase Dnmt3b, underscoring the interplay between DNA methylation, H3K9 and H3K4 methylation (Du, Johnson et al. 2015). These studies together with our experiments indicate that the three proteins (Kdm5c, Ehmt2 and Dnmt3b) are indispensable for producing permanent silencing of these loci. In particular, both the removal of H3K4me3 and the incorporation of methyl groups at H3K9 (to produce H3K9me2) seem to be instructive for DNA methylation. If either one of these processes is disrupted, these loci will not be inactivated by CpG methylation and will consequently remain transcriptionally active. Further supporting Auclair's results and our data, hypomorphic mutant mice for Dnmt3b were found to induce the illegitimate activation of several germline genes in somatic tissues that appeared to be linked directly to their hypomethylation in mutant embryos. Interestingly, Dnmt3b seems to directly regulate these genes upon recruitment by the transcriptional repressor E2F6 (Velasco, Hube et al. 2010, Walton, Francastel et al. 2014).

The ablation of other transcriptional repressors, such as Myc-associated Factor X (Max) and polycomb group ring finger 6 (Pcgf6), also leads to robust de-repression of germ cell-related genes (Maeda, Okamura et al. 2013, Endoh, Endo et al. 2017). Max

interacts with histone H3K9 methyltransferases and represses germ cell-specific genes in ESCs (Maeda, Okamura et al. 2013, Endoh, Endo et al. 2017). Interestingly, Okuda and colleagues found that MAX-depleted ESCs and germline cells present a meiosis-like phenotype (Suzuki, Hirasaki et al. 2016, Suzuki, Hirasaki et al. 2017). In addition, Pcgf6 seems to interact with RING1A/B and E2F6 associated factors to form a non-canonical polycomb repressive complex 1 (PRC1) and repress a subset of germ cell-related genes in ESCs, affecting cell growth and viability. Accordingly, MAX/MGA heterodimer is the TF complex responsible to recruit Pcfg6 to its target loci (Maeda, Okamura et al. 2013, Endoh, Endo et al. 2017). Furthermore, it has recently been demonstrated that Pcgf6 has a similar genomic profile of the tritorax group proteins and, contrary to PRC1 and 2, it acts as a positive regulator of transcription and binds predominantly to promoters bearing active chromatin marks. Its action seems to directly regulate Oct4, Nanog, Sox2, and Lin28 expression to maintain ESC identity and avoid differentiation (Zdzieblo, Li et al. 2014).

Most of the proteins under investigations in all these studies (Max, Pcgf6, Ring1a, E2f6, Ehmt2) seem to interact with Kdm5c (Tahiliani, Mei et al. 2007, Outchkourov, Muino et al. 2013, Endoh, Endo et al. 2017), indicating that most probably a large multimeric repressive complex is responsible for the silencing of germline genes in somatic cells and Max could be the driving force for Kdm5c recruitment at specific targets (**Figure 9**).

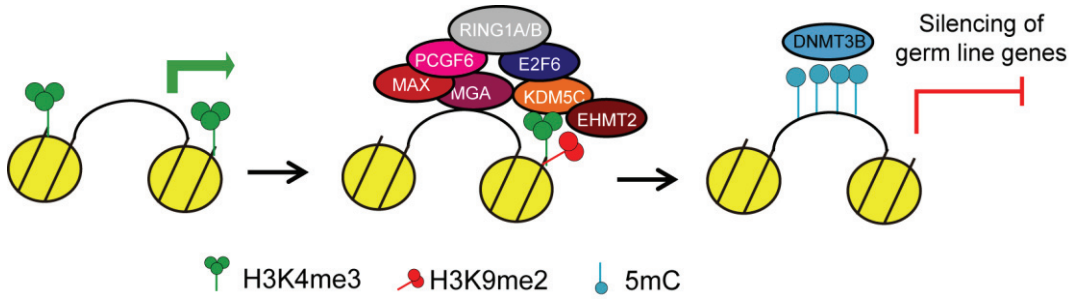

Figure 9. Model for silencing of germ line genes during early development. Representation of the possible repressive complex recruited by MAX/MGA TFs, responsible for inducing the permanent silencing of germ line loci by DNA methylation.

Confirming this view, pyrosequencing analyses in Kdm5c-KOs evidenced a significant decrease in promoter methylation at the two loci analysed suggesting a partial loss of DNA methylation. However, this reduction did not represent a complete loss of methylation and the loci remained much more methylated than in germ line cells, which would explain their overall low expression compared to other genes. This observation suggests the existence of other proteins and complexes involved in the silencing of the loci so that the absence of Kdm5c is not enough to fully prevent their methylation and silencing. An appealing explanation for the limited demethylation of these loci would be mono-allelic silencing, i.e., one of the two alleles (maternal or paternal) is silenced in a Kdm5c-independent manner. Interestingly, DNA methylation and histone modifications mark also Imprinting Control Regions (ICRs) and are established in gametes in an allele-specific manner. They have to be stably maintained during development and in somatic cells to ensure the correct mono-allelic expression of imprinted genes. The histone H3 lysine 9 methyltransferase Ehmt1/2 complex (also called G9a/GLP) is required for stable maintenance of imprinted DNA methylation in ESCs although its catalytic activity and consequent H3K9me2 mark are dispensable for imprinting maintenance. On the other hand, H3K9me2-depleted cells present the genome-wide loss of non-imprinted DNA methylation suggesting a different role for

Ehmt1/2 in the two types of loci. It seems that the G9a/GLP complex protects imprinted DNA methylation by recruitment of *de novo* DNA methyltransferases, which antagonize TET dioxygenase-dependent erosion of DNA methylation at ICRs (Zhang, Termanis et al. 2016).

Intriguingly, several of the genes under study (such as *D1Pas1* and *Naa11*) have a retrosposon origin. For example, *D1Pas1* is a retrosposon (repetitive DNA fragment which is inserted into a chromosome after being reverse transcribed from a RNA molecule) located in the chromosome 1 and paralogue of the X-linked *Ddx3x*, also found to be mutated in a form of ID (Snijders Blok, Madsen et al. 2015). Similarly, *Naa11* encodes for an acetyltransferase and is located in the chromosome 11 but constitutes the paralogue of *Naa10* in the chromosome X, also linked to ID (Arnesen, Betts et al. 2006, Rope, Wang et al. 2011, Casey, Stove et al. 2015, Popp, Stove et al. 2015). Kdm5c could silence these genes just because they share similar promoter sequences with X-linked targets (although the paralogues does not seem to be directly affected in Kdm5c KO male hippocampi) or because they are the result of an aberrant retrotransposition, dangerous for the cell, and Kdm5c is acting as genome surveillor by repressing them (Kaessmann 2010). However, it is striking that the X-linked paralogues of these two genes have been also related to ID.

In female mammals, most genes on one X chromosome are silenced as a result of XCI. However, some genes escape X-inactivation and are expressed from both the active and inactive X chromosome. Such genes are potential contributors to sexually dimorphic traits, to phenotypic variability among females heterozygous for X-linked conditions, and to clinical abnormalities in patients with abnormal X chromosomes (Carrel and Willard 2005). Kdm5c is considered a sexual dimorphic gene for escaping XCI and having a different expression levels between females and males; thus its action

can contribute to the sexual dimorphic traits. This fact suggests a potential role for Kdm5c in establishment and/or maintenance of XCI. ATRX, for example, associates with the Xi following the onset of random XCI, consistent with a potential role in maintenance of XCI. These results have important implications regarding a previously described escape from imprinted XCI in ATRX-deficient mice as well as cases of skewed XCI in patients with ATRX syndrome (Baumann and De La Fuente 2009). Maybe Kdm5c is behaving similarly in females and it is important for establishment or maintaining of the inactive X as recently suggested by Iwase and colleagues (Gayen, Maclary et al. 2017).

As explained in the Introduction, gametogenesis is an essential process to ensure the evolutionary transfer of genetic information from one generation to the next. Primordial germ cells (PGCs) undergo meiosis to become functional haploid gametes (sperm and oocyte), and, prior to that, they undergo epigenetic imprinting which involves the erasure and the reestablishment of DNA methylation. Also, histone modifications and XCI have important roles during germ cell development (Sun, Wang et al. 2017). Moreover, retention of H3K4me3 at critical developmental promoters in the germ line might be a means of preventing DNA methylation at these important regulatory sites (Lesch and Page 2014). Therefore, it is not excluded that Kdm5c could have a role also in this process. Interestingly, a recent paper provided evidence that KDM5C may participate in gonadal dysgenesis by impairing ovarian development through regulation of miR-486-5p or miR-320a (Sun, Zhang et al. 2017). According to this speculation, a recent study in Drosophila pointed out that depletion of dKDM5/LID results in a perturbation of the oocyte's epigenome with consequently temporal dysregulation of meiotic transcription and decrease in female fertility (Navarro-Costa, McCarthy et al. 2016).

The loss of DNA methylation is not a feature only found in CJ-XLID patients (Grafodatskaya, Chung et al. 2013). It has been also detected in other IDDs and genomic imprinting-related diseases (Bérubé and Kramer 2014). Several recent studies on other epigenetic regulators such as Kdm1a/Lsd1 and Kdm1b relate them to the regulation of genomic imprinting (Ciccone, Su et al. 2009, Katz, Edwards et al. 2009, Siklenka, Erkek et al. 2015). Moreover, similarly to KDM5C, LSD1 is considered an epigenetic transcriptional corepressor, interacting with CoREST and histone deacetylases HDAC1/2 (Shi, Matson et al. 2005) and has been recently linked to both ID (Pilotto, Speranzini et al. 2016) and germline transcription (Katz, Edwards et al. 2009).

An example of KMT for H3K4 that also seems to be involved in this kind of regulation is Mll2. Mll2 is known to be important in development and germ cell lineage, regulating in testes genes like *Sycp3*, *Ddx4* and *Stra8*. Its loss causes in these genes reduction of H3K4me3 and gain of H3K27me3 and DNA methylation, resulting in impaired spermatogenesis (Glaser, Lubitz et al. 2009), although it is not known if Mll2 would have the same role in neurons or other somatic cells. Other germ cell related genes such as *Stra8*, *Boule* and *Dazl* are related to Max KO-induced phenotype in fact their overexpression leads to transformation of bone marrow mesenchymal stem cells to putative male germ cells (Li, Yan et al. 2017).

We still ignore how deleterious is this spurious activation and aberrant transcription of germ line genes in the frame of neuronal differentiation, maturation and ID. Indeed, these genes are very low-expressed even after Kdm5c loss and we did not prove the production of functional protein products from these transcripts. Therefore, it is very difficult to hypothesize the role they could have in neurons and neuronal progenitors before differentiation because all the previous studies focused on their

expression in testis and gonadal organs. Nonetheless, it is striking that several epigenetic enzymes linked to neurodevelopmental defects target the same or similar loci (i.e. Ehmt2, Dnmt3b, Kdm1a, Atrx) (Hansen, Wijmenga et al. 1999, Hagleitner, Lankester et al. 2008, Katz, Edwards et al. 2009, Schaefer, Sampath et al. 2009, Auclair, Borgel et al. 2016). This suggests that their defective silencing may play a role in the etiology of ID, a hypothesis that defines an interesting future area of investigation.

Altogether, these hypothesized mechanisms remain speculative and several questions arisen from our study still need to be investigated, such as: what is the relevance of testis-specific genes misexpression in brains of KO male mice? What is the relationship with ID? Why is so important to silence these loci before neuronal differentiation? Is this occurring also before differentiation to other cell types? What is the role of Kdm5c in germ cells where those genes should stay on? And what is the role of Kdm5c in female XCI?

5.2.2. Kdm5c-dependent regulation of activity-driven enhancers

Another group of genes identified in our differential expression analysis that is potentially relevant for the pathoetiology of neurological phenotypes is that associated with neuron-related Gene Ontology (GO) terms. In this group we find the genes encoding for the phosphatases Dusp5, Dusp18 and the RNA polymerase II transcription inhibitors Hexim1 and Hexim2 that were modestly upregulated, and the synaptic components Grik3, Nrp2, Syt2 that were significantly, although also modestly, downregulated. As Kdm5c acts mostly as a transcriptional repressor and no decrease in H3K4me3 was detected at the TSSs of the downregulated genes, we believe that the latter group is more likely to be subjected to indirect regulation. These results are consistent with those presented by Shi and colleagues who demonstrated that several

key pathways that play essential roles in the development and function of neuronal circuitry were aberrantly expressed in amygdala and prefrontal cortex of KOs, and that these alterations correlated with dendritic morphology defects. Among the misregulated pathways there were neurotransmitter systems (i.e. dopamine-, glutamate- and gaba-related), calcium transmission, cilia function and androgen-regulated pathways (Iwase, Brookes et al. 2016) although, similarly to our study the magnitude of the changes was small.

One of the main novel findings of this thesis is the involvement of Kdm5c in the fine-tuning of activity-driven gene expression during neuronal maturation. In fact, this idea was hypothesised long time ago due to the involvement of Kdm5c in neuronal development (Iwase, Lan et al. 2007) but nobody had demonstrated it before. Notably, we did not initially detect significant difference in the expression of plasticity-related genes such as *Arc*, *Fos*, *Npas4*, *Bdnf* and *Homer1* in the basal state, given the stringent p-value and fold change thresholds used in our DE screen, but we noticed a small and consistent upregulation affecting many IEGs. Since these differences could be magnified after neuronal induction, we next examined *in vivo* hippocampal neuronal activation by exposing animals to a novel environment (NE). This is known to trigger transcription of several IEGs involved in neuronal plasticity and memory (Flavell (Flavell and Greenberg 2008, Lyons and West 2011). KO animals were able to induce the NE-dependent program but the magnitude of the induction was smaller as a result of the higher expression of IEGs in naïve condition. Changes in activity-regulated transcription have been also associated with abnormal neuronal excitability (e.g., slight increase in cells responsiveness) and this could be the cause of the structural changes or dendritic/spines defects that possibly prevent cells from firing correctly after activity. In

turn, altered dendritic spines can very importantly contribute to the reported neurological disturbances.

IEGs seem to be affected by the germinal absence of Kdm5c but not after Kdm5c deletion in adult mice. The *in vitro* conditional model permitted to validate which was the developmental window in which Kdm5c plays a role modulating the ground state of expression of these genes. It seems that, during *in vitro* neuronal maturation, Kdm5c, most probably by regulating enhancer histone methylation and eRNA transcription, fine-tunes expression levels of these genes (*Npas4, Arc, Fos* and *Bdnf*) and establish what is the their ground expression. Interestingly, neurons are very plastic during development so Kdm5c removal at this stage provokes a rapid and, most probably transient, increase of IEGs expression that is restored by the addition of wild type hKDM5C. Therefore, Kdm5c is possibly fine-tuning the ground activity of these enhancers during maturation while, at later stages, other proteins are responsible for the induction of IEG upon different stimuli. Our results also indicate that the basal activity state is later perpetuated (maintained) epigenetically during the life of the animal in the absence of Kdm5c (**Figure 10**).

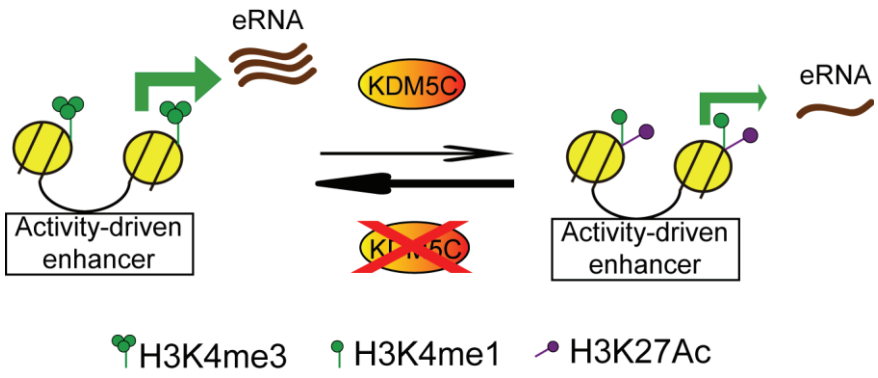

Figure 10. Model for regulation of enhancer transcription by Kdm5c. Scheme depicting the unbalance of H3K4 tri- vs mono-methylation upon lack of Kdm5c. When Kdm5c demethylase activity is impaired during neuronal maturation, IEGs enhancers, normally enriched for H3K4me1 and H3K27Ac present increased H3K4me3 signal causing an enhanced eRNA transcription.

Recent studies have demonstrated that enhancers are often major sites of extragenic noncoding transcription (Andersson 2015, Li, Notani et al. 2016, Paralkar, Taborda et al. 2016). Our experiments clarify previous conflicting results regarding the function of Kdm5c at enhancers (Outchkourov, Muino et al. 2013, Shen, Xu et al. 2016). In fact, in (Outchkourov, Muino et al. 2013), enhancers had been shown to be positively regulated by Kdm5c. However, conversely to those findings and similarly to our study, Shen et al. demonstrated a negative regulation of enhancers (Shen, Xu et al. 2016). The lack of Kdm5c would unset H3K4me3 (a HPTM strongly associated with active promoters) and H3K4me1 (which is enriched at enhancers) levels, thereby favoring eRNA transcription from Kdm5c-regulated enhancers. Interestingly, a recent study has highlighted the importance of the KMTs Mll3/Mll4 for eRNA synthesis and RNA PolII recruitment, despite their H3K4 methyltransferase activity (Dorighi, Swigut et al. 2017).

Tuning down activity-driven genes is essential for the maturation of dendritic arbors and the control of neural circuit responses to upcoming signals (Yang, Yamada et al. 2016). We can speculate that some of the phenotypes observed in KOs that recapitulate clinical symptoms, such as epilepsy propensity and learning disability, could be caused by the mistuning of activity-regulated enhancers.

It is important to remind that the abnormal expression of IEGs could be also the cause of cognitive deficits in other IDDs. The chromatin regulator KDM1A/LSD1, which as Kdm5c regulates H3K4 methylation, has been implicated in cognitive processes via direct control of neuronal plasticity transcriptional programs, prompting aberrant IEGs transcriptional modulation in relationship with another IDD (namely, CPRF). In particular, the neuronal isoform Lsd1n together with Lsd1 seem to be

involved in a neuronal-restricted mechanism of fine transcriptional modulation of plasticity related IEGs, including *Fos*, *Egr1*, *Npas4*, *Nr4a1* and *Arc,* through the interaction with the activity-regulated transcription factor SRF (Toffolo, Rusconi et al. 2014, Rusconi, Grillo et al. 2016). In addition, very recent evidences suggest that in post-mitotic neurons, loss of LSD1 result in premature activation of enhancers and genes that are normally induced after neuronal activation (Agarwal, Garay et al. 2017).

5.2.3. Understanding spurious transcription in ID

The specificity of cognitive deficits in ifKO males (restricted to a learning delay in MWM task) and their apparent absence in ifKO females indicate that the biological relevance of Kdm5c in neuronal physiology declines over time, probably in parallel with the progressive locking of the cell type-specific epigenetic status (Smith and Meissner 2013). The epigenomic and transcriptomic changes still detected after Kdm5c ablation in adult forebrain neurons is largely restricted to the ectopic expression of non-neuronal genes and aberrant transcription from cryptic promoters or regulatory sites at intra- and intergenic regions. More ncRNAs were discovered after novel transcriptome assembly suggesting that non-annotated genes can also contribute to the impairments observed in ifKOs. We globally refer to these events as spurious transcription (Hennig and Fischer 2013).

Therefore, Kdm5c seems to retain a life-long surveillance role in mature neurons preventing extemporaneous transcription from numerous intragenic and intergenic regulatory sites with transcriptional potential. Such unscheduled gene activation can be deleterious for normal brain function, but not at the point of causing neuronal death or severe malfunction. We ignore if these aberrant transcript are degraded by the RNA exosome complex or if they are capped, polyadenylated, and translated into aberrant

proteins. Overall, this form of deregulation seems to be well tolerated in the CNS, and most of the Kdm5c-sensitive loci and associated lncRNAs that undergo spurious transcription have an unknown function and likely are aberrantly activated in all tissues due to a lower evolutionary pressure respect to that acting on protein coding genes.

However, some of these Kdm5c-regulated ncRNAs may play regulatory roles and cause more or less severe phenotypes in specific cell types during different developmental stages. For example, one of the genes indirectly downregulated in KO screens was *Zfhx2,* a gene encoding a TF expressed mainly in differentiating neurons in the mouse brain (**Chapter I, Figure 7I**). This gene has an antisense transcript *Zfhx2os* that was upregulated in both KO strains (Komine, Nakamura et al. 2006). Interestingly, it has been found that Zfhx2-deficient mice display behavioural abnormalities, namely, hyperactivity, enhanced depression-like behaviors, and an aberrantly altered anxiety-like phenotype (Komine, Takao et al. 2012). Thus, the misregulation observed in both KOs and ifKOs could have more severe consequences for cognition and adaptive behavior during development than in the adult brain. Furthermore, the consequences of the spurious activation of intergenic sites may be more severe in humans than mice since a recent study linked the expression of thousands of unannotated, non-exonic differentially expressed regions (DERs) in the human prefrontal cortex to a higher risk of developing brain disorders and variations in neuronal phenotype (Jaffe, Shin et al. 2015).

The abnormal germ line genes transcription discussed above (section 5.2.1.) can also be considered an example of spurious transcription. Interestingly, preventing transcription from cryptic promoters is also important in Drosophila testis tissue in which a similar spurious activation of cryptic promoters occurs in absence of a cell type specific multiple Zn Finger protein (Kim, Lu et al. 2017).

Misregulation of many different histone modifications has been linked to aberrant transcription (Smolle and Workman 2013). It is conceivable that spurious transcription is genome-wide connected to abnormal DNA methylation by DNMTs. In fact, gene body methylation reduces transcriptional noise associated with spurious transcription of genes. Heavy methylation of vertebrate genomes may have evolved as a global regulatory mechanism to control for transcriptional noise (Huh, Zeng et al. 2013). In contrast, promoter H3K4 methylation exhibits positive correlations with the level of transcriptional bursts. Cells have evolved mechanisms to preserve chromatin integrity during transcription, thereby preventing the emergence of cryptic transcripts from spurious promoter sequences. Bidirectional transcription from TSSs has been demonstrated in different studies but recent evidences showed that widespread divergent transcription at protein-encoding gene promoters over short distances is common for active promoters and may help promoter regions maintain a state poised for subsequent regulation (Seila, Calabrese et al. 2008). Moreover, polyadenylated cryptic transcripts running both sense and antisense relative to genes have been found for instance in a mutant of *S. cerevisiae* (Uwimana, Collin et al. 2017), underscoring the evolutionary importance of repressing and controlling this kind of transcription.

The life-long role of Kdm5c in genome surveillance, described in this work, might not only explain its retained expression in adult neurons and the deficits observed in ifKOs, but also pinpoints new possible mechanisms of pathoetiology. GO analyses of the common DEGs revealed a significant enrichment for muscle and extracellular matrix associated terms. Intriguingly, Kdm5c seem to have a life-long role in mature neurons repressing these genes. This geneset has been related with a tumorigenic phenotype in a recent article by Lan's group in which the lack of KDM5C induced xenografts to become breast cancer cells (Shen, Xu et al. 2016). In a similar way, it is

also possible that more prominent phenotypes could emerge in *Kdm5c*-ifKOs upon exposure to specific situations or challenges.

Although CJ-XLID has not been associated with a higher incidence of tumors, as commented in the introduction, the inactivation of KDM5C is observed in ccRCC, and the patients presenting KDM5C-inactivating mutations have a poorer prognosis than those without (Rondinelli, Rosano et al. 2015). Importantly, these cancer cells showed frequent chromosomal rearrangements and aberrant overexpression of lincRNAs, similarly to our adult Kdm5c-KO neurons. Indeed, KDM5C has been found to be mutated or overexpressed in tumours which often exhibit intragenic DNA hypomethylation, suggesting that this epigenetic mechanism may promote the expression of abnormal transcripts in cancer (Roy, Walsh et al. 2014). Interestingly, intragenic spurious transcription has been also recently linked to impaired Dnmt3b-dependent DNA methylation and genome instability in cancer cells (Neri, Rapelli et al. 2017), which suggests that illegitimate transcription is an essential feature of different pathologies with epigenetic etiology. Moreover, recent studies have underscored the relevance of genome instability in brain pathology, particularly in the context of aging and age-related neurodegenerative disorders (McKinnon 2013, Madabhushi, Pan et al. 2014) but also in ID. Indeed, *Ehmt1/G9a* deficiency in the forebrain led to ID phenotype and derepression of non-neuronal genes, suggesting a role of the Ehmt1/G9a complex in protecting neurons from transcriptional noise (Schaefer, Sampath et al. 2009). Furthermore it is known that EHMT1/EHMT2 methylation activity specifically recruits core members of PRC, namely enhancer of zeste homologue 2 (EZH2) that catalyzes the deposition of H3K27me3 negative mark to silence important genomic targets, encoding developmental and neuronal regulators (Mozzetta, Pontis et al. 2014). Another ID-related chromatin modifying enzyme (WHSC1/NSD2) was described to

associate with TFs and to prevent the inappropriate transcription that can lead to various pathophysiologies (Nimura et al, 2009). The transcriptional noise as well as the slight expression changes are seen also in other mouse models for XLID such as Rett syndrome (Lyst and Bird 2015, Ludwig, Zhang et al. 2017).

Important questions related to this finding should be answer in future studies. For example, is there a common TF or TFs that recruit Kdm5c to these loci to regulate H3K4 methylation? Are spurious transcription and Kdm5c recruitment cell-type specific?

5.3. Therapy prospects and concluding remarks

The experiments presented in this thesis provide novel insight into the etiology of CJ-XLID and unveiled new aspects of the role and specific genomic actions of Kdm5c in neurodevelopment. This progress opens up new avenues for therapy and pinpoints specific genes as potential biomarkers for this disorder. Moreover, the studies in ifKOs and ifKO PNCs permitted the dissection of the neurological defects that have a neurodevelopmental origin delimiting the window for reversal therapies, although more investigation is needed for answering several open and outstanding questions.

Furthermore, Kdm5c targets partially overlap with other chromatin regulators' targets and identification of dysregulated genes and epigenetic marks in these chromatin disorders is the subject of ongoing research. Understanding the role and the broad impact of H3K4 hypermethylation on gene enhancers and promoters in pathological contexts and its relationship with other histone modifications could also help to better comprehend and possibly cure other IDDs. Therefore, the impact of our findings goes beyond CJ-XLID and can provide insight into other chromatin-related IDDs that affect

common gene networks. This seems to be the case, for example, of activity-driven transcription which as afore discussed is altered in different IDD mouse models.

Importantly, the use of not only animal models but also iPSCs and organoids models will increase the number of observations as well as the possibilities of application of new therapies. Unfortunately, at the moment there is no approved treatment for H3K4 linked-IDDs. It would be of great interest to develop pharmacological therapies based on the use of H3K4 KMT inhibitors or KDM facilitators. Other epigenetic drugs, such as HAT inhibitors or small molecules that normalize activity of gene products overexpressed due to loss of H3K4 demethylation, could be also assessed (Millan 2013, Wang and Patel 2013, Cacabelos 2014, Ricq, Hooker et al. 2016). Complementing classical pharmacological approaches, novel tools for therapy, such as genome and epigenome editing in combination with replacement therapies will have a prominent role in trying to ameliorate the cognitive deficits and to overcome the side effects characteristic of the classical drugs used for curing the symptoms (**Figure 11**).

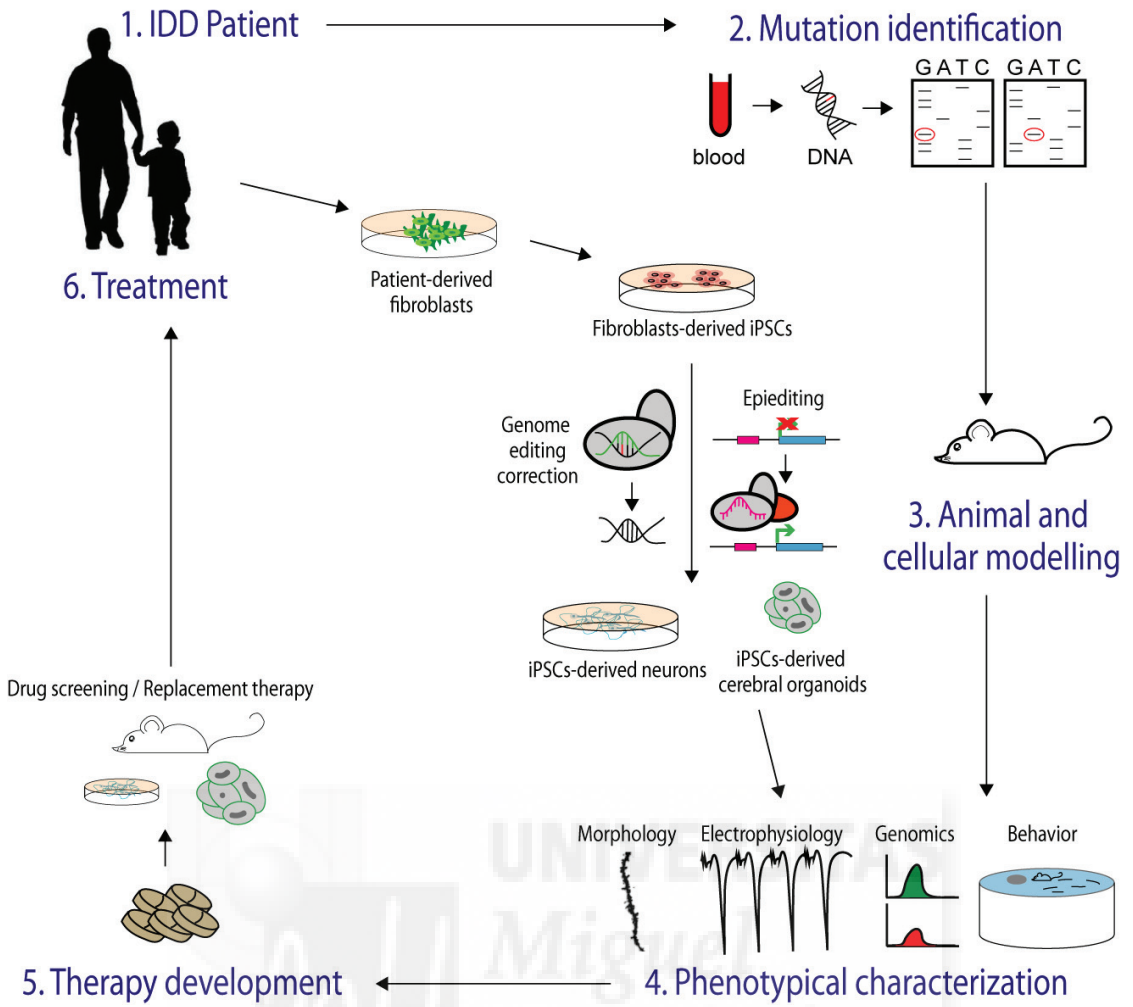

Figure 11. Scheme depicting the flow from the disease to the treatment. After detecting the disease-causing mutation, animal models are developed. On the other hand, cells derived from the IDD-patient are reprogrammed to iPSCs to develop neuronal cultures and cerebral organoids patient-specific. Before reprogramming, genomic and epigenomic editing techniques can be used to correct the mutation or the transcriptional alterations. Animal and cellular models are used to characterize and study the disease with the aim of discovering new therapies/drugs to treat the affected individuals.

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CONCLUSIONS

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1. Kdm5c-KOs recapitulate neurological, cognitive and emotional alterations observed in children diagnosed with CJ-XLID, including microcephaly, epilepsy propensity, motor impairments, impulsivity, altered emotionality, hyperreflexia and cognitive impairments.
2. Heterozygous female mice display milder cognitive defects than males resembling those reported in carrier human females.
3. Forebrain-restricted inducible KO (ifKO) males show mild impairment in spatial navigation learning.
4. The comparison of ifKO and KO mice allowed the dissection of the developmental and adult component of the syndrome demonstrating that Kdm5c has a more relevant role during development of the CNS than in the adult brain.
5. Transcriptional changes exclusive of KO mice comprise aberrant expression of a subset of germline genes pinpointing Kdm5c as a critical factor required for DNA methylation-induced silencing of those genes before neuronal differentiation.
6. The loss of Kdm5c in germinal cells or immature neurons is associated with upregulation of IEGs at the basal state and enhanced H3K4me3 levels at IEG-associated enhancers, which indicates that Kdm5c participate in the fine-tuning of the activity of these enhancers during neuronal maturation.
7. Complementary transcriptomic and epigenomic screens revealed the de-repression of non-neuronal promoters in both KOs and ifKOs demonstrating a life-long role for Kdm5c preventing illegitimate transcription at those sites.
8. Taken together, the dissection of the different genomic actions of Kdm5c along development provides novel insight into the specific molecular function of this chromatin enzyme, pinpoints possible mechanisms of pathoetiology, and identifies possible biomarkers and therapeutic targets of the syndrome.

9. The production of the first cerebral organoids derived from CJ-XLID patients represents a promising new tool for studying ID-related processes during human brain development.
10. Overall, the findings derived from the present thesis lay an innovative conceptual framework for future investigations on epigenetic regulators and ID, and provide novel tools and resources to deepen in the pathoetiology of CJ-XLID and to design and evaluate therapeutic interventions.

Conclusiones (Español)

1. Los ratones KO para Kdm5c recapitulan las graves alteraciones neurológicas, cognitivas y emocionales observadas en los niños diagnosticados con CJ-XLID, incluyendo la microcefalia, propensión a la epilepsia, alteraciones motoras, impulsividad, alteraciones emocionales, hipereflexia y discapacidad intelectual.
2. Las hembras heterocigotas muestran defectos cognitivos más leves que los machos, recapitulando por tanto los modestos defectos cognitivos reportados en mujeres portadoras de mutaciones asociadas a CJ-XLID.
3. Los ratones ifKO machos muestran un deterioro significativo del aprendizaje de la navegación espacial.
4. La comparación de ratones KO e ifKO ha permitido la disección de las componentes de desarrollo y adulta del síndrome y demuestra que Kdm5c tiene un papel más relevante durante el desarrollo del sistema nervioso central que en el cerebro adulto.
5. Los cambios transcripcionales exclusivos de los ratones KO incluyen la expresión aberrante de un subconjunto de genes de línea germinal, lo cual identifica a Kdm5c como un factor crítico en la inducción del silenciamiento de esos genes mediante la metilación del ADN de forma previa a la diferenciación neuronal.
6. La pérdida de Kdm5c en línea germinal y en neuronas inmaduras se asocia con una modesta regulación positiva de genes de respuesta inmediata temprana y con un aumento de los niveles de H3K4me3 en regiones reguladoras proximales (enhancers), lo que sugiere que Kdm5c contribuye a definir los niveles basales de actividad de esas regiones reguladoras durante la maduración neuronal.
7. Los análisis de transcriptómica y epigenómica han revelado la des-represión de promotores no neuronales tanto en KOs como en ifKOs, lo cual demuestra una

función permanente de Kdm5c previniendo la transcripción ilegítima en esos sitios.

8. En conjunto, la disección de las diferentes acciones genómicas de Kdm5c durante el desarrollo proporciona una visión novedosa de las funciones moleculares de esta enzima, apunta posibles mecanismos de patoetiología e identifica genes como posibles biomarcadores o dianas terapéuticas del síndrome.
9. La producción de los primeros organoides cerebrales derivados de pacientes con CJ-XLID proporciona una nueva y prometedora herramienta para investigar los procesos relacionados con la discapacidad intelectual (DI) durante el desarrollo del cerebro humano.
10. En general, los hallazgos derivados del presente estudio establecen un innovador marco conceptual para investigaciones futuras sobre el papel de reguladores epigenéticos en DI, proporciona nuevas herramientas y recursos para profundizar en la patoetiología del CJ-XLID, y para el diseño y evaluación de posibles intervenciones terapéuticas.

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ANNEX I

UNIVERSITAS
Miguel
Hernández

I obtained a second first-author-publication during the Ph.D. period. Because of the differences between the subject investigated in that article and the main focus on my thesis project, KDM5C and histone methylation, I decided to present this work as an annex to the thesis:

Fine-tuned SRF activity controls asymmetrical neuronal outgrowth: implications for cortical migration, neural tissue lamination and circuit assembly. **Marilyn Scandaglia**, Eva Benito, Cruz Morenilla-Palao, Anna Fiorenza, Beatriz del Blanco, Yaiza Coca, Eloísa Herrera, and Angel Barco. Sci Rep. 2015 Dec 7;5:17470. doi: 10.1038/srep17470.

In this article, we investigated the role of the transcription factor Serum Response Factor (SRF) in neuronal morphology, cortical migration and axonal connections through loss and gain of function approaches. I performed and analyzed most of the experiments in primary neuronal cultures and all the experiments in utero-electroporated mice, and wrote the paper in collaboration with Dr. Eloisa Herrera and Dr. Ángel Barco who supervised the work. Several researchers also contributed to this study. In particular, Eva Benito prepared the original lentivirus construct expressing the constitutive active form of SRF; Cruz Morenilla-Palao and Yaiza Coca performed and analysed the experiments in the retina system; Anna Fiorenza conducted neuronal death assays; and Beatriz del Blanco assisted me in the ChIP-assay experiments.

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Fine-tuned SRF activity controls asymmetrical neuronal outgrowth: implications for cortical migration, neural tissue lamination and circuit assembly

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The stimulus-regulated transcription factor Serum Response Factor (SRF) plays an important role in diverse neurodevelopmental processes related to structural plasticity and motile functions, although its precise mechanism of action has not yet been established. To further define the role of SRF in neural development and distinguish between cell-autonomous and non cell-autonomous effects, we bidirectionally manipulated SRF activity through gene transduction assays that allow the visualization of individual neurons and their comparison with neighboring control cells. *In vitro* assays showed that SRF promotes survival and filopodia formation and is required for normal asymmetric neurite outgrowth, indicating that its activation favors dendrite enlargement versus branching. In turn, *in vivo* experiments demonstrated that SRF-dependent regulation of neuronal morphology has important consequences in the developing cortex and retina, affecting neuronal migration, dendritic and axonal arborization and cell positioning in these laminated tissues. Overall, our results show that the controlled and timely activation of SRF is essential for the coordinated growth of neuronal processes, suggesting that this event regulates the switch between neuronal growth and branching during developmental processes.

The assembly of neuronal networks is accomplished through a precise sequence of developmental processes that are highly dependent on cellular motile functions, including cell migration, axon versus dendrite specification, neurite outgrowth, axon guidance and synaptic targeting. It is thought that the stimulus-regulated transcription factor (TF) Serum Response Factor (SRF) plays a role in these processes by regulating actin dynamics[1,2]. SRF binds to a specific DNA sequence referred to as "SRF Response Element" or SRE (also termed "CArG box"), but its transactivation activity depends on the interaction with two families of coactivators, the MRTFs (myocardin-related transcription factors) and the TCFs (ternary complex factors), which are engaged in the Rho/actin and Ras/MAPKs pathways, respectively[3–5]. This position, at the convergence of different activity-regulated signal transduction cascades and upstream of gene programs controlling cytoskeletal changes, pinpoints SRF as an important regulator of complex adaptive responses involving changes of neuronal morphology[6].

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From the early gastrulation defects observed in conventional knockout (KO) mice to the most specific phenotypes found in tissue-restricted conditional mutants, the deficits associated with SRF's loss-of-function (LOF) have been consistently related to cytoskeletal perturbations[1,2,7,8]. In the nervous system, conditional, late-prenatal deletion of *Srf* impairs tangential cell migration along the rostral migratory stream and causes the ectopic accumulation of progenitor cells in the subventricular zone (SVZ)[9]. Furthermore, *Srf* loss in forebrain neurons abolishes mossy fiber segregation and causes ectopic fiber growth in the hippocampus[10], as well as hippocampal and cortical lamination defects that affect both the positioning of cell bodies and layer-restricted termination of commissural and mossy fiber axons[11]. More recently, experiments in conditional KO (cKO) mice lacking SRF in neural progenitor cells also revealed deficits in cortical axonal projections, including corticostriatal, corticospinal, and corticothalamic tracts, that were often associated with the loss of the corpus callosum[12]. Of note, the synaptic plasticity defects observed after SRF ablation in the adult brain[13–16] might also originate from defective regulation of cytoskeleton dynamics[6]. Although the aforementioned LOF studies have demonstrated that SRF activity is essential for normal brain development, the precise mechanism by which this TF regulates neuronal growth and circuit formation, as well as the consequences of increasing SRF activity during development *in vivo*, are still unknown.

Here, we investigated the cell-autonomous role of SRF in neuronal growth and neurodevelopment by combining *in vitro* and *in vivo* experiments in which SRF activity was bidirectionally manipulated. Our experiments in neuronal cultures reveal that SRF controls the asymmetric outgrowth of neurites favoring primary dendrite enlargement versus branching. In turn, this cellular function influences different neurodevelopmental processes, including neuronal migration, tissue lamination and circuit assembly, which highlights the critical importance of fine-tuned SRF activity in the development of the nervous system.

Results

SRF regulates dendritic outgrowth, synaptogenesis and neuroprotection in neuronal cultures. To precisely investigate the impact of chronic enhancement or interference of SRF function in neuronal growth, we produced lentivirus-based bicistronic constructs that co-express GFP with different SRF variants under the control of the neuronal-specific synapsin promoter (Fig. 1A). For gain-of-function (GOF) studies, we expressed a constitutively active SRF variant (caSRF) that results from fusing the DNA binding domain (DBD) of SRF with the potent acidic transactivation domain of the viral protein VP16 (Fig. 1B)[17]. For LOF experiments, we expressed a truncated SRF variant that encompasses the DBD of SRF, but lacks any transactivation domain and thereby is expected to act as a competitive inhibitor for SRF binding to its targets (from now referred to as ciSRF)[18]. As control, we used a construct encoding for GFP alone or co-expressing GFP and the VP16 domain (without DBD). Luciferase reporter assays demonstrated the functionality of the SRF-based constructs (Fig. 1C).

We next combined lentiviral infection of primary hippocampal cultures from E17.5 mouse embryos with the transfection of a plasmid encoding dsRed (Fig. 1D). The lower efficiency of transfection compared to infection, together with the ability of dsRed to fill the whole neuron with red fluorescence, allowed a clear visualization of the impact of the chronic manipulation (7 days) of SRF activity on the morphology of individual neurons (Fig. 1E). These experiments led to several conclusions. First, most of the caSRF-infected neurons exhibited bipolar morphology with two or more main/apical dendrites that contrasted with the typical pyramidal shape observed in neurons infected with the control construct (one apical dendrite and several basal dendrites) (Fig. 1E). Furthermore, Sholl analysis evidenced that caSRF neurons present longer but less branched dendrites (Fig. 1F). These observations were confirmed by the quantification of the primary dendrites terminal distance (higher in caSRF neurons, Fig. 1G) and the number of endings (lower in caSRF neurons, Fig. 1H). CaSRF-expressing neurons also had smaller somas (Fig. 1I), which is consistent with the recent observation of enlarged somas in neurons from SRF-deficient mice[19]. Second, the Sholl analysis of ciSRF-transfected cells revealed an overall shape similar to control neurons (GFP) but with slightly reduced dendrite growth and consequently less developed dendritic trees (Fig. 1E–G). This result suggests only a partial interference with endogenous SRF function indicating that SRF/ciSRF heterodimers are likely to retain transactivation activity. Third, caSRF-expressing neurons exhibited a significant increase in spine density (Fig. 1E,J). These spines were thin and filopodia-like indicating that they are likely immature. We did not observe significant changes in spine density in the case of ciSRF-infected neurons. Forth, despite of the abnormal morphology, neither caSRF nor ciSRF expression were *per se* toxic. Moreover, as previously reported[20–22], we found that caSRF expression was neuroprotective against excitotoxic insult and serum deprivation (Supplementary Fig. S1).

SRF regulates dendritic and axonal arborization, as well as neuronal positioning in the developing cortex. SRF is expressed in cortical neurons both during embryonic development and postnatally (Fig. 2A). To investigate its function in neuronal growth and cortical development *in vivo*, we transduced the lentiviral constructs described above in the somatosensory cortex of E14.5 embryos by *in utero* electroporation and examined the brain of electroporated mice at P20 (Fig. 2B). These constructs drove transgene expression *in vivo* as efficiently as in culture. As a result, GFP fluorescence completely filled the cytoplasm of transduced postmitotic neurons, allowing us to analyze dendritic trees in the electroporation site, the crossing fibers at the corpus callosum and axonal arborization at the contralateral side.

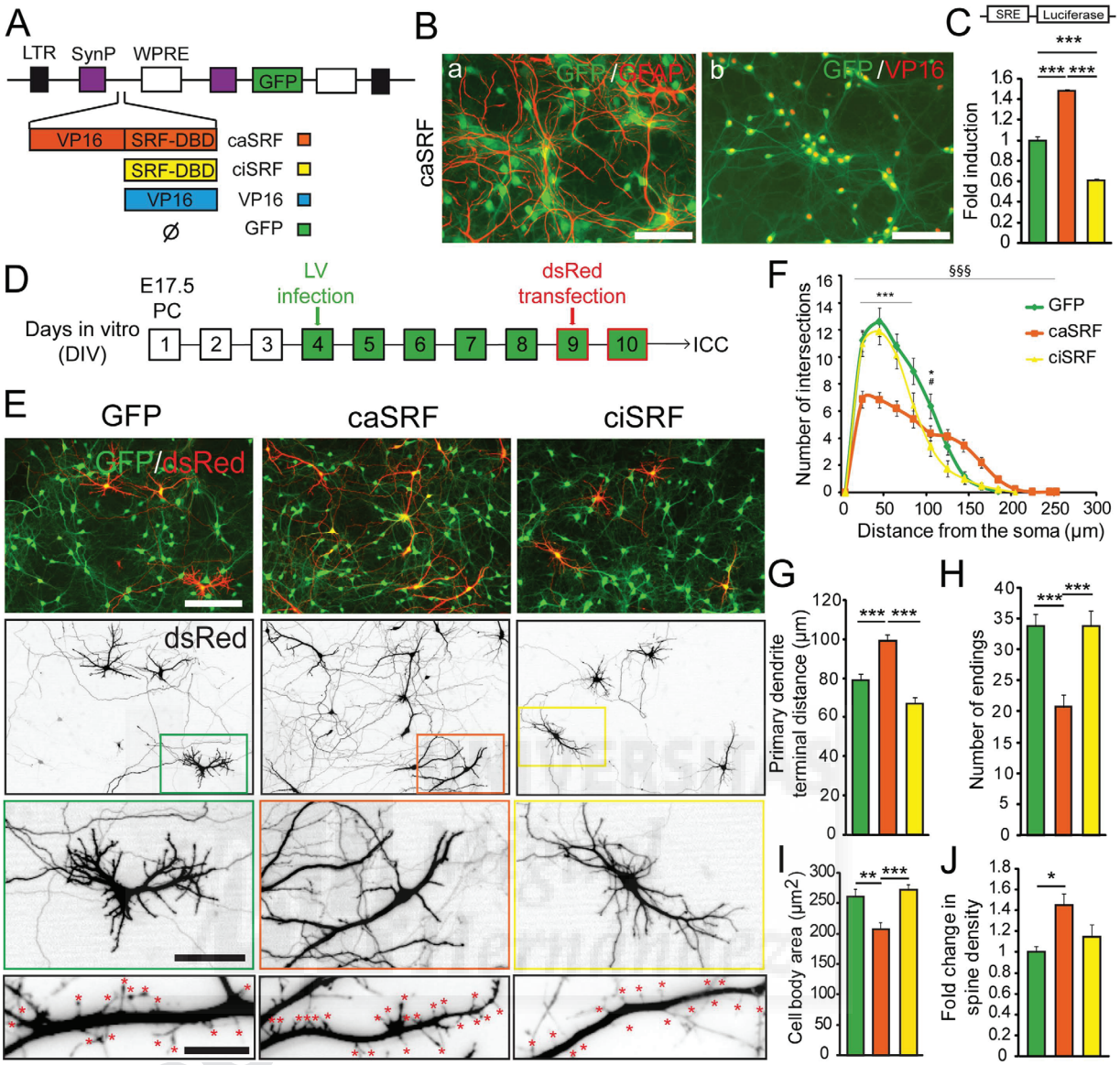

Figure 1. SRF regulates dendritic outgrowth and polarity in neuronal cultures. (**A**) Scheme of lentiviral (LV) constructs and their color code used in this study. LTR, Long-termination repeats; SynP, synapsin promoter; WPRE, woodchunk hepatitis virus post-transcriptional regulatory element. (**B**) Hippocampal neurons infected with caSRF were stained for GFP, GFAP and VP16. (a) GFP does not co-localize with the astrocytic specific marker GFAP, (b) Co-expression of VP16 and GFP and presence of VP16-SRF in the nucleus. Scale bar: 100 µm. (**C**) Luciferase reporter assay in HEK293 cells using an SRE-driven luciferase reporter. ***$p < 0.0005$, 1-way ANOVA followed by Bonferroni's multiple comparison test ($n = 4$). (**D**) Scheme of the *in vitro* experiment. PC, primary culture; ICC, immunocytochemistry. (**E**) Hippocampal cultures infected with the different viruses and transfected with dsRed show GFP expression in all neurons (green) and dsRed expression (red) in a small percentage of cells (scale bar: 100 µm). The two middle rows present representative images of neurons that are both infected and transfected (scale bars: 20 µm). The bottom row shows representative images of dendritic branches in neurons that are both infected and transfected (scale bar: 5 µm). Neurons expressing caSRF show more filopodia-like processes (red asterisks). (**F**) Sholl analysis presenting number of intersections versus distance to soma. Data are expressed as means ± SEM and compared using 2-way ANOVA followed by Bonferroni's multiple comparison test. Error bars are shown every 20 µm. The (*) and (#) symbols indicate statistical difference between caSRF and GFP or ciSRF and GFP, respectively; (§) symbols indicate significant interaction between the transduced protein and distance for caSRF or ciSRF and GFP. $^{*,\#}p < 0.05$; ***$p < 0.0005$; $^{§§§}p < 0.0005$. (**G**) Quantification of primary dendrite terminal distance. (**H**) Quantification of number of endings. (**I**) Quantification of the cell body area. For **G–I**, **$p < 0.005$, ***$p < 0.0005$, 1-way ANOVA followed by Bonferroni's multiple comparison test. For **F–I**, $n = 19–26$ in all groups. (**J**) Quantification of synaptic density. *$p < 0.05$, 1-way ANOVA followed by Bonferroni's multiple comparison test ($n = 9$).

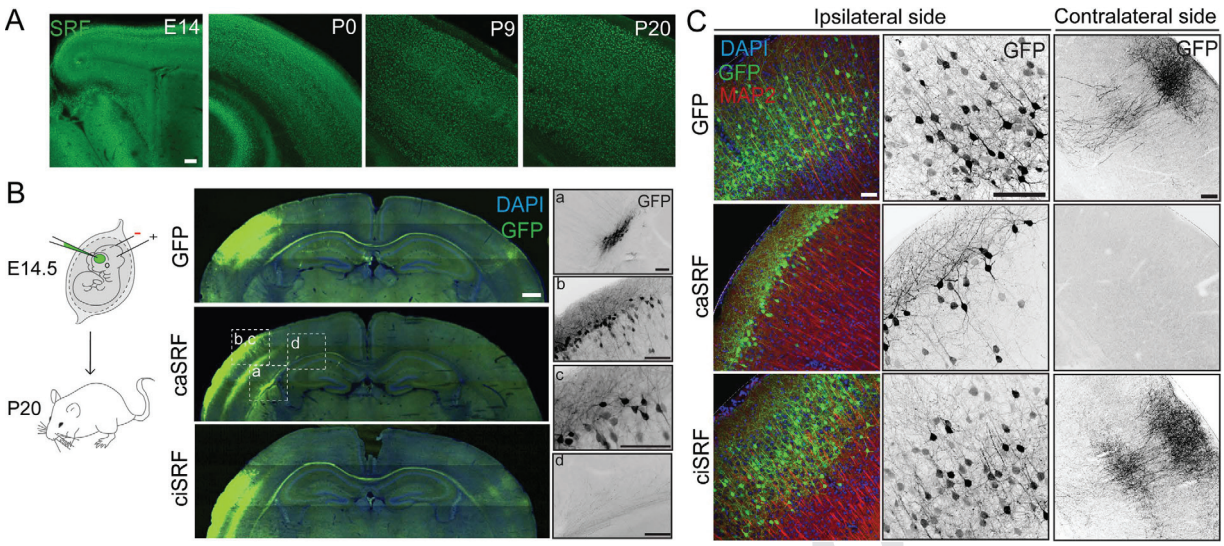

Figure 2. Enhanced SRF activity causes aberrant cell positioning and arborization in the developing cortex. (**A**) Immunostaining against SRF indicates that SRF is expressed during cortex development. Scale bar: 100 μm. (**B**) Left panel: Scheme of the *in utero* electroporation experiment. Center panels: composite images of coronal brain sections of P20 mice electroporated at E14.5 with GFP alone (GFP) or in combination with caSRF or ciSRF. Brain slices were stained with anti-GFP antibody and counterstained with DAPI. Scale bar: 500 μm. Right panels: Four phenotypes associated with caSRF expression: (a) impaired migration of some neurons to the cortical layers. (b) aberrant layer positioning, (c) misoriented apical dendrite, (d) axon mistargeting along corpus callosum. Scale bar: 100 μm. (**C**) Higher magnification images of electroporated neurons at the ipsilateral side after triple staining with DAPI (DNA counterstaining), anti-GFP and anti-MAP2 (neuronal dendritic marker). Scale bar: 100 μm.

Enhanced SRF activity caused four prominent phenotypes (Fig. 2B,C). First, some neurons failed to migrate to the cortical layers and were retained at the SVZ (Fig. 2B-a); second, those cells that migrated were mispositioned along the laminar structure of the cortex and, compared to control GFP-expressing neurons, were much more superficially located (Fig. 2B-b); third, the dendrites of caSRF-expressing cortical neurons were poorly arborized and frequently grew horizontally to the cortical plate instead of projecting an apical dendrite towards the surface as observed in the control condition (Fig. 2B-c); and fourth, whereas the cortico-cortical projections of GFP neurons developed an exuberant arborization in the contralateral side, the axons of caSRF-expressing neurons did not. In fact, some axons were not able to reach their target area in the other hemisphere defasciculating after crossing the midline of the corpus callosum (Fig. 2B-d), which indicates that callosal axon extension and pathfinding are affected in a cell-autonomous manner. In contrast, the expression of ciSRF had a subtler effect in dendrite and axonal arborization than caSRF, although both dendritic ramifications in the ipsilateral side and callosal projections in the contralateral side were thinner and less exuberant than in controls (Fig. 2B,C).

The first two phenotypes are consistent with impaired radial migration. During cortical development, subsequent waves of newborn neurons migrate along radial glial fibers to form the cortical plate. Each wave of migrating cells travels past its predecessor forming layers in an inside-out manner so that the youngest neurons are the closest to the surface[23]. Therefore, the presence of transduced cells in the SVZ and in the proximity of the cortical surface both indicate that caSRF-expressing neurons have a radial migration defect; either they do not migrate and accumulate at the SVZ or they migrate slower and consequently locate at the most superficial layer. The quantification of the relative position of electroporated neurons along the cortical layers is consistent with this interpretation. Whereas GFP-expressing neurons in control cortices collected at P20 were located across layers 2/3 and 4 (Fig. 3A), migrating caSRF-expressing neurons were found almost exclusively in the upper part of layer 2 (Fig 3A). Interestingly, ciSRF-expressing neurons also located at more superficial positions than control neurons suggesting a slighter impairment in radial migration (Fig. 3A). Further demonstrating the fine sensitivity of radial migration to changes in SRF activity, the co-expression of both SRF variants ameliorated the developmental effects induced by caSRF (Fig. 3B).

To gain additional insight into the effect of chronic cell-autonomous SRF activation during radial migration, we conducted a new series of *in utero* electroporation experiments at E14.5 in which we sacrificed the mice at earlier time points (Fig. 3C). At E17.5, numerous control GFP-expressing cells have already moved to the intermediate zone (IZ), whereas most caSRF-expressing neurons remained in the proximity of the SVZ. At P0, most GFP-expressing neurons in control cortices have reached the cortical plate (CP), while in caSRF-electroporated cortices a lower percentage of labeled neurons has reached

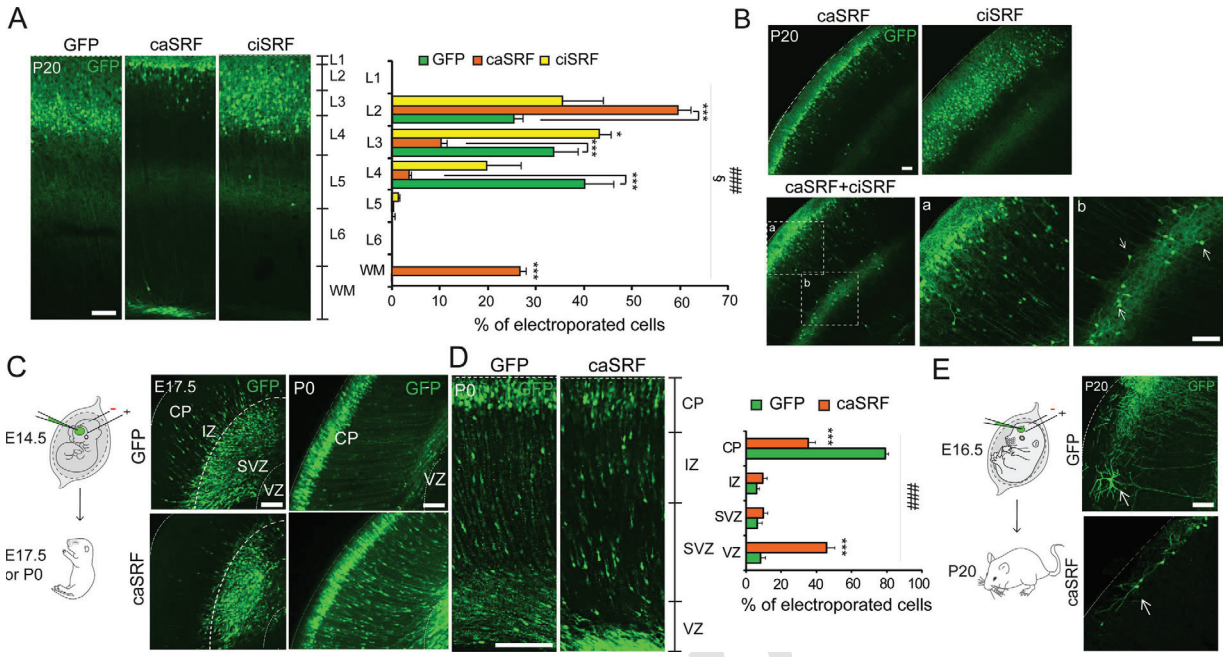

Figure 3. Enhanced SRF activity slows down migration and impairs cortical lamination. (A) Quantification of the relative position of the somas of electroporated neurons at P20 in GFP, caSRF and ciSRF conditions revealed the impaired positioning in the cortical layers. The left panels show representative images of electroporated cortices. L1-L6: layer 1- layer 6, WM: white matter (**B**) Partial rescue of caSRF-induced changes in cortical development by co-electroporation with ciSRF. (a) Detail showing the modest impact of ciSRF co-expression in the lamination defect caused by caSRF. (b) The white arrows label multipolar cells-like neurons that were probably halt in the migration process. (**C**) Scheme of the experiment and representative images of coronal brain sections of E17.5 and P0 cortices electroporated at E14.5 with caSRF or GFP. CP: cortical plate, VZ: ventricular zone, IZ: intermediate zone, SVZ: subventricular zone. The right bar graph shows the quantification of the relative position of electroporated neurons at P0, showing the delayed migration and accumulation in the VZ of caSRF-expressing neurons. (**D**) Scheme of the experiment and representative images of coronal brain sections (P20) showing the morphology of electroporated neurons located in layer 2 (white arrows). Scale bars: 100 μm. Note that data in bar graphs are expressed as means of the percentage of GFP^+ cells ± SEM and were compared using 2-way ANOVA for repeated measure, followed by the Bonferroni's multiple comparison test. The (*) symbols indicate statistical difference between caSRF or ciSRF and GFP control conditions; (#) and (§) symbols indicate statistical interaction between the electroporated construct and layer distribution for caSRF and ciSRF, respectively. $^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$; $^{§}p < 0.05$; $^{###}p < 0.0005$ (n = 3).

this position, with more than 40% of electroporated cells still located at the VZ. These results conclusively demonstrate that radial migration in caSRF-expressing neurons is slower than in control neurons. This migration delay could be explained by the alteration of cell morphology associated with caSRF expression. During radial migration, migrating neurons acquire a characteristic asymmetrical bipolar morphology in which the longer leading process is directed towards the pia and the shorter trailing process is extended below, and failures in acquiring this morphology have been consistently associated with migration defects[24,25].

Intriguingly, these electroporation experiments could also suggest that caSRF expression causes the mispositioning of the soma by mechanisms that are independent of the radial migration defect. A significant percentage of caSRF-expressing neurons have reached the cortical plate at P0 (Fig. 3C, ~40%) occupying position equivalent to those found for electroporated neurons in control cortices. However, caSRF-expressing neurons were still located at the cortical surface 20 days later while, at that time, most control neurons have relocated to inner layers (Fig. 3A), indicating that, after migration, another developmental process might be affected by chronic SRF activation. Numerous studies indicate that the characteristic shape and extent of the dendritic arbor in the neurons of each layer result from the interaction between intrinsic developmental programs and local environmental cues, but relatively little is still known about how dendritic outgrowth influences the final position of the neuronal soma in the cortical plate[24,26,27]. Like for radial migration, the cortical lamination defect can be a consequence of the abnormal dendritic growth because the underdevelopment of the apical dendrite might prevent the correct positioning of the soma after migration. Consistent with this view, when the electroporation was

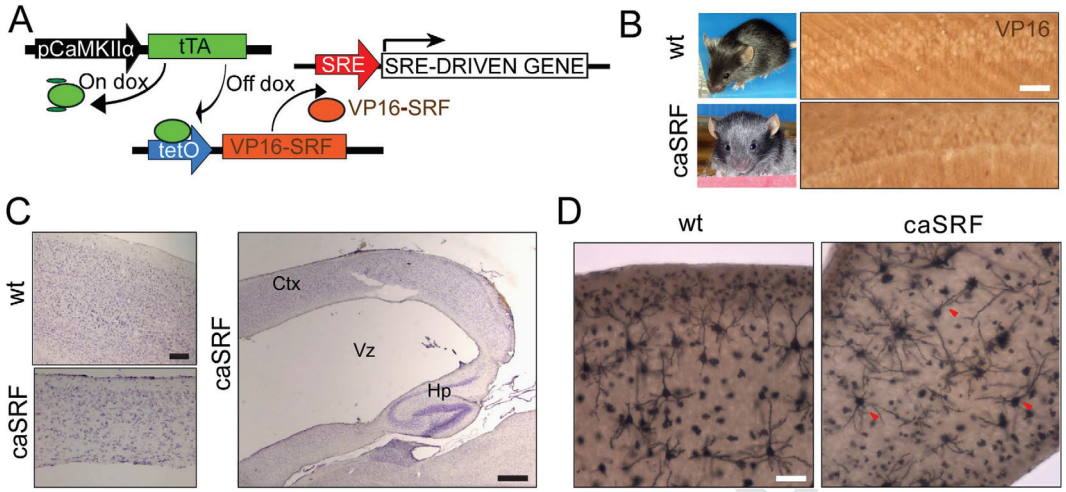

Figure 4. caSRF transgenic mice show severe neurological defects. (**A**) Scheme of the double transgenic approach. (**B**) Images of control and bitransgenic mice (left) and immunostaining of the CA1 subfield using α-VP16 antibody (right). Scale bar: 20 μm (**C**) Nissl staining of wt and caSRF animals. Left panels: Gross abnormalities of cortical somas in caSRF mice. Scale bar: 100 μm. Right panels: Hydrocephalia in caSRF animals. Ctx: cortex, Vz: ventricle, Hp: hippocampus. Scale bar: 500 μm. (**D**) Golgi staining showing the abnormal orientation of apical dendrites in principal cortical neurons (red arrowheads) of caSRF bitransgenics. Scale bar: 100 μm.

conducted at E16.5, a time in which control neurons find its final location in layer 2, the soma of many caSRF-expressing neurons located even more superficially than when electroporation occurred at E14.5. Furthermore, in these animals we often observed dendrites that projected parallel to the pial surface (Fig. 3D). Together, these results suggest that defects in dendritic growth/arborization can contribute to the strong lamination defect observed in caSRF-electroporated cortices, although alternative interpretations are also possible. For example, caSRF-expressing neurons might not respond to migration stop signals.

Notably, the abnormal orientation of the apical dendrite in neurons expressing caSRF was also observed in a strain of bitransgenic mice generated using the inducible CamKIIα-tTA system (Fig. 4A). In these mice, the expression of caSRF was observed at early-postnatal stages in specific layers of the cerebral cortex and the hippocampus (Fig. 4B) and was associated with severe neurological phenotypes including hydrocephalia (Fig. 4C), reduced body weight and premature death when the animals were 3–4 weeks old (100% of the bitransgenic mice died before reaching 1 month). Golgi staining in brain slices revealed a normal pyramidal morphology in neurons from control mice while those in transgenic mice often had a less polarized morphology (Fig. 4D).

SRF also regulates lamination, and dendritic and axonal arborization in the visual system. To determine whether SRF has a similar role in other neural circuits, we turned to the visual system because this is one of the best-established models to investigate dendritic arborization, and axon growth, guidance and targeting[28]. We first analyzed the endogenous expression of SRF in the retina by immunohistochemistry. SRF is first expressed in retinal ganglion cells (RGCs) around perinatal stages and its level progressively increases after birth at the time when RGC dendrites grow and axons reach their main target tissues to arborize (Fig. 5A). To specifically transduce RGCs[29], we next electroporated the retina of E13.5 embryos with the different constructs and sacrificed the pups at P9 (Fig. 5B) when the visual system is nearly mature[28]. GFP fluorescence completely filled the electroporated neurons revealing the dendritic trees in the retina and axonal arborization in the superior colliculus (SC), which is one of the main targets of retinal projections.

In the control GFP-electroporated retinas, neurons located in the RGC layer (RGCL) had a prominent basal dendrite that separates the soma from the inner plexiform layer (IPL) (Fig 5C, panels a, d–g). However, caSRF-expressing neurons frequently showed more than a principal dendrite emerging parallel to the IPL and concomitant mispositioning of the soma. Furthermore, the dendrites of caSRF-expressing neurons occupied larger areas but exhibited fewer ramifications than the controls (Fig. 5C, panels b, h–j). Although the axons of both GFP- and caSRF-expressing RGCs reached the corresponding topographical area in the SC, the first ones developed exuberant arborization in the target area, whereas the second ones exhibited a grainier appearance, did not arborize properly and never reached the surface of the SC (Fig. 5D, panels a, b). In conclusion, like in the developing cortex, caSRF expression caused lamination defects and the mislocation of somas in the retina. Also consistent with our observations in the cortex,

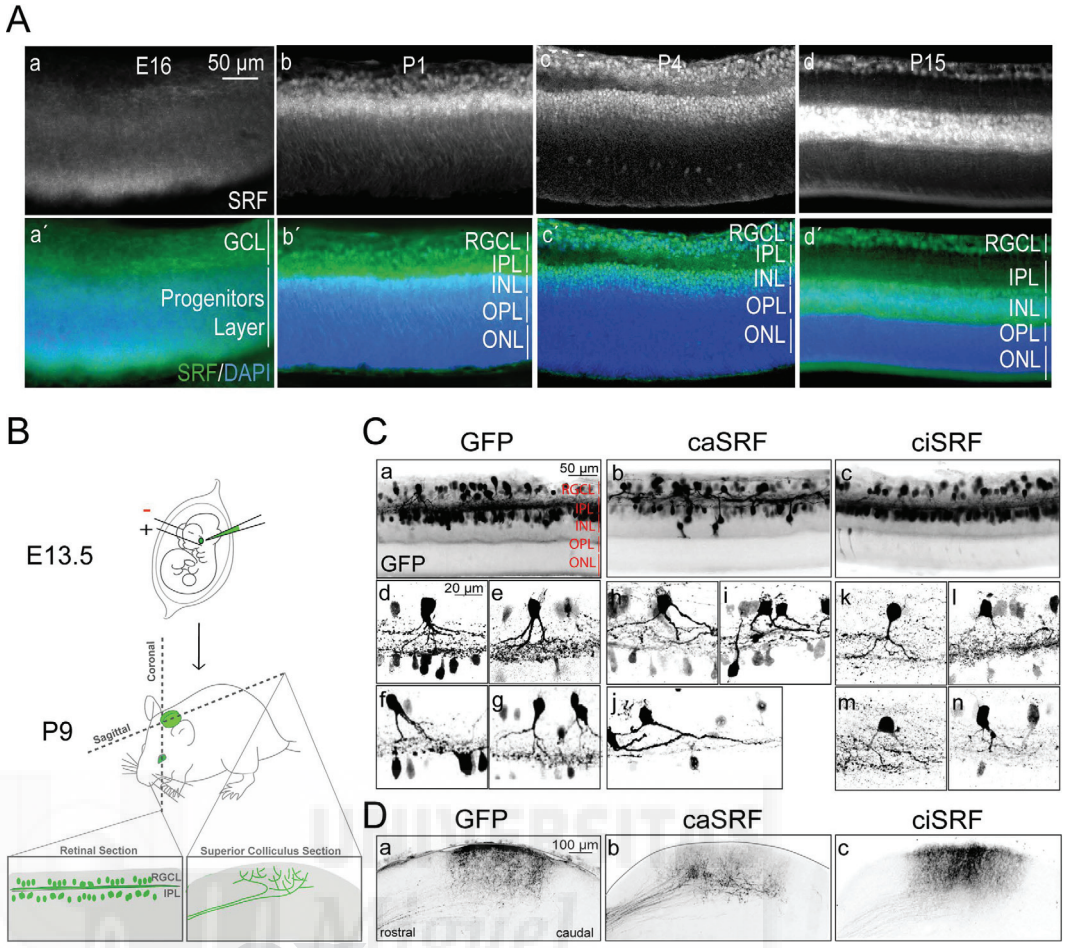

Figure 5. Altered SRF activity also causes growth defects in the developing visual system. (**A**) Expression pattern of SRF in the developing retina at different ages: E16, P1, P4 and P15. (a–d) Immunostaining of SRF in retinal sections from developing or postnatal mice. SRF expression is upregulated after birth. At P4, SRF expression is visible in the retinal ganglion cells and the inner nuclear layers. Two weeks after birth, SRF is still expressed in the RGC layer and strongly expressed in the INL. (a′–d′) Same sections counterstained with DAPI to better visualize the different retinal layers. GCL, ganglion cells layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. (**B**) Scheme of the experiment. (**C**) SRF modulates retinal dendrites arborization. (a–c) Retinal sections from P9 mice electroporated at E13.5 with plasmids encoding for GFP, caSRF and ciSRF. (d–n) Higher magnification images of retinal neurons from GFP (d–g), caSRF (h–j), or ciSRF (k–n) conditions. (**D**) SRF controls axon branching at the superior colliculus (SC). Sagittal sections through the SC of P9 in the three conditions: GFP (a), caSRF (b) and ciSRF (c).

the dendrites in ciSRF-electroporated RGCs seemed thinner than in GFP-expressing control neurons (Fig. 5C, panels c, k–n), and their axons developed smoother arborization at the SC than those of control neurons, although axonal pathfinding was apparently not affected (Fig. 5D, panel c).

SRF drives the expression of genes related to neuronal growth polarity. In order to identify candidate effector genes downstream of SRF responsible for these phenotypes, we turned to the transcriptomics screen presented in[17]. This screen showed that caSRF induces a gene program in hippocampal cultures that is remarkably enriched for SRE motifs (Fig. 6A) at promoter sequences[17] and for genes related to synaptic transmission, axon guidance and neuronal growth according to Gene Ontology (GO) classification of biological processes (Fig. 6B). This set included genes involved in neuronal growth, such as those encoding the RAS protein-specific guanine nucleotide-releasing factor (Rasgrf1), the neuroepithelial cell transforming protein 1 (Net1), and the semaphorins Sema3a, Sema3d and Sema7a. Intriguingly, although the analysis of TF binding sites enrichment did not reveal enrichment for SRE sites among downregulated genes, this small gene set was also enriched for axon guidance and cytoskeleton remodeling genes, including *Slit2*, *Dcc*, *Nrp1*, *Rnd2, Alcam*, *Epha7*, *Epha3* and *Epha5*.

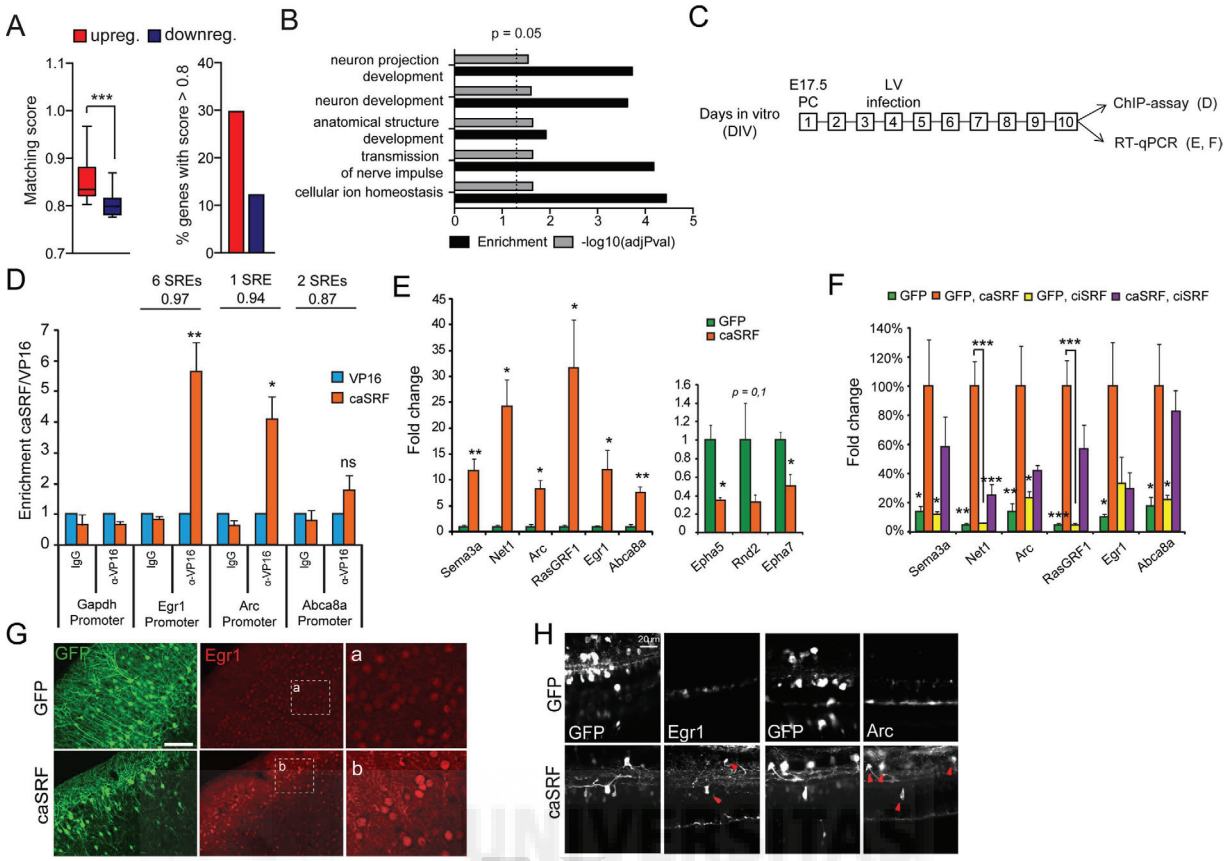

Figure 6. SRF regulates a gene program involved in neurite growth and polarity. (**A**) Left panel: distribution of SRE site prediction scores (>0.8) for SRF-upregulated genes and an equal number of SRF downregulated genes. Right panel: percentage of genes with an SRE matching score >0.8 within up- and downregulated genes. (**B**) Salient GO categories enriched in SRF-upregulated genes. Depicted are the enrichment over baseline and the transformed adjusted p-value. The vertical line indicates the 0.05 significance threshold. (**C**) Scheme of the experiments. Cultures infected at DIV4 and samples collected at DIV10. (**D**) ChIP assays confirm the direct binding of caSRF to the promoter of Arc and Egr1. ChIP-qPCR assays for specific promoters using primers close to the SRE-containing region. The number of SRE sites and the highest Transfac matrix similarity score for SRF binding (obtained with PSCAN) is indicated. α-VP16: specific antibody, IgG: preimmune. *$p < 0.05$; **$p < 0.005$: ns: not significant; Student's t test (n = 4–6). (**E**) RT-qPCR assays confirmed the upregulation (left) and downregulation (right) of candidate genes retrieved in the transcriptomics analysis. *$p < 0.05$, **$p < 0.005$ referred to GFP. Student's t test (n = 3–4). (**F**) The co-infection of neuronal cultures with ciSRF and caSRF shows that ciSRF interferes with caSRF-dependent transcription. Data in bar graphs are expressed as means of the fold changes ± SEM and were compared using 1-way ANOVA, followed by Bonferroni's multiple comparison test. The (*) symbols indicate statistical difference between all the conditions and caSRF; *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$ (n = 3–4). (**G**) Immunohistochemistry against Egr1 in cortices of P20 mice electroporated with GFP alone (GFP) or in combination with caSRF (caSRF). Scale bar: 100 μm. (**H**) GFP- and caSRF-electroporated retina showing cells with upregulated Egr1 and Arc upon caSRF expression (red arrowheads).

To examine whether some of these upregulations were the result of the direct binding of caSRF to the gene promoter, we conducted chromatin immunoprecipitation (ChIP) assays in infected hippocampal cultures that confirmed the direct binding to the *Arc* and *Egr1* promoters (Fig. 6C,D). For other candidate genes, we did not detect binding to the predicted regulatory sites, suggesting that either caSRF binds to more distant sites or the regulation is indirect. In parallel, RT-qPCR experiments confirmed the upregulation and downregulation of relevant candidate genes in caSRF-transduced cultures (Fig. 6E). Consistent with our functional assays (Fig. 3B), these experiments also showed that caSRF had a stronger effect in gene expression than ciSRF, although ciSRF counteracted the transcriptional changes promoted by caSRF when both variants were co-expressed (Fig. 6F). Finally, also supporting the consistency between in culture and *in vivo* results, immunohistochemistry analyses in electroporated mice

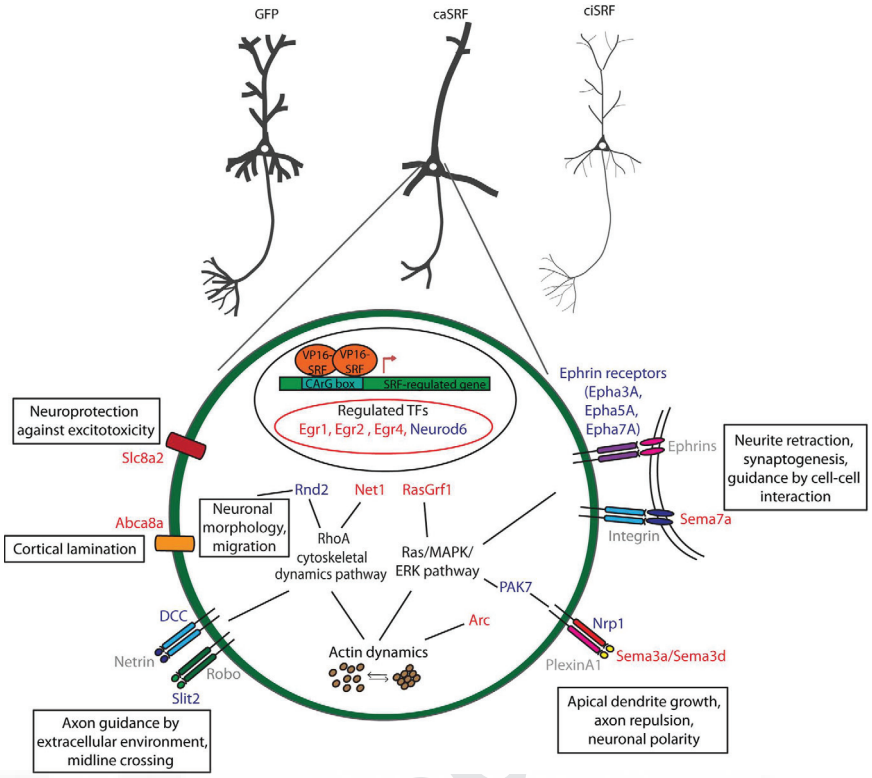

Figure 7. SRF target genes and biological processes affected by caSRF. Scheme highlighting SRF target genes and biological processes. Putative effector proteins upregulated or downregulated upon caSRF expression are shown in red or blue, respectively. Non affected proteins in our transcriptomics analysis are shown in grey.

confirmed the upregulation of relevant candidate genes, such as *Egr1*, after ectopic expression of caSRF in cortical neurons (Fig. 6G) and RGCs (Fig. 6H).

Discussion

We demonstrate here that the manipulation of SRF activity in neurons has important consequences in cell morphology, survival and growth *in vitro* and in the formation of neuronal circuits *in vivo*. Our results are congruent with previous LOF studies in SRF deficient mice indicating that interfering with SRF function alters neuronal growth and neurodevelopment[10–12] and with *in vitro* GOF experiments indicating that enhancing SRF function promotes neuronal growth[10,11,21,30] and protects against insults[20–22]. In addition, our GOF experiments using different approaches (viral transduction, in utero electroporation and transgenesis) revealed for the first time a role for SRF regulating asymmetrical outgrowth: caSRF expression favored primary dendrites enlargement versus branching both *in vitro* and *in vivo*. Axonal arborization was also severely reduced both in cortex and retina. This altered neuronal polarity, in turn, affected neuronal migration and the correct positioning of the soma in laminated structures like the cortical plate and the retina. Consistent with the cell-autonomous effect of caSRF expression reported here, recent studies have shown that ectopic expression of a different constitutive active form of SRF caused the mislamination of the photoreceptor layer in the retina of young transgenic animals[31], whereas SRF deficient mice exhibited defects in hippocampal and cortical lamination[11] and craniofacial development defects associated with cell migration deficits[32]. How can LOF and GOF manipulations produce similar phenotypes? We hypothesize that complex processes, such as circuit formation and tissue lamination, require fine-tuned SRF activity and are similarly affected by both positive and negative manipulations. Unbalanced or extemporaneous SRF activity both cause growth and arborization defects in a cell-autonomous manner, indicating that caution is needed in evaluating restorative strategies in the nervous system targeted to this TF[30].

Our recent transcriptomics analysis[17] retrieved important candidate genes that were validated here (Fig. 6), and contribute to explain how SRF can regulate the affected processes. In addition to previously reported SRF targets, such as the immediate early gene (IEG) *Arc*, transgelin (*Tagln*) and several members of the *Egr* family (*Egr1*, *Egr2* and *Egr4*), we identified a number of novel downstream genes related to the various phenotypes associated with enhanced SRF-driven transcription (Fig. 7). Thus, among the gene expression changes identified in our transcriptomics screen, we find the downregulation of the ephrins receptors *Epha7*, *Epha3* and *Epha5* and the upregulation of the semaphorin genes *Sema3a*,

Sema3d and *Sema7a*, all of which are known to play a critical role regulating neuronal migration and/or growth. Of note, experiments in forebrain-restricted *Srf* cKO mice had already suggested that the dysregulation of Ephs and Semaphorin guidance cues might contribute to the reduction of neurite outgrowth and to mossy fiber segregation defects[10,33]. In particular, the downregulation of ephrin receptors observed in caSRF-expressing cells might reduce the sensitivity to ephrins and cause the mistargeting of axons observed in our *in utero* experiments, while changes in the expression of semaphorins can provide an explanation for the axon mistargeting and lamination defects[34]. For instance, Sema3a can function as either a chemorepulsive agent, inhibiting axonal outgrowth, or as a chemoattractive agent, stimulating the growth of apical dendrites[35,36]. Less is known about Sema7a, but this protein has been shown to promote axonal growth in the embryonic olfactory bulb[37], and spreading and dendrite outgrowth in melanocytes[38]. Interestingly, the third altered semaphorin, Sema3d, which is known to induce the collapse and paralysis of neuronal growth cones, binds to neuropilin 1 which was also found altered (downregulated) in caSRF-expressing neurons[39].

Other genes whose function in neurons is less understood can also contribute to the altered morphology. For instance, the downregulation in caSRF-expressing neurons of the small Rho GTPase encoded by *Rnd2* suggests the activation of a homeostatic response to refrain SRF activation. Interestingly, it has been demonstrated that Rnd2 is essential in controlling the multipolar to bipolar transition during the migratory process of pyramidal neurons in the cortex[40], and in utero electroporation of Rnd2 shRNA in the cortex causes migration and lamination defects similar to those reported here for caSRF[41]. Another interesting candidate is Net1, a RhoGEF protein that regulates cytoskeleton dynamics. Intriguingly, exon-level analysis of transcriptomics data revealed that the upregulation of *Net1* in caSRF-expressing neurons was exclusively caused by the overexpression of a shorter, N-terminally truncated variant known as Net1A (Supplementary Fig. S2), which localizes in the cytosol and is thought to stimulate actin polymerization more efficiently than the full-length protein[42,43]. In turn, Na+/Ca2+ exchanger (NCX) encoded by *Slc8a2*, a low affinity, high capacitance calcium anti-porter membrane protein that can switch to 'reverse mode' under excitotoxicity and other forms of cellular stress, is a likely candidate to mediate the neuroprotective effect of caSRF[44]. Of note, *Slc8a2* and *Abca8a* (which encodes a lipid transporter and is also upregulated) are two of the genes specifically expressed in the cortical subplate at E15.5[45], suggesting that their overexpression may contribute as well to the abnormal cortical lamination.

In conclusion, although the list of putative transcriptional targets and their biological role are highly consistent with the phenotypes associated with SRF manipulations, the direct binding to most of these targets remains to be determined and unspecific caSRF-DNA interactions are not excluded. It is expected that several effector genes downstream of SRF redundantly or synergically contribute to the alterations associated with enhanced SRF activity. Functional assays for each of these candidate genes overexpressed either individually or in combination would be required to determine the precise molecular underpinning of the different phenotypes described here.

Little is still known about how the precise regulation of neurite outgrowth by intrinsic and extrinsic signals leads to the intricate branching pattern unique to each neuronal class observed *in vivo*. During development, dendrites and axons of central nervous system neurons grow and refine by addition and retraction of thin branches. This process, reproduced in neuronal cultures, is highly dynamic and only a small fraction of newly added branches remain as part of the mature dendritic and axonal trees. It is thought that the growth rates of axons and dendrites result from the interplay between pre-programmed TFs levels and intrinsic length-sensing mechanisms[46]. Transcription is now thought to primarily occur in the form of pulsatile bursts that rely on continuous oscillations in the expression level or subcellular localization of TFs[47]. Such oscillatory activity is likely disrupted by the expression of a constitutively active variant like caSRF. Pulsatile bursts of SRF activity during neuronal morphogenesis may regulate the asymmetric outgrowth of neuronal processes by balancing enlargement versus branching, thereby providing a link between genome expression and environment-regulated neuronal growth. This view helps to understand how SRF can influence a variety of neurodevelopmental processes, from neuronal migration and tissue lamination to circuit assembly.

Materials and Methods

Lentiviral production. To generate the caSRF variant, the DNA binding domain (DBD) of SRF was fused with the acidic transactivation domain of viral protein 16 (VP16) of herpes simplex virus[48]. To produce the ciSRF variant, we expressed the DBD of SRF alone[18]. The VP16-expressing construct, used as control in ChIP assay experiments, carries the transactivation domain of VP16 and lacks any DBD[17]. Both constructs were cloned into the synapsin promoter-bearing lentiviral vector LenLox 3.7[49] and used either in electroporation experiments or in the production of lentiviral pseudovirions as described[17]. Viral stocks were tittered by RT-qPCR and concentrated through ultracentrifugation.

Mouse strains. TetO-caSRF transgenic mice were generated by microinjection of the linear construct as previously described[50]. Analysis of transgenic and founder mice was performed by Southern blotting using a VP16 probe. The founder mice were backcrossed to C57BL6/J mice to generate the transgenic line used in our study. We designated as caSRF mice those bitransgenic animals resulted of the crossing of pCaMKII-tTA mice (line B)[50], and tetO-VP16-SRF transgenics and as wild-type mice those littermates carrying either pCaMKII-tTA, tetO-VP16-SRF or none transgene. Note that the tetO-caSRF transgenic

strain is no longer available. The observations reported here are based on the analysis of the bitransgenic mice born in 2005 (5 out of 5 mice showed hydrocephalus). Albino ICR mice were used for primary neuronal culture and in utero electroporation experiments in the cerebral cortex, whereas B6D2F1/J mice were used for in utero electroporation experiments in the retina because pigmented eyes are easier targets for DNA injection. All mice were maintained according to animal care standards and experimental protocols were approved by the Institutional Animal Care and Use Committee.

Culture, infection and transfection of hippocampal neurons. Primary hippocampal neurons were obtained from E17.5–E18.5 embryos. Hippocampi were dissected and processed as described[17]. The day of plating was considered day *in vitro* 1 (DIV1). Neurons were incubated for at least 3 additional days (DIV4) before they were used in any experiment. Primary hippocampal neurons were infected at the times indicated by adding the necessary volume of the concentrated viral preparation to achieve an effective multiplicity of infection of 1–10, which provided a percentage of neuronal infection close to 100%. In co-infection experiments, equal volumes of each virus were mixed. In transfection experiments, the plasmid pDsRed-Express2-C1 (Clontech 632538) driving dsRed expression was transfected using Lipofectamine 2000 (Invitrogen) as described by the manufacturer.

Cell culture assays and treatments. For luciferase assays, HEK293 cells were grown in 90% DMEM supplemented and 10% fetal calf serum supplemented with 2 mm glutamine and penicillin/streptomycin (100 U/ml to 100 μg/ml) (Invitrogen). HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) with efficiency larger than 90%. Plasmids were transfected in a ratio of 500:50:1 (construct of interest: Firefly reporter: Renilla reporter) with a total DNA amount of 1 μg per 100.000 cells. The Firefly and Renilla luciferase reporter plasmids used in this study were pSRE-Luc (Clontech 631911) and pRL-SV40 (Promega E2231), respectively.

Luciferase activity was measured at 24 h after transfection, using the Dual Luciferase Assay kit (Promega) as reported before[17]. For cell death assays, primary hippocampal neurons infected at DIV4 were treated with NMDA at DIV9 at different concentrations. After 12 hours LDH assay was performed using the Cytotoxicity Detection Kit Plus (LDH) (Roche) following manufacturer instructions. For B27 deprivation complete maintaining media was substituted at DIV7 by B27-deprived media.

In utero electroporation in retina and cortex. Cortex in utero electroporation were performed in timed-pregnant ICR mice anesthetized with isofluorane, their abdominal cavity cut, open, and the uterine horns exposed. Approximately 1–2 μl of DNA solution (1 μg/μl) was injected into the lateral ventricle of E14.5 or E16.5 embryos using a pulled glass micropipette. In co-electroporation experiments we used the same amount of total DNA (i.e., the concentration of the individual plasmids was 0.5 μg/μl). Each embryo was placed between tweezers-type electrodes (CUY650-P5, NEPA Gene). The angle of inclination of the electrode paddles with respect to the horizontal plane of the brain was zero for targeting the cortical ventricular zone (VZ). Square electric pulses (45 V; 50 ms) were passed five times at 950 ms intervals using an electroporator (CUY21; NEPA Gene). Retina in utero electroporation experiments were performed in E13.5 B6D2F1/J embryos as described in[29]. Each experiment was repeated 7 times for a total of 13–18 retinas and SC analyzed. In both cases, only a few embryos were exposed outside the abdominal cavity at a time to prevent excessive temperature loss. The wall and skin of the abdominal cavity were sutured-closed, and embryos were allowed to develop normally till E17.5, P0, P9 or P20.

Immunocytochemistry and immunohistochemistry. Immunostainings in primary hippocampal cultures were conducted as described in[17]. For Sholl analysis, the dendrites of transfected neurons were drawn and analyzed with Neurolucida neuron tracing Software (MBF Bioscience) in a blind manner. The number of intersections was determined every 2 μm, starting from a radius of 5 μm to the center of the neuronal soma. For immunohistochemistry, mice were perfused with 4% paraformaldehyde in PBS. Coronal vibratome sections (50 or 100 μm) were obtained from electroporated brains; washed in PBS and PBS−0.1% Triton X-100 (PBT) and incubated for 0,5 h at room temperature with 3% BSA-PBT. The primary antibodies used in this study are α-GFP (Aves Labs, GFP-1020), α-MAP2 (Sigma, M9942), α-GFAP (Sigma-Aldrich, G9269), α-VP16 (mouse monoclonal from Santa Cruz Biotechnology sc-7545; rabbit polyclonal from Sigma-Aldrich V4388), α-Egr1 (Santa Cruz Biotechnology, sc-110), α-SRF (Santa Cruz Biotechnology sc-335), α-dsRed (Clontech, 632496) and α-Arc (Synaptic Systems, 156-003). Nuclei were counterstained with a 1 nM DAPI solution (Invitrogen) before mounting. Images were taken with Olympus Confocal Inverted Microscope or a Leica Microscope.

RNA extraction, RT-qPCR and microarray analysis. Total RNA from hippocampal cultures was extracted with TRI reagent (Sigma-Aldrich) and reverse transcribed to cDNA using the RevertAid First-Strand cDNA Synthesis kit (Fermentas). qPCR was performed in an Applied Biosystems 7300 real-time PCR unit using Eva Green qPCR reagent mix. The primer sequences used in the RT-qPCR assays are shown in Supplementary Table 1. Each independent sample was assayed in duplicate and normalized using GAPDH levels. New bioinformatical analyses were performed using the datasets presented in[17] and accessible at ArrayExpress database (accession number E-MEXP-3167). The enrichment for TFBS was calculated with PSCAN using JASPAR (Fig. 6A) or Transfac (Fig. 6D) as the reference

databases, and exploring from −950 to +50 bp region of promoters[51]. Webgestalt[52] was used to perform the GO analysis of caSRF-regulated gene (FDR 0.1, >2 genes per category).

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) experiments were conducted as previously described[53] with some modifications. Primary hippocampal neurons infected with caSRF or VP16 at DIV4 were washed with PBS at DIV10, and cross-linked by the addition of 1% formaldehyde (v/w) and incubated for 10 min at room temperature. The reaction was stopped by adding glycine to 0.125 M and incubating for 5 min at RT. After two washes in PBS, neurons (2×10^6) were scraped and resuspended in 400 μl 1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), and protease inhibitors and incubated for 10 min on ice. The cell suspension was sonicated using a Branson sonicator at 10% of capacity, 6 cycles of 15 sec. Chromatin was used for ChIP by incubation for 16 h at 4 °C with 5 μg specific anti-VP16 (from Sigma V4388) or isotype-matched controls Abs, followed by the addition of 30 μl Dynabeads of protein G an additional 2.5 h incubation at 4 °C. The DNA was resuspended in 30 μl of water for subsequent PCR analysis as a template to evaluate the presence of regulatory regions of caSRF targets. The primer sequences used in the ChIP-qPCR assays are listed in Supplementary Table S1. Paired sample *t*-tests were used to determine the statistical significance between values.

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Author Contributions

M.S. conducted most of the experiments described in this article and analyzed the data; E.B. generated lentiviral constructs and performed preliminary experiments in culture and some bioinformatics analyses; B.B. and A.F. conducted biochemical and molecular assays in primary cultures and analyzed the data; C.M.-P. and Y.C. performed the in utero electroporation experiments in retina. A.B. and E.H. supervised the work and designed experiments. M.S., A.B. and E.H. wrote the paper. All authors discussed the results and reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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SUPPLEMENTARY INFORMATION

Title: Fine-tuned SRF activity controls asymmetrical neuronal outgrowth: implications for cortical migration, neural tissue lamination and circuit assembly

Authors: Marilyn Scandaglia, Eva Benito, Cruz Morenilla-Palao, Anna Fiorenza, Beatriz del Blanco, Yaiza Coca, Eloísa Herrera and Angel Barco

Inventory of supplementary information:

• Legend for Supplementary Figures S1 and S2.

• Supplementary Figure S1. caSRF promotes neuronal survival.

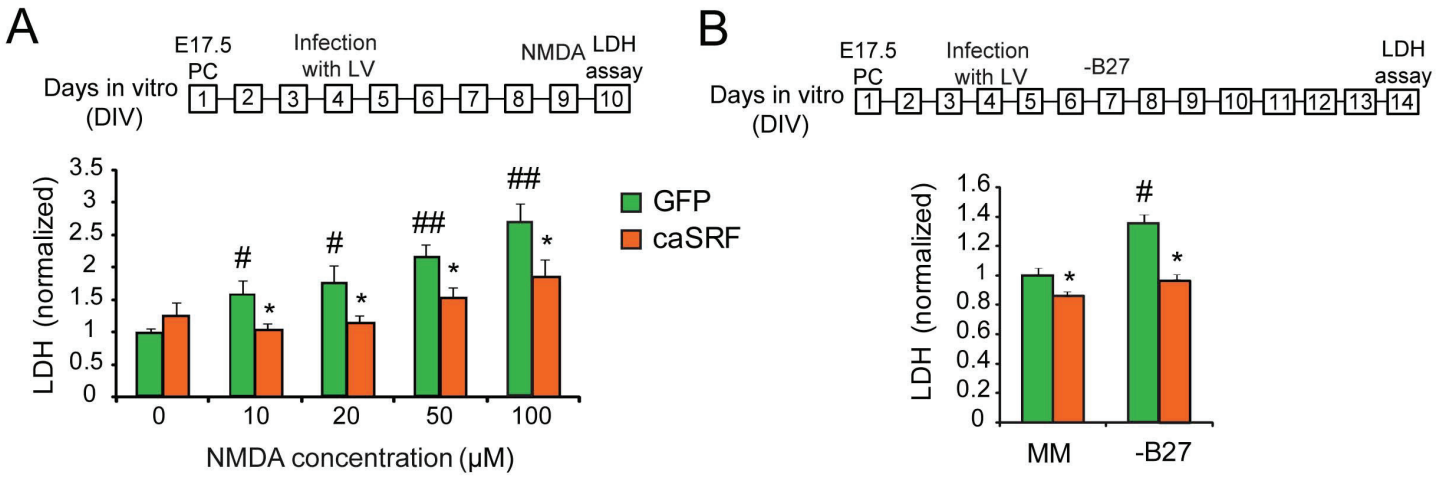
• Supplementary Figure S2. Net1A is selectively upregulated in caSRFexpressing cultures.

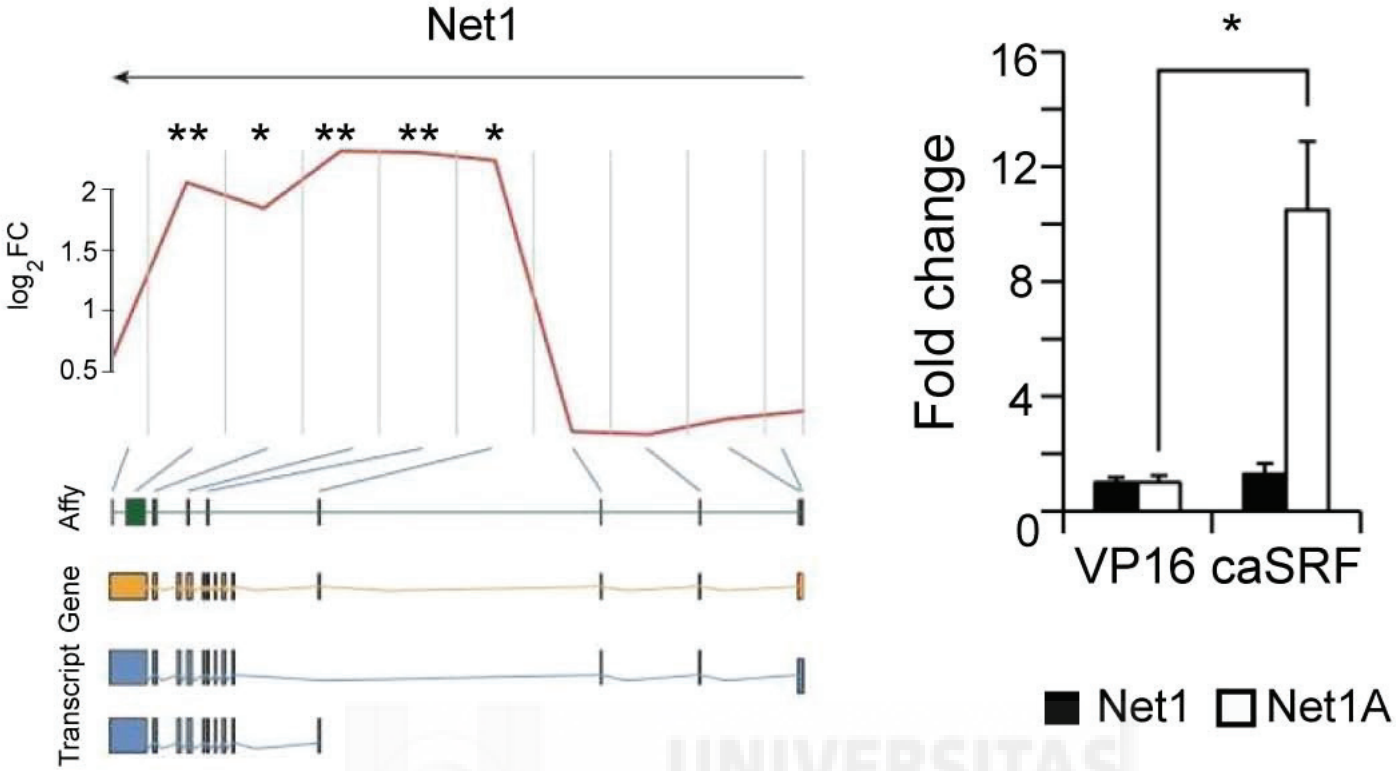
• Supplementary Table S1. Primers used in this study.

SUPPLEMENTARY FIGURE LEGEND

Supplementary Figure S1. caSRF promotes neuronal survival. A. Scheme of the experiment and quantification of LDH activity in LV-infected cultures at different NMDA concentrations. caSRF expression ameliorates NMDA-induced neurotoxicity (n = 9). **B.** Scheme of the experiment and quantification of LDH activity after 7 days of trophic deprivation. caSRF expression also reduces neuronal death induced by serum deprivation indicated as -B27 (n = 8). MM: complete maintaining media. #, *: $p < 0.05$; ##: $p < 0.005$, Student's t-test (* symbol used for statistical difference between lentiviral vectors infected cultures, # symbol used for statistical difference between treatments).

Supplementary Figure S2. Net1A is selectively upregulated in caSRF-expressing cultures. Left: Exon-level analysis of microarray data for the Net1 gene. Right: RT-qPCR confirmed the specific upregulation of the short variant Net1A.





Supplementary Table S1: Primers used in this study.

Primer sequences used in the RT-qPCR

	Forward (5'-3')	Reverse (5'-3')
Sema3a	CTTACAGCCAGAGCAAACTATG	GAAGGAAGGTGTGGTAACTGG
Net1	AAGGACGATCCGAGTCTTAGATG	AGCTAGAGGCCGAACTCTCTTATTG
Arc	GCAGGAGAACTGCCTGAACAG	AAGACTGATATTGCTGAGCCTCAA
RasGRF1	GACCTTGGAGTTGAGGACCG	CGCCTCATGTTCTGTAGCCA
Egr1	CCGTCCTGTTCCCTTTGACTT	GAACTTGGACATGGCTGTTTCAG
Abca8a	GGGGTCATATTCACCAACGC	TGAGCTGTGTGGTCCTGATG
Epha5	ACCTTGGAGGATTTGAGGCG	TCCCGTTTACCATCTGCACC
Rnd2	AAGATCGTAGTGGTGGGGGA	GATCTCGAAGCTGGCAGTGT
Epha7	GGTGTTCATGGTGTTCGGCT	AACTGACAGGTGCTCATTTGTTAC
Gapdh	CTTCACCACCATGGAGAAGGC	CATGGACTGTGGTCATGAGCC

Primer sequences used in the ChIP-qPCR

	Forward (5'-3')	Reverse (5'-3')
Arc	TGTTGCCAGGGAATCGGAAG	GCAGAGGAAAGCAAGATGCC
Egr1	TTGGATGGGAGGGCTTCAC	CTCCGCCGTGACGTACAT
Abca8a	GAGCTCAGAGCCTTTTAAGGGA	CCTCCCCCAAAAGAAATTGACAG
Gapdh	TTCACCTGGCACTGCACAA	CCACCATCCGGGTTCCTATAA

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ANNEX II

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Other publications co-authored during the thesis period

- *Blocking miRNA biogenesis in adult forebrain neurons enhances seizure susceptibility, fear memory, and food intake by increasing neuronal responsiveness.* Anna Fiorenza, Jose P. Lopez-Atalaya, Victor Rovira, **Marilyn Scandaglia**, Emilio Geijo-Barrientos, and Angel Barco. Cereb Cortex. 2016 Apr;26(4):1619-1633. doi: 10.1093/cercor/bhu332. Epub 2015 Jan 16

- *Epigenetic Etiology of Intellectual Disability.* Shigeki Iwase, Nathalie G. Berubé, Zhaolan Zhou, Nael Nadif Kasri, Elena Battaglioli, **Marilyn Scandaglia**, and Angel Barco. J Neurosci. 2017 Nov 8;37(45):10773-10782. doi: 10.1523/JNEUROSCI.1840-17.2017