Identifying the role of the imprinted gene Pw1/Peg3 in the central nervous system
Anne-Lyse Denizot

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Identifying the role of the imprinted gene $Pw1/Peg3$ in the central nervous system

Par Anne-Lyse DENIZOT

Thèse de doctorat de Biologie Moléculaire et Cellulaire

Dirigée par David SASSOON

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*Tibo, je serai toujours là pour toi. Qu’il pleuve sur moi.*

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### LIST OF ABBREVIATIONS

**A**
- Airn: antisens of IGF2R non-coding RNA
- APeg3: antisens Peg3 non-coding RNA
- AS: Angelman syndrome

**B**
- BAC: bacterial artificial chromosome
- BBB: blood-brain barrier
- BDNF: brain-derived neurotrophic factor
- BET: ethidium bromide
- βGAL: β-galactosidase
- bFGF: basic fibroblast growth factor
- BMP: bone morphogenic protein
- bp: base pair
- BSA: bovine serum albumin
- BV: blood vessel
- BWS: Beckwith-Wiedemann syndrome

**C**
- CA: cornu ammonis
- Cdkn1c/p57: cyclin-dependent kinase inhibitor 1c
- cDNA: complementary DNA
- CNS: central nervous system
- CP: choroid plexus
- CpG: cytosine - phosphate - guanine
- CSF: cerebrospinal fluid
- CTCF: CCCTC-binding factor

**D**
- DAPI: 4',6'-diamidino-2-phenylindol
- DG: dentate gyrus
- Dio3: thyroxine deiodinase type III
- Dlk1: delta-like 1 homolog
- DMR: differentially methylated region
- DNA: deoxyribonucleic acid
- DNMT: DNA methyltransferase

**E**
- E: ependymal cell
- ECM: extra cellular matrix
- ECR18: evolutionarily conserved region 18
- EDTA: ethylenediaminetetraacetic acid
- EGF: epidermal growth factor
- ES cell: embryonic stem cell
- E17.5: embryonic stage E17.5
F
FACS: fluorescence-activated cell sorting
FFA: free fatty acid
FGF: fibroblast growth factor
FISH: fluorescent in situ hybridization

G
GABA: gamma-aminobutyric acid
GC: granule cell
gDNA: genomic DNA
GFAP: glial fibrillary acidic protein
Gnas: stimulating guanine-nucleotide binding protein
Gnasxl: extra large stimulating guanine-nucleotide binding protein
Grb10: growth factor receptor-bound protein 10

H
HSPG: heparan sulfate proteoglycans

I
ICR: imprinting control region
Ig: immunoglobulin
IGC: immature granule cell
Igf2: insulin-growth factor type 2
Igf2r: insulin-growth factor type 2 receptor
IGN: imprinted gene network
IPC: intermediate progenitor
IRES: internal ribosome entry site
IUGR: intra-uterine growth restriction

K
Kb: kilobases
Kcnq1: potassium channel voltage gated KQT-like
Kcnq1ot1: Kcnq1 antisens non-coding RNA
KO: knockout

L
LacZ: gene encoding a β-galactosidase
LV: lateral ventricle
LOI: loss of imprinting
LT-HSC: long-term hematopoietic stem cells

M
Magel2: melanoma antigen family L2
MAT: maternally-inherited Pw1\textsuperscript{IRES\textsubscript{LacZ}} allele
MEF: mouse embryonic fibroblast
Mest: mesoderm specific transcript
Mimt1: MER1 repeat-containing imprinted transcript 1
miR: micro RNA
mL: milliliters
ML: molecular layer
MRF: myogenic regulatory factor
MRI: magnetic resonance imaging
mRNA: messenger RNA

N
ncRNA: non-coding RNA
Ndn: gene encoding NECDIN protein
NFκB: nuclear factor kappa B
NGS: next generation sequencing
NS: neurosphere
NSC: neural stem cell

O
ORF: open reading frame
OT: oxytocin
OTB: ovarian time-bomb hypothesis

P
PAT: paternally-inherited $Pw1^{IRES\text{LacZ}}$ allele
PCR: polymerase chain reaction
PBS: phosphate buffer saline solution
Peg3: paternally expressed gene 3
PGK: phosphoglycerate kinase
PICs: PW1+ interstitial cells
Plagl1 (alias Zac1): pleiomorphic adenoma gene-like 1
PP: postpartum
Pre-mRNA: primary transcript messenger RNA
PTH: parathyroid hormone
PVN: paraventricular nucleus
PWS: Prader-Willi syndrome
$Pw1^{+/-}$: wild-type $Pw1$ alleles
$Pw1^{m/-/p^+}$: maternal allele deletion of $Pw1$
$Pw1^{m+/p^-}$: paternal allele deletion of $Pw1$
$Pw1^{1/-}$: deletion of both parental $Pw1$ alleles

R
RA: radial astrocyte
Rasgrf1: Ras-specific guanine nucleotide-releasing factor 1
RMS: rostral migratory stream
RNA: ribonucleic acid
Rpm: rotation per minute
RT-qPCR: reverse transcription quantitative PCR

S
SDS: sodium dodecyl sulfate
Sec: seconds
SGZ: subgranular zone
SHH: sonic hedgehog
SNC: central nervous system
Snrpn: small nuclear ribonucleoprotein polypeptide N
Snx14: sorting nexin 14
SO: supraoptic nucleus
SRS: Silver-Russell syndrome
SVZ: subventricular zone

T
TGF: tumor growth factor
TNF: tumor necrosis factor
TRAF2: TNF receptor-associated factor 2

U
Ube3a: ubiquitin protein ligase E3A
UPD: uniparental disomy
Usp29: ubiquitin specific peptidase 29
UV: ultraviolet

X
XCI: X-chromosome inactivation

Z
Zfp264: zinc finger protein 264
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Etude du rôle du gène d'empreinte Pw1/Peg3 dans le système nerveux central

Résumé:
Chez les mammifères, une centaine de gènes sont soumis à une régulation épigénétique où seule la copie maternelle, ou paternelle, est exprimée. Ce phénomène appelé empreinte parentale alimente encore différentes théories liées à la reproduction, notamment celles du conflit parental et de la coadaptation entre mère et enfant. Pw1/Peg3 est un gène d’empreinte paternellement exprimé. Cependant, à l’aide de deux modèles de souris bien distincts, une souris rapporteur (Pw1IREsnLacZ) et une nouvelle souris knockout pour Pw1/Peg3, nous avons détecté des transcrits Pw1/Peg3 maternels dans le cerveau périnatal. Plus précisément, nous avons mis en évidence une expression bi-allélique du gène rapporteur Pw1IREsnLacZ restreinte aux deux futures nèches de cellules souches neurales adultes. In vitro, nous avons conclu, via des cultures primaires de cellules souches neurales, que l’expression bi-allélique endogène de Pw1/Peg3 est un évènement ponctuel rare. D’ailleurs lors de la caractérisation de notre modèle de souris Pw1/Peg3 knockout, nous avons observé un retard de croissance uniquement lors de la délétion de l’allèle Pw1/Peg3 paternel. Ce phénotype n’est pas lié à un problème de prise alimentaire chez les nouveaux-nés et contrairement à ce qui a été précédemment décrit, nous n’avons détecté aucun défaut de comportement maternel chez les femelles mutantes pour Pw1/Peg3. La lactation n’est pas non plus impactée par la délétion de Pw1/Peg3. Ces résultats démontrent que Pw1/Peg3 favorise intrinsèquement la croissance postnatale et que, désormais, ce gène d’empreinte ne peut plus être utilisé afin d’illustrer la théorie de coadaptation entre mère et enfant.

Mots clés: Pw1/Peg3, empreinte parentale, expression allèle-spécifique, cellules souches neurales, comportement maternel.

Identifying the role of the imprinted gene Pw1/Peg3 in the central nervous system

Abstract:
In mammals, a hundred of genes are preferentially expressed from one specific parental allele; a phenomenon referred as genomic imprinting. Establishing theories to explain the emergence of such a gene dosage strategy is challenging. Pw1/Peg3 is a paternally expressed gene. Using both a reporter mouse model and a novel constitutive knockout mouse model, we detected Pw1/Peg3 transcription from the maternal allele, which is normally silent, in the perinatal brain. Specifically, we observed that a putative Pw1/Peg3 bi-allelic expression is mainly restricted to the two future adult neural stem cells niches. In vitro experiments on primary neural stem cells allowed us to conclude that imprinting relaxation of the Pw1/Peg3 maternal allele is a rare event. Whether it affects the mouse phenotype is currently under investigation. In parallel, consistent with previously established mutant mouse models we confirmed that paternal Pw1/Peg3 deletion leads to growth retardation. However we did not find any impairment in maternal behaviors upon heterozygous or homozygous loss of Pw1/Peg3. Lactation was also not disrupted and mutant pups exhibited a normal suckling ability. Taken together, PW1/PEG3 promotes growth intrinsically and can no longer be used to illustrate the popular coadaptation theory between mother and infant.

Keywords: Pw1/Peg3, genomic imprinting, allele-specific expression, neural stem cells, maternal behavior.
RÉSUMÉ DE LA THÈSE

Il existe chez les mammifères une centaine de gènes qui se trouvent exprimés différemment selon leur origine parentale (Ferguson-Smith, 2011). Ces gènes dits *gènes d'empreintes* sont uniquement exprimés soit via la copie paternelle soit via la copie maternelle, l’allèle parental correspondant et non exprimé étant mis sous silence par une méthylation différentielle de l'ADN. Chacun de ces gènes possède des caractéristiques propres quant à leur profil d’expression, leur localisation subcellulaire et leur fonction. Pourquoi ces gènes ont-ils besoin d’une régulation aussi sexiste? Deux principales théories s’opposent. Celle du *conflit parental* (ou *Kinship* en anglais) témoigne de l’affrontement des deux génomes parentaux pour garantir soit la grandeur et la force des enfants sous l’influence du génome paternel, soit la santé et la préservation des ressources de la mère, par le biais du génome maternel (Moore and Haig, 1991). La théorie dite de *coadaptation*, quant à elle, a pour but d’optimiser les chances d’obtenir une progéniture viable en coordonnant les échanges entre mère et enfant(s) in utero et durant toute la période d’allaitement (Keverne and Curley, 2008).

Certains tissus ou certains types cellulaires échappent cependant à la restriction allélique imposée par l’empreinte parentale. C’est le cas dans le cerveau où *Igf2*, un gène où la copie maternelle est normalement inactive, est exprimé bi-alléliquement dans les plexus choroïdes et les leptoméninges (DeChiara et al., 1991, Charalambous et al., 2004, Hagege et al., 2006). *Dlk1* est un gène paternellement exprimé (Kobayashi et al., 2000, Schmidt et al., 2000, Wylie et al., 2000) mais Ferrón *et al* ont récemment démontré qu’après la naissance, une expression bi-allélique de *Dlk1* dans la niche des cellules souches neurales est essentielle au maintien de la neurogènèse chez l’adulte (Ferron et al., 2011). L’empreinte parentale, est désormais définie comme une régulation épigénétique qui est dynamique à l’échelle de l’organisme.

Comme *Dlk1*, de nombreux gènes d'empreintes sont impliqués dans le contrôle des cellules souches. *Cdkn1c/p57*, un gène maternellement exprimé, permet le maintien du statut quiescent des cellules souches neurales dans l’hippocampe (Furutachi et al., 2013). Venkatraman *et al* ont montré que la perte de l’expression allèle-spécifique des gènes *Igf2* et *H19* induit l’appauvrissement du pool de cellules souches
hématopoïétiques (Venkatraman et al., 2013). En 2011, Berg et al ont identifié une surreprésentation des gènes d'empreinte dans une analyse transcriptomique des cellules souches somatiques adultes réalisée dans le système hématopoïétique, la peau et le muscle squelettique. Ainsi, tout un réseau de gènes d'empreinte, appelé *Imprinted Gene Network* (IGN), est spécifiquement co-régulé dans les cellules souches, une hypothèse qui avait été émise par Varrault et al notamment au cours du développement embryonnaire (Varrault et al., 2006).

Au sein de notre laboratoire, le gène d'empreinte *Pw1*, alias *Peg3*, est l'un de nos intérêts majeurs. *Pw1* est un gène paternellement exprimé (Kuroiwa et al., 1996, Relaix et al., 1996). De précédentes études chez la souris ont permis de mettre en évidence l'implication de *Pw1* dans la croissance pré et postnatale, le comportement maternel et l'allaitement, de la production de lait à la prise alimentaire des nouveaux-nés (Li et al., 1999, Curley et al., 2004, Champagne et al., 2009). Ainsi, ces travaux ont permis d'établir l'un des tous premiers liens entre l'empreinte parentale, le comportement et une reproduction sexuée garantie. Au cours du développement embryonnaire, *Pw1* est fortement exprimé dans les tissus d'origine ectodermique et mésodermique ainsi que dans les tissus extra-embryonnaires (Relaix et al., 1996). Après la naissance, l'expression de *Pw1* diminue progressivement jusqu'à devenir restreinte, chez l'adulte, aux cellules souches de multiples tissus tels que le muscle squelettique, la peau et le système hématopoïétique (Mitchell et al., 2010, Besson et al., 2011, Bonfanti et al., 2015), d'où son appartenance à l'IGN (Varrault et al., 2006). En revanche, bien que *Pw1* soit transcrit dans les cellules souches neurales adultes son expression reste importante dans tout le lignage neuronal, notamment les neurones hypothalamiques (Kuroiwa et al., 1996, Relaix et al., 1996, Li et al., 1999, Besson et al., 2011).

**Dans la première partie de notre étude, nous avons concentré nos recherches sur l'identification de cellules exprimant *Pw1* bi-alléliquement.**

L'utilisation d'un modèle de souris transgénique rapporteur *Pw1*IRE*SnLacZ* nous a permis d'observer une transcription active de l'allèle *Pw1*IRE*SnLacZ* maternel, en particulier au stade fœtal E17.5, dans les deux futures niches de cellules souches neurales adultes. Ce phénomène qui n'avait jusqu'alors jamais été décrit pour *Pw1*
suggère que certaines cellules souches/progénitrices nécessitent une expression bi-allélique de *Pw1*. Afin de déterminer si cette modulation de l’empreinte parentale est bien effective au niveau du locus *Pw1* endogène, nous avons réalisé des expériences de pre-mRNA-FISH pour détecter à l’échelle de chaque cellule si un ou deux allèles parentaux étaient transcrits. Pour cela nous avons opté pour un système *in vitro*: la culture primaire de cellules souches neurales sous forme de neurospHERes (Reynolds and Weiss, 1992, 1996, Temple, 2001). Tout d’abord, nous avons montré que les neurosphères ont un contenu très hétérogène : la moitié des cellules expriment l’allèle *Pw1* paternel seulement tandis que l’autre moitié expriment les deux allèles *Pw1* bi-alléliquement. A deux stades de développement différents, foetal E17.5 et postnatal 1 mois, le pool de cellules exprimant le gène rapporteur *Pw1* bi-alléliquement augmente après passage des neurosphères ce qui est caractéristique des cellules souches neurales. Cependant, nous avons pu constater que la β-galactosidase, de par sa stabilité, surestime le nombre de cellules PW1+ à un instant *t*. Les résultats de pre-mRNA-FISH nous démontrent que le nombre total réel de cellules souches neurales exprimant les deux allèles *Pw1* parentaux est inférieur à 2%. Ce faible taux ne nous permet pas à l’heure actuelle de confirmer les données obtenues *in vitro* grâce au modèle de souris rapporteur *Pw1* bi-alléliquement. En revanche, l’utilisation de notre nouvel outil génétique, la souris knockout (KO) pour *Pw1*, nous a permis de clairement mettre en évidence une activation de l’allèle *Pw1* paternel endogène lorsque l’allèle *Pw1* maternel est muté dans le cerveau périnatal. Ce résultat concorde en partie avec une étude récente menée par Perera *et al* (Perera *et al.*, 2015) qui décrit une expression bi-allélique de *Pw1* dans l’hypothalamus et les plexus choroïdes, à la naissance et à l’âge adulte. La signification biologique d’une telle relaxation de l’empreinte pour *Pw1* n’est cependant pas encore connue.

**Dans la seconde partie de notre étude nous nous sommes intéressé à la fonction de *Pw1* via la caractérisation d’un nouveau modèle de souris KO constitutif pour *Pw1***.

PW1 participe à deux voies de signalisation liées au stress cellulaire, p53 et TNF/NFκB, permettant de réguler le destin de la cellule, respectivement la mort par apoptose ou la survie (Relaix *et al.*, 1998, Relaix *et al.*, 2000). En stabilisant β-caténine,
PW1 inhibe la voie de signalisation Wnt (Jiang et al., 2010). PW1 agit également comme facteur de transcription; Thiaville et al ont identifié, dans le cerveau adulte, des gènes cibles impliqués dans le métabolisme cellulaire (Thiaville et al., 2013a). Nous avons alors émis l’hypothèse que PW1 est un médiateur du stress cellulaire principalement dans les cellules souches/progénitrices. Afin de déterminer si ce rôle est indépendant de la régulation de la croissance ou du comportement maternel documentés précédemment, nous avons généré une souris KO conditionnelle pour Pw1. Contrairement aux autres modèles de souris mutantes pour Pw1, nous avons ciblé les exons 8 et 9 codants pour plus de 91% de la protéine avec excision de la cassette de sélection Néomycine. Afin de comparer notre mutant avec les précédents modèles établis, nous avons en parallèle généré un modèle Pw1 KO constitutif en croisant la souris Pw1 conditionnelle avec une souris PGK-Cre (Lallemand et al., 1998). Les travaux de thèse présentés ici se sont d’ailleurs principalement axés sur la caractérisation du modèle Pw1 knock-out constitutif.

Dans les conditions testées, la délétion de Pw1 n’impacte en aucun cas le comportement maternel des femelles vierges et primiparous (Denizot et al, publication soumise à PLoS Genetics). Toutes les femelles Pw1+/+, Pw1m−/p+, Pw1m+/p− et Pw1−/− ont, lors du test de comportement, ramenés les nouveaux-nés dans le nid à une vitesse moyenne équivalente. Elles ont également fabriqué un nid de même qualité. Nous n’avons observé aucune mortalité accrue dans les portées des femelles Pw1m+/p− et Pw1−/−. Nous n’avons pas non plus détecté de problèmes d’éjection de lait chez les femelles Pw1m+/p− comme dans l’étude de Li et al (Li et al., 1999); leur progéniture Pw1+/+ ne démontrant aucun retard de croissance comparé aux nouveaux-nés Pw1+/+ nés d’une femelle Pw1+/+. Li et al (Li et al., 1999) avaient en partie associé le trouble du comportement maternel et le déficit en éjection de lait des femelles Pw1m+/p− à un faible nombre de neurones synthétisant l’oxytocine dans l’hypothalamus. L’oxytocine est un neuropeptide qui favorise différentes étapes du processus de maternité: les contractions intra-utérines lors de l’accouchement, l’éjection de lait dans les glandes mammaires pour l’allaitement et le lien affectif entre la mère et l’enfant (Pedersen and Prange, 1979, Pedersen et al., 1982, Richard et al., 1991, Gimpl and Fahrenholz, 2001, Insel and Young, 2001, Donaldson and Young, 2008, Marlin et al., 2015). En concordance avec nos résultats, nous n’avons pas détecté de différences significatives en terme de nombre de
neurones exprimant l’oxytocine et de concentration plasmatique en oxytocine parmi les femelles $Pw1^{+/+}$, $Pw1^{m+/p+}$, $Pw1^{m+/p-}$ et $Pw1^{-/-}$. Néanmoins, les résultats démontrent, en accord avec la littérature, un retard de croissance des souris $Pw1^{m+/p-}$ et $Pw1^{-/-}$ qui tend à débuter à la naissance et à se maintenir à l’âge adulte. Les nouveaux-nés $Pw1^{m+/p-}$ ont une capacité de prise alimentaire semblable aux $Pw1^{+/+}$ ce qui suggère que le retard de croissance est intrinsèque aux animaux $Pw1^{m+/p-}$ et $Pw1^{-/-}$. Tous les organes des souris adultes $Pw1^{m+/p-}$ et $Pw1^{-/-}$ sont proportionnellement plus petits à l’exception du cerveau, dont la taille est similaire aux souris adultes de type sauvage et $Pw1^{m+/p+}$.

Les différences observées entre notre étude et celles précédemment établies (Li et al., 1999, Curley et al., 2004, Kim et al., 2013, Perera et al., 2015) peuvent s’expliquer d’une part sur la stratégie génétique utilisée. En effet, les groupes de Surani et Kim ont générés des mutants en insérant une cassette βgeo dans l’exon 5 et l’intron 5 de $Pw1$, respectivement (Li et al., 1999, Kim et al., 2013). Cette cassette contient le gène rapporteur LacZ ainsi que le gène de sélection Néomycine. Lors de la validation de notre modèle de souris KO conditionnel pour $Pw1$ nous avons constaté que la présence du gène Néomycine perturbait fortement l’expression de $Pw1$. En absence de recombinaison, le niveau de transcription de $Pw1$ était diminué de 70% et la protéine n’était plus détectable à partir du stade embryonnaire E14.5, et cela de manière irréversible. L’interférence liée à l’insertion de séquences codantes annexes telle que la Néomycine a été répertorié à de nombreuses reprises (Rijli et al., 1994, Gerard et al., 1999, Muller, 1999); les phénotypes engendrés par la délétion du gène d’intérêt s’en trouvent ainsi modifiés. Nous avons par conséquent excisé le gène Néomycine de nos modèles de souris $Pw1$ KO (Rodriguez et al., 2000). D’autre part, le fond génétique utilisé peut expliquer des variabilités phénotypiques observées pour une seule et même mutation. Li et al (Li et al., 1999) ont réalisé leur étude du comportement maternel sur des souris de souche 129Sv. Cependant, quelques années plus tard, Champagne et al (Champagne et al., 2009) ont reproduit leurs résultats sur un fond C57Bl/6J, identique au nôtre, ce qui favorise l’hypothèse de la stratégie de mutation. Ces auteurs ont d’ailleurs introduit une notion intéressante de ‘compensation’ où le défaut de comportement maternel tend à disparaître au fil des générations.
Conclusion

Les résultats présentés ici remettent en cause une hypothèse majeure du rôle de l'empreinte parentale chez les mammifères. *Pw1* ne peut plus donner de crédit à la théorie de coadaptation entre mère et enfant du moins après la naissance. Enfin, nos efforts fournis pour l'identification d'une transcription de l'allèle *Pw1* maternel ont aboutis via l'utilisation de notre nouveau modèle de souris KO pour *Pw1*. 
CHAPTER 1. GENOMIC IMPRINTING

1. THE DISCOVERY OF GENOMIC IMPRINTING

Genomic imprinting refers to an epigenetic process whereby genes are regulated differently as a function of whether they are inherited from the mother or the father. To date, about one hundred genes undergo this form of epigenetic regulation (Ferguson-Smith, 2011). In the animal kingdom, genomic imprinting is restricted to mammals suggesting that this form of epigenetic regulation plays a specific role in mammals; however, the evolutionary selective advantage of genomic imprinting is still under debate. Over 30 years ago, teams led by Solter and Surani assessed the equivalency of parental genomes (Surani and Barton, 1983, McGrath and Solter, 1984, Surani et al., 1984). They carried out pronuclear transplantation experiments and found that embryos generated from the fusion of two maternal genomes, called gynogenotes or parthenogenotes, gave rise to the development of embryonic tissues at the expense of extraembryonic tissues. In contrast, embryos derived from two paternal genomes, referred as androgenotes, mainly generated extraembryonic lineages. In both cases, embryonic lethality was demonstrated leading to the consensus that both maternal and paternal genomic contributions are necessary to support normal embryonic development. In parallel, Cattanach, Searle and colleagues generated embryos with uniparental disomy at particular chromosomal regions. They demonstrated the existence of multiple regions that carried parent-specific effects. The phenotypes they observed ranged from growth and behavioral abnormalities to death (Searle and Beechey, 1978, Cattanach et al., 1982, Cattanach and Kirk, 1985, Cattanach, 1986). Accordingly, several human disease conditions were suspected to rely on genomic imprinting defects due to their penetrance, which exhibit a parental-sex bias (Fig. 1) (Lubinsky et al., 1974). In 1991, the first imprinted genes were identified in the mouse: Igf2 (insulin-like growth factor 2), Igf2r (IGF2 receptor), and H19 (non-coding transcript) corresponding to one paternally and two maternally expressed genes, respectively (Barlow et al., 1991, Bartolomei et al., 1991, DeChiara et al., 1991). It was proposed that the expression of specific genes in a parent-of-origin manner ensured successful sexual mammalian reproduction.
**Figure 1: Imprinting disorder pedigree.** Inheritance of a paternally expressed gene: a schematic view. Family members are affected when the gene mutation is paternally transmitted. A similar pedigree is generated in reverse if the gene is maternally transmitted.
2. The Establishment of Genomic Imprinting

Genomic imprinting is driven by DNA methylation of specific regions of the genome of the progeny (Fig. 2) (Bartolomei et al., 1993, Ferguson-Smith et al., 1993, Stoger et al., 1993). This process is established during gametogenesis by DNA methyltransferase (DNMT) 3A coupled with DNMT3L (Okano et al., 1999, Bourc’his et al., 2001, Hata et al., 2002, Kaneda et al., 2004) and maintained upon cell division by DNMT1 (Li et al., 1993). De novo DNA methylation takes place on specific CpG rich islands to mark the parental origin. At this stage, differentially methylated regions (DMRs) called germline DMRs appear. CpG islands are methylated either on the paternal or maternal allele depending upon the locus. After fertilization, additional CpG islands undergo allele-specific methylation (referred as secondary DMRs). These epigenetic markings of parent-of-origin specific transcription are developmentally erased in the germ cells of the progeny and followed by de novo genomic imprinting.

3. Imprinted Gene Clusters

As exemplified in figure 3, imprinted genes are often found in clusters of 2-12 genes comprising of both maternally and paternally expressed genes. For several imprinted domains such as H19-Igf2, Igf2r, Dlk1-Dio3, Gnas, and Snrpn, mouse genetic studies have led to the identification of imprinting control regions (ICRs) (Fig. 3) (Wutz et al., 1997, Thorvaldsen et al., 1998, Yang et al., 1998, Lin et al., 2003, Williamson et al., 2006). Deletions of the germline DMRs have strong effects on allele-specific expression and transcriptional levels of the associated imprinted genes; hence the term ICR. DNA-binding sites for transcription factors such as CTCF1 participate in the ICR-dependent imprinting. For example, in the Igf2-H19 domain, imprinted genes rely upon a single enhancer. Paternal methylation of the ICR inhibits the binding of the insulator protein CTCF, which directs the enhancer to act upon the Igf2 promoter (Bell and Felsenfeld, 2000, Hark et al., 2000).

\[^1\] CCCTC-binding factor
**Figure 2: Imprinting life cycle.** Methylation marks at imprinting control region (ICR) are erased in the primordial germ cells and subsequently established according to the parental sex (blue: male; red: female). ICR1 and ICR2 are shown in grey (methylated) or white (non-methylated) as an example. Their status is maintained in all somatic cells of the offspring. Adapted from Reik and Walter (Reik and Walter, 2001).
Figure 3: Imprinted genes are found in clusters. A. Gnas domain. B. Snrpn/Ube3a domain. C. Dlk1/Dio3 domain. D. Igf2/H19 domain. A methylated ICR corresponds to either a maternal germline DMR (A and B), located at promoters, or a paternal germline DMR (C and D), located at intergenic regions. The clusters and genes are not drawn to scale. C: centromere. T: telomere. From Peters (Peters, 2014).
In contrast, the maternal ICR is unmethylated, which favors CTCF binding leading to a conformational change that prevents enhancer interaction with the \textit{Igf2} promoter. Thus, the \textit{H19} promoter is active only on the maternally inherited allele.

Imprinted gene clusters also harbor imprinted non-coding RNAs (ncRNAs) that act in cis to induce neighboring gene repression and this mechanism has been shown to operate in the cases of \textit{Airn} in the \textit{Igf2r} domain (Sleutels et al., 2002), \textit{Ube3a-as} in the Snrpn domain (Meng et al., 2012), and \textit{Kcnq1ot1} in the \textit{Kcnq1} domain (Mancini-Dinardo et al., 2006).

Lastly, even though imprinted genes are often found in clusters and share cis regulatory elements, each gene exhibits a specific spatio-temporal expression pattern. The brain is a major site of imprinted gene expression and displays a diverse and complex pattern of imprinted genes (Fig. 4). For example, several imprinted genes such as \textit{Dlk1} (delta-like 1 homolog) and \textit{Rasgrf1} (Ras-specific guanine nucleotide-releasing factor 1) switch or relax their allele-specific expression status at key developmental stages of brain development and in this case serves to regulate gene dosage that has been shown to be important for neuronal development and lifelong adult neurogenesis (Plass et al., 1996, Drake et al., 2009, Ferron et al., 2011).

4. IMPRINTED GENE FUNCTIONS

a. EMBRYONIC AND POSTNATAL GROWTH

While imprinted genes are often co-expressed in multiple tissues as well as in overlapping cell types, they encode a wide variety of proteins with intrinsically different cellular functions. Despite the variety of imprinted gene products, the characterization of mutant mouse models for imprinted genes revealed a common phenotype affecting both pre- and postnatal growth. Maternally expressed genes, such as \textit{Grb10} (growth factor receptor-bound protein 10), act to reduce growth whereas paternally expressed genes such as \textit{Igf2}, \textit{Rasgrf1}, \textit{Peg1/Mest} (mesoderm specific transcript), \textit{Gnasxl} (extra large stimulating guanine-nucleotide binding protein), and \textit{Dlk1}, act to promote growth (DeChiara et al., 1990, DeChiara et al., 1991, Itier et al., 1998, Lefebvre et al., 1998, Moon et al., 2002, Plagge et al., 2004, Garfield et al., 2011).
**Figure 4:** Schematic representation of imprinted gene expression pattern in the developing and adult brain. Each imprinted gene possesses unique characteristics for allele-specific expression within the brain during development and in the adult. E: embryonic. P: postnatal. CP: choroid plexus. Paternal allele expression is shown in blue. Maternal allele expression is shown in pink. Bi-allelic expression is shown in purple. For some genes, such as *Dlk1*, a subtype of cells exhibits loss of imprinting (purple middle line). For *Ube3a*, neuronal subtypes only exhibit parent-of-origin expression (pink middle line). Glia encompasses astrocytes, oligodendrocytes, and/or microglial cells. The asterix means that gene expression is restricted to specific nuclei.
In most cases, in utero growth defects do not lead to embryonic lethality unless imprinted genes are crucial for placenta development such as *Mash-2*, alias *Ascl2*, and *Peg10* (Guillemot et al., 1994, Guillemot et al., 1995, Ono et al., 2006). However, at birth, many mutant lines display a decrease in postnatal survival. For example, loss of *Magel2* (melanoma antigen family L2) impairs muscle function leading to impairment in pup suckling due to nipple attachment failure (Kozlov et al., 2007, Schaller et al., 2010). Newborns deficient for *Zac1* exhibit intrinsic growth retardation from stage E16.5; while their placenta is intact some organs are disproportionately bigger or smaller such as the brain and the lungs, respectively (Varrault et al., 2006). A high rate of early postnatal lethality is observed due to bone and lungs malformations. Importantly, even if some mutant mice catch-up weight postnatally, defects in fetal growth impact health later in life.

b. Metabolism

While paternally expressed genes enhance growth, they do not all converge toward the same metabolic features in adulthood. Obesity can arise upon loss of *Dlk1* and *NdN* due to their role in adipogenesis. Specifically, *Dlk1* inhibits adipocyte differentiation, whereas *NdN* regulates the number of adipocytes (Moon et al., 2002, Plagge et al., 2004). In contrast, a lean phenotype is observed when expression of *Dio3*, *Rasgrf1*, or *Gnasxl* paternal allele is disrupted (Clapcott et al., 2003, Font de Mora et al., 2003, Hernandez et al., 2006, Xie et al., 2006, Krechowec et al., 2012). A role for maternally expressed genes has also been found in fat metabolism. Increased fat occurs upon reduced energy expenditure, as seen in *Gnas* loss-of-function mouse models (Yu et al., 2000, Kelly et al., 2009), whereas a maternal deletion of *Grb10* leads to an increase in lean mass reflecting a resistance to obesity (Smith et al., 2007).

c. Behavioral effects

As mentioned in the previous section (b), the brain is a key site of expression of many imprinted genes.
**Figure 5: Imprinted genes involved in behavior and neuronal functions.** Peg: paternally expressed genes (blue). Meg: maternally expressed genes (pink). Minus (-): gene impacting negatively the corresponding phenotype. Plus (+): gene impacting positively the corresponding phenotype. The asterix denotes that anxiety in adulthood is decreased specifically due to the placental \( Igf2 \) P0 isoform. Adapted from Peters, 2014 (Peters, 2014).
Since the first reports in the late 90’s, a role for imprinted genes in the regulation of behavior have accumulated using mutant mouse models (Fig. 5). The first behavioral study carried out for an imprinted gene revealed that loss of Peg1/Mest affected maternal behaviors (Lefebvre et al., 1998). Specifically, mutant females did not adequately care for their pups, did not build a nest, and did not eat the placenta (placentophagia), which are all traits found in wildtype female mice (Kristal et al., 2012).

At present, imprinted genes have been associated to multiple neurological functions including neurogenesis, memory, cognition, sleep, olfaction, sociability, and neuronal excitability (Fig. 5). For example, Grb10 exhibits paternal allele expression specifically in the brain, whereas in other tissues Grb10 is maternally expressed. Upon paternal deletion of Grb10, mice display an increased social dominance resulting in facial barbering (Garfield et al., 2011). Giese et al (Giese et al, 2001) and Darcy et al (Darcy et al., 2014) demonstrated that Rasgrf1 knockout mice exhibit learning disabilities due to defects in the maturation of newborn neurons in the hippocampus. In some cases, loss of gene function leads to an “imprinting phenotype cascade” as seen following the deletion of the Igf2 P0 placenta-specific isoform that in turn results in an in utero nutrient imbalance leading to anxiety in adulthood (Mikaelsson et al., 2013).

d. STEM CELLS

Ferrón et al (Ferron et al., 2011) showed that Dlk1 gene dosage was necessary to maintain a quiescent pool of neural stem cells throughout life. Neural stem cells communicate with niche astrocytes in the subventricular zone2; both cell types switch Dlk1 expression status from mono- to bi-allelic postnatally (referred to as ‘relaxation of imprinting’), which leads to an increase in DLK1 protein levels and neural stem cell quiescence. Niche astrocytes secrete DLK1, whereas neural stem cells express the membrane-bound DLK1 isoform. Using cultured primary neural stem cells, grown as neurospheres, it was demonstrated that DLK1 regulated neural stem cell state extrinsically. In addition, neural stem cells in the dentate gyrus of the hippocampus3 exhibited maternal expression of Cdkn1c/p57. Upon loss of Cdkn1c/p57, the pool of

\[2\] One of two adult neural stem cell niches.

\[3\] The second adult neural stem cell niche
neural stem cells was also exhausted due to an increase in activation and proliferation (Furutachi et al., 2013).

Aside from neurogenesis, other adult tissues show a potential role for imprinted genes in the regulation of adult somatic stem cells. In particular, Venkatraman and colleagues showed that imprinting of the Igf2-H19 locus is essential to maintain the quiescent state of adult haematopoietic stem cells (Venkatraman et al., 2013). By deleting the H19-Igf2 germline DMR maternally, they induced bi-allelic expression of Igf2 and silencing of H19, which decreased the hematopoietic stem cell pool over time. Interestingly, using Zac1 loss-of-function mouse model Varrault and colleagues hypothesized that imprinted genes could be co-regulated as part of an imprinted gene network (IGN) during embryonic development (Varrault et al., 2006). Berg et al extended this putative co-regulation to adult somatic cells, including long-term hematopoietic stem cells (LT-HSCs), skin stem cells, and muscle stem cells (Berg et al., 2011). By differential gene expression analyses, they showed that members of the IGN are highly expressed during embryonic and early postnatal development, followed by a decline in expression levels in adult tissues with the exception of stem cells. In particular, upon acute and chronic stress leading to activation of LT-HSCs, the transcript level of imprinted genes dropped. Zacharek and colleagues also illustrated a co-regulation within the IGN using a Bmi14 knockout mouse in lung stem cells (Zacharek et al., 2011). Loss of Bmi1, known to abrogate self-renewal, indirectly induced a drastic change in imprinted gene expression. Specifically, imprinted genes part of the IGN, including Cdkn1c, H19, Dlk1, Zac1, Igf2, Grb10, Mest, and Ndn, were all upregulated. By silencing Bmi1 and some of these paternally and maternally expressed genes altogether they could obtain a partial phenotype rescue.

Mutant mouse model phenotypes are generally consistent with the symptoms seen in human imprinting disorders. Less than 10 major human imprinting syndromes with usually different etiologies are described thus far.

\[^{4}\text{Non imprinted polycomb ring finger oncogene}\]
In the mid 50's, the Silver-Russell syndrome (SRS) was described as an intrauterine and postnatal growth restriction condition (Fig. 6A and B) (Silver et al., 1953, Russell, 1954). Affected children also display asymmetric body growth associated with features such as protruding forehead at the age of 1-3 years and a triangular face. Genetic mutations associated with SRS are only known in two out of three patients. In 10% of cases, maternal uniparental disomy (UPD) at the IGF2/H19 locus leads to bi-allelic expression of H19 and absence of IGF2 expression. In 50-60% of cases, hypomethylation at the ICR of the IGF2/H19 locus leads to the repression of the normally active paternal IGF2 allele, while H19 undergoes a switch from maternal to bi-allelic expression.

In 1956, Prader-Willi syndrome (PWS) was described as a multisystem disorder characterized by hypotonia and neonatal feeding impairment followed by an excessive food intake leading to morbid obesity later in life (Fig. 6D) (Prader, 1956). In addition, patients show an intellectual developmental delay and variable cognitive disabilities. Due to genital hypoplasia, PWS patients undergo an incomplete puberty frequently associated with infertility as well as a short stature linked to growth hormone deficiency. PWS arises upon mutations at the SNRPN/UBE3A imprinted locus. In 70% of cases, a paternal deletion at chromosome 15q11-q13 is observed, while a maternal UPD (29%) or an imprinting defect (1%) are detected in the remaining cases.

Angelman syndrome (AS) was originally reported in 1965 (Hart, 2008). Children were compared to "puppets" due to ataxia, a condition detectable during the first years of life. Aside from this motor impairment, severe mental retardation, lack of speech, and moments of inappropriate laughter are characteristics of AS patients. The causal mutation abrogates UBE3A maternal allele expression. Four molecular etiologies were identified. Maternal deletion of UBE3A on chromosome 15q11-q13 accounts for 70% of AS cases. Paternal UPD is responsible for AS in 7% of patients. In 3% of cases, an epimutation or methylation defect leads to the repression of UBE3A expression off the maternal allele. In 11% of case, mutations within the maternal UBE3A copy itself are found.

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5 Lack of muscle control during voluntary movements.
In the late 1960’s, the Beckwith-Wiedemann syndrome (BWS) was characterized by three main phenotypes: exomphalos\(^6\), macroglossia\(^7\), and body overgrowth (Fig. 6C) (Wiedemann, 1969). Hemihyperplasia is also part of BWS features (Engstrom et al., 1988). Importantly, up to 8 years of age, affected individuals show an increase of tumors of embryonic origin including Wilms’ tumor\(^8\), hepatoblastoma, neuroblastoma, and rhabdomyosarcoma (Rump et al., 2005). The molecular causes of BWS originates on chromosome 11p15. A paternal isodisomy was first described by a loss of the maternal allele and duplication of the paternal allele concomitant with a loss and gain of methylation at the ICR2 (CDKN1C/KCNQ1) and ICR1 (IGF2-H19), respectively. Loss of methylation at the ICR2 induces an indirect transcriptional repression of CDKN1C alias p57. Gain of methylation detected on the maternal ICR1 leads to bi-allelic IGF2 expression.Mutations in CDKN1C leads to BWS in 5% of patients.

In addition to SRS, PWS, AS, and BWS, other imprinting disorders have been identified. Mutations at the ZAC1 locus on chromosome 6q24 can induce PLAG1 and HYMAI\(^9\) overexpression, whichs lead to transient neonatal diabetes (TND) with type II diabetes later in life and growth retardation (Mackay and Temple, 2010). Mutations at the DLK1 locus on chromosome 14q32 may induce development of two syndromes. Maternal UPD causes loss of expression of both DLK1 and RTL1 giving rise to intrauterine and postnatal growth retardation, early puberty and obesity (da Rocha et al., 2008). In contrast, paternal UPD engenders an increased RTL1 expression that induces dysmorphism and placentomegaly. On chromosome 20q13.3, mutations at the GNAS locus lead to GNAS downregulation. The symptoms of type 1 hypoparathyroidism include dysmorphism, cognitive deficit, obesity, and resistance to the parathyroid hormone (PTH) (Kelsey, 2010). Recently, a novel paternally expressed gene was identified named Snx14 (Huang et al., 2014). Using an in vitro approach, Huang and colleagues showed that SNX14 plays a key role in neuronal excitability and synaptic transmission. Mutations of SNX14 in humans lead to cerebellar atrophy and ataxia (Thomas et al., 2014, Akizu et al., 2015). Taken together, clinical conditions of humans with imprinted syndromes provide the basis for proposing a role for imprinted genes

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\(^6\) Abdominal wall weakness leading to prominent bowel and liver.

\(^7\) Large tongue that prevents correct feeding.

\(^8\) Rare type of kidney cancer.

\(^9\) Hydatidiform mole-associated and imprinted.
and their specific regulation in growth, metabolism, neuronal development, behavior, and tumorigenesis.

6. EVOLUTIONARY-BASED THEORIES TO EXPLAIN PARENTAL IMPRINTING

Gene dosage compensation is a process that is best exemplified by sex chromosomes. Males and females express X-linked genes at the same level to ensure proper development and reproduction. In Caenorhabditis elegans, females undergo a 2-fold downregulation of their X chromosomes while male sex chromosomes remain unchanged (Meyer, 2000). In Drosophila melanogaster, males undergo a 2-fold upregulation of their single X chromosome, whilst females do not modulate expression of their two copies of the X chromosome (Akhtar, 2003). In mammals, females silence one of the two X chromosomes, a process known as X chromosome inactivation (XCI) (Lyon, 1961). Genomic imprinting appeared in mammals along with XCI. These two epigenetic regulations share similarities in terms of mechanisms to modify histones and silence genes; it was notably proposed that they have co-evolved (Lyon, 1999). However, imprinted genes are autosomal. They display unique expression patterns and diverse phenotypes when mutated. The fact that some genes are strictly expressed from the paternal or the maternal allele in some cells, tissues, or whole individuals does not lead to an obvious selective advantage for this form of epigenetic regulation. Many hypotheses emerged to explain why this extra epigenetic regulation occurred in mammals in such a restricted number of genes.

In the evolvability model, silencing specific genes may favor selection of mutations over multiple generations (McGowan and Martin, 1997, Beaudet and Jiang, 2002). Mammals would thus increase genomic variability and interactions of new mutations. However, significant criticism suggests that it is unlikely that this process would be restricted to very few genes and also restricted to mammals. It is believed that a selective pressure began with a common ancestor of monotreme, marsupial and eutherian mammals to imprint genes involved in early postnatal feeding (Reik and Lewis, 2005). Concomitantly with the appearance of placental mammals, genomic imprinting expanded to growth-related genes. This hypothesis is consistent with the idea that imprinted genes control
resource allocation between mother and offspring to balance intra-uterine development and postnatal breast-feeding.

Weisstein suggested in 2002 that genomic imprinting protects unfertilized eggs from spontaneously initiating development and implantation in the absence of fertilization (Weisstein et al., 2002). This idea, referred to as the ovarian time bomb hypothesis (OTB), does not satisfactorily explain the silencing of paternally transmitted genes as many paternally expressed genes are not involved in trophoblast growth.

In 1991, Moore and Haig proposed the kinship theory (Moore and Haig, 1991). The idea is that both parental genomes are in conflict with regard to expending maternal resources during gestation and nursing. In this model, the mother’s drive is to produce multiple progenies from different fathers to maximize her genetic heirs by surviving multiple pregnancies though a balance of in utero and postnatal resource expenditure. In contrast, the father “wants” his own offspring to be as big and as strong as possible. The conflict arises from maximizing pup size at the expense of the mother's survival. Accordingly, paternally expressed genes promote growth. In contrast, the mother will spare her resources for an equal distribution between all her progenies. Many maternally expressed genes are growth suppressors. This hypothesis is appealing, but if not carefully regulated, the two genetic strategies can kill the mother. Vrana and colleagues (Vrana et al., 1998) assessed the kinship theory by studying imprinting in two distinct *Peromyscus* species. *Peromyscus polionotus* is a monogamous species whereas *Peromyscus maniculatus* is a polyandrous one and only the latter exhibits sexual dimorphism. Hybrids of these two *Peromyscus* species show strong growth defects. Crossing a male *P. polionotus* with a female *P. maniculatus* leads to an important growth restriction as if maternally expressed genes were bi-allelically expressed. Crossing a female *P. polionotus* with a male *P. maniculatus* leads to a lethal oversize phenotype as if paternally expressed genes were bi-allelically expressed. Thus, monogamous *P. polionotus* was thought to demonstrate loss-of-imprinting in a parental conflict-free environment. However, authors showed using SNP sequencing analysis that at least three genes, *Igf2*, *H19*, and *Igf2r*, were similarly imprinted in both *Peromyscus* species meaning that the kinship theory may not be applicable in this specific context.
A related and second well accepted theory of imprinting is called coadaptation (Curley et al., 2004, Keverne and Curley, 2008). In 2004, Curley and colleagues (Curley et al., 2004) confirmed that the paternally expressed gene \textit{Pw1/Peg3} acts as a growth enhancer since mutant pups showed growth restriction and a lower milk intake. They also validated maternal care and nursing impairments in mutant mothers. While all these phenotypes fit the parental conflict theory, authors also noticed that the paternal deletion of \textit{Pw1/Peg3} in both mother and offspring was drastically increasing pup death rate compared to pup-specific or mother-specific \textit{Pw1/Peg3} mutation. At weaning age, only 6% of the progeny survived instead of 70%, respectively. In this study, Curley \textit{et al} (Curley et al., 2004) claim that \textit{Pw1} plays an essential role in mother-offspring interaction during pregnancy. Notably, \textit{Pw1} expression in the offspring enhances maternal weight gain during pregnancy while \textit{Pw1} expression in the mother enhances pre- and postnatal growth (Curley et al., 2004). Resources allocation is thus optimized between mother and offspring to ensure successful mammalian reproduction.

Additionally, as the allele-specific expression is a dynamic process for some imprinted genes such as \textit{Dlk1}, it has been proposed that genomic imprinting allows adaptation to environmental changes \textit{in utero} as well as in adulthood (Ferguson-Smith, 2011, Ferron et al., 2011).

These hypotheses on genomic imprinting in evolution were primarily based on integrating functions of paternally versus maternally expressed genes. As the majority of imprinted genes clusters are controlled by maternal DMRs, it is possible that genomic imprinting reflects a bias towards maternal control (Arnaud, 2010). Paternal DMRs are attributed to only three imprinted loci: \textit{H19/Igf2}, \textit{Rasgrf1}, and \textit{Dlk1}.
CHAPTER 2. PW1/PEG3, A PATERNALLY EXPRESSED GENE

1. THE PW1/PEG3 GENE CLUSTER

In 1996, two independent studies led to the identification of Pw1, also named as Peg3 for paternally expressed gene 3 (Kuroiwa et al., 1996, Relaix et al., 1996). Kuroiwa and colleagues (Kuroiwa et al., 1996) screened cDNA libraries from parthenogenetic and wild-type E9.5 embryos to identify genes with a parent-of-origin specific regulation. Relaix et al (Relaix et al., 1996) performed a differential screen to isolate transcripts expressed early in the myogenic lineage, prior to the expression of the lineage determination genes for muscle known as the myogenic regulatory factors (MRFs). Pw1/Peg3 was the first imprinted gene described on mouse proximal chromosome 7 with its human PW1 homolog on chromosome 19q13.4 (Kim et al., 1997). It is now known to be part of a cluster of imprinted genes in this chromosomal region (Flisikowski et al., 2010, Ling et al., 2011, He and Kim, 2014). The mouse Pw1 imprinted domain contains 4 protein-coding genes (Zim1, Zim2, Pw1, and Usp29), and 3 non-coding RNAs (APeg3, Zim3, and Zfp264) (Fig. 7). The Pw1 domain comprises 500Kb of genomic sequences that encode for proteins that contain Kruppel type (C2H2) zinc fingers (Fig. 7). Notably, Zim1 is a zinc finger gene preferentially expressed from the maternal allele, but bi-allelically expressed in the neonatal and adult brain (Kim et al., 1999). While the function of Zim1 remains unknown, Pw1 expression has been shown to downregulate Zim1 (Ye et al., 2014). Using embryonic fibroblasts (MEFs) derived from the Pw1 mutant mouse, Ye and colleagues demonstrated recently that Pw1 binding to the largest exon of Zim1 is associated with repressive H3K9me3 marks on Zim1. Zim2 is a second zinc finger gene of unknown function, maternally expressed in the neonatal mouse brain but bi-allellically expressed in testis (Kim et al., 2000a, Kim et al., 2004). A detailed description of Pw1 is presented in section 2.3.

10 Cys(2)His(2) is the most frequent DNA-binding motif among transcription factors found in eukaryotes.
**Figure 7:** *Pw1* locus, an imprinted gene cluster. *Pw1, APeg3, Usp29,* and *Zfp264* are paternally expressed. *Zim1, Zim2,* and *Zim3* are maternally expressed. Bottom panel represents *Pw1* exons in light blue.
**Usp29**, alias **Ocat**, is a paternally expressed gene encoding a ubiquitin-specific peptidase (Kim et al., 2000b, Szeto et al., 2000). **Usp29** expression is predominantly detected in embryonic and fetal tissues, notably in the developing bones (Szeto et al., 2000). In the adult mouse brain, **Usp29** marks neuronal cells in the hippocampus, hypothalamus, cerebral cortex, habenula\(^{11}\), ependymal cells, and choroid plexus cells (Kim et al., 2000b). Recent studies have revealed a potential tumor suppressor role for **Usp29**. Upon oxidative stress, **USP29** gets actively transcribed, via JTV1/FBP\(^{12}\) co-activation, to deubiquitinate and thus stabilize p53 (Liu et al., 2011). Martin and colleagues also showed that **USP29** is important for Claspin stability, a protein involved in DNA damage checkpoint and control of DNA replication (Martin et al., 2015).

In addition to protein-coding genes, the **Pw1** domain contains 3 non-coding RNAs. **APeg3**, paternally expressed, is oriented antisense to **Pw1**. **APeg3** expression is specific to vasopressinergic magnocellular neurons in the hypothalamus (Glasgow et al., 2005). It is co-regulated with **Pw1** upon osmotic challenge and can downregulate **Pw1** transcription and protein levels *in vitro* (Glasgow et al., 2005, Choo et al., 2008). **Zim3**, a maternally expressed gene, is oriented antisense to **Usp29**. **Zim3** is expressed primarily in the testis; its function is unknown, but **Zim3** may downregulate **Usp29** (Kim et al., 2001). **Zfp264** is a non-coding paternally expressed RNA in the neonatal and adult brain with an unknown role (Kim et al., 2001).

In 2011, Ling *et al* identified a non-imprinted member of the **Pw1** cluster: **miR-3099** (Fig. 7) (Ling et al., 2011). This micro-RNA, located within **Usp29** large intron, is broadly expressed at embryonic stage E11 and becomes restricted to the external cortical layer of the brain at E17.5. In adult mice, **miR-3099** expression is high in the pancreas, maintained in the brain, and low in many other organs (Ling et al., 2011).

Two bidirectional promoters have been identified in the **Pw1** domain; one - for **Pw1** and **Usp29** transcription and another - for **Zim3/Zfp264** transcription. Bidirectional promoters are identified as “head-to-head” configuration genes with an opposite transcriptional direction. Importantly, around 10% of the coding genes have

\(^{11}\) **Habenula** is a brain region involved in decision-making.

\(^{12}\) FBP means far upstream element binding protein
bidirectional promoters that are unique to the mammalian genome (Wakano et al., 2012). Many of the genes driven by bidirectional promoters closely participate in DNA repair, chromosome assembly, cell cycle control and metabolism regulation. The assumption is that the genes related to one bidirectional promoter are co-regulated to coordinate a related biological process (Adachi and Lieber, 2002). Zim3 and Zfp264 are not expressed from the same parental allele and no correlation has been established thus far. However, as it is described in section 2.1, Pw1 and Usp29 share a similar expression pattern in the adult brain and likely participate in a related biological process.

The human PW1 domain shows some striking structural differences as compared to the murine domain. Specifically, ZIM1 is not conserved but is found in mammals with large litter sizes, including rodents (Kim et al., 2007). ZIM2 is switched from a maternal to paternal allele-specific expression. The human ZIM2 shares a promoter and the first seven exons with PW1 (Kim et al., 2000a, Kim et al., 2004). USP29 does not have the same open reading frame (ORF) and is restricted to the larger exon of mouse Usp29 (He and Kim, 2014). The human ZIM3 and ZNF264 retain ORFs and thus serve as protein-coding genes. Their expression pattern is nonetheless conserved across species (Kim et al., 2001). MIMT1, also called Mim1 for MER1 repeat-containing imprinted transcript 1, is a paternally expressed non-coding RNA; it corresponds to the two first mouse Usp29 exons (Kim et al., 2007). In cattle, MIMT1 is essential for fetal development and perinatal offspring survival (Flisikowski et al., 2010, Flisikowski et al., 2012, Venhoranta et al., 2013). Overall, Pw1 and APeg3 are structurally the most conserved genes in the Pw1 imprinted domain between mouse and human.

2. IMPRINTING MECHANISMS

Since the discovery that multiple imprinted genes surround the Pw1 locus, several groups have examined the chromatin marks that could be responsible for parent-of-origin allele-specific expression of each Pw1 domain gene. It was unknown whether a shared imprinting control center (ICR) was regulating the entire cluster. Three DMRs have been identified in the Pw1 domain on Pw1, Zim2, and Zim3. However, during oogenesis, the only CpG island undergoing methylation is the Pw1-DMR (Huang and Kim,
2009). Consequently, the *Pw1* germline DMR is referred to as a maternal DMR. In 2012, Kim and colleagues demonstrated that *Pw1*-DMR acted as an ICR for the entire *Pw1* cluster (Figure 8) (Kim et al., 2012, Thiaville et al., 2013b). By deleting a 2.5Kb region containing multiple YY1-binding sites in the first *Pw1* intron, which corresponds to a third of the entire *Pw1*-DMR, they showed that expression of several imprinted genes of the *Pw1* domain were affected. Specifically they observed that *Pw1* expression was downregulated. Furthermore, the imprinting status of Zfp264 switched from paternal to maternal expression in the neonatal brain. Zim2 became bi-allelically expressed and *Zim1* expression was upregulated. Partial embryonic lethality was described and mice exhibited a smaller body weight. However, these changes were observed only for paternally derived mutant mice, whereas maternal deletion did not alter gene expression. These results are surprising since one may expect bi-allelic expression of *Peg3* and *Usp29* in addition to *Zfp264*. Nonetheless embryos with a homozygous loss of *Pw1*-DMR were not viable (Kim et al., 2012). What emerges from these studies is that the regulation and mechanistic control of the ICR is complex: it controls both imprinting status and expression of multiple genes.

As *Pw1* was the first imprinted gene identified in this cluster and is conserved across mammalian species, it has been the most extensively studied gene within the *Pw1* domain. Three main team leaders focused their research on it: Surani A. associated with Keverne E.B. in Cambridge (UK), Kim J. associated with Teruyama R. in Baton Rouge (Louisiana, USA), and Sassoon D. associated with Marazzi G. here in Paris (France).

### 3. *Pw1*, a Protein-Coding Zinc Finger Gene

As reviewed in section 2.1, the first published studies on *Pw1* appeared almost 20 years ago (Fig. 9A).
Figure 8: Imprinting control of the Pw1 domain. Model proposed by He and Kim, G&I, 2014 (He and Kim, 2014). The shared enhancer ECR18 directs transcription of Pw1 domain genes depending on the methylation status of the Pw1-DMR. On the paternal allele, Pw1-DMR is not methylated allowing the dominant use of ECR18 of the paternally expressed genes (Thiaville et al., 2013b). On the maternal allele, Pw1-DMR is methylated abrogating Pw1 and Usp29 expression. ECR18 can consequently target additional genes including Zim1 and Zim2. Whether this model is applicable to Zim3, Zfp264 and A Peg3 requires further investigation.
Figure 9: Scientific publications linked to Pw1 expression and function. A. Chronology of scientific reports associated with Pw1. This is a non-exhaustive list of studies carried out on human samples (upper panel) and animal models, principally mice (bottom panel). B. Keyword density reflecting Pw1 functions based on published works on both human and rodents since 1996.
Pw1 transcription starts upon gastrulation in the ectoderm, mesoderm, and extraembryonic tissues (Relaix et al., 1996). At embryonic day E9.5, Pw1 is expressed in the forming diencephalon, branchial arches, somites, and gut primordium (Kuroiwa et al., 1996, Relaix et al., 1996). Concomitantly Pw1 is downregulated in the heart. At stage E12.5 Pw1 expression is detected in the hypothalamus, pituitary gland, facial skeleton, tongue, vertebral cartilage, and gut. In mouse and human placenta, PW1 expression is detected in trophoblast cells (Hiby et al., 2001). From birth through adulthood, the mouse brain retains a high level of Pw1 expression with marked expression in the hypothalamus, pituitary gland, choroid plexus, amygda13, and lateral septum14; Pw1 is weakly expressed in the dentate gyrus, cortex, and Purkinje cell layer in the cerebellum. In the brain, apart from the choroid plexus epithelial cells, Pw1 is mostly restricted to the neuronal lineage (Kuroiwa et al., 1996, Relaix et al., 1996, Li et al., 1999). The adult heart shows Pw1 expression in the atria and in the future epicardium (Relaix et al., 1996). Importantly, skeletal muscle maintains Pw1 expression during embryonic and postnatal life, although levels are markedly reduced in postnatal tissue. In adult skeletal muscle, PW1 identifies the two muscle stem cell populations known as satellite cells and PW1+ interstitial cells (PICs) (Mitchell et al., 2010, Pannerec et al., 2013, Formicola et al., 2014). In 2011, we generated a transgenic Pw1IRESnLacZ mouse model as a tool to easily follow Pw1 expression without loss of function (Besson et al., 2011). These studies revealed that PW1 expression is restricted to adult stem and progenitor cells in a wide range of tissues including the skin, testis, hematopoietic system, and central nervous system, in particular the subventricular zone which is a site of neural stem cells (Besson et al., 2011). In parallel, Berg et al showed that Pw1 was exclusively expressed in several adult somatic stem cells as one of 10 imprinted genes that was referred to as the imprinted gene network (IGN) (Varrault et al., 2006, Berg et al., 2011).

13 Amygdala is involved in fear response; it integrates emotions
14 Lateral septum is associated with social behavior, anxiety, fear conditioning, and reward
b. Functions

The primary sequence of the Pw1 mRNA encodes a large protein containing 12 zinc fingers (Kuroiwa et al., 1996, Relaix et al., 1996). Consistent with the presence of zinc fingers, PW1 has been shown to localize to the nucleus of cells in which it is expressed (Relaix et al., 1996). These data suggest that PW1 acts, at least in part, as a transcription factor (Fig. 10). Thiaville and colleagues identified PW1 target genes in the adult mouse brain using chromatin immunoprecipitation followed by next generation sequencing (NGS) (Thiaville et al., 2013a). They identified putative target genes involved in development (Dusp1, Mapk14, Wnt9a), mitochondrial activity (Ndufs7, Ndufs8, Ppargc1b, Sdhb), body growth, and behavior (Grb10).

In the late 90’s, Li and colleagues (Li et al., 1999) generated the first Pw1 mutant mouse model. To abolish Pw1 expression and to follow Pw1 allele-specific expression pattern, they inserted a βgeo15 cassette into Pw1 coding exon 5. By detecting reporter gene expression solely upon paternal inheritance of the mutated allele they confirmed Pw1 imprinting status. More recently, Kim et al established a second Pw1 mutant mouse model using the same strategy but targeting Pw1 intron 5 (Kim et al., 2013). Lastly, the Teruyama group recently published a study on a third Pw1 mutant mouse, a constitutive Pw1 knockout mouse model (Perera et al., 2015). Their resulting findings are discussed in section 6 as they do not address Pw1 function.

✓ Growth and Metabolism

From fetal stage E17.5 loss of Pw1 lead to a reduced postnatal growth (Li et al., 1999, Curley et al., 2005, Kim et al., 2013, Perera et al., 2015). At postnatal day 28, mutant pups showed a 35% decrease in body weight with a proportional reduction in the size of all organs as compared to wild-type, with recovery of the weight defect later in adulthood (Li et al., 1999).

15 βgeo cassette contains a LacZ reporter gene and an ES-cell selection Neomycin gene
**Figure 10: Schematic representation of PW1 domains.** *Pw1* encodes 12 unusually spaced zinc fingers, aa: amino acid. PHRD: Pro-His repeat domain. PRD: Pro repeat domain. RER: Glu-Arg repeat. SCAN: leucine rich, protein interaction motif. ZF: zinc finger. Adapted from Relaix et al, 1996 (Relaix et al., 1996).
Concomitant with the postnatal growth restriction, *Pw1* mutant females reached puberty slower than wild-type (Curley et al., 2004, Curley et al., 2005). Despite a reduced body weight, one-year old *Pw1* mutant mice showed increased white abdominal and subcutaneous fat with a concomitant decrease in muscle weight (Curley et al., 2005). Six-months old mutant mice exhibited lower oxygen consumption and lower body temperature, suggesting a lower metabolic activity (Curley et al., 2005). While they did not detect any changes in plasma level of insulin, glucose, free fatty acid (FFA), corticosterone, IGF1, T3 and T4 hormones, they observed a drastic increase in leptin hormone levels in *Pw1* mutant mice consistent with increased body fat (Curley et al., 2005). Leptin is secreted by adipocytes in the blood stream to target hypothalamic neurons in the arcuate nucleus. The hypothalamus regulates energy homeostasis, food intake and expenditure in response to leptin levels (Schwartz et al., 2000, Spiegelman and Flier, 2001). This metabolic control is lost in obese patients since their high plasma leptin levels induce leptin resistance leading to an energy imbalance, and a similar scenario is proposed to occur in *Pw1* mutant mice. In addition, Curley and colleagues found that neuropeptides regulating appetite including POMC16, NPY17, MCH18, and orexin were differentially expressed upon loss of *Pw1* (Curley et al., 2005). However they did not find the exact functions that *Pw1* serves in growth and metabolism and as such remains to be determined if these changes are direct or indirect consequences of loss of *Pw1* function.

**BEHAVIOR**

*Pw1* has been linked to maternal care (see figure 5). Upon loss of *Pw1*, Li *et al* (Li et al., 1999) reported that mutant females did not properly care for their offspring leading to an increased pup death rate and compromised postnatal weight gain. Loss of *Mest/Peg1* function also was shown to lead to maternal behavior defects (Lefebvre et al., 1998). Subsequently, Champagne and colleagues showed that loss of *Pw1* reduces exploratory level and increases both anxiety and aggressivity (Champagne et al., 2009). Given that PW1 binds Grb10 promoter in the brain and that *Grb10* is downregulated in absence of

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16 POMC: pro-opiomelanocortin precursor polypeptide
17 NPY: neuropeptide Y
18 MCH: melanin-concentrated hormone
Pw1 (Thiaville et al., 2013a), the aggressivity observed in Pw1 mutant mice is consistent with the increased social dominance seen upon paternal loss of Grb10 (Champagne et al., 2009, Garfield et al., 2011). We note that only the paternal Grb10 allele is expressed in the brain and that in contrast to Pw1 loss-of-function mouse model, maternal loss of Grb10 leads to an overgrowth phenotype. Thus, PW1 may act as a transcriptional activator of Grb10 specifically in the brain.

✓ LACTATION AND NURSING

In addition to maternal care, Li et al (Li et al., 1999) reported that Pw1 function is required for milk ejection in postpartum females. Wild-type pups born to Pw1 mutant females exhibited a reduced postnatal growth up to weaning, however they showed a significant growth after weaning consistent with a delayed postnatal growth due to nursing defects. This defect in milk ejection was explained by a reduced number of oxytocin neurons in the paraventricular nuclei (PVN) and the supraoptic nuclei (SON) of the hypothalamus, which was proposed to also lead to impairment in maternal behavior. Oxytocin is a neuropeptide enhancing social behavior and bonding (Pedersen and Prange, 1979, Pedersen et al., 1982, Richard et al., 1991, Insel and Young, 2001, Donaldson and Young, 2008, Marlin et al., 2015). It is synthesized by the magnocellular neurons in the hypothalamus and released in pulses from two specific compartments (Fig. 11). In the brain, parenchyma oxytocin is released via the dendrites where it stimulates maternal behavior, social interaction, and confidence. In the pituitary gland, oxytocin is released into the blood stream to promote contraction of myoepithelial cells in the mammary gland and smooth muscle cells in the uterus (Richard et al., 1991, Gimpl and Fahrenholz, 2001). Hence parturition and nursing are optimized for progeny survival. However, a defect in oxytocin release alone is unlikely to lead to a complete loss of maternal care behaviors since oxytocin knockout mice as well as mice deficient for the oxytocin receptor do not exhibit an impairment in maternal behavior and parturition (Nishimori et al., 1996, Winslow et al., 2000, Ferguson et al., 2001, Takayanagi et al., 2005, Higashida et al., 2010). Thus, the reduced number of oxytocin neurons in Pw1 mutant mice, potentially correlated with a global reduced oxytocin synthesis, may solely explain the milk ejection defect. In addition to potential defects in the ability of the mother to eject milk, Curley and colleagues also showed that Pw1
mutant pups were unable to suckle properly, which was proposed to contribute to their perinatal lethality (Curley et al., 2004, Kim et al., 2013). Whether this phenotype is due to a defect in muscles necessary for suckling or feeding stimulation in the pups remains unaddressed. Interestingly, in case of mid-gestation starvation, a critical environmental stress condition, Pw1 is important for sparing fetal brain functions via a compensatory gene expression of the placenta to the fetal hypothalamus (Broad and Keverne, 2011, Radford et al., 2012).

Taken together, Surani and Kim groups favored the idea that Pw1 is essential for a successful mammalian reproduction. While both groups focused their research on metabolic, behavioral and neuronal functions, other teams including ours investigated Pw1 function in different lineages such as skeletal muscle in vivo and in vitro.

✓ **CELL STRESS**

Pw1 has been demonstrated to participate in two cell stress signaling pathways, p53 and TNF/NFκB, promoting cell death or survival, respectively (Relaix et al., 1998, Relaix et al., 2000, Coletti et al., 2002, Schwarzkopf et al., 2006). Relaix and colleagues showed that PW1 binds to TRAF2 to induce NFκB activation and nuclear translocation in cultured cells (Relaix et al., 1998). They also demonstrated that PW1 interacts with Siah1a, and that in presence of p53 and c-myc, PW1 directs cells towards apoptosis (Relaix et al., 2000). In addition, it has been demonstrated that PW1 mediates Bax translocation from the cytosol to the mitochondria to promote a p53/TNFα-dependent block of myogenic differentiation that was proposed to contribute to cachexia19 (Deng and Wu, 2000, Coletti et al., 2002, Schwarzkopf et al., 2006). Nicolas et al generated a dominant negative mutant mouse model for Pw1 (ΔPw1) targeting specifically the skeletal muscle lineage (Nicolas et al., 2005). The ΔPw1 transgene included the first 592 amino acids encoded by Pw1 exon 9 out of 1380, which blocks p53-mediated apoptosis and TNFα-mediated NFκB activation (Relaix et al., 1998, Relaix et al., 2000). Mutant pups showed severe muscle atrophy and a dramatic postnatal growth reduction leading to postnatal lethality by 3 weeks of age.

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19 Cachexia or muscle wasting is a public health issue in several chronic disease conditions and aging.
Figure 11: Oxytocin release: multiple targets for driving parturition and motherhood. Oxytocin (OT) expressing magnocellular neurons are located in the paraventricular nuclei (PVN) and the supraoptic nuclei (SON) of the hypothalamus. Each oxytocin neuron has 2-4 dendrites, which contain 15,000 vesicles of oxytocin. The axon extends into the posterior pituitary where it divides into a large number of terminals (2,000/cell) with a total of 500,000 vesicles. Upon activation, oxytocin is released into the brain parenchyma via the dendrites and into the blood via the axons. Pituicytes are wrapped around terminals that control access to the blood vessel. Adapted from Berridge MJ, Cell Signalling Biology, 2012.
They also identified an altered muscle stem cell behavior in vivo. In addition, Yamaguchi and colleagues showed Pw1 is upregulated in peri-ischemic neurons as a downstream cell death-mediator of p53 in response to brain ischemia (Yamaguchi et al., 2002). Under hypoxic conditions in vitro, Pw1 overexpression similarly reduced neuronal cell viability. Taken together, these data are consistent with a role for Pw1 as a mediator of both intrinsic and extrinsic cell stress responses.

**Cancer**

The involvement of PW1 in NFkB activation and p53-mediated apoptosis and growth arrest suggests a role for PW1 as a tumor suppressor. Consistent with this notion, Pw1 was found to be silenced in gynecologic cancer cell lines (Dowdy et al., 2005). In addition, Feng and colleagues (Feng et al., 2008) showed that PW1 was one the most downregulated genes in human ovarian cancers. Cervical cancer invasiveness was correlated with Pw1 hypermethylation (Nye et al., 2013). Otsuka et al identified changes in Pw1 methylation in human glioma biopsies (Otsuka et al., 2009); Pw1 expression levels were lower in the most severe grade tumors as compared to lower grades. Pw1 was also shown to inhibit the Wnt signaling pathway by promoting β-catenin stabilization, regulating glioma growth (Jiang et al., 2010). Interestingly, Pw1 promotes Decorin-mediated autophagy in mouse and human endothelial cells (Buraschi et al., 2013). Decorin is a secreted proteoglycan that inhibits angiogenesis and tumorigenesis. While these data point to a tumor suppressor role for Pw1, the primary cellular target(s) as well as the sequence of transforming events leading to a cell cycle dysregulation remain unresolved.

**Stem Cells**

Mesoangioblasts have the unique potential to home and treat muscle injury and disease upon systemic injection (Tajbakhsh, 2009, Tedesco et al., 2010). Bonfanti and colleagues (Bonfanti et al., 2015) demonstrated that Pw1 expression is required for the stem cell competence of human, mouse, and dog mesoangioblasts. They showed that silencing PW1 in mesoangioblasts blocked their myogenic differentiation in vitro and abrogated their ability to cross the vessel wall upon systemic delivery in mice. Mechanistically,
they revealed that PW1 binds to the junctional adhesion molecule-A (JAM-A) promoter as a transcriptional repressor allowing trans-vessel migration. Aside the skeletal muscle stem cell niche, multiple adult mouse tissues were shown to contain PW1 positive stem and progenitor cells, including the central nervous system (Besson et al., 2011). In this particular context, Pw1 function has not been yet investigated. However, it was shown through engrafting experiments that only PW1+ bulge cells of the hair follicle are capable of stem cell self-renewal when compared to bulge cells that do not express PW1 (Besson et al., 2011).

As mentioned earlier Pw1 is the best studied gene within the Pw1 domain. According to its complex expression pattern during embryonic development and postnatal life, it is not surprising to see that PW1 roles range from animal behavior, to growth, lactation, metabolism, stem cells, cell stress and cancer. While some of these functions are clearly related, ie. cell stress and cancer, it is still hard to integrate and understand how and when all these processes are disrupted in absence of PW1. In addition, appart from mesoangioblasts and bulge stem cells, Pw1 is expressed in many other stem/progenitor cells where its function has not been elucidated yet, in particular in neural stem/progenitor cells.
CHAPTER 3. NEURAL STEM CELLS

More than a decade ago, the brain was thought to be a quiescent pool of highly specialized cells with little to no regenerative capacities. However, Reynolds and Weiss (Reynolds and Weiss, 1992, 1996) demonstrated that cells derived from the embryonic mouse brain have stem cell potentials in vitro. By growing cells in suspension with specific growth factors, they were able to generate neurospheres and to amplify neural precursors that were capable of being passaged and expanded in culture. The acceptance that the CNS contains competent stem cells was reinforced with the identification of multipotent cells in the adult rodent brain (Doetsch et al., 1999, Seri et al., 2001). One major focus of research has been the stem cell microenvironment or ‘niche’ where neural stem cells reside.

1. THE ADULT NEURAL STEM CELL NICHEs

Two distinct neural stem cell niches have been identified in the CNS of adult rodents: the ventricular-subventricular zone (SVZ) next to the lateral ventricles and the subgranular zone (SGZ) in the hippocampus (Doetsch et al., 1999, Seri et al., 2001). Both niches have different structures and functions.

a. ANATOMY

✓ THE SVZ NEURAL STEM CELL NICHE

The adult neural stem cell of the SVZ is referred to as B1 cell (Fig. 12). Each B1 cell spans three domains (Fuentealba et al., 2012). In the proximal/apical domain, B1 cells directly contact the cerebrospinal fluid (CSF) of the lateral ventricles by a single non-motile primary cilium (Mirzadeh et al., 2008, Shen et al., 2008). This process is surrounded by multiciliated ependymal cells that line the ventricular walls (Mirzadeh et al., 2008). The intermediate domain contains the B1 cell body as well as transit amplifying cells and neuroblasts.
Figure 12: The ventricular-subventricular neural stem cell niche (SVZ). A: neuroblast; As: astrocyte; B1: neural stem cell; BV: blood vessel; C: transit amplifying cell; E: ependymal cell; M: ramified microglia; P: pericyte; RMS: rostral migratory stream; SVZ: subventricular zone; V: lateral ventricle; VZ: ventricular zone. Double arrow: neuronal projections of non-neurogenic regions such as raphe nuclei, substantia nigra, ventral tegmental area, and striatum. A linear orange process bridging blood vessel and ependymal cells represents fractones.
Microglia, the resident immune cells specific to the CNS, are also present. Finally, the distal domain links the B1 cell with the vasculature (Shen et al., 2008). In fact, the V-SVZ niche is located between two dynamic fluid compartments: the cerebrospinal fluid and an extensive vascular plexus all along the distal domain of the niche. The blood-brain barrier (BBB) is composed of endothelial cells, pericytes, and astrocyte end feet. Surprisingly, the BBB appears to be leaky where clusters of B1 and transit amplifying cells are found (Tavazoie et al., 2008). This punctual lack of glial end feet and pericyte coverage is a feature unique to the SVZ. Of note, like B1 cells, fractones bridge apical and distal domains with a close contact with each SVZ cell. These laminin-rich structures containing heparan sulfate proteoglycans (HSPGs) are part of the niche extracellular matrix (ECM) (Mercier et al., 2002).

✓ The SGZ neural stem cell niche

This second neural stem cell niche is distinguished from the SVZ by its location and organization. The neural stem cells of the SGZ, called radial astrocyte (RAs), are present in the dentate gyrus of the hippocampus. The RAs are highly polarized and also span at least three domains (Fig. 13). The proximal domain, facing the hippocampal hilus, contains the RA primary cilium, blood vessels, and is in direct contact with RAs, and to some extent hilar mossy cells and interneurons. Compared to the SVZ, angiogenesis is highly active in this domain with intense vascular remodeling (Palmer et al., 2000). In the intermediate domain of the dentate gyrus, RAs extend long processes that contact other neural stem cells (Fuentealba et al., 2012). RAs also possess small horizontal processes that may allow interactions with the neighboring cells. In fact, the differentiated progeny surrounds each RA cell body and its long shaft. One RA successively generates intermediate progenitors type 1 and 2 (IPC), which are often found next to RA cell bodies (Kempermann et al., 2003). These IPCs give rise to immature granule cells (IGC) that differentiate into fully mature granule cells. In contrast to the SVZ, newly born neurons do not migrate away from their niche and instead they reside in the dentate gyrus. Microglia have a strategic position in the granule cell layer. Finally, in the distal domain, RA possesses a large set of thin branches within the molecular layer of the hippocampus.
Figure 13: The subgranular zone neural stem cell niche (SGZ). A: astrocyte; BV: blood vessel; CA3: cornu ammonis; DG: dentate gyrus; GC: granule cell; GCL: granule cell layer; I: inhibitory interneuron; IGC: immature granule cell; IPC: intermediate precursor cell; M: ramified microglia; ML: molecular layer; Mo: mossy cell; P: pericyte; RA: radial astrocyte; SGZ: subgranular zone. Double arrows: synaptic inputs from a non-neurogenic region of the entorhinal cortex.
Although B1 cells and RA are found in a different microenvironment and generate different types of neurons, they do share key features. Both progenitors have ultrastructural characteristics of astrocytes with long processes. They express astroglial markers such as the glial fibrillary acidic protein GFAP and are found in close vicinity with the vasculature (Fig. 12, Fig. 13, Fig. 14). Thus local and systemic changes can modulate both neural stem cell niches.

b. Functions

During homeostasis, all the neuroblasts continuously produced in the SVZ migrate in chains through the rostral migratory stream (RMS) (Fig. 12) (Doetsch and Alvarez-Buylla, 1996). They differentiate into olfactory interneurons once they reach the olfactory bulb. Importantly, diverse subtypes of olfactory neurons are specifically derived from SVZ microdomains (Merkle et al., 2007).

In the dentate gyrus, the SGZ neural stem cells generate granule cells locally (Fig. 13). These newly born neurons contribute to spatial memory, learning, and more specifically to pattern separation function, a characteristic not shared by the neighboring fully mature granule cells (Lacar et al., 2014). Pattern separation is a process by which comparable, but not identical input patterns are considered as distinct. This means that the harder is the discrimination between two events or two input patterns the more advantageous is the adult SGZ neurogenesis. Mature granule cells as opposed are biased toward judging similar input patterns as identical. Newly born granule cells at 3-4 weeks of age possess a unique circuitry and physiology for a limited period of time, hence their restricted role in pattern separation (Nakashiba et al., 2012). The position of radial astrocytes in the entire dentate gyrus, from the most studied septal to temporal, may also influence neural stem cell fate and function due to inputs from different brain areas.

A tight regulation must be achieved within the niche in order to maintain neurogenesis throughout life. A balance between proliferation and the number of differentiated cells generated is orchestrated by several mechanisms at specific sites within the niche.
Although a single signaling pathway is not restricted to one particular domain. Below are some examples of interactions occurring within the neural stem cell niches.

### c. INTERACTIONS AND CELL FATE CONTROL

The CSF contains multiple soluble factors such as TGFs, IGFs, BMPs, Wnts, SHH, Slits, PDGFs and retinoic acid (Redzic et al., 2005, Lehtinen et al., 2011). The main source of CSF production is the choroid plexus and the CSF composition reflects the physiological state of the animal (Redzic et al., 2005). Importantly, even if B1 primary cilium is thought to integrate CSF soluble factors, highly concentrated growth factors within the lateral ventricles are able to penetrate the ependymal wall and bind to fractones (Kerever et al., 2007). According to their position and storing capacity of EGFs, FGFs and BMPs such as BMP7, fractones can deliver growth factors to each niche cell (Mercier et al., 2002). For example, neural progenitor cells express EGF receptor (EGFR) important for their proliferation (Doetsch et al., 2002).

Soluble factors can also originate from ependymal cells, SVZ cells, afferent axons and systemic signals. Ependymal cells secrete noggin, a BMP antagonist, leading to V-SVZ progenitor proliferation and an increase in neuroblast generation (Lim et al., 2000). Quiescence and thus maintenance of the neural stem cell pool involves in a highly active manner the canonical Notch signaling pathway (Imayoshi et al., 2010). Notch ligands are expressed within the whole SVZ niche. As an example, transit-amplifying cells express ASCL1, which increases the expression of Notch ligands and so in turn inhibits B1 cell proliferation. Neurotransmitters play an important role within the niche. Newly born neuroblasts spontaneously release gamma-aminobutyric acid (GABA) (Liu et al., 2005). Both transit amplifying cells and B1 cell, possessing GABA receptors, start to depolarize, which inhibits their cell cycle progression. Microglia are part of the niche actors (Walton et al., 2006). They secrete TGF-α and BDNF that enhance proliferation and neural differentiation. Non-neurogenic regions can also influence the neural stem cell niche. Dopamine is released by neuronal projections of the substantia nigra and the ventral...
Tegmental area. This input gives rise to an increase proliferation of transit amplifying cells (Silva-Vargas et al., 2013). The neural stem cell position within the niche is critical. Notably, endothelial cells closely interact with progenitor cells. Disrupting neural stem cell vascular adhesion leads to proliferation defects in vivo (Kokovay et al., 2010).

**The SGZ neural stem cell niche**

Like in the SVZ, canonical Notch signaling is active in the dentate gyrus neurogenic niche. Radial astrocytes express the downstream effectors of Notch HES5 and RBPjk. The latter binds directly to Sox2 promoter and activates its transcription, which is essential for RA quiescence (Ehm et al., 2010).

Again neurotransmitters play a critical role in neurogenesis. GABA, synthesized by parvalbumin interneurons, induces depolarization of IPCs toward a neuronal differentiation. At 1-2 weeks of age, they receive synaptic GABAergic inputs. The following week they start to express glutamatergic receptors and get hyperpolarized. At 4 weeks of age, they receive glutamatergic inputs from the entorhinal cortex and undergo fewer inhibitory inputs from the interneurons compared to mature cells. At this stage the young granule cells are hyperexcitable (Marin-Burgin et al., 2012). Finally, around 6-8 weeks of age, granule cells are fully mature and inhibition take over. Both young and mature granule cells connect interneurons directly or via the mossy cells.

Surprisingly, in normal condition most of the IPCs die 1 to 4 days after being generated via an apoptotic process (Sierra et al., 2010). Microglia located within the dentate gyrus play an important role in this neurogenesis regulation. These resident immune cells specific to the central nervous system engulf apoptotic IPCs avoiding any inflammatory response. In this homeostasis control, microglia act silently in a highly efficient manner (Sierra et al., 2010). The cells do not get activated and keep their ramified morphology. Microglia also secrete factors such as IGF-1 and BDNF influencing proliferation, differentiation, and survival of the newborn cells (Morgan et al., 2004, Ziv and Schwartz, 2008).
How and when neural stem cells and their niche are established in the developing brain has been the focus of numerous studies, especially for the SVZ niche.

2. NICHE DEVELOPMENT

The onset of neurogenesis at embryonic day 9-10 is followed by the appearance of highly proliferative radial glia in the cerebral cortex (Fig. 15). Radial glia cells are considered as the bona fide neural stem cells in the embryonic ventricular zone (Kriegstein and Alvarez-Buylla, 2009). These bipolar pseudostratified neuroepithelial cells possess a primary cilium in direct contact with the ventricle and begin to express astrocyte-specific markers, such as the glutamate transporter GLAST, brain lipid-binding protein (BLBP), and Tenascin C (TN-C). Thanks to their long basal radial fiber reaching the pia matter basement membrane radial glia serves as a scaffold for neuronal progeny migration through the cortex. Once cortical neurogenesis completes, gliogenesis initiates. Radial glia switches to astrocyte, exit the ventricular zone and its radial process regresses. Concomitantly, the primary SVZ vascular plexus takes place between embryonic day 16 and 18 with the first specialized neural stem cell contacts (Takahashi et al., 1990). Then, a transition from radial glia derived astrocyte to B1 cell is achieved postnatally with the establishment of a single ependymal cell layer lining the ventricles (Merkle et al., 2004). In some vertebrates, radial glia cells are maintained postnatally as the source of neurogenesis without any conversion. It is the case in songbirds, fish, and reptiles (Alvarez-Buylla et al., 1998, Garcia-Verdugo et al., 2002, Zupanc, 2006). The developmental origin of the SGZ neural stem cells in the hippocampus is not clear. Nonetheless, as already mentioned earlier, RA shares key features of radial glia such as bipolarity and primary cilium (Altman and Bayer, 1990).

3. ADULT NEUROGENESIS IN HUMAN

Neurogenesis in the human SVZ is controversial (Curtis et al., 2007, Sanai et al., 2011). An innovative strategy using carbon dating on human tissues recently concluded that there was no sign of newly born olfactory neurons (Bergmann et al., 2012, Spalding et al., 2013). Ernst and colleagues demonstrated that human neural stem/progenitor cells were able to generate striatal interneurons.
Figure 15: The glial nature of neural stem cells (NSCs) in the developing and the adult SVZ. Solid arrows are supported by experimental evidence in rodents; dashed arrows are hypothetical. B cells: neural stem cells; IPC: intermediate progenitor cell; MA: mantle; MZ: marginal zone; NE: neuroepithelium; nIPC: neurogenic progenitor cell; oIPC: oligodendrocytic progenitor cell; RG: radial glia; SVZ: subventricular zone; VZ: ventricular zone. From Kriegstein and Alvarez-Buylla (Kriegstein and Alvarez-Buylla, 2009).
However, it seems that those cells are generated by local astrocytes (Ernst et al., 2014). The situation is more straightforward in the human dentate gyrus. It is well accepted that it acts as a long-lasting neurogenic niche (Eriksson et al., 1998, Spalding et al., 2013). As opposed to rodents, humans have smaller olfactory bulbs and a different hippocampal anatomy with larger ventral dentate gyrus. This could reflect human-specific functions for adult-born neurons.

4. Stem cell potential outside the neurogenic niches

- **NG2 glia**

NG2 glia is a specific glia cell type scattered in the brain parenchyma. Almost all of these cells are NG2+PDGFRα+ and undergo a slow rate of division, more than once a month, to self-renew and produce myelinating oligodendrocytes (Dimou et al., 2008, Rivers et al., 2008, Psachoulia et al., 2009). These represent a very limited progenitor cell type that renews the supporting glia cells but does not contribute to neurons.

- **Reactive astrocytes**

Astrocytes are the support cells of the CNS. Among other functions, they modulate neuronal synapses and vasculature. Even if they share expression signatures with neural stem cells, astrocytes do not divide in normal condition. However, following brain injury such as ischemic stroke, astrocytes near the lesion undergo astrogliosis after several days. This process is characterized by a pronounced change in morphology and gene expression that leads to reactive astrocyte proliferation and acquisition of neural stem cell properties (Buffo et al., 2008). This astrocytic response has both beneficial and detrimental impact on tissue repair. Notably, the astrocytes produce a scar to protect the tissue surrounding the lesion from receiving additional inflammatory signals. However, the scar later inhibits axonal regeneration and neurogenesis (Pekny and Pekna, 2004, Sofroniew and Vinters, 2010). While many of the signals released upon brain injury overlap with those found in the neural stem cells, the astrocytes are not capable of forming multiple cell types such as the progenitors found in the SVZ and SGZ (Buffo et al., 2008).
While there are specific niches with multipotent stem cells in the brain, they appear to serve very specific functions. The subventricular zone, next to the lateral ventricles, produces new neurons that will differentiate once they reach the olfactory bulb. In contrast, the dentate gyrus of the hippocampus locally generate neuroblasts that will enhance learning and memory. Whether neural stem cells and glia cells with a restricted potential can be manipulated to effect more substantial repair in the brain is currently one of the main research focuses in this field. As mentioned in Chapter 2, in the neonatal and adult brain Pw1 expression is mostly restricted to the neuronal lineage including neural stem/progenitor cells in the subventricular zone (Kuroiwa et al., 1996, Relaix et al., 1996, Li et al., 1999, Besson et al., 2011). Yamaguchi et al (Yamaguchi et al., 2002) showed that under hypoxic conditions primary cultured cortical neurons and astroglial cells upregulate Pw1. Whether this phenomenon potentially reflects cellular activation towards a stem cell fate is not known. A very first question needs to be answered: does PW1 confer stem cell capacities to neural stem/progenitor cells?
RATIONALE AND OBJECTIVES OF THE THESIS

Parental imprinting is a mammalian-specific form of epigenetic regulation that affects ~100 genes, in which one allele is silenced and the other allele is expressed as a function of parental origin (Ferguson-Smith, 2011). Multiple theories and explanations as to why some genes are imprinted have been proposed, but to date, none satisfy the diverse functions of these genes or explain why some genes inherited from the mother or father are preferentially expressed. While genes that are identified as 'paternally expressed' or 'maternally expressed' show a clear bias in allele-specific expression, several reports have demonstrated that loss of imprinting in specific cell-types at specific key stages leads to bi-allelic expression of imprinted genes (DeChiara et al., 1991, Charalambous et al., 2004, Hagege et al., 2006, Ferron et al., 2011). This phenomenon, referred to as 'relaxation of imprinting' participates in specific biological processes such as maintenance of the neural stem cell pool (Ferron et al., 2011), adding an extra level of complexity to the epigenetic regulation and the general role of the imprinted genes.

My thesis work has been focused on Pw1 (also known as Peg3), which is a paternally expressed gene that was identified over 20 years ago by both our laboratory and that of Surani (Kuroiwa et al., 1996, Relaix et al., 1996). I have addressed the following lines of research in my studies: the role of Pw1 in neural stem cells (addressed in Chapter 5), the potential role of bi-allelic expression (addressed in Chapter 5), and the impact of the loss of Pw1 function on brain and behavior (addressed in Chapter 6). The first part followed our discovery that Pw1 expression is highly restricted to adult stem cells in many tissue types including the CNS (Besson et al., 2011). Using the Pw1\textsuperscript{IRES\textsubscript{N}\textsubscript{LacZ}} reporter mouse model, our preliminary experiments suggested that a subset of stem cells in the adult skin stem cell niche underwent a relaxation of imprinting in response to injury. I initially studied the expression pattern of both paternally and maternally transmitted reporter as a surrogate for endogenous allele-specific expression of Pw1 during embryonic and fetal development. The underlying hypothesis was that Pw1 bi-allelic expressing cells are required for the establishment of adult stem cell niches.
Previously established Pw1 mutant mice revealed phenotypes associated with growth, behavior, and metabolism. Notably, paternal loss of Pw1 was shown to lead to impaired maternal care and milk ejection defects leading to decreased offspring survival prior to weaning. Additionally, pup lacking the paternal copy of Pw1 exhibited an impaired suckling ability. However, these reports were carried out on mouse models that targeted exons with little coding sequence and without the excision of the ES cell-selection neomycin cassette, which has been reported to lead to unanticipated phenotypic outcomes in several mouse models due to cis-gene effects (Gerard et al., 1999, Muller, 1999). We generated a new mutant floxed allele for Pw1 that targets the largest coding exon (exon 9 containing >90% of the coding domain), which we used to generate a constitutive loss-of-function mouse line in order to compare phenotypes with those previously reported.

Several parentally imprinted genes have been shown to play a role in regulating behaviors including aggression, anxiety, and parental care of the young (Lefebvre et al., 1998, Li et al., 1999, Champagne et al., 2009, Garfield et al., 2011). Other parentally imprinted genes, including those implicated in behavioral control, have also been shown to play a role in regulating body metabolism and specifically, in maternal-fetus and maternal-offspring balancing of nutritional resources (Curley et al., 2004, Curley et al., 2005, Smith et al., 2007, Keverne and Curley, 2008, Kim et al., 2013). The fact that Pw1 (as well as many other imprinted genes) remains highly expressed in the brain is consistent with a role in governing animal behavior. The expression of Pw1 (as well as a group of 9 other parentally imprinted genes) in adult stem cells suggests that these genes play a role in stem cell regulation and function (Varrault et al., 2006, Berg et al., 2011). How all these processes link to the control of body growth and metabolism presents a major challenge to the field of developmental biology as well as towards understanding why a small subset of genes are parentally imprinted and what function this form of epigenetic regulation plays. The underlying hypothesis was that Pw1 expression and function in adult stem cells is centrally regulated and that a stressful condition leading to mouse anxiety leads to a generalized impairment of stem cell niches.
CHAPTER 4. MATERIALS AND METHODS

1. MOUSE MODELS

a. \( PW1^{IRESnLacZ} \) REPORTER MOUSE MODEL

Transgenic mice were generated using bacterial artificial chromosome (BAC) recombineering technology (Lee et al., 2001). An IRESnLacZ cassette has been inserted into the \( Pw1 \) coding exon 9 of a 129Sv BAC clone (ID#508P6, 180Kb) (Fig. 16). A single copy of this BAC-containing transgene has been randomly integrated into the C57Bl/6J mouse genome (Besson et al., 2011).

![Figure 16: \( PW1^{IRESnLacZ} \) reporter mouse construct.](image)

The transgenic BAC was randomly inserted into the mouse C57Bl/6J genome. One single insertion was verified by fluorescent in situ hybridization (FISH).

b. \( PW1 \) CONSTITUTIVE KNOCKOUT MOUSE MODEL

A \( Pw1^{loxneolox} \) mouse was first generated by homologous recombination (Fig. 17). Two \( loxP \) sites were inserted to flank coding exons 8 and 9. The ES cell-selection neomycin cassette was excised by crossing \( Pw1^{loxneolox} \) mouse with a \( Flippase \) mouse that recognizes and cleaves \( Frt \) sites. The subsequent \( Pw1^{floxed} \) was finally bred to a constitutive \( PGK-Cre \) mouse in order to obtain a constitutive \( Pw1 \) knockout mouse that was expanded on C57Bl/6J background.
**Figure 17: Pw1 knockout mouse construct.** Coding exons 8 and 9 were excised corresponding to >91% of PW1 protein.

### c. Genotyping

Genotyping was carried out from mouse tails or embryo yolk sacs using MyTaq Extract-PCR kit according to manufacturer’s protocol (Bioline). Cycling conditions were as follows: 95°C for 3 minutes followed by 35 cycles of amplification (95°C for 20s, 57°C for 20s and 72°C for 20s). In order to discriminate between Pw1+/+, Pw1−/+ and Pw1−/− mice both Pw1 wild-type and knockout primers were pooled together as a unique polymerase chain reaction. Primers sequences and amplicons size are listed in the table below (Table 1).

**Table 1. Primers sequences for mouse genotyping**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Primer ID</th>
<th>Sequence 5’-3’</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pw1IRESmLacZ</td>
<td>dLacZ (Forward)</td>
<td>CCGCTACAGTCAACAGCAAC</td>
<td>880</td>
</tr>
<tr>
<td></td>
<td>24040 (Reverse)</td>
<td>CCACATTCTTACTCAACGC</td>
<td></td>
</tr>
<tr>
<td>Pw1 wild-type allele</td>
<td>23210 (Forward)</td>
<td>AAGGCCACCTCATCGAGGTCCAAGAGAATGCC</td>
<td>830</td>
</tr>
<tr>
<td></td>
<td>24040 (Reverse)</td>
<td>CCACATTCTTACTCAACGC</td>
<td></td>
</tr>
<tr>
<td>Pw1 knockout allele</td>
<td>38207 (Forward)</td>
<td>ACATGCCTGGAAGGACTCCAGTG</td>
<td>707</td>
</tr>
<tr>
<td></td>
<td>28250 (Reverse)</td>
<td>ACCTTCACAGGACTATCTAGAGGTAGGG</td>
<td></td>
</tr>
</tbody>
</table>
2. PRIMARY CULTURE OF NEUROSHERES

The subventricular zone was dissected out for the generation of both fetal and postnatal neurospheres after animal decapitation or cervical dislocation, respectively. Fetal starting material was mechanically dissociated in cold PBS (MgCl₂-free, Ca²⁺-free) while postnatal SVZ was enzymatically dissociated using NeuroCult® Kit according to manufacturer's instructions (StemCell Technologies). Cells were grown in proliferation media containing DMEM/F12 (1:1, Gibco) supplemented with B27 (Life Technologies), N-2 (Life Technologies), EGF (20ng/mL; Life technologies), basic-FGF (20ng/mL; PeproTech), D-()+-Glucose (6mg/mL; Sigma), HEPES buffer (5mM; Life Technologies), human insulin (25µg/mL; Sigma) and gentamycin (50µg/mL; Life Technologies). To expand neurospheres, cells were plated at 20,000 cells/mL. For clonal analyses, cells were plated at very low density (2.5 cells/µL). Culture conditions were at 37°C, 10% CO₂ in a humidified incubator.

Upon passage neurospheres were centrifuged 5 minutes at 1,100rpm. Cell pellet was trypsinized (Gibco) for 5 minutes at 37°C. Warm DMEM (10% FBS, 80U/mL DNase I) was added and a mechanical dissociation was carried out in PBS (MgCl₂-free, Ca²⁺-free).

**NB: For adherent growth of neural stem cells, single cells were plated at 1,000 cells/mL on poly-L-ornithine (0.01%; Sigma) and laminin (10µg/mL; Sigma) coated plates. Media used was the neurosphere proliferation media detailed above except for the concentrations of EGF and basic-FGF (10ng/mL only) (Pollard et al., 2006).**

3. DIFFERENTIATION OF NEUROSHERES

Upon neurosphere dissociation, 20,000 single cells were plated onto a 12mm diameter coverslip coated with poly-D-lysine (10µg/mL; Millipore) and laminin (10µg/mL; Sigma). Differentiation media (DMEM/F12 (1:1, Gibco) supplemented with B27 (Life Technologies), N-2 (Life Technologies), D-()+-Glucose (6mg/mL; Sigma), HEPES buffer (5mM; Life Technologies), human insulin (25µg/mL; Sigma) and gentamycin (50µg/mL; Life Technologies) was supplemented with 5% fetal bovin serum (Invitrogen).
4. HISTOLOGY AND IMMUNOFLUORESCENT STAININGS

For histochemistry, neurospheres were collected in a 50mL Falcon tube and fixed 4 minutes in 0.2% glutaraldehyde at 4°C. After reacting neurospheres for 2 hours at 37°C with a chromogenic X-gal substrate solution (PBS 1X, K3Fe(CN)6 4mM, K4Fe(CN)6 4mM, MgCl2 2mM, NP40 0.02%, X-gal 0.4mg/mL), β-galactosidase-positive nuclei were revealed in blue. A post-fixation was performed for 10 minutes at RT in 4% paraformaldehyde before mounting.

For immunofluorescence, settled down neurospheres onto a poly-D-lysine (10µg/mL; Millipore) coated coverslip and adherent cells were fixed in 4% paraformaldehyde for 20 minutes at RT. Permeabilization was performed in ice cold methanol for 6 minutes. Blocking in PBS supplemented with 4% IgG-free bovin serumalbumin (Jackson ImmunoResearch) was carried out 1 hour at RT before incubation with primary antibodies as described below.

For embryonic analyses, females were killed by cervical dislocation and embryos were individually fixed in 4% paraformaldehyde for 2 hours at 4°C. Cryoprotection was performed overnight in 20% sucrose PBS before embedding in OCT compound (Leica), sectioning and immunostaining as described below.

All images were acquired using a Leica DM fluorescence and light microscope or Leica SPE confocal microscope.

5. Pw1 TRANSCRIPT AND PROTEIN LEVELS

At passage 0, 1, 2 or 3, primary neurospheres cultured for 7 days in T75cm² flasks at 25,000 cells/mL were centrifuged 5 minutes at 1,100rpm at +4°C. After one wash in cold PBS, neurospheres were once more centrifuged 5 minutes at 1,100rpm at +4°C. Upon supernatant removal, the pellet was used for RNA or protein extraction as described in the attached publication methods (see Chapter 6).
Protocols for pre-mRNA and DNA Fluorescent In Situ Hybridization were kindly provided by Edith Heard at Curie Institute (Paris) (Chaumeil et al., 2008).

Requirements for designing pre-mRNA FISH probes include the spanning of a minimum of 3kb intronic sequences. In this way the probe specifically targets primary transcripts. Five couples of primers were designed for generating *Pw1* probe. One couple of primers was designed for *Nestin* probe. Synthesis was carried out using 100ng of template C57Bl/6J genomic DNA provided by Frédéric Auradé and the Long PCR Enzyme Mix (Thermo Scientific) containing 8% DMSO. Cycling conditions were as followed: initial denaturation of 3 minutes at 94°C; 10 cycles of denaturation/annealing/extension (20s 94°C, 30s 57°C, 4 minutes 68°C); 10 cycles of denaturation/annealing/extension (20s 94°C, 30s 57°C, 5 minutes 68°C); 10 cycles of denaturation/annealing/extension (20s 94°C, 30s 57°C, 6 minutes 68°C) with a final extension for 10 minutes at 68°C. At this stage only two couples of primers for *Pw1* were satisfying called Peg3 #1 and Peg3 #4 (Table 2, Fig. 18). Amplified DNA was ligated for 3 hours or overnight at 25°C into a pGEM®-T Easy Vector (Promega) following manufacturer’s protocol with a ratio vector/insert 1:3 or 1:10. Transformation was then carried out using TOP10 one shot chemically competent cells (Life Technologies). In details, 3µL of ligation reaction was added to 100µL of competent cells for an incubation of 30 minutes on ice. Heat shock was performed for 40s at 42°C without shaking. Cells were quickly placed on ice for 2 minutes before adding 400µL of SOC media. Under agitation cells were incubated 30 minutes at 37°C and plated onto LB/Ampicillin/Xgal plates. After an overnight incubation at 37°C, white colonies were individually selected for minicultures in LB/ampicillin media overnight at 37°C. Plasmid purification was performed using NucleoSpin Plasmid kit (Macherey-Nagel) according to manufacturer’s protocol. Digestions with EcoRI, KpnI and Ncol were carried out using 10X Fast Green buffer for 1 hour at 37°C to check for DNA probe insertion. Probe-specific clones were finally validated upon sequencing using universal T7/Sp6 and internal primers (GATC). Midipreps of plasmidic DNA was performed for one validated clone per probe following Nucleobond Xtra Midi kit manufacturer’s protocol (Macherey-Nagel).
Table 2. Primers for pre-mRNA-FISH probes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Probe ID</th>
<th>Sequence 5’-3’</th>
<th>Transcript content</th>
<th>Size (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pw1/Peg3</td>
<td>Peg3 #1</td>
<td>FWD - GGCTCTGCATCTAGTGCTGTGGGA&lt;br&gt;REV - TGCTGTGGGATCGGCTTGTCACTCACCTCG</td>
<td>exon 7 (103bp) + exon 8 (116bp)</td>
<td>3,84</td>
</tr>
<tr>
<td>Pw1/Peg3</td>
<td>Peg3 #4</td>
<td>FWD - TTCAAGGTGCAAGGGCCAGGAGTAGC&lt;br&gt;REV - TGCCAGCAAGCTATGTCTACCCCCA</td>
<td>*</td>
<td>3,59</td>
</tr>
<tr>
<td>Nestin</td>
<td>Nestin</td>
<td>FWD - AGGAAGGGGTGGGGCAGGCAAACACT&lt;br&gt;REV - AGGATGTGGGCTGAGGACAGGGAGCA</td>
<td>exon 2 (125bp) + exon 3 (74bp) + exon 4 (84bp)</td>
<td>3,48</td>
</tr>
</tbody>
</table>

*Peg3 #4 probe spans a putative coding exon 2 isoform.

Figure 18: Probe design for pre-mRNA-FISH. Probes must span more than 3Kb of intronic sequences. For Pw1, two probes were generated: P1 for Peg3 #1 and P4 for Peg3 #4. For Nestin, only one probe was cloned called N1.

Probes were labeled using 2µg of plasmidic DNA, 0.2mM Orange-dUTP (Enzo Life Sciences) and 5µL of nick translation enzyme per 50µL of reaction for 4 hours at 15°C following manufacturer’s instructions (Abbott Molecular). They were kept at -20°C until use. As positive controls, Edith Heard group provided us ready-to-hybridize probes for Eya3 and Atrx primary transcripts. A Pw1-containing BAC clone (ID#508P6, 180 Kb) was used as Pw1 DNA-FISH probe. Nick translation was in this case carried out for 7 hours at 15°C using 0.2mM Green-dUTP.

On the day of hybridization 5µL of nick translated probes were 100% ethanol precipitated with 10µg of salmon sperm DNA, 1µg of COT-1 DNA, 0.1 volume of 3M sodium acetate pH5.5. After a brief vortexing, probes were incubated on dry ice for 15 minutes and centrifuged at 12,000rpm for 30 minutes at 4°C. Two washes of the pellet in cold 70% ethanol were carried out to remove unincorporated nucleotides. After pellet drying at RT for 15 minutes, probes were resuspended thoroughly in formamide for 15-
30 minutes at 40°C. Probes were denatured 7 minutes at 75°C. After adding 1 volume of 2X Hybridization buffer probes were then kept on ice; ready to be hybridized; for up to 30 minutes.

7. PRE-MRNA FLUORESCENT IN SITU HYBRIDIZATION

Neurospheres were dissociated as described above. Single cell suspension was prepared at 12,500 cells/µL in cold 1X PBS. On a 14mm diameter glass coverslip pre-coated with 10µg/mL of poly-D-lysine (Millipore), 50µL of single cells was gently deposited. After settling down of the cells for 5 minutes at +4°C, a slow spinning down of 5 minutes at 50g +4°C was carried out to enhance cell attachment. After 1X PBS removal, cells were permeabilized on ice for 5 minutes in cold CSK buffer pH6.8 (100mM NaCl, 300mM Sucrose, 3mM MgCl2, 10mM PIPES) supplemented with 0,5% Triton X-100 and 2mM Vanadyl Ribonucleoside complex (New England BioLabs). Cells were fixed in 3% paraformaldehyde for 10 minutes at room temperature. Then, cells were washed twice 5 minutes in 70% ethanol. At this stage, the coverslip could be stored in 70% ethanol at -20°C for several months.

On the day of hybridization the coverslip was dehydrated in 80%, 95% and 100% ethanol for 3 minutes each at room temperature and air-dried. The denatured probe was deposited onto an RNase-free glass slide and the coverslip was placed on top; cells facing down. Hybridization occurred overnight at 37°C in a dark humid chamber made with 50% formamide/2X SSC. Cells were washed three times for 5 minutes in pre-heated 50% formamide/2X SSC pH7.2 at 42°C. Then, cells were washed twice for 5 minutes in 2X SSC at 42°C. Cells were counterstained for 2 minutes in 2X SSC containing 0,2mg/mL DAPI. Two additional washes in 2X SSC were carried out before mounting onto a glass slide using fluoromount G. Slides were scanned at the oil objective 40X for DAPI and TRITC channels using a Nikon Eclipse Ti microscope.

To validate RNA specificity of fluorescent dots an RNase A-treated (Life technologies) control was run in parallel for each experiment. A minimum of 500 cell nuclei per condition was assessed to determine the percentage of mono- and bi-allelic expressing cells (Table 3).
Probes used for DNA-FISH were prepared exactly as described in the pre-mRNA FISH section with an additional step of competition. After denaturation, probes were incubated at 37°C waterbath for 45 minutes prior to hybridization.

Only a few steps vary from the pre-mRNA FISH hybridization protocol. Prior to dehydration, cells were treated with RNase A for 1 hour at 37°C. After dehydration, cells were air-dried and denatured in pre-warmed 70% formamide/2X SSC pH7.2 for 30 minutes at 80°C. Two washes in cold 2X SSC and an ice-cold ethanol serié were very quickly performed for dehydration before depositing the probe. Hybridization was carried out overnight at 42°C.

To validate DNA specificity of fluorescent dots a DNase I-treated (Roche) control was run in parallel for each experiment. A minimum of 500 cell nuclei per condition was assessed to determine the percentage of dots-positive cells.

9. Statistical analyses

All statistical analyses were performed using GraphPad Prism software, version 6.0. Tests used are pointed out in figure legends; they include student t-tests, one- or two-way ANOVA tests. Non-parametric tests were used when data are not normally distributed (D’Agostino-Pearson omnibus normality test and/or Kolmogorov-Smirnov test). No outliers were removed. Data are presented as the mean ± standard error of the mean (s.e.m.) *P<0.05, **P<0.01, ***P<0.001.
**NB:** Methods used for transcript levels, protein levels, maternal behavior, milk intake, oxytocin immunohistochemistry, and oxytocin plasma levels analyses are detailed in the attached publication (see chapter 6).
CHAPTER 5. PW1 EXPRESSION IN NEURAL STEM/PROGENITOR CELLS

The initial aim of this thesis project was to identify a potential role for $Pw1$ gene dosage based upon my observations that suggested that the maternal allele of $Pw1$ was expressed in neural stem cells in the brain. I took advantage of the $Pw1^{IRESnLacZ}$ reporter mouse model (Besson et al., 2011) to study allele-specific $Pw1$ reporter gene expression at different stages in different tissues. The BAC carrying the reporter gene contains $Pw1$ domain ICR (Kim et al., 2012). Consequently, we confirmed that the $Pw1^{IRESnLacZ}$ transgenic mouse model recapitulates the $Pw1$ imprinting pattern where strong reporter gene expression was detected upon paternal inheritance only. However, during embryonic and fetal development, we observed the expression of maternally inherited $Pw1^{IRESnLacZ}$ at several stages, notably E12.5 and E17.5 (Fig. 19). Overall, every tissue analyzed thus far that showed maternal $Pw1^{IRESnLacZ}$ expression also showed paternal $Pw1^{IRESnLacZ}$ expression. This result strongly suggests that there is either mono-(paternal) or bi-allelic $Pw1^{IRESnLacZ}$ expression in specific tissues but no allelic exclusion (i.e. maternal expression alone). Stage E17.5 was studied extensively due to strong and broad PW1 expression; while paternal $Pw1^{IRESnLacZ}$ allele expression was present in almost all tissues the maternal $Pw1^{IRESnLacZ}$ showed a highly restricted expression pattern (Fig. 19-panel III). In particular, brain, tongue and mandible showed strong bi-allelic reporter gene expression in specific regions of interest that became the initial focus of my studies.

1. $Pw1^{IRESnLacZ}$ ALLELE-SPECIFIC EXPRESSION IN THE ESTABLISHING NEURAL STEM CELL NICHES

$Pw1^{IRESnLacZ}$ bi-allelic expression in the fetal brain corresponded to regions which will form the neural stem cell niches. Specifically, bi-allelic expression was found in the subventricular zone and the dentate gyrus of the hippocampus (Fig. 19-panel III). We note that PW1 expression was detected in the adult neural stem cells present in the subventricular zone, therefore suggesting that $Pw1$ bi-allelic expression participates in the establishment of adult neural stem cells (Besson et al., 2011). It was first necessary to confirm that this relaxation of imprinting leading to $Pw1^{IRESnLacZ}$ bi-allelic expression
in restricted areas of the brain was occurring in the endogenous Pw1 locus and to also understand its biological significance. A similar phenomenon was described for the paternally expressed gene Dlk1 in the adult neural stem cell niche where a bi-allelic expression of Dlk1 is required to maintain life-long neurogenesis (Ferron et al., 2011). However, no such report was known until very recently for Pw1 (Perera et al., 2015). At the time of this preliminary observation, the Pw1 conditional mutant allele was still being generated, therefore, I adopted an in vitro neural stem cell culture model in which neural stem cells could be grown and expanded as neurospheres as a central approach. Primary neural stem cells were generated from fetal stage E17.5 subventricular zone and first analyzed for PW1 expression at the cellular level (Fig. 20).

2. Pw1 is expressed in primary fetal neural stem cells

Established by Reynolds and colleagues, the neurosphere assay allows for monitoring proliferation and differentiation capacities of neural stem/progenitor cells in vitro (Reynolds and Weiss, 1992). Upon tissue dissociation, cells with self-renewal potential will grow in suspension and form spheres that contain a heterogeneous population of neural stem/progenitor and differentiating cells. Upon multiple passages loosing cells with low self-renewal capacities enrich the pool of neural stem cells in culture (Temple, 2001). After 7 days of proliferation, primary fetal neurospheres contained 70% of PW1+ cells. This population expressed PW1 heterogeneously with half of the cells that co-expressed the proliferative marker Ki67 (Fig. 20A and B). As a second control, I also generated adherent primary neural stem cells, which also expressed PW1 widely (Fig. 20C). Of note, some NESTIN+ cells that correspond to neural stem cells did not show PW1 expression. These results suggest that PW1 marks a subpopulation of NESTIN+ fetal neural stem cells in vitro in both neurospheres and adherent cell culture conditions. These analyses validate the use of the neurosphere assay as a tool to study the role of Pw1 and its putative bi-allelic expression in neural stem cells in vitro. I therefore examined neurospheres generated from fetuses in which the Pw1IRESnLacZ allele was inherited maternally (MAT) or paternally (PAT) (Fig. 21).
Figure 19: Pw1IREsLacZ bi-allelic expression during embryonic and fetal development. Panel I. Pw1IREsLacZ reporter mouse transgenic construct. Panel II. Paternal (PAT) and maternal (MAT) Pw1IREsLacZ reporter gene expression at embryonic stage E12.5. Whole embryos were stained with Xgal. Panel III. Paternal (PAT) and maternal (MAT) Pw1IREsLacZ reporter gene expression at fetal stage E17.5. Coronal and sagittal sections were stained with Xgal for 3 hours and counterstained with Nuclear Fast Red. A, C, E, G. Coronal sections of the subventricular zone with low (C and E) and high magnifications (A and G). D and F. Coronal sections of the dentate gyrus of the hippocampus. B and H. Whole sagittal sections of E17.5 fetuses. In some tissues, such as the central nervous system, both paternal and maternal Pw1IREsLacZ reporter alleles are found expressed. This strongly suggests that restricted cell types express Pw1 bi-allelically.
Figure 20: PW1 is expressed in neural stem cells grown as neurospheres. A and B. Quantification of the number of cells expressing PW1 and Ki67 from fetal neurospheres immunostained as shown in B (n=1). B. Immunostaining for PW1 and Ki67 on wild-type neurospheres. C. Immunostaining for PW1 and NESTIN on adherent fetal neural stem stem cells at 0, 1, 3, 5, and 7 days in vitro. Taken together, PW1 is expressed in fetal neural stem cells grown both as neurospheres and as adherent cell culture.
3. P_{W1}^{IRES\text{LacZ}} ALLELE-SPECIFIC EXPRESSION IDENTIFIES SUBPOPULATIONS OF PRIMARY FETAL AND POSTNATAL NEURAL STEM/PROGENITOR CELLS IN VITRO

Following 7 days in culture (passage 0), all the PAT fetal neurospheres contained >98% of cells expressing the P_{W1} reporter gene (Fig. 21A and D). In contrast, only a subset of cells within MAT neurospheres expressed the P_{W1} reporter gene. Based upon these observations, I assumed that neurospheres are composed of cells expressing both paternal and maternal P_{W1}^{IRES\text{LacZ}} alleles and cells expressing the paternal P_{W1}^{IRES\text{LacZ}} allele alone. The percentage of cells expressing both P_{W1} reporter alleles was variable from one neurosphere to another. The majority of the fetal P0 neurospheres contained ~50% bi-allelic reporter expressing cells. Surprisingly, following a single passage (P1), almost all fetal neurospheres displayed an increase in bi-allelic reporter expression to levels near 98%. This pool was maintained over multiple passages but tended to decrease at passage 5 and 10 (Fig. 21A and D). To see whether this percentage of β-galactosidase-positive (βGAL+) cells per neurosphere was stage-dependent I generated primary neurospheres from 4 weeks old mice. After 7 days in culture (passage 0), the majority of the postnatal neurospheres contained less than 25% of bi-allelic reporter expressing cells (Fig. 21B and D). Again, this pool increased with passages but much slower as it never reached the same amount than for fetal neurospheres in the conditions tested. Taken together, we propose that the pool of bi-allelic reporter expressing cells within neurospheres is stage-dependent and that the bi-allelic expressing cells represent a subpopulation of neural stem cells with different stem cell potential as compared to mono-allelic cells. If the increase in bi-allelic expression reflects bona fide transcription from the endogenous P_{W1} alleles, we would expect to see an increase in P_{W1} transcription and protein levels. I therefore analyzed wild-type fetal neurospheres after 7 days of culture at passage 0, 1, 2, and 3. Using real time RT-PCR, I did not detect significant differences (Kruskal-Wallis test, P=0.0707) in P_{W1} transcript levels with the exception of a trend towards an increase following the first passage (unpaired t-test, P=0.0635 between passage 0 and 1) (Fig. 21C). PW1 protein levels were similarly unchanged between passage 0, 1, and 2 (Kruskal-Wallis test, P=0.6275) (Fig. 21E). As we did not detect any strong correlation between the number of bi-allelic P_{W1}^{IRES\text{LacZ}} expressing cells and total P_{W1} transcript level, we wondered whether the total number of bi-allelic reporter expressing cells was overestimated.
Figure 21: The maternal $Pw1^{IRESn LacZ}$ reporter allele shows chimeric expression within primary fetal and postnatal neurospheres. All neurospheres were generated from the subventricular zone. A. X-gal staining of $Pw1^{IRESn LacZ}$ maternally-derived (MAT) and paternally-derived (PAT) fetal neurospheres at passage 0 (P0) and passage 1 (P1) (n=3). B. X-gal staining of $Pw1^{IRESn LacZ}$ maternally-derived 4 weeks old neurospheres at passage 0 (P0) (n=3). C. Real-time RT-PCR showing $Pw1$ transcript level in fetal wild-type neurospheres over passage (n=3). Kruskal-Wallis test did not reveal any significant differences (P value=0.0707). D. Expression pattern of both maternal (MAT) and paternal (PAT) $Pw1^{IRESn LacZ}$ reporter alleles within fetal (E17.5) and postnatal (4 weeks old) neurospheres over passage. Percentage corresponds to the approximate number of reporter expressing cells within neurospheres (n=3). P=passage, NS=neurosphere. Two-way ANOVA analysis revealed a significant increase in neurospheres containing ≈50% and >98% of MAT βGAL⁺ cells from P0 to P1 at both fetal and postnatal stages. E. Western Blot analyses of fetal wild-type neurospheres for PW1 and GAPDH (n=3). No significant differences were found upon blot quantitation using Kruskal-Wallis test. This in vitro analysis showed that primary fetal neurospheres contain a sub-population of $Pw1^{IRESn LacZ}$ bi-allelic expressing cells, which decreases in number at 4 weeks of age. In both stages, the percentage of bi-allelic expressing cells per neurosphere significantly increases after the first passage meaning that the pool of bi-allelic $Pw1^{IRESn LacZ}$ expressing cells is enriched and maintained over the first neurosphere passage in vitro. This observation tends to correlate with an increase in $Pw1$ transcript level from P0 to P1 in fetal wild-type neurospheres (p value=0.064 using Unpaired t test).
A major caveat with the use of βGAL as a reporter gene is that it is very stable and the product is not under the same post-translational control as PW1. Indeed, I observed that while >98% of fetal neurosphere cells display strong paternal reporter expression, only 70% of the total cells express detectable levels of PW1 protein (Fig. 20A and B, Fig. 21A and D). It is also possible that the maternal Pw1IRESnLacZ allele is expressed at very low levels compared to the paternal allele or that activity from the maternal allele may not be linked to RNA or protein levels but reflect gene activity from neighboring genes (i.e. cis effects).

4. PRE-MRNA-FISH EXPERIMENTS DEMONSTRATE A VERY FEW NUMBER OF ENDOGENOUS Pw1 BI-ALLELIC EXPRESSING CELLS

Defining the endogenous Pw1 bi-allelic expression was a critical first step prior to the study of the biological significance of bi-allelic expression. To address this issue, I carried out primary transcript mRNA fluorescent in situ hybridization (pre-mRNA-FISH) experiments on wild-type fetal neural stem cells in order to visualize at the single-cell level Pw1 bi-allelic expressing cells.

The procedure for passaging neurospheres involves an inherent selection after sphere dissociation, which is reflected by a marked decline in the total cell number during the first 2 days of culture in suspension (Fig. 22A). After 2 days, cells reinitiate division and by day 7, the neurospheres proliferate exponentially. At the different phases of neurosphere growth, the total number of PW1 expressing cells does not exhibit any overt changes (Fig. 22B). Heterogeneity in PW1 expression level is as follows: only 10 to 20% of total PW1+ cells express PW1 strongly (PW1high) and only 0.2 to 3.4% of mitotic PH3+ cells co-express PW1. As previously shown in adherent neural stem cells, the majority of neurosphere cells (~70%) that express the neural stem cell marker NESTIN show a co-expression with PW1 at culture day 7 (Fig. 22C and D). Therefore, in order to optimize the probability of endogenous Pw1 bi-allelic expression, I performed pre-mRNA-FISH on fetal neurosphere cells daily from passage 2 to passage 3 using wild-type fetal stage neurospheres as these conditions corresponded to the peak of bi-allelic expression as deduced from the maternally derived reporter neurospheres.
Figure 22: PW1+ cell number is maintained over neurosphere expansion. A. Neurosphere growth curve from passage 2 to passage 3 represented as the daily total cell number after neurosphere dissociation (n=3). B. Quantification of the number of cells expressing PW1 and the mitosis marker phospho-histone 3 (PH3) from passage 2 to passage 3 neurosphere cells (n=1). C. Immunostaining for NESTIN and PW1 on dissociated fetal neurospheres at passage 2. D. Quantification of the number of cells expressing PW1 and NESTIN from dissociated fetal neurospheres immunostained as shown in B (n=3). Overall, the number of PW1+ cells did not change dramatically over neurosphere growth compared to PH3+ cells that seemed to increase at culture day 5. A mentioned earlier at culture day 7, 70% of the fetal neurosphere cells heterogeneously express PW1. Approximately 10% of the total neurosphere cells express strong levels of PW1. The majority (95%) of PW1+ cells co-express the neural stem cell marker NESTIN.
Pre-mRNA-FISH allows for the direct visualization of a gene transcription at the single cell level. By generating fluorescent probes specific to the primary gene transcript, one can identify whether a cell is transcriptionally active at one or both loci. Under the guidance of Edith Heard (Institut Curie - Paris), I established pre-mRNA-FISH technique in our laboratory. I first designed pre-mRNA-FISH probes: 2 probes for Pw1 and 1 probe for the non-imprinted gene Nestin (Fig. 23A). Targeting intronic regions ensures probe specificity for the primary transcript (pre-mRNA). Meanwhile I also worked out the pre-mRNA-FISH protocol, notably, the way I would process the neurospheres during the whole proliferation period. I opted for a quick enzymatic dissociation of neurospheres to facilitate pre-mRNA-FISH experiment and the subsequent analysis on single cells.

At all stages analyzed, both Pw1 probes revealed 8% to 20% of Pw1 transcriptionally active cells within growing neurospheres (Fig. 23B and C). Importantly, both Pw1 probes showed between 0.9% to 2.6% (P1) and 0.7% to 2.3% (P2) of Pw1 bi-allelic expressing cells, or more specifically, 8% to 15% of Pw1 transcriptionally active cells within neurospheres display Pw1 endogenous bi-allelic expression. Results obtained with the Pw1 P1 probe highlight a significant increase in the total number of cells that fired Pw1 transcription from culture day 1 to day 4, 6, and 7 using Two-way ANOVA (not significant using Kruskal-Wallis test on total Pw1+ cells only, P=0.0620) (Fig. 23C). However, this was not reproduced using Pw1 P4 probe. Taken together we conclude that the percentage of Pw1 bi-allelic expressing cells was not correlated with any specific phase of neurosphere growth. Nestin pre-mRNA-FISH also showed a steady level of 60% of Nestin transcriptionally active cells at all stages of neurosphere growth of which one-third show bi-allelic Nestin transcription (Fig. 23B and D). As Nestin is a non-imprinted gene, we anticipated that nearly all the cells would display Nestin transcription from both parental alleles at any given time. My results reveal that transcription is not a stable process from both alleles at all times but is far more stochastic. Alternatively, there remains the risk that the sensitivity and fidelity of the approach used, defined as probe/protocol efficiency and target-specificity respectively, is neither optimal nor reliable and could lead to the wrong interpretation. Therefore, I used two positive control probes, Eya3 and Atrx, (kindly provided by Edith Heard) to ensure that the pre-mRNA-FISH protocol was working efficiently (Fig. 23E).
Figure 23: Bi-allelic expression of the endogenous Pw1 gene in neural stem cells.
A. Probes used for primary transcript detection by pre-mRNA-FISH. Two probes were designed for Pw1 (P1 and P2). One probe only was designed for Nestin (N1). B. Confocal microscopy images of pre-mRNA-FISH hybridization for Pw1 (left panel) and Nestin (right panel) on primary cultured fetal neural stem cells. One red fluorescent dot per nuclei indicates a mono-allelic expression. Two red fluorescent dots per nuclei indicate a bi-allelic expression. C and D. Pw1 and Nestin transcriptional firing in neural stem cells from passage 2 to passage 3 neurospheres.
was determined daily by scoring fluorescent signals (dots) per nuclei following pre-mRNA-FISH as shown in D (n=3). Cells were also treated with RNase (RNase+) on culture day 7 to assess RNA-specificity of the probes (n=3). 10T1/2 fibroblasts cells do not express PW1 and NESTIN. They were used as a negative control cell line to assess target gene specificity of the probes (n=3). For both *Pw1* and *Nestin*, the percentage of mono- and bi-allelic expressing cells was stable over the 7 days of neurosphere proliferation. *Pw1* P1 probe revealed a significant increase in the total percentage of cells that fire *Pw1* transcription at day 4, 6, and 7 of culture. However, this increase was not seen with the *Pw1* P4 probe. Two third of the *Nestin* transcriptionally active cells are bi-allelic whereas 10% only of the *Pw1* transcriptionally active cells are bi-allelic. This result fits with *Pw1* imprinting status. Overall, 0.7% to 2.6% of the total neural stem cells fire *Pw1* transcription from both parental alleles. This result is much lower than what we would expect according to *Pw1IRESnLacZ* reporter gene expression. For *Pw1* (P1), statistical analysis was performed using two-way ANOVA (F(12,42)=1.085, P value=0.03969). For *Pw1* (P4), statistical analysis was performed using two-way ANOVA (F(12,42)=0.2544, P value=0.9932). For Nestin, statistical analysis was performed using two-way ANOVA (F(12,42)=0.2657, P value=0.9917). E. *Eya3* transcriptional firing in neural stem cells was assessed as a positive control for bi-allelic gene expression (n=3). In female only neural stem cells, the transcriptional firing of the X-linked gene *Atrx* was assessed as a positive control for mono-allelic gene expression since one X chromosome is inactive (n=2). F. Fluorescent microscopy image showing *Pw1* loci detectable by DNA-FISH in neural stem cells used in B-D. F. Accessible *Pw1* DNA loci were scored by the number of fluorescent dots per nuclei following DNA-FISH as shown in H (n=2). The yellow arrow shows a cell with no *Pw1* locus accessibility. The red arrow shows a cell with one accessible *Pw1* allele only. The white arrow shows a cell where the two parental *Pw1* alleles were accessible. This result suggests either that the DNA-FISH technique has to be improved or that probes do not have access to *Pw1* loci in some cells. It could also explain why transcriptional firing detected by pre-mRNA-FISH as shown in B and C is not seen at the same percentage than protein levels as shown by immunostaining.
Eya3 expression is known to reflect bi-allelic active transcription in >80% of ES cell-derived neural stem cells (Gendrel et al., 2014). In the conditions tested thus far, I observed, in fetal neurospheres cultured for 7 days, only 40% of Eya3 transcriptionally active cells including one third that are firing Eya3 bi-allelically. Atrx is an X-linked gene known to show mono-allelic expression in female neural stem cells due to X-chromosome inactivation (Gibbons et al., 1995, Baumann and De La Fuente, 2009). Therefore, as an additional control, I generated neurospheres derived from a 4 weeks old female brain and I observed >60% of cells that fired Atrx transcription mono-allelically (Fig. 23E) and only 0.5% of the total cells showed bi-allelic signal. These control results proved that the pre-mRNA-FISH technique is working. I was able to discriminate mono- versus bi-allelic expression; however, this approach may not be optimal given the low number of Eya3 transcriptionally active cells detected. Hence the percentage of cells that fire Pw1 transcription from both parental alleles revealed earlier was potentially underestimated.

I next carried out an assessment of RNA target-specificity for each probe used in these experiments. To verify that the signal obtained reflected pre-mRNA, I treated culture day 7 neurosphere cells with RNAse A, which should eliminate the signal. In all cases, I observed that RNAse treatment reduced the signals obtained for Nestin, Pw1 P1, and Pw1 P4 probes for either mono-allelic transcription (1.6%, 1.2%, and 1%, respectively) or bi-allelic expression (0.1%, 0%, and 0.1%, respectively) (Fig. 23C and D). I next performed pre-mRNA-FISH on 10T1/2 fibroblasts cells that do not express either Pw1 or Nestin. I observed that the Nestin probe did not detect any significant signals for mono-allelic transcription (1.6%) or bi-allelic expression (0%) (Fig. 23D). In contrast, Pw1 P1 and Pw1 P4 probes showed a high background of signals either mono-allelic (6.4% and 6.1%, respectively) or bi-allelic dots (0.6% and 0.9%, respectively) (Fig. 23D). Taken together, RNAse treatment demonstrated that all three homemade probes are specific to RNA, however, using 10T1/2 cells only the Nestin probe appeared highly specific. This is surprising since there are no Pw1 paralogs in the mouse genome, and thus the source of this background remains unresolved. Thus, while the various approaches used strongly support that bi-allelic Pw1 expression occurs in neuronal stem cells, we cannot arrive at this conclusion with certainty.
In parallel to these investigations, I worked out the DNA-FISH technique as a tool to verify that the two dots revealing bi-allelic Pw1 transcription in a nuclei cell upon pre-mRNA-FISH are at the exact same location as the two corresponding Pw1 genomic DNA loci. The goal was to perform first pre-mRNA-FISH, analyze, and subsequently carry out DNA-FISH and superimposed RNA and DNA-FISH signals in geo-localized cells using a BAC-derived Pw1 probe that spans 180Kb of Pw1 genomic DNA locus. Using this approach I observed a heterogeneous signal after DNA-FISH alone. Specifically 36% of the total neural stem cells showed accessibility to both Pw1 genomic DNA loci (Fig. 23F and G) and 17% of the total cell population did not show any Pw1 genomic DNA loci. This unanticipated preliminary result may reflect technical issues such as DNA denaturation or cell permeabilization efficiency or may reflect that the accessibility to either one or both Pw1 loci depends on the neural stem cell state.

5. NEURAL STEM CELL CAPACITIES ARE NOT IMPAIRED UPON PATERNAL DELETION OF Pw1 IN VITRO

While investigating endogenous Pw1 bi-allelic expression I started to assess whether the allele-specific expression of Pw1 is necessary for neural stem cell proliferation and differentiation. I thus took advantage of our recently generated Pw1 constitutive knockout mouse model described in Chapter 6 to delete respectively the paternal or the maternal Pw1 allele.

Primary neurospheres derived from Pw1+/+ and Pw1m+/p− newborns did not show any significant differences in terms of neurosphere size and number (Fig. 24A-C). They were both rich in NESTIN+ cells, and at later stages, postnatal days 7 and 60, primary neurosphere number remained unchanged upon paternal loss of Pw1. Upon neurosphere passage, I observed the same outcome in secondary and tertiary neurospheres for all stages analyzed thus far (data not shown). Primary neurospheres derived from Pw1+/+ and Pw1m+/p+ newborns littermates did not show either significant differences in terms of neurosphere number and size (data not shown). I cannot really conclude on differentiation capacities of Pw1+/+ and Pw1m+/p− newborn-derived neurospheres as I did not repeat the experiment but both neurospheres types gave rise to the three expected differentiated cell types: astrocytes mainly, neurons and
oligodendrocytes (Fig. 24D and E). As a control I checked Pw1 transcript levels in newborn neurospheres and observed, as in newborn and adult brains, that the truncated Pw1 allele was expressed in Pw1\textsuperscript{m+/p} neurospheres but not in Pw1\textsuperscript{m-/p+} neurospheres (Fig. 24F). Importantly, in contrast to the newborn brain (see Chapter 6), I was not able to detect any wild-type Pw1 transcripts in Pw1\textsuperscript{m+/p} neurospheres in the conditions tested. This result led us to the conclusions that either RT-PCR is not a sensitive enough technique or that Pw1 pre-mRNA-FISH probes were not specific enough to Pw1 primary transcript misleading the endogenous detection of Pw1 bi-allelic expressing cells within neurospheres.

\textit{NB}: It would have been worth generating neurospheres from one-year-old mice to see whether we obtain differences upon loss of Pw1. Indeed, at postnatal day 60 large error bars did not allow us to find a significant increase (Fig. 24C).

Preliminary results strongly suggested that the maternal Pw1 allele could be activated in very specific tissues and cell types, notably the developing neural stem cell niches. Accordingly, this first axis of research relied on studying Pw1 allele-specific expression and function in neural stem/progenitor cells grown as neurospheres. The Pw1\textsuperscript{IRESnLacZ} reporter mouse model allowed me to identify a subpopulation of bi-allelic reporter expressing cells within neurospheres. The pre-mRNA-FISH technique was chosen to prove this novelty at the endogenous Pw1 locus but I could not firmly validate it and the generation of Pw1\textsuperscript{m+/p} mutant neurospheres did not reveal any Pw1 transcripts of the maternal allele. In addition, I found that loss of PW1 does not affect neurosphere size and number at the three stages analyzed thus far. Due to the time limitations in completing a Ph.D. and the numerous technical obstacles encountered in addressing the question of bona fide bi-allelic PW1 expression in neural stem cells, I abandoned this project in order to obtain clear results to satisfy the requirements for a Ph.D. including, most notably, a primary scientific communication from the Ph.D. candidate. Nonetheless, I propose that there is now a stronger and more developed framework that I have established that can form the basis for future efforts on this question and then ultimately, what is the biological significance of bi-allelic expression in the neonatal brain.
Figure 24: Paternal loss of Pw1 does not affect neurosphere generation. A. Primary neurospheres derived from Pw1+/+ and Pw1m+/p- newborns subventricular zone after 7 days in vitro. B. Immunostaining for NESTIN of Pw1+/+ and Pw1m+/p- on newborn neurospheres at passage 2. C. Primary neurosphere number at postnatal day 0, 7 and 60 derived from Pw1+/+ and Pw1m+/p- subventricular zone. Neurospheres derived from both genotypes were comparable in terms of size and number. No significant differences were found thus far up to passage 3 (data not shown) (n≥3). D. Immunostaining for GFAP (astrocytes), Tuj1 (neurons) and, O4 (oligodendrocytes) on Pw1+/+ and Pw1m+/p- neural stem cells differentiated for 5 days. E. Quantification of the number of cells expressing GFAP, Tuj1 or O4 from differentiated Pw1+/+ and Pw1m+/p- neural stem cells immunostained as shown in D (n=1). Neurospheres derived from both genotypes were able to generate the three major lineages of neural stem cells: astrocytes, neurons and oligodendrocytes. F. Pw1 wild-type and truncated (knockout) allele expression level in Pw1+/+, Pw1m+/p- and, Pw1m+/p+ newborn primary neurospheres analyzed by semi-quantitative RT-PCR. 10T1/2 fibroblasts cells do not express PW1; they were used as a negative control. Pw1m+/p- brains showed complete absence of Pw1 wild-type allele expression, which suggest that primary newborn neurospheres do not express Pw1 off the maternal allele. We can notice that the truncated Pw1 allele is still transcribed when inherited paternally. Pw1m+/p- brains only show Pw1 expression from the paternal untruncated allele. Accordingly, the Pw1 endogenous bi-allelic expression observed in fetal primary neurosphere using pre-mRNA-FISH is questioned.
CHAPTER 6. PW1 FUNCTION IN THE CENTRAL NERVOUS SYSTEM

1. Generation of a novel PW1 knockout mouse model

One of the most powerful genetic tool for examining gene function is the use of genetically modified animal models including mice, notably the directed deletion of a gene sequence referred to as knockouts and the insertion of interfering sequences into a gene referred to as knockins. Two mutant alleles for Pw1 have been generated previously. The group of Surani generated the first mutant allele for Pw1 in 1999 (Li et al., 1999) followed by the group of Kim in 2013 (Kim et al., 2013). Both groups used the same targeting strategy, which involved the insertion of a large cassette containing the LacZ reporter gene and the Neomycin selection cassette into coding exon 5 or intron 5, respectively (Li et al., 1999, Kim et al., 2013). It has become increasingly recognized that the residual sequences left in the genome as a result of site directed gene recombination can lead to changes in normal gene expression that lie near the inserted DNA (Gerard et al., 1999, Muller, 1999). As discussed in more detail below, the neomycin selection cassette used for screening recombinant ES cells has specifically been found to lead to unanticipated outcomes in later analyses of the resultant mutant mice (Muller, 1999).

Lastly, >90% of the coding domain for PW1 is located in exon 9 which also has a putative transcriptional start site and can generate a functional protein (Relaix et al., 1996, Relaix et al., 1998), thus, it remained possible that the recombined gene can generate a transcript that can successfully produce a functional protein. Given these concerns coupled with the fact that neither mouse model could be obtained, we generated a novel mutant allele (see Chapter 6.2). We therefore designed and generated a Pw1loxneolox (Pw1 floxed Neo+) mouse line containing loxP sites flanking Pw1 coding exons 8 and 9 thus targeting the major coding exon. The characterization of this mouse model was central to the efforts of the entire team and is described here.

We first verified the pattern of PW1 expression following the insertion of the loxP sequences and neomycin ES cell-selection cassette prior to flippase crossing. Expression analyses of the Pw1loxneolox mice revealed a complete loss of PW1 expression in adult tissues. Analyses of Pw1loxneolox embryos revealed the expected high levels of expression
at stage E11.5 that was indistinguishable from wild-type embryos (Fig. 25). In contrast, by stage E14.5, expression levels of PW1 in Pw1loxneolox embryos markedly and precociously declined to near undetectable levels in multiple tissues (Fig. 25). As mentioned previously, numerous studies have shown that the presence of the neomycin selection cassette can interfere with correct cis gene expression (Gerard et al., 1999, Muller, 1999). Therefore, we took advantage of the Frt sites flanking the neomycin gene and crossed the Pw1loxneolox mouse with a Flippase mouse (Rodriguez et al., 2000) in order to excise the neomycin gene. The resulting Pw1floxed mouse (floxed Neo’) was characterized and we found completely normal levels and patterns of PW1 expression indistinguishable from wild-type mice. While the precise mechanism of the interference with normal PW1 expression is unclear, these results underscore the importance of removing residual sequences that can pose cis-mediated transcriptional interference. While the final mouse line we generated has a major advantage of being a conditional knockout line that can be crossed to a large array of Cre and Cre-inducible lines, we first used this line to generate a constitutive knockout in order to compare our results with the Pw1 mutant mouse phenotypes previously described in the literature (Li et al., 1999, Kim et al., 2013). We therefore crossed the Pw1floxed mouse with a constitutive PGK-Cre mouse (Lallemand et al., 1998) (see Chapter 6 section 2). While described in detail in Chapter 2 section 2b, the two major phenotypes previously ascribed to loss of PW1 expression are a reduced body size and highly compromised maternal behavior (Li et al., 1999). Once I had established a constitutive knockout mouse for Pw1, I oversaw the expansion of the colony during which I had informally observed that the mutant mothers appeared to properly nurse and care for their pups. This observation had a potentially high impact on the field of parental imprinting since the potential selective advantages of imprinting and potentially shared roles for parentally imprinted genes remains highly debated. The seminal observations by Surani and colleagues (Li et al., 1999) that Pw1 was critical for proper maternal behavior had become a cornerstone in the field. We therefore decided, in concert with my thesis committee, that a rigorous examination of maternal behavior should be carried out.

NB: The following results will only focus on our Pw1 constitutive knockout mouse model.
Figure 25: Neomycin insertion shuts down PW1 expression during embryonic development. Immunostaining for PW1 on E11.5, E14.5, and E16.5 $\text{Pw1}^{+/+}$ and $\text{Pw1}^{+\text{loxneolox}}$ embryos (8µm thick sections). A. Umbilical cord. B. Brain. C. Abdominal wall. PW1 became completely silenced in $\text{Pw1}^{+\text{loxneolox}}$ embryos between E11.5 and E14.5 (n=1).
2. *Pw1* regulates postnatal growth but does not impact maternal behavior

Please find hereafter the publication entitled: "A novel mutant allele of *Pw1/Peg3* challenges the maternal behavior theory" submitted to PLoS Genetics Journal. In contrast to previously established *Pw1* mutant mouse phenotypes, the present results show that *Pw1* deletion does not have any impact on maternal care, lactation or pup suckling.
A Novel Mutant Allele of *Pw1/Peg3* Challenges the Maternal Behavior Theory

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Abstract

Parental imprinting is a mammalian-specific form of epigenetic regulation in which one allele of a gene is silenced depending on its parental origin. Parentally imprinted genes play a role in growth, metabolism, cancer, and behavior. Although the molecular mechanisms underlying this unique form of epigenetic regulation have been largely elucidated, the potential selective advantage of parental imprinting remains unclear. The mutant phenotype of the imprinted gene, Pw1/Peg3, provides a key example to illustrate the widely held hypothesis on a coadaptation between mother and offspring, in which Pw1/Peg3 is required for a set of essential maternal behaviors, such as nursing, nest building, and postnatal care. We have generated a novel Pw1/Peg3 mutant allele that results in a complete loss of Pw1/Peg3 expression. This allele is different from previous mutant alleles in that it targets the major coding exon of Pw1/Peg3 and does not contain residual sequences used for targeting or reporters. In contrast to previous reports, we observe that maternal behavior and lactation are not disrupted upon loss of Pw1/Peg3. Both paternal and homozygous Pw1/Peg3 mutant females nurse and feed their pups properly and no differences are detected in either oxytocin neuron number or oxytocin plasma levels. In addition, suckling capacities are normal in mutant pups. Consistent with previous reports, we observe a reduction of postnatal growth. These results challenge the coadaptation theory of parental imprinting and instead support a general role in the regulation of body growth.
Author summary

Parental genomic imprinting is a mammalian-specific form of epigenetic control that regulates genes differently depending upon whether they are paternally or maternally inherited. The selective advantage of genomic imprinting is poorly understood and serves as a subject of numerous theories. In the last several decades, mouse genetic studies have revealed that imprinted genes regulate embryonic and postnatal growth, metabolism, stem cells, neuronal functions, and most notably, behavior. The paternally expressed gene Pw1/Peg3 was one of the first imprinted genes shown to influence maternal behaviors essential for pup survival and growth. Several key studies have demonstrated that Pw1/Peg3 is required for proper nursing and milk ejection by the mother and suckling by the offspring. These previous observations have provided a strong support for the coadaptation theory of imprinting, which proposes that imprinted genes regulate the use of resources between mother and progeny to optimize their survival and future reproductive success. Here we describe that Pw1/Peg3 mutant females exhibit intact maternal behaviors and do not display milk ejection defects. In addition, mutant pups are able to nurse properly.
Introduction

Parental imprinting is a form of epigenetic regulation that results in an allele-specific expression of a gene according to its parental origin and is restricted primarily to placental mammals (McGrath and Solter, 1984, Surani et al., 1984, Cattanach and Kirk, 1985). Since 1991, about 100 parentally imprinted genes have been identified and mutant mice have been generated for many of these genes (reviewed in (Ferguson-Smith, 2011, Peters, 2014, Plasschaert and Bartolomei, 2014)). The selective advantages of parental imprinting remain unclear. Analyses of mice carrying mutations in parentally imprinted genes as well as genetic diseases corresponding to parentally imprinted genes in humans have shown that many of these genes play key roles in regulating body growth, metabolism, and adult behaviors (Itier et al., 1998, Lefebvre et al., 1998, Li et al., 1999, Curley et al., 2005, Varrault et al., 2006, Garfield et al., 2011, Charalambous et al., 2012).

*Pw1/Peg3* was identified initially in 1996 and was shown to be expressed primarily from the paternally inherited allele (Kuroiwa et al., 1996, Relaix et al., 1996). PW1/PEG3 expression initiates upon gastrulation and persists at high levels in multiple embryonic tissues (Kuroiwa et al., 1996, Relaix et al., 1996). Most postnatal and adult tissues exhibit restricted PW1/PEG3 expression in a small cell population with a notable exception of the brain, which maintains higher levels of expression in the neuronal lineage throughout adult life (Relaix et al., 1996, Li et al., 1999, Besson et al., 2011). A constitutive Pw1/Peg3 knockout mouse mutant was generated previously and analyses of the paternal mutants (*Pw1/Peg3*<sup>m+/p−</sup>) revealed perinatal growth retardation (Li et al., 1999). In addition, *Pw1/Peg3*<sup>m+/p−</sup> females displayed impairments in nest building, pup retrieval, and decreased milk ejection leading to decreased offspring survival (Li et al., 1999, Chiavegatto et al., 2012, Kim et al., 2013). Li and colleagues (Li et al., 1999) identified a decrease in oxytocin-expressing neurons as one primary mechanism underlying these maternal defects. Loss-of-function of another parentally imprinted gene, *Mest/Peg1*, also results in maternal behavior defects (Lefebvre et al., 1998). These observations, made more than 20 years ago, were viewed within the context of two prevailing theories regarding the role of parentally imprinted genes and their unique epigenetic control. The first theory, referred to as the ‘parental conflict hypothesis’ (also known as the ‘kinship theory’) proposes that the paternal contribution to the offspring drives embryonic and postnatal growth, whereas the maternal contribution limits growth of the offspring, yet promotes the conservation of maternal resources that would favor maternal
survival (Moore and Haig, 1991). The second proposal, referred to as the ‘coadaptation theory’, posits that these genes are critical for optimizing maternal-offspring survival (Keverne and Curley, 2008). These two theories are compatible in that the paternal drive of the offspring growth would be of a little value if the mother either did not survive pregnancy or failed to adequately care for her young, implying that a balance of these two directives is essential for survival.

*Pw1*/Peg3 was discovered in an effort to identify paternally expressed (Peg) or maternally expressed (Meg) genes in the mammalian genome (Kuroiwa et al., 1996) and independently in the same year from a screen for upstream regulators of stem cell specification (Relaix et al., 1996). Consistent with the latter strategy, it was found that *PW1/PEG3* is expressed in adult somatic stem cells in a wide range of tissues including skeletal muscle, skin, gut, testis, and hematopoietic system, and central nervous system (Mitchell et al., 2010, Besson et al., 2011, Pannerec et al., 2013). More recently, it has been demonstrated that *PW1/PEG3* is required for stem cell competence in mesoangioblasts (Bonfanti et al., 2015). The regulatory role of *PW1/PEG3* is likely to be complex as previous studies demonstrated that *PW1/PEG3* participates in the two cell-stress signaling pathways - p53 and TNF/NFκB - leading to either cell death or survival, respectively (Relaix et al., 1998, Relaix et al., 2000). *PW1/PEG3* has also been shown to inhibit the Wnt signaling pathway by promoting β-catenin stabilization (Jiang et al., 2010). In addition, *PW1/PEG3* functions a transcription factor that regulates multiple genes involved in cellular metabolism (Thiaville et al., 2013a). Taken together, these results suggest that *PW1/PEG3* serves as a mediator of cell stress in adult stem/progenitor cells. Whether a role for *PW1/PEG3* in stem cells is independent of a function in regulating maternal behaviors and body growth requires the generation of a conditional *Pw1*/Peg3 allele. Perera and colleagues recently generated a conditional *Pw1*/Peg3 allele targeting the coding exon 6 (Perera et al., 2015). They first induced a constitutive recombination of their mouse model and showed that paternal *Pw1*/Peg3 deletion alone up-regulates the paternally expressed gene *Zim1* and results in postnatal growth defects. Interestingly, they reported *Pw1*/Peg3 expression from the maternal allele in restricted regions of the neonatal and adult brain including the hypothalamus (Perera et al., 2015).

We report here the generation of a novel mutant allele for *Pw1*/Peg3 (referred to henceforth as *Pw1*). Similarly to Perera *et al*, our approach takes advantage of the technological progress since the generation of the initial *Pw1* mutant allele in 1999, allowing for a clean excision of the targeting construct ES-cell selection cassette. We targeted the last
*Pw1* exon with loxP sites that contains >90% of the coding sequence and a putative transcription start site (Relaix et al., 1996), whereas all the other *Pw1* mutants previously reported leave this major coding exon intact. In order to compare our allele with previously reported results for constitutive loss-of-function models, we crossed our *Pw1* floxed mice with PGK-Cre mice to obtain offspring with germ-line mutation of *Pw1*. These mice were then used to establish a colony of constitutive mutant mice. Analyses of paternal, maternal, and homozygous mutant mice were carried out. We confirmed that our targeting strategy eliminated PW1 protein expression during development and in postnatal tissues including the hypothalamus, and as seen in previous studies, mutant mice are viable but display postnatal growth defects. Despite this phenotypic overlap with previous models in postnatal growth, a detailed analysis of paternal, maternal and homozygous *Pw1* mutant females showed that all aspects of maternal behavior (nest building, pup retrieval, crouching, pup sniffing, milk ejection) were indistinguishable from wild-type nulliparous and primiparous adult females. In addition, wild-type pups showed no difference in postnatal growth when nursed by wildtype or mutant mothers and no difference in mutant pup milk intake was observed between wildtype and mutant pups. Lastly, the number of oxytocin expressing neurons in oxytocin-producing hypothalamic nuclei were unaffected and the circulating levels of oxytocin were unchanged. Taken together, our data show that *Pw1* does not play a role in maternal behaviors, but does play a role in the regulation of postnatal growth. The implications of these findings with regard to prevailing theories on the evolutionary benefits of parental imprinting are discussed.

**Results**

**Generation and characterization of a *Pw1* constitutive knockout mouse**

In this study, we have generated a *Pw1* conditional knockout mouse targeting *Pw1* exons 8 and 9 (Fig. 1A). The neomycin ES-cell selection cassette was excised by crossing with a mouse line carrying flippase, which recognizes and recombines Frt sites (Rodriguez et al., 2000). Using these mice, we also generated a constitutive *Pw1* knockout mouse model by crossing our *Pw1* floxed mouse with a constitutive PGK-Cre mouse. The mutant mice were viable and survived to adult stages. As a first step to validate our constitutive *Pw1* knockout mouse model, we examined the *Pw1* transcript and protein levels in the whole brain. We
detected a truncated $Pw1$ transcript from the recombined paternal allele suggesting that the targeted allele underwent correct transcriptional regulation (Fig. 1B). No expression from the maternal $Pw1$ allele was detected in 2 months-old $Pw1^{m+/p-}$ mutant brains (Fig. 1B-E). In contrast, we detected a low level of maternal $Pw1$ transcription in $Pw1^{m+/p-}$ P0 brains samples using semi-quantitative RT-PCR (Fig. 1B).

**Paternal loss of $Pw1$ leads to reduced postnatal growth**

Given the shared phenotype reported for the previously generated $Pw1$ mutant mice for reduced postnatal growth (Li et al., 1999, Kim et al., 2013, Perera et al., 2015), we monitored the postnatal growth of our $Pw1^{m+/p-}$, $Pw1^{m-/p+}$, and $Pw1^{-/-}$ mice. We found that postnatal growth of $Pw1^{m+/p-}$ and $Pw1^{-/-}$ mice displayed a significant reduction by postnatal day 21 (Fig. 1F), whereas maternal $Pw1$ deletion had no detectable effect upon postnatal growth. After weaning, the growth defect observed in $Pw1^{m+/p-}$ and $Pw1^{-/-}$ mice persisted through adulthood with 15 to 20% weight reduction by 2 months of age in both sexes. While overall body sizes were decreased, the weight of $Pw1^{m+/p-}$ and $Pw1^{-/-}$ brains did not show any significant differences between all genotypes. Since the body sizes of the mutant mice were smaller in both $Pw1^{m+/p-}$ and $Pw1^{-/-}$ mice, the brains were proportionally larger (S1 Fig.). Of note, $Pw1^{-/-}$ mice did not show any differences in postnatal growth compared to $Pw1^{m+/p-}$ mice. Hence, the $Pw1$ maternal allele expression detected in $Pw1^{m+/p-}$ newborn brains is not sufficient to rescue body growth.

**Reproduction, litter size, and maternal behaviors are not affected in $Pw1$ mutant mice**

We monitored the matings of $Pw1^{m+/p-}$, $Pw1^{m-/p+}$, and/or $Pw1^{-/-}$ mice and did not detect any impact on the percentage of post coitum pregnancies and all crosses carried out gave rise to the expected Mendelian ratios (Table 1). Importantly, mutant mothers appeared to properly care for their pups and litter sizes recorded at postnatal day 0 were similar in all cases with no significant differences in pup mortality indicating that the mutant mothers were not impaired in bringing their pups to weaning age (Table 1, S2 Fig.). While statistically non-significant, we noted that pup mortality tended to be lower with $Pw1$ mutant female mothers as compared to wild-type mothers; although whether this is due to pup genotype ($Pw1^{+/+}$ and $Pw1^{m+/p+}$) or female genotype remains unclear (S2 Fig.). Given the previous reports that maternal behavior, and more specifically, maternal care for pups is impaired in $Pw1$ mutant female mice, we examined a range of maternal behaviors in our mutant allele. Maternal behaviors were assessed by measuring pup retrieval latency, nest building latency, nest quality, and the time
spent crouching over the pups in nulliparous (virgin) and primiparous female mice. In addition, we measured the time nulliparous females took to acknowledge pup presence by recording pup sniffing latency, to establish a baseline between all genotypes. Among all the behaviors recorded, we did not detect any impairment in maternal care in nulliparous and primiparous Pw1 mutant females (Fig. 2A-B, S1 Movie). Additionally, the percentage of non-retrieved pups born to any group of primiparous mothers did not exceed 8.6% (Fig. 2B). We note that nulliparous females did not show any significant differences in pup sniffing latency among all genotypes thereby allowing for a direct comparison of maternal behaviors for all genotypes examined (Fig. 2A).

Table 1. Reproduction parameters of Pw1 mutant mice are comparable to Pw1+/+ mice.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Female genotype</th>
<th>Male genotype</th>
<th>% Birth (plug efficiency)</th>
<th>Litter size (Litter number)</th>
<th>Mendelian inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pw1+/+</td>
<td>Pw1+/+</td>
</tr>
<tr>
<td>Pw1+/+</td>
<td>Pw1+/+</td>
<td>73.8% ±18</td>
<td>8.1 ±0.5 (14)</td>
<td>100% ±0</td>
<td>0% ±0</td>
</tr>
<tr>
<td>Pw1−/−</td>
<td>Pw1+/+</td>
<td>69.1% ±7</td>
<td>8.6 ±0.4 (9)</td>
<td>46.1% ±6</td>
<td>53.9% ±6</td>
</tr>
<tr>
<td>Pw1−/−</td>
<td>Pw1+/+</td>
<td>80.0% ±19</td>
<td>8.0 ±0.3 (20)</td>
<td>54.3% ±4</td>
<td>45.7% ±4</td>
</tr>
<tr>
<td>Pw1−/−</td>
<td>Pw1+/+</td>
<td>62.7% ±8</td>
<td>7.9 ±1.0 (7)</td>
<td>24.5% ±7</td>
<td>49.2% ±6</td>
</tr>
<tr>
<td>Pw1−/−</td>
<td>Pw1+/−</td>
<td>69.4% ±3</td>
<td>8.1 ±0.4 (10)</td>
<td>0% ±0</td>
<td>100% ±0</td>
</tr>
<tr>
<td>Pw1−/−</td>
<td>Pw1+/+</td>
<td>61.7% ±24</td>
<td>7.7 ±0.3 (15)</td>
<td>0% ±0</td>
<td>100% ±0</td>
</tr>
<tr>
<td>Pw1−/−</td>
<td>Pw1+/−</td>
<td>62.5% ±19</td>
<td>8.5 ±0.4 (13)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pw1−/−</td>
<td>Pw1+/−</td>
<td>ND</td>
<td>ND</td>
<td>0% ±0</td>
<td>100% ±0</td>
</tr>
</tbody>
</table>

Values represent mean ± s.e.m. ND = not determined. Total litter number analyzed for litter size is indicated in brackets for each breeding type. Total pup number analyzed for Mendelian inheritance is indicated for each breeding type in the far right column.

Oxytocin circulating level in females is not decreased upon Pw1 deletion

Oxytocin is a peptide hormone synthesized in the hypothalamus. It has two different sites of release. In the brain parenchyma, oxytocin induces social bonding, notably maternal care (Pedersen and Prange, 1979, Caldwell et al., 1987, Young et al., 1997, Donaldson and Young, 2008). Circulating oxytocin targets myoepithelial cells in the mammary gland to stimulate milk ejection and smooth muscle contraction in the uterus upon parturition (Nishimori et al., 1996). Li and colleagues reported a decrease in the population of oxytocin-
expressing neurons and suggested this was the most likely basis for the decrease in maternal care in \( Pw1^{m+/p^-} \) females described in their study (Li et al., 1999). Consistent with a decrease in oxytocin expressing neurons, they also showed that \( Pw1^{m+/p^-} \) females displayed a decreased capacity for milk ejection in nursing mutant female mice (Li et al., 1999). We therefore examined both oxytocin neuron number and circulating levels of oxytocin. We quantified the number of oxytocin-expressing neurons in the paraventricular nuclei and the supraoptic nuclei and found no differences between all four genotypes (Fig. 3A-E). Consistent with these data, we found that oxytocin plasma levels in nulliparous females were comparable among all genotypes (Fig. 3F). As expected, oxytocin plasma levels increased slightly upon parturition. Compared to wildtype, \( Pw1^{m+/p^-} \) and \( Pw1^{m-/p+} \) mutant females, both nulliparous and primiparous, show no significant change in oxytocin systemic release (Fig. 3F).

**Lactation and suckling in \( Pw1 \) deficient mice is unchanged.**

Li and colleagues previously showed that \( Pw1^{m+/p^-} \) mothers were deficient in milk ejection and suggested that this defect contributed to growth retardation of wild-type pup progeny (Li et al., 1999). In addition, Curley et al (Curley et al., 2004) and Kim et al (Kim et al., 2013) showed impaired suckling of \( Pw1^{m+/p^-} \) pups born to wild-type mothers. However, we observed that wild-type progeny born to \( Pw1^{m+/p^-} \) mothers did not show an impaired postnatal growth as compared to wild-type pups born to wild-type mothers (Fig. 4A). Similarly, \( Pw1^{m-/p+} \) progeny born to \( Pw1^{m+/p^-} \) or \( Pw1^{-/-} \) mothers did not display any postnatal growth differences (Fig. 4B). Taken together, these results demonstrate that mutant mothers, carrying either a paternal mutant or homozygous mutant alleles for \( Pw1 \), do not show any defects in milk let-down or in milk ejection. Additionally, on the day of birth, pups born to mother specific genotype can show the same milk spot size (Fig. 4D). By day 2, all the pups born to \( Pw1^{+/+} \), \( Pw1^{m+/p^-} \), \( Pw1^{m-/p+} \), and \( Pw1^{-/-} \) mothers show a milk spot of a similar size (data not shown). As paternal loss of \( Pw1 \) leads to growth reduction of pups that is detectable during weaning, we wondered if there was a decrease in milk intake in \( Pw1^{m+/p^-} \) pups born to \( Pw1^{+/+} \) mothers by measuring weight gain of the pups. At postnatal day 7, \( Pw1^{m+/p^-} \) pups displayed no detectable differences in milk intake as compared to wild-type pups (Fig. 4C). We conclude that \( Pw1^{p-/m^+} \) pups do not have any detectable suckling defects.
Discussion

Three *Pw1* mutant mouse models have been previously and independently generated. While the studies of Kim *et al* (Kim et al., 2013) and Perera *et al* (Perera et al., 2015) did not examine behavioral phenotypes, Li *et al* (Li et al., 1999) reported that the mothers did not properly care for their young and failed to eject milk correctly. Their study suggested that defects in postnatal growth were due to impairments in maternal function, since the wild-type pups cared by mutant mothers also showed a decreased postnatal growth prior to weaning (Li et al., 1999). However, unlike mutant pups, wild-type pups caught up to normal body size once weaned (Li et al., 1999). These data strongly supported a critical role for *Pw1* in maternal function. Moreover, Curley *et al* (Curley et al., 2004) and Kim *et al* (Kim et al., 2013) demonstrated that mutant pups have a decreased suckling ability, which leads to a decreased neonatal survival rate. Taken together, these studies showed that *Pw1* plays a role in maternal behavior, suckling, and body growth (Li et al., 1999, Curley et al., 2004, Champagne et al., 2009, Kim et al., 2013) and provided genetic evidence for the coadaptation theory of parentally imprinted gene function.

In the present study, we have generated a novel conditional *Pw1* allele and as a first step, we generated a constitutive loss-of-function line in order to compare the outcome with previously published results. While we confirm a postnatal growth defect in both paternal and homozygous *Pw1* mutant mice, we observe that all previously measured features of maternal behavior are indistinguishable from wild-type mice. This is surprising since *Pw1* is one of two genes, including *Mest/Peg1*, that have been shown to lead to maternal defects when mutated, and consequently these mutant phenotypes have been frequently cited as a key support for the coadaptation theory that states that there is a mammalian-specific growth demands of the pups that is genetically balanced with the survival needs of the mother (Keverne and Curley, 2008). How might we account for these phenotypic differences among different *Pw1* mutant models? In the course of our analyses, we first generated a conditional mouse line that retained the neomycin selection (*Pw1^loxneolox*). We observed that the presence of the neomycin gene silenced PW1 expression as early as embryonic stage E12-13 (S3 Fig.). A similar disruption of neighboring gene expression by neomycin insertion has been observed previously in multiple studies (Olson et al., 1996, Zakany et al., 1997). Notably, the imprinted gene *Necdin*, encoding a DNA-binding protein is a maternally imprinted gene highly expressed in many tissues during embryonic and fetal development (MacDonald and Wevrick, 1997, Watrin et
A Necdn mutant mouse was generated by knocking-in the LacZ gene with and without the neomycin cassette (Gerard et al., 1999). The authors observed that reporter gene expression was silenced in LacZ\textsuperscript{+}Neo\textsuperscript{+} E16.5 embryos whereas when the neomycin cassette was removed, they observed a rescue of normal embryonic expression (Gerard et al., 1999). While Li et al (Li et al., 1999) and Kim et al (Kim et al., 2013) inserted a βgeo cassette containing LacZ and Neomycin genes into Pwl coding exon 5 and intron 5 respectively, Perera et al (Perera et al., 2015) deleted Pwl coding exon 6. In the first two mutants, the authors reported that a paternal deletion of Pwl led to a complete loss of PW1 protein expression. In contrast, Perera and colleagues (Perera et al., 2015) detected PW1 protein expression from both paternal and maternal alleles in the neonatal and adult brain. We therefore removed the neomycin cassette from our Pwl conditional mouse following a cross with the mouse carrying flippase (Rodriguez et al., 2000), that lead to a complete restoration of normal Pwl expression during development and in the adult. A Pwl constitutive knock out mouse line was then obtained by crossing the Pwl\textsuperscript{floxed} mouse with a PGK-Cre mouse (Lallemand et al., 1998) and bred onto a C57Bl/6J background. These observations revealed that removal of the selection cassette is essential for subsequent phenotype and behavioral analyses and prompted us to examine our novel allele in light of the reported mutant alleles of Pwl. In addition, our strategy targeted coding exons 8 and 9 for Pwl thereby eliminating >91% of the coding sequences (Relaix et al., 1996). Relaix et al (Relaix et al., 1996) showed that an alternative Pwl transcription start site is located at the 5'-end of Pwl coding exon 9. Consequently, previously generated Pwl mutant mouse models might represent hypomorphs (Li et al., 1999, Kim et al., 2013, Perera et al., 2015). Notably, Perera et al (Perera et al., 2015) detected PW1 protein in the hypothalamus and choroid plexuses of Pwl\textsuperscript{m+/p} neonatal and adult brains; however, they did not verify a loss of PW1 protein expression signal in homozygous Pwl mutant mouse brains. Additionally, housing conditions can also impact on animal behavior. In Surani's studies mice were housed in a reversed light cycle and experiments were performed during dark period (Li et al., 1999). Due to animal facility practices we performed our experiments during light cycle. Nonetheless, light and dark phase testing have been shown to score similar social behaviors (Yang et al., 2008). Lastly, variability between mouse strains may account for phenotype differences. While we used C57Bl/6J mice, Li et al (Li et al., 1999) generated their Pwl mutant mice in 129Sv mouse strain (Li et al., 1999). Nonetheless, they later derived their mutant mouse model in a C57Bl/6J background and reproduced the maternal behavior phenotype (Champagne et al., 2009). The authors suggested that there was compensation over
time since their original 129Sv *Pwl* mutant mice showed less maternal behavior defects after multiple generations (Champagne et al., 2009). Thus, it is likely that the different targeting strategies used to generate the various *Pwl* mutant mice account for the differences we see compared to the reported behavioral phenotypes (Li et al., 1999, Kim et al., 2013), although specific strain backgrounds may also contribute to differences observed.

We note that growth restriction is a central defect that is common to all *Pwl* mutant mouse models previously established including the novel mutant allele reported in this study (Li et al., 1999, Kim et al., 2013, Perera et al., 2015). Even though we observe that *Pwl* and *Pwl*--/-- pups are significantly smaller around postnatal day 21, they tend to have a decreased weight by postnatal day 2. Indeed Li and colleagues (Li et al., 1999) demonstrated a growth restriction starting at E17.5. Thus, promoting growth may be one of the most important and clearly the most robust function regulated by *Pwl*. However, even in this regard our results differ with the observations reported (Curley et al., 2004, Kim et al., 2013), since we observe that *Pwl*--/-- pups do not exhibit any suckling defect suggesting that growth restriction is completely intrinsic to *Pwl*--/-- and *Pwl*--/-- mice. Postnatal growth retardation of our *Pwl* knockout mouse model is similar to *Rasgrf1* mutant mice, a paternally expressed gene specific to the central nervous system that shows bi-allelic expression from postnatal day 21 (Itier et al., 1998). *Pwl* and *Rasgrf1* also exhibit similar expression patterns in the adult brain (Li et al., 1999, Lein et al., 2007). Itier and colleagues (Itier et al., 1998) identified growth hormone defects in *Rasgrf1* mutant mice. As *Pwl* deletion does not have any impact on *Rasgrf1* transcript levels when examined at birth and in the adult, we conclude that both genes are involved in independent pathways (S4 Fig. A-B).

We note that we did not observe *Pwl* maternal allele expression upon deletion of the *Pwl* paternal allele in the adult brain in our model. Thus, there is no loss of imprinting in the conditions tested. However, we detect *Pwl* paternal allele transcription at postnatal day 0 *Pwl*--/-- brains. Whether this weak expression of the normally silenced copy is due to *Pwl* paternal deletion or reflects a normal relaxation of imprinting at birth remains to be resolved. Nonetheless, our observations are consistent with recent findings of Perera et al (Perera et al., 2015) in the neonatal brain. However, this *Pwl* expression detected from the maternal allele is not sufficient to rescue postnatal growth defects. While the role, if any, of the maternal allele remains to be discovered, we note that we detected transcription from the truncated paternal allele in *Pwl*--/-- and *Pwl*--/-- brains at high levels. As our PW1 antibody was generated to the coding domain present in the recombined 9th exon (Relaix et al., 1996), we cannot determine if a truncated PW1 protein is generated. In addition, Ye and colleagues previously showed
that PW1 is a transcriptional repressor of Zim1 (Ye et al., 2014). Upon paternal loss of Pw1 they detected an increase in Zim1 transcript level and validated PW1 binding to Zim1 gene by ChIP analyses in the neonatal brain. However, in the present study we did not see any change in Zim1 expression in Pw1 knockout postnatal day 0 brains (S4 Fig. C, S3 Fig. B).

In conclusion, seminal studies examining Pwl function had revealed the novel observation that an imprinted gene regulates behavior, specifically those related to maternal care (Li et al., 1999, Curley et al., 2004). These findings have been cited in support of the coadaptation theory to explain genomic imprinting and whether imprinted genes serve coordinated functions (Keverne and Curley, 2008). However, the results presented here using a novel Pw1 knockout mouse model demonstrate that Pw1 does not play a role in maternal behavior, milk letdown, or pup suckling ability, but serve primarily to regulate postnatal body growth. Radford and colleagues (Radford et al., 2012) showed that Pw1 is a stress-response gene during embryonic and fetal development. Specifically, upon maternal nutritional restriction, PW1 expression increases in the brain and liver of fetuses as a compensatory mechanism to ensure fetal growth. This stress response in the whole brain is consistent to previous studies showing that PW1 participates in cell stress-responses leading to cellular growth arrest and cell death (Relaix et al., 1998, Relaix et al., 2000). PW1 expression persists in all stem/progenitor cells from all tissues examined to date including muscle resident stem cells, the hematopoietic system, brain, and skin (Besson et al., 2011). Thus, Pw1 may act as a coordinator to modulate growth cues to the whole body through direct action upon stem cells that underlie tissue growth representing a co-adaptation at the organismal level.
Materials and Methods

Mice

A *Pw1* conditional knockout mouse line was generated by inserting two LoxP sites flanking *Pw1* exons 8 and 9 (Fig. 1A). Two founder lines were generated by homologous recombination. One founder was chosen for further analyses. The Frt site flanked neomycin ES-cell selection cassette was excised by crossing with a mouse carrying flippase (Rodriguez et al., 2000). A constitutive *Pw1* knockout mouse model was subsequently obtained by crossing the *Pw1* floxed mouse with a constitutive PGK-Cre mouse (Lallemand et al., 1998) and expanded onto a C57Bl/6J background (Elevage Janvier). The genotypes tested in this study were *Pw1*+/+(wildtype), *Pw1*p-/m+(paternal deletion), *Pw1*p+/m- (maternal deletion) and *Pw1*−/− (homozygous deletion) mice. All work with mice was carried out in adherence to French government and European guidelines.

RNA extraction, RT-PCR and RT-qPCR

RNA extracts were prepared using RNeasy Mini Kit (Qiagen) according to manufacturer's guidelines. RNA was treated with RNase-free DNase I (Qiagen) following the manufacturer's protocol. cDNAs were synthesized using random hexamers (SuperScript First-Strand Synthesis System; Life Technologies). Semi-quantitative polymerase chain reactions were carried out on a ProFlex™ PCR System. Cycling conditions were as follows: 95°C for 5 min followed by 30 (*18S*) to 34 (*Pw1*) cycles of amplification (95°C for 30 s, 56°C (truncated *Pw1*) or 60°C (*18S*) for 30 s and 72°C for 1 min), followed by a final incubation at 72°C for 10 min. Quantitative polymerase chain reaction was performed on a LightCycler 480 (Roche) using SYBR® green (Thermo Fisher Scientific). Cycling conditions were as followed: 95°C for 5 min followed by 42 cycles of amplification (95°C for 15 s, 60°C for 15 s and 72°C for 20 s), then 95°C for 5 s followed by a final incubation at 65°C for 1 min.

Primers sequences used were: *Pw1* wild-type allele (semi-quantitative PCR) FWD 5'-AAGGCCACTCATCGAGGTCCAAGAGAACTGCC-3' and REV 5'-CCACATTCTTACACTCAAAGC-3'; *Pw1* knockout allele FWD 5'-ACATGCCTGGAACTCCAGTGC-3' and REV 5'-ACCTTCACAGGACTATCTAAGAGGTAGGGG-3'; *18S* FWD 5'-
CGGCTACCACATCCAAGGAA-3' and REV 5'-TATACGCTATTGGAGCTGGAA-3'; Pw1 wild-type allele (qPCR) FWD 5'-TGGGAGTCCAGCTTGCCGAAGA-3' and REV 5'-CCACGCTGTGGGATGGCTTT-3'; Hprt1 FWD 5'-AGGGCATATCCAACAAACTT-3' and REV 5'-GTAAAGCAGTACAGCCCCAAA-3'. For real time PCR, levels of Pw1 expression were normalized to Hprt1 gene expression.

**Western analyses**

Freshly isolated brains were homogenized in lysis buffer (150mM NaCl, 50mM Hepes pH7.6, 1% NP-40, 0,5% sodium deoxycholate, 5mM EDTA) supplemented with 1mM PMSF, Complete (Roche), 20mM NaF, 10mM b-glycerophosphate, 5mM Na-pyrophosphate, and 1mM Na-orthovanadate. Equal amounts of protein were separated by electrophoresis (Novex® NuPAGE® Bis-Tris protein gel 4-12% or 5% home-made Bis-Tris gel) and transferred to a PVDF membrane in 20% methanol transfer buffer. Membranes were probed with polyclonal rabbit antibodies to PW1 (rabbit, 1:10,000) (Relaix et al., 1996) and GAPDH (Abcam). Antibody binding was visualized using horse-radish peroxidase (HRP)-conjugated species-specific secondary antibodies (Jackson ImmunoResearch) followed by enhanced chemiluminescence (Pierce).

**Histological analyses**

For immunofluorescence and immunohistochemistry experiments, animals were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde (PFA), pH 7.4. Brains were post-fixed 18 hours at 4°C, cryoprotected overnight in 20% sucrose in PBS at 4°C, and snap frozen in isopentane at -50°C. Coronal cryosections (8mm) were processed for immunostaining. Permeabilization was performed in PBST (PBS, 0,1% Triton X-100) for 10 minutes. For PW1 staining alone, antigen retrieval was carried out in 0.01M citric acid, pH6.0, with two consecutive incubations of 5 minutes at 95°C. Sections were blocked by incubation for 1 hour in PBS supplemented with 4% IgG-free BSA (Jackson ImmunoResearch). Primary antibodies used were: PW1 (rabbit, 1:3,000) (Relaix et al., 1996), OXYTOCIN (rabbit, Abcam, 1:10,000). For immunofluorescence, antibody binding was visualized using rabbit-specific secondary antibody coupled to Alexa Fluor 488 (Life Technologies). Nuclei were counterstained with DAPI (Sigma). For immunohistochemistry, antibody binding was reacted using rabbit-specific secondary antibody coupled to biotin followed by streptavidine coupled to HRP (Jackson ImmunoResearch). The DAB enzymatic
reaction was carried out using nickel enhancement according to manufacturer's instructions (VECTOR Laboratories). Oxytocin-positive neurons were quantified in the paraventricular nuclei by counting every other section from interaural coordinate 2.680 to 3.400 (from 2.680 to 3.900 for the supraoptic nuclei). Images were acquired using a Leica DM fluorescence and light microscope or Leica SPE confocal microscope.

Maternal and reproduction behavior analyses

Maternal behavior was assessed based upon previously established protocols (Li et al and Champagne et al) (Li et al., 1999, Champagne et al., 2009). Observations were performed during the light period of a non-reversed light cycle cage facility. Females were accustomed to the experimental room at least 30 minutes prior testing. In their home-cage, each female was individually monitored for pup retrieval latency, time crouching over the whole pup set, nest quality and nest building latency. Pup retrieval was assessed as the transfer of a pup into the nest. Nest quality was scored from 0 (absence of nest building activity) to 3 (perfectly built nest with no remaining nest material outside the nest). Accordingly, all cages were enriched with Cell Sizzle (SAFE) nest material for housing and maternal behavioral analyses. Observations and records were performed blind to genotype. The mean of each pup sniffing and retrieving latencies was used for both nulliparous and primiparous females.

Nulliparous (virgin) two months-old females were briefly removed from the test cage. Three newborn pups (0-3 days) were randomly chosen and placed interspaced in the test cage at the opposite side of the nest. In order to assess nest quality, the nest was gently disturbed by placing some nest material far from the nest. Nulliparous females were then transferred back to the test cage. Pup sniffing latency and maternal behaviors were recorded for 20 minutes. Nest quality was scored at the end of the test period. For primiparous females, three to four months old primary timed-pregnant females were isolated at gestation day 17 into a new cage with fresh nest material. On the day of birth (2-6pm), nest quality was scored before removing the female from the cage. All pups were placed interspaced at the opposite side of the nest before disturbing the nest gently. Upon retransfer of the female back to her pups, maternal behavior was monitored and recorded for 7 minutes. Behavioral analysis was carried out as described above except that the female was tested with her own litter. A GoPro Hero3+ camera was fixed on top of the cage in order to better track maternal behavior. In addition, the number of dead pups (excluded from the test) and litter size were recorded.
Controlled matings were performed to assess reproductive competence. Around 5-6pm, two females were introduced to a male overnight. The following morning (9-10pm), females were checked for vaginal plug and separated from the male accordingly. Plugged females were monitored for gestation up to 17 days post-coitum. For each cross, at least three different males and a dozen of females were used for a total of 15 plugs analyzed. For this study, all animals were 2 to 6 months old.

**Milk intake**

Pup milk intake ability was measured to assess suckling ability in postnatal day 7 wild-type and paternal mutant pups born to wild-type mothers in order to avoid potential contribution of the maternal genotype to suckling behavior. Pups were placed into a warm incubator for 2 hours allowing for the weight to stabilize followed by re-introduction to the mother. Individual pup weights were recorded hourly for 4 hours and again at 24 hours.

**Oxytocin measurements**

Blood was collected from the tail of 3 months old nulliparous and 3-4 months old primiparous females using kalium-EDTA coated tubes (Sarstedt). Plasma were isolated according to manufacturer's instructions and stored at -80°C. Oxytocin plasma levels were measured using the Oxytocin ELISA kit (Enzo Life Sciences), following the manufacturer's protocol. Optical densities were measured on a FlexStation 3 (Molecular Devices) at 405nm, with correction at 580nm.

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism software, version 6.0. Tests carried out are mentioned in figure legends; they include one- or two-way ANOVA tests. Data are presented as the mean ± standard error of the mean (s.e.m.) *P<0.05, **P<0.01 and ***P<0.001. The number of animals used for each experimental condition (n) is indicated in the figure legends or in the above methods.

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References


Fig. 1: Pwl knockout strategy and characterization. A. Pwl knockout construct. Pink, blue, and green arrows-arrowheads correspond to location of Pwl primers. B. Expression levels of Pwl wildtype and Pwl truncated knockout alleles from semi-quantitative RT-PCR analysis in postnatal day 0 (P0) and 2 months old (2 mo) Pwl^{+/+} (+/+), Pwl^{m/+p} (+/-), Pwl^{m+p} (+/+), and Pwl^{-/-} (-/-) brains (n=3). C. Expression level of Pwl wild-type allele from real time PCR normalized to Hprt1 gene (n=3). D. PW1 immunofluorescence on 3-4 months old postpartum female hypothalamus (retrochiasmatic area) (n≥4). E. Western blot analysis showing levels of PW1 at P0 in Pwl^{+/+} (+/+), Pwl^{m/+p} (+/-), Pwl^{m+p} (-/-), and Pwl^{-/-} (-/-) brains (n=3). F. Postnatal growth of Pwl^{+/+} (n=26), Pwl^{m/+p} (n=12), Pwl^{m+p} (n=5), and Pwl^{-/-} (n=5) female mice. Paternal loss of Pwl leads to a reduced postnatal growth. In all graphs, values represent mean ± s.e.m. Statistical analysis was performed using two-way ANOVA test. *P<0.05, **P<0.01, and ***P<0.001. NS: non-significant.
Fig. 2: Maternal care is not impaired in Pw1 mutant mice. A. Assessment of maternal behavior in 2 months old nulliparous (virgin) females (n≥12). B. Assessment of maternal behavior in 3 to 4 months old primiparous females on the day of delivery (n≥12). Nest quality is scored as followed: 0= no nest building activity/no nest built; 1= quick nest building activity, few nest materials/twigs have been retrieved; 2= consequent nest building activity with some twigs remaining outside the nest. 3= perfect nest without any twig left outside the nest. In all graphs, values represent mean ± s.e.m. Statistical analysis was performed using nonparametric one-way ANOVA (Kruskal-Wallis test). No significant differences were found between any of the four genotypes.
Fig. 3: *Pw1* deletion does not result in significant decrease in oxytocin production and release. **A.** Left panel: schematic sagittal section of the adult mouse brain showing sectioning direction (arrow) on interaural coordinates. Right panel: schematic coronal section of the adult mouse brain showing the paraventricular nuclei (PVN) and the supraoptic nuclei (SON) in pink. **B-C.** Immunohistochemistry for oxytocin-expressing neurons in the PVN (B) and SON (C) of postpartum female brains (n=5). Scale bar: 50µm. **D.** Total number of oxytocin (OT) positive neurons per nuclei as stained as in Figure 3B and C. No significant differences were found between all four genotypes. **E.** Number of oxytocin-positive neurons per section as stained as in Figure 3B and C. **F.** Oxytocin plasma level in virgin (V) and postpartum (PP) females (n=6). *Pw1*⁻⁻ postpartum females tend to have a lower oxytocin plasma level but this observation is not statistically significant. In all graphs, values represent mean ± s.e.m. except in Figure 3E where values represent mean only. Statistical analysis was performed using nonparametric one-way ANOVA (Kruskal-Wallis test) (Fig. 3D) or two-way ANOVA test (Fig. 3F).
Fig. 4: Lactation is not compromised in Pwl mutant mice. A. Early postnatal growth of wild-type progeny to Pwl\textsuperscript{m+/p} mothers is comparable to the wild-type progeny of Pwl\textsuperscript{1/+} mothers. Weights have been measured at postnatal days 2, 7, 10, 14, and 21, prior to weaning (n=12, n=6, n=9, n=3 for breedings Pwl\textsuperscript{1/+} x Pwl\textsuperscript{1/+}, Pwl\textsuperscript{1/+} x Pwl\textsuperscript{m+/p}, Pwl\textsuperscript{m+/p} x Pwl\textsuperscript{1/+}, Pwl\textsuperscript{m+/p} x Pwl\textsuperscript{m+/p}, respectively). No significant differences were found. B. Early postnatal growth of Pwl\textsuperscript{m-/p+} progeny to Pwl\textsuperscript{m+/p} and Pwl\textsuperscript{1/-} mothers crossed with a Pwl\textsuperscript{1/+} male are comparable. Weights have been measured at postnatal days 2, 7, 10, 14, and 21, prior to weaning (n=9, n=11, for breedings Pwl\textsuperscript{m+/p} x Pwl\textsuperscript{1/+} and Pwl\textsuperscript{1/-} x Pwl\textsuperscript{1/+}, respectively). No significant differences were found. C. At postnatal day 7, milk intake has been assessed by measuring the gain of weight. Milk intake of Pwl\textsuperscript{m+/p} pups is similar to Pwl\textsuperscript{1/+} (n≥8). No significant differences appeared. The two-sided arrow indicates the 2 hour time-window were the pups were starved. D. Milk spot in day 0 pups (arrow). Mother genotype has no apparent effect on milk ejection. In all graphs, values represent mean ± s.e.m. Statistical analysis was performed using two-way ANOVA test.
Supporting Information

S1 Movie. Maternal behavior of primiparous $Pw1^{+/+}$, $Pw1^{m+/p-}$, $Pw1^{m-/p+}$ and $Pw1^{-/-}$ females. One representative female per genotype is shown. Video has been provided accelerated. Normal total test duration is 7 minutes.

![S1 Movie](image1)

S1 Fig. $Pw1$ mutant and $Pw1^{+/+}$ brains show the same weight and size with respect to the body weight and size, respectively. A. Brain weights of 2 months old $Pw1^{+/+}$, $Pw1^{m+/p-}$, $Pw1^{m-/p+}$ and $Pw1^{-/-}$ males. B. Representative picture of 2 months old $Pw1^{+/+}$ and $Pw1^{-/-}$ brains. C. Brain over body weights ratio of 2 months old $Pw1^{+/+}$, $Pw1^{m+/p-}$, $Pw1^{m-/p+}$ and $Pw1^{-/-}$ males. In all graphs, values represent mean ± s.e.m. Statistical analysis was performed using one-way ANOVA (n≥4). *P<0.05, **P<0.01 and ***P<0.001.

![S1 Fig.](image2)

S2 Fig. Progenies of $Pw1$ mutant mothers do not show increased death rate within the first 12 hours of life. Values represent mean ± s.e.m. Statistical analysis was performed using one-way ANOVA Kruskal-Wallis test (n≥12 litters).

![S2 Fig.](image3)
S3 Fig. Neomycin insertion downregulates *Pw1* expression. A-C. Postnatal day 0 brains were analyzed for *Pw1* (A), *Zim1* (B), and *Usp29* (C) expression levels by RT-qPCR. Values represent fold increase ± s.e.m. normalized to *Hprt1* expression level. Statistical analysis was performed using one-way ANOVA Kruskal-Wallis test (n=3). *P<0.05, **P<0.01 and ***P<0.001. D-F. Immunostaining for PW1 on E11.5, E14.5, and E16.5 *Pw1*+/+ and *Pw1*+/loxneolox embryos (8mm thick sections). D. Umbilical cord. E. Brain. F. Abdominal wall. In *Pw1*+/loxneolox embryos, neomycin insertion irreversibly shuts down PW1 expression between E11.5 and E14.5.

S4 Fig. *Rasgrf1* and *Zim1* transcript levels do not change upon loss of *Pw1*. A. *Rasgrf1* isoform 1 real time RT-PCR on postnatal day 0 brains (n=3). B. *Rasgrf1* isoform 1 real time RT-PCR on 2 months old brains (n=3). C. *Zim1* real time RT-PCR on postnatal day 0 brains (n=3). Gene expression levels are normalized using *Hprt1* gene expression.
S5 Fig. Mother genotype does not affect wild-type offspring birth weight. All values were normally distributed (P value > 0.1000, alpha = 0.05, Kolmogorov-Smirnov test). Using one-way ANOVA (F$_{2.25}$=0.538; P value=0.5904) no significant differences were found between all three genotypes (n=7, 8, and 13 pups, respectively).
4. Statistical details missing in the submitted version

*NB:* No outliers were removed prior analyses. Alpha=0.05 for all tests (D'Agostino and Pearson omnibus and Kolmogorov-Smirnov tests: if P<0.05 data do not pass the normality test).

**Fig. 1: Pwl knockout strategy and characterization. C.** Statistical analysis was carried out using two-way ANOVA (F3,16=7.159; P=0.0029). **F.** All values were normally distributed (P > 0.0646, D'Agostino and Pearson omnibus normality test). Statistical analysis was carried out using ordinary two-way ANOVA (F_{48,639}=4.068; P<0.0001).

**Fig. 2: Maternal care is not impaired in Pwl mutant mice. Panel A. Pup retrieval latency:** Values were not all normally distributed (P ≥ 0.0041, D'Agostino and Pearson omnibus normality test). Using Kruskal-Wallis test (P=0.4171) no significant differences were found between all four genotypes. **Nest building latency:** Values were not all normally distributed (P ≥ 0.0383, D'Agostino and Pearson omnibus normality test). Using Kruskal-Wallis test (P=0.1032) no significant differences were found between all four genotypes. **Nest quality:** Values were all normally distributed (P > 0.2142, D'Agostino and Pearson omnibus normality test). Using ordinary one-way ANOVA (F_{3,46}=1.954; P=0.1341) no significant differences were found between all four genotypes. **Time spent crouching:** Values were all normally distributed (P > 0.1567, D'Agostino and Pearson omnibus normality test). Using ordinary one-way ANOVA (F_{3,46}=0.8627; P=0.4669) no significant differences were found between all four genotypes. **Pup sniffing latency:** Values were not all normally distributed (P ≥ 0.0011, D'Agostino and Pearson omnibus normality test). Using Kruskal-Wallis test (P=0.3885) no significant differences were found between all four genotypes. **Panel B. Pup retrieval latency:** Values were not all normally distributed (P < 0.0001, D'Agostino and Pearson omnibus normality test). Using Kruskal-Wallis test (P=0.1346) no significant differences were found between all four genotypes. **Nest building latency:** Values were all normally distributed (P > 0.5123, D'Agostino and Pearson omnibus normality test). Using ordinary one-way ANOVA (F_{3,46}=0.8627; P=0.4669) no significant differences were found between all four genotypes. **Nest quality:** Values were not all normally distributed (P < 0.0001, D'Agostino and Pearson omnibus normality test). Using Kruskal-Wallis test (P=0.5854) no significant differences were found between all four genotypes. **Time spent crouching:** Values were not all normally distributed (P ≥ 0.0027, D'Agostino and Pearson omnibus normality test). Using Kruskal-Wallis test (P=0.1856) no significant differences were found between all four genotypes. **Non-retrieved pups:** Values were not all normally distributed (P < 0.0001, D'Agostino and Pearson omnibus normality test). Using Kruskal-Wallis test (P=0.4047) no significant differences were found between all four genotypes.
Fig. 3: *Pw1* deletion does not result in significant decrease in oxytocin production and release. **D. Upper panel - PVN.** All values were normally distributed (P > 0.1000, Kolmogorov-Smirnov test). Using one-way ANOVA (F_{3,16}=1.798; P=0.1881) no significant differences were found between all four genotypes. Using unpaired t test (P=0.1146) no significant differences were found between *Pw1*^{+/+} and *Pw1*^{m+/p-} genotypes. **Bottom panel - SON.** All values were normally distributed (P > 0.0848, Kolmogorov-Smirnov test). Using one-way ANOVA (F_{3,16}=0.2851; P=0.8354) no significant differences were found between all four genotypes. Using unpaired t test (P=0.2910) no significant differences were found between *Pw1*^{+/+} and *Pw1*^{m+/p-} genotypes. **F.** Only one value was found not normally distributed (Primiparous *Pw1*^{-/-} females; P = 0.0143, Kolmogorov-Smirnov test). Using two-way ANOVA (Interaction: F_{3,40}=0.6892; P=0.5640) no significant differences were found. Using one-way ANOVA (F_{1.559,7.793}=0.2627; P=0.7231) no significant differences were found between all four genotypes within the virgin group. Using Kruskal-Wallis test, no significant differences were found between all four genotypes within the primiparous group. Using one-way ANOVA (F_{3,20}=2.891; P=0.0.0608) no significant differences were found between all four genotypes within the primiparous group. However, using Mann-Whitney test primiparous *Pw1*^{-/-} females showed a significant reduction in oxytocin plasma level compared to *Pw1*^{+/+} (P=0.0260); *Pw1*^{-/-} vs. *Pw1*^{m+/p-} (P=0.0411); *Pw1*^{-/-} vs. *Pw1*^{m+/p+} (P=0.0173).

Fig. 4: Lactation is not compromised in *Pw1* mutant mice. **A.** Statistical analysis was carried out using two-way ANOVA (F_{12,130}=0.6009; P=0.8383). **B.** Statistical analysis was carried out using two-way ANOVA (F_{4,84}=0.5958; P=0.6667). **C.** Statistical analysis was carried out using two-way ANOVA (F_{6,112}=0.5823; P=0.7438).

S1 Fig. *Pw1* mutant and *Pw1*^{+/+} brains show the same weight and size with respect to the body weight and size, respectively. **A.** Statistical analysis was carried out using ordinary one-way ANOVA (F_{3,16}=1.938; P=0.1642). **C.** Statistical analysis was carried out using ordinary one-way ANOVA (F_{3,16}=26.42; P<0.0001). Using unpaired t test *Pw1*^{+/+} and *Pw1*^{m+/p+} brain/body weight ratio are significantly very different (P<0.0001); *Pw1*^{+/+} and *Pw1*^{m+/p+} brain/body weight ratio are not significantly different (P=0.4081). *Pw1*^{m+/p-} and *Pw1*^{-/-} brain/body weight ratio are not significantly different (P=0.7438).

S2 Fig. Progenies of *Pw1* mutant mothers do not show increased death rate within the first 12 hours of life. Values were not all normally distributed (P ≤ 0.1033, D'Agostino and Pearson omnibus normality test). Using Kruskal-Wallis test (P=0.4261) no significant differences were found between all four genotypes. Using Mann-Whitney test pups born to *Pw1*^{+/+} mothers are not significantly different from pups born to *Pw1*^{m+/p-} mothers (P=0.4483).
S3 Fig. Neomycin insertion downregulates *Pw1* expression. A. Statistical analysis was carried out using ordinary one-way ANOVA (F2,9=53.23; P<0.0001). B. Statistical analysis was carried out using ordinary one-way ANOVA (F2,6=0.2396; P=0.7941). C. Statistical analysis was carried out using ordinary one-way ANOVA (F2,6=1.713; P=0.2580).

S4 Fig. *Rasgrf1* and *Zim1* transcript levels do not change upon loss of *Pw1*. A. Statistical analysis was carried out using ordinary one-way ANOVA (F3,8=0.5367; P=0.6701). B. Statistical analysis was carried out using ordinary one-way ANOVA (F3,8=1.049; P=0.4222). C. Statistical analysis was carried out using ordinary one-way ANOVA (F3,8=0.0107; P=0.9983).
This second research axis initiated with the generation of a Pw1 floxed mouse model with the aim of deciphering PW1 functions. While characterizing this novel genetic tool we found that the Neomycin ES-cell selection cassette alone was disrupting Pw1 expression. This observation encouraged us to generate in parallel a constitutive Pw1 knock-out mouse model and confront it to previously established Neomycin+ Pw1 mutant mouse models. A completely new project emerged then since upon characterization and expansion of the Pw1 constitutive knock-out mice I noticed that in contrast to the literature none of the mutant mice exhibit maternal care defects. This finding was confirmed by carrying out proper behavioral analyses. The number of oxytocin expressing neurons, the oxytocin plasma level, and the milk ejection capacity in Pw1 mutant females were similar to wild-type females. In addition, Pw1 mutant pups did not show any suckling impairment suggesting that the postnatal growth restriction observed upon loss of PW1 is an intrinsic phenotype; independent of the mother genotype. Taken together, these results contradict a previously established theory of parental imprinting, the coadaptation theory. Whether these discrepancies are due to mutant mouse-specific construct strategies are discussed. The coadaptation theory mostly relies on previous Pw1 studies, which showed that Pw1 regulates nutritional resources between mother and offspring as an optimization process to ensure successful mammalian reproduction. As researchers are continuously debating on the emergence of parental imprinting, this present work worth a scientific publication. It was recently submitted to PLOS Genetics and sent out for review.


CHAPTER 7. DISCUSSION

_Pw1_ bi-allelic expression: does it occur in the CNS?

_Pw1_ has been described previously as a paternally expressed gene (Kuroiwa et al., 1996). The _Pw1_<sup>BRESnLacZ</sup> reporter mouse model was generated using a modified BAC containing the _Pw1_ locus in which _LacZ_ was introduced into the exon 9 and this modified BAC was used to generate transgenic mice (Besson et al., 2011). This BAC contains a significant amount of flanking genomic DNA that includes critical sequences for specifying parental imprinting (Kim et al., 2012, He and Kim, 2014). We observed that paternal transmission of the transgene gave rise to reporter gene expression that overlapped with endogenous PW1 expression whereas maternal transmission gave rise to reporter gene expression that was either absent or highly reduced depending upon tissue and stage of development. While we cannot follow paternally and maternally transmitted expression on the same tissue sample, given the fact that all cells that express PW1 protein show reporter expression regardless of the sex of parental transmission, we conclude that the maternally transmitted expression occurs in a subset of cells that also express the paternally transmitted reporter. This raises the possibility that bi-allelic expression occurs from the endogenous allele. As our previous studies showed that PW1 is required for stem cell competence, we wondered whether bi-allelic expressing cells possess an even greater stem cell capacity. I addressed this question using the neurosphere assay that was initially very promising but ultimately did not allow me to clearly identify endogenous _Pw1_ bi-allelic expression _in vitro_. The pool of neurosphere cells that showed bi-allelic _Pw1_ reporter expression may reflect a higher stability of β-galactosidase versus endogenous _Pw1_ expression (Muller, 1999). In fact, while >98% of the neurosphere cells showed paternal reporter expression only 70% of the cells have detectable PW1 protein levels, confirming that β-galactosidase protein and activity is still present even when PW1 is below the level of detection. While these data would imply that there is no firm evidence for maternal allele expression of _Pw1_, I was able to detect _bona fide_, albeit low levels of wild-type _Pw1_ transcripts from the maternal allele using PCR-based RNA analysis on postnatal day 0 _Pw1_ paternal mutant brains (_Pw1<sup>m+/p-</sup>_).
Perera and colleagues (Perera et al., 2015) have reported recently that Pw1 is expressed bi-allelically in adult brains in specific areas including the choroid plexus and the hypothalamus (Table 4). We have been unable to replicate this observation and note that Perera et al (Perera et al., 2015) did not use a homozygous Pw1 mutant mouse as a negative control. Taken together, whether Pw1 bi-allelic expressing cells are present in the CNS remains unanswered, however the levels and extent of maternal expression are likely very low.

Identifying Pw1 function

Previously reported Pw1 mutant mouse models by Li (Surani lab) and Kim (Teruyama lab) revealed that Pw1 is required for proper maternal care, milk ejection, subsequent pup survival, pup suckling, body growth, and metabolism (Table 4) (Li et al., 1999, Curley et al., 2004, Curley et al., 2005, Champagne et al., 2009, Kim et al., 2013). Both groups used a similar strategy to target the Pw1 locus by inserting a LacZ+ Neomycin+ βgeo cassette into coding exon 5 or intron 5, respectively. In contrast, we replaced coding exon 8 and 9, which eliminated more than 91% of Pw1 coding sequence including all the zinc finger domains. In contrast to Li and Kim (Li et al., 1999, Kim et al., 2013), we did not detect any impairment in maternal behavior, nursing by mutant females, or suckling by mutant pups.

During the generation of our conditional Pw1 knockout mouse model, we first generated a mouse containing the neomycin selection cassette which was then crossed to the Flippase mouse (Rodriguez et al., 2000). Nonetheless, we characterized the Pw1loxneolox mouse and observed that the presence of the neomycin cassette resulted in a loss of Pw1 expression between embryonic stage E11.5 and E14.5. These data confirm previous observation by others that the presence of the neomycin selection gene impacts expression of neighboring genes and modifies the phenotypic outcomes (Rijli et al., 1994, Fiering et al., 1995, Pham et al., 1996, Gerard et al., 1999, Muller, 1999).
## Table 4. Pw1 mutant mouse models

<table>
<thead>
<tr>
<th>Reference</th>
<th>Construct</th>
<th>Analyzed Genotypes</th>
<th>Phenotypes</th>
<th>Loss of Imprinting</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Li LL, Keverne EB, Apricicio SA, Ishino F, Barton SC, Saurin MA. Regulation of maternal behavior and offspring growth by paternally expressed Peg. Science, 1999</td>
<td>Peg1&lt;sup&gt;+/&lt;/sup&gt;</td>
<td>Growth retardation from fetal stage E17.5; At 4 weeks old, 15% weight loss</td>
<td>None</td>
<td>Expression of the paternal allele only</td>
<td>Hypermorphic model; No obvious structural abnormalities observed in the brain</td>
</tr>
<tr>
<td>Curley JP, Pinock SB, Dickson SL, Thresher R, Miyoshi N, Saurin MA, Keverne EB. Increased body fat in mice with a targeted mutation of the paternally expressed imprinted gene Peg. FASEB J, 2005</td>
<td>Peg1&lt;sup&gt;+/&lt;/sup&gt;</td>
<td>Adult mice show increased body fat (white fat) and higher body weight</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curley JP, Barton S, Saurin A, Keverne EB. Co-adaptation in mother and offspring regulated by a paternally expressed imprinted gene Peg. Proc Biol Sci. 2004</td>
<td>Peg1&lt;sup&gt;+/&lt;/sup&gt;</td>
<td>Reduced maternal and offspring behavior</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Champagne FA, Curley JP, Sweeney WT, Hasen NS, Keverne EB. Paternal influence on female behavior: the role of Peg in exploration, olfaction, and neuroendocrine regulation of maternal behavior in female mice. Behav Brain Res. 2006</td>
<td>Peg1&lt;sup&gt;+/&lt;/sup&gt;</td>
<td>Reduction in exploratory behavior and increased anxiety</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pereira BP, Irnayya R, Kim J. Y1 gene dosage effect and bi-allelic expression of Peg. PLoS ONE. 2015</td>
<td>Peg1&lt;sup&gt;+/&lt;/sup&gt;</td>
<td>Y1Y deletion upregulates Peg1 transcript levels</td>
<td>Yes, maternal allele expression reported in the neonatal and adult brain (hypothalamus, midbrain and cerebral cortex) RNA and protein</td>
<td>No maternal behavior investigation</td>
<td></td>
</tr>
<tr>
<td>Denkert AJ, Besson V, Combes RM, Mazziola A, Marzetti G, Starson DA. A Novel Mutant Allele for Pw1 Mutant Challenges the Maternal Behavior Theory. Submitted.</td>
<td>Peg1&lt;sup&gt;+/&lt;/sup&gt;</td>
<td>Postnatal growth retardation (15-20%) weight loss</td>
<td>Yes, maternal allele expression reported in the neonatal brain only RNA</td>
<td></td>
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*LacZ-reporter gene, Neurocanesion-gene.*
We note that the *Pw1* mutant mouse models generated previously contain the *Neomycin* sequence, which may contribute to the differences in behavioral phenotypes observed in our model. The expression of imprinted genes in the *Pw1* domain could be affected by *Neomycin* insertion such as *Usp29*, *Zim1*, *Zim2* that are expressed in the hypothalamus, *Zim3*, and *Zfp264* (*Kim et al., 1999, Kim et al., 2000b, Kim et al., 2001, Kim et al., 2004*). However, there are currently no reports assessing their function apart from *Usp29* that has been identified as a potent tumor suppressor *in vitro* (*Liu et al., 2011, Martin et al., 2015*). *APeg3* is expressed as an antisense RNA in vasopressinergic magnocellular neurons of the supraoptic nucleus (*Glasgow et al., 2005, Choo et al., 2008*). According to our *Pw1* knockout mouse construct, *APeg3* transcripts are truncated upon paternal inheritance of our *Pw1* mutant allele. However, *APeg3* has been described to downregulate *Pw1* transcripts and protein levels (*Frey and Kim, 2014*) suggesting that *APeg3* disruption does not alter the final phenotype that we observe. A fourth *Pw1* mutant mouse model was generated recently by Perera *et al* (*Kim and Teruyama lab*) where they knocked-out coding exon 6 and removed the neomycin selection cassette (*Perera et al., 2015*) however, no behavioral studies nor examination of nursing capacities by mutant mothers were performed. We note that in this study, a defect in postnatal growth following the paternal loss of *Pw1* was confirmed.

Apart from *Pw1* mutant mouse construct specificities, experimental design may impact phenotypic outcomes, notably breeding strategies. According to Curley, Broad, and Keverne (*Curley et al., 2004, Broad and Keverne, 2011*), *Pw1* acts synergistically at the maternal hypothalamus, the placenta, and the fetal hypothalamus to ensure reproductive success. *Pw1* expression in the placenta and fetal hypothalamus enhances maternal care and lactation, which suggests that offspring genotype affects mother phenotype. Quoting Curley *et al* (*Curley et al., 2004*), "when the mutation was in the foetus, wild-type mothers ate less and failed to increase their food intake in the last week of pregnancy, suggesting an impairment of placental endocrine signals that are, in part, responsible for regulating maternal food intake." If such a generational impact is verified, the genotype of the offspring (n+1) is critical. In our behavioral analysis, females were bred to *Pw1+/+* males meaning that offsprings were either *Pw1+/+* or *Pw1m-/+p+,* which does not lead to PW1 loss. Thus, we can accurately compare all female genotypes altogether. Importantly, according to Curley and colleagues (*Curley et al.,
results, during pregnancy, the maternal weight gain of \( Pw1^{+/+} \) mothers carrying \( Pw1^{m+/p} \) fetuses is decreased, at the same extent than \( Pw1^{m+/p} \) mothers carrying \( Pw1^{+/+} \) pups. Accordingly, they suggested that \( Pw1 \) functions are synchronized and optimized between mother and infant as a coadaptation phenomenon; \( Pw1 \) expression in the fetus enhances \( Pw1 \) functions in the mother. However, they built this hypothesis relying on maternal weight gain only during pregnancy while they could have analyzed maternal behavior, nursing, thermogenesis in \( Pw1^{+/+} \) mothers carrying \( Pw1^{m+/p} \) fetuses to check whether they find the same defects than in \( Pw1^{m+/p} \) mothers carrying \( Pw1^{+/+} \) pups.

Additionnally, Curley et al (Curley et al., 2004) showed that wild-type pups born to \( Pw1^{m+/p} \) mothers have a decreased birth weight and a reduced postnatal growth up to weaning. Using our \( Pw1 \) mutant mouse model we do not reproduce this result meaning that in the conditions tested so far, the mother genotype does not impact pre- and postnatal growth of the pups (Chapter 6, S5 Fig.). Moreover we do not see any increased perinatal death rate upon paternal and homozygous \( Pw1 \) deletion in both mother and offspring.

Aside from genetically modified mouse models, Chiavegatto and colleagues correlated a decreased hypothalamic \( Pw1 \) expression with maternal care defects using a specific intercross of two inbred mouse strains (Chiavegatto et al., 2012). They suggested that \( Pw1 \) mutations in \( LG/J \) mouse strain underlie the single-locus QTL (quantitative trait loci), which they identified on chromosome 7 in association with maternal behavior. However, on the two QTLs analyzed, on chromosome 2 and 7, they focused only on 3 candidate genes based on previous studies including \( Pw1 \). The previous acceptance that \( Pw1 \) controls maternal behaviors is therefore in need of reconsideration. Almost two decades following the first reports linking \( Pw1 \) function to maternal behavior, we conclude that these observations are either in error or are not sufficiently robust to be reproduced. Like \( Pw1 \), \( Peg1/Mest \) mutant mouse model also retains the \( Neomycin^+ \) selection cassette (Lefebvre et al., 1998) and thus, as suggested for the original \( Pw1 \) mutant mice, it is possible that the resultant phenotypes concerning maternal behaviors reflect the disruption of other neighboring genes.

Reduced body growth is undoubtedly the most robust phenotype observed in all \( Pw1 \) mutant mouse models generated thus far including our allele although even in this
regard, we observe some discrepancies (Table 4). Whereas postnatal growth analyses revealed a significant growth reduction by postnatal day 21, paternal loss of \textit{Pw1} leads to a decreased birth weight associated with a lower fiber number in skeletal muscles (Cerrera \textit{et al}, in preparation). However, total \textit{Pw1}^{p-/m+} and \textit{Pw1}^{-/-} brain weights at birth and in adulthood are comparable to wild-type and \textit{Pw1}^{p+/m-} animals meaning that upon paternal loss of \textit{Pw1} the brain is proportionally bigger. This disproportion reflects asymmetric intra-uterine growth restriction (IUGR) where the brain is spared from limited placental nutritional resources. In human, asymmetric IUGR account for 70-80% of the total fetal growth restriction cases and are usually detected in the third trimester of pregnancy leading to perinatal mortality (Lin \textit{et al}, 1991, Militello \textit{et al}, 2009). It is now commonly accepted that fetal growth restriction, usually followed by a postnatal ‘catch-up', is correlated with an increase in adult onset diseases such as neurobehavioral disorders, obesity, type 2 diabetes mellitus, and cardiovascular problems increasing morbidity in adulthood. IUGR has been linked to many paternally expressed genes loss-of-function (DeChiara \textit{et al}, 1990, Takahashi \textit{et al}, 2000, Moon \textit{et al}, 2002, Gicquel and Le Bouc, 2006, Varrault \textit{et al}, 2006, Netchine \textit{et al}, 2013). In addition, Mikaelsson and colleagues showed that IUGR could impact animal behavior much later in life (Mikaelsson \textit{et al}, 2013). Specifically, they showed increased anxiety in adult mice that lacked a placental \textit{Igf2} isoform prenatally; synonym of biased feto-placental exchanges.

Another consistent phenotype regarding \textit{Pw1} mutant mouse models is that paternal loss of \textit{Pw1} induces metabolic dysfunction in adult mice (Curley \textit{et al}, 2005). In fact, similarly to Curley and colleagues, we found increased body white fat in adult \textit{Pw1}^{p-/m+} and \textit{Pw1}^{-/-} mice (Tanaka \textit{et al}, in preparation). Ongoing experiments will allow us to better understand the origin of \textit{Pw1}-dependent metabolic functions.

Even though it is generally accepted that offspring \textit{in utero} development needs to be well controlled and optimized as regard as mother resources, researchers are still wondering why parental imprinting would be selectively advantageous. Independently of Wolf and Hager (2006, 2008), which described an evolutionary mathematical model, Curley and Keverne (Curley \textit{et al}, 2004, Keverne and Curley, 2008) proposed a coadaptation theory of parental imprinting. Using a \textit{Pw1} mutant mouse model they found, in addition to maternal care and lactation defects in mutant females, suckling
impairment in mutant pups. Broad and Keverne (Keverne and Curley, 2008, Broad and Keverne, 2011) also showed that Pw1 was regulating the co-expression of genes in the placenta, the fetal hypothalamus, and the mother hypothalamus. Accordingly, they suggest that Pw1 is a master regulator of reproduction, specifically priming care and nursing of the mother but also suckling, consequently growth, and future maternal care in pups adult life. Pw1 expression in the placenta and offspring hypothalamus would also act synergistically on the mother hypothalamus itself ensuring parenting.

Paternal behaviors are common in many taxa other than mammals. For example, the majority of bird species equally share the responsibility of parental care and in teleost fish, males are even more involved in parenting than females (Dulac et al., 2014). It is convincing to believe that parental imprinting emerged in mammals to guarantee placentation and lactation. The fact that Pw1, as an imprinted gene, would additionally regulate maternal behavior is a comprehensive argument. In mammals, mothers are commonly in charge of nursing and carrying the young; while, except in humans, fathers usually ignore or attack the progeny (Dulac et al., 2014). Previous Pw1 findings supporting coadaptation theory show nonetheless some weaknesses. First, Pw1 has a broad and complex expression pattern throughout embryonic and fetal development and is not simply restricted to the placenta and the hypothalamus. Thus, to support their coadaptation hypothesis Curley and Keverne may require to delete Pw1 specifically in the placenta and/or the hypothalamus of fetuses. Studying imprinting in marsupials, Stringer et al (Stringer et al., 2014) could not find any Pw1 orthologues in the australian tammar wallaby despite a rich genome content in zinc finger genes. This points out that in a species where lactation corresponds to 80% of the total maternal investment from fercodation to weaning (40% in rat), Pw1 does not appear to be conserved. In addition, Keverne's theory does not explain why paternally and maternally expressed genes impact other adult animal behaviors including sleep, learning, memory, and sociability.

Our present results refute the involvement of Pw1 in maternal caring and nursing in females, and milk suckling in pups (Fig. 26, Fig. 27). Thus far, we also fail to show genotype interactions between mother and offspring. The roles ultimately attributed to Pw1 during prenatal development remains to be determined. We note that controlling body growth is a consistent and robust phenotype observed following paternal loss of
*Pw1.* Several questions arise: to what extents the IUGR is responsible for the postnatal and adult onset phenotypes of our *Pw1* knockout mouse? As a consequence of a critical environmental stress condition during fetal development, such as mid-gestation starvation, is the absence of *Pw1* deleterious? Why does *Pw1* display conflicting functions, acting both to promote growth and suppress tumors? Are there cell-specific *Pw1* roles? It is hoped that the characterization of this novel *Pw1* mutant allele along with the *Pw1* floxed mouse model will provide important tools to address these critical questions.
Figure 26: Schema of our \textit{Pw1} knockout mouse phenotypes. Experiments are in process to deepen and correlate together all phenotypes observed thus far. Our team members are currently analyzing skeletal muscle regenerative capacity (Rosamaria Correra), metabolism (Karo Tanaka), heart physiology (Kateryna Kyrylkova), and skin (Sergiy Kyryachenko). Our collaborators are working on the pancreas (Paula Bonfanti, UCL, UK) and vasculature (Elisabetta Dejana, IFOM-IEO, Italy).
Figure 27: Imprinted genes involved in behavior and neuronal functions. Peg: paternally expressed genes in blue. Meg: maternally expressed genes in pink. Question marks correspond to Pw1 functions that were not yet investigated on our constitutive Pw1 knock-out mouse model. A stop sign means that we did not reproduce the previously established Pw1 phenotype. Minus (-): gene impacting negatively the corresponding phenotype. Plus (+): gene impacting positively the corresponding phenotype.
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