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Inducible uniparental chromosome disomy to probe genomic imprinting at single-cell level in brain and beyond

Florian M. Pauler [a,1], Quanah J. Hudson [b,1], Susanne Laukoter [a,1], Simon Hippenmeyer [a,*]

[a] Institute of Science and Technology Austria, Am Campus 1, 3400, Klosterneuburg, Austria
[b] Department of Obstetrics and Gynecology, Medical University of Vienna, Vienna, Austria

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ABSTRACT

Genomic imprinting is an epigenetic mechanism that results in parental allele-specific expression of ~1% of all genes in mouse and human. Imprinted genes are key developmental regulators and play pivotal roles in many biological processes such as nutrient transfer from the mother to offspring and neuronal development. Imprinted genes are also involved in human disease, including neurodevelopmental disorders, and often occur in clusters that are regulated by a common imprint control region (ICR). In extra-embryonic tissues ICRs can act over large distances, with the largest surrounding *Igf2r* spanning over 10 million base-pairs. Besides classical imprinted expression that shows near exclusive maternal or paternal expression, widespread biased imprinted expression has been identified mainly in brain. In this review we discuss recent developments mapping cell type specific imprinted expression in extra-embryonic tissues and neocortex in the mouse. We highlight the advantages of using an inducible uniparental chromosome disomy (UPD) system to generate cells carrying either two maternal or two paternal copies of a specific chromosome to analyze the functional consequences of genomic imprinting. Mosaic Analysis with Double Markers (MADM) allows fluorescent labeling and concomitant induction of UPD sparsely in specific cell types, and thus to over-express or suppress all imprinted genes on that chromosome. To illustrate the utility of this technique, we explain how MADM-induced UPD revealed new insights about the function of the well-studied *Cdkn1c* imprinted gene, and how MADM-induced UPDs led to identification of highly cell type specific phenotypes related to perturbed imprinted expression in the mouse neocortex. Finally, we give an outlook on how MADM could be used to probe cell type specific imprinted expression in other tissues in mouse, particularly in extra-embryonic tissues.

1. Aims and scope of this review

Genomic imprinting is an epigenetic mechanism controlling parental-specific gene expression. An imprinted gene is expressed from either the maternal or the paternal allele only. Much of the interest in imprinted genes originates from the finding that many imprinted genes are important regulators of organismal development and disease, which has been excellently summarized elsewhere (Monk et al., 2019; Peters, 2014; Tucci et al., 2019). Since imprinting restricts gene expression to one parental allele, the biological relevance of imprinted expression should be tested by experiments that increase expression, ideally by approximating biallelic expression. However, our current understanding of imprinted gene function stems largely from classical gene deletion experiments that were often not cell type specific. Since less than 1% of all genes in mouse and human show imprinted expression, and many genes show developmental defects when deleted, it is possible that imprinted genes are not more or less biologically relevant than any other gene. Additionally, some imprinted expression may be a result of an 'innocent bystanders' effect. In such cases the imprinted silencing mechanism required for the correct function of one or more genes may be imprecise and extend to neighboring genes where imprinted expression has no effect on their function (Wilkins and Haig, 2003). Finally, imprinted genes might not act alone, but rather synergize with each other in larger imprinted gene networks to exert their biological function (Patten et al., 2016). In order to gain a clear picture of the role of genomic imprinting in development and disease, a cell type specific map of imprinted expression and tools to analyze the functional consequences of imprinted expression are required. To this end, we will discuss in this review the latest developments in mapping and

* Corresponding author.
E-mail address: simon.hippenmeyer@ist.ac.at (S. Hippenmeyer).
[1] equal contribution.

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Abbreviations

Igf2r	Insulin-like growth factor type 2 receptor
Igf2	Insulin-like growth factor 2
Dlk1	Delta like non-canonical Notch ligand 1
Grb10	Growth factor receptor bound protein 10
Cdkn1c	Cyclin-dependent kinase inhibitor 1C
Ube3a	Ubiquitin protein ligase E3A
Phdla2	Pleckstrin homology like domain, family A, member 2
Meg3	Maternally expressed 3, long non-coding RNA
Snrpn	Small nuclear ribonucleoprotein N
Peg3	Paternally expressed 3
Mest	Mesoderm specific transcript
Osbpl5	Oxysterol binding protein-like 5
Tssc4	Tumor-suppressing subchromosomal transferable fragment 4
Bax	BCL2-associated X
gDMR	gametic Differentially Methylated Region
ICR	Imprint Control Region
UPD	Uniparental chromosome disomy
AS	Angelman Syndrome
PWS	Prader-Willi Syndrome
MADM	Mosaic Analysis with Double Markers
matUPD	unimaternal chromosome disomy
patUPD	unipaternal chromosome disomy
FACS	Fluorescence activated cell sorting
SNP	single nucleotide polymorphism

interrogating the function of imprinted gene expression at the single-cell level.

2. Background

Genomic imprinting has independently evolved at least three times: in insects (Matsuura, 2020), plants (Batista and Köhler, 2020) and therian mammals (Barlow and Bartolomei, 2014). The epigenetic mechanism and biological relevance of imprinting has been most intensely studied in mammals, which is the focus of this review. Like all diploid organisms, mammals possess two sets of chromosomes, one inherited from the mother and one from the father. Multiple mechanisms regulate gene expression on these two alleles, with most presumably acting similarly on both alleles, given that the DNA sequence is identical or highly similar. For some specific gene groups such as the olfactory receptor or protocadherin genes, transcriptional regulation involves monoallelic gene expression, but in most cases the choice of the silent allele is random (reviewed in (Khamlichi and Feil, 2018)). The exception to such feature is genomic imprinting where either the maternal or paternal allele is consistently silenced. Since it was first described in the 1960s, genomic imprinting has attracted constant attention of many researchers (see (Tucci et al., 2019) for a brief historic overview). Many imprinted genes are key developmental regulators that are necessary for normal growth as well as neural development, which we discuss in detail below. Since loss-of-imprinting can cause developmental abnormalities, imprinted genes are also relevant to the fields of regenerative medicine and assisted reproductive technologies (Perrera and Martello, 2019). Besides developmental functions, genomic imprinting has also been implicated in biological processes such as circadian rhythm, sleep, obesity and behavior (Peters, 2014). Insights into these functions have come from diverse studies using *in vitro* systems, animal models and from human patients with imprinting disorders. However the role of imprinted expression of many imprinted genes remains under-investigated, particularly at the cell type specific and single cell level.

3. Mechanism of genomic imprinting

Genomic imprinting is an epigenetic process instructing somatically stable and heritable gene regulatory mechanisms to act differentially on parental alleles without apparent differences in DNA sequence (Goldberg et al., 2007). The DNA sequence-independent function of genomic imprinting is best demonstrated in inbred mouse strains, where both parental alleles show identical DNA sequence, yet genomic imprinting is faithfully maintained (Barlow and Bartolomei, 2014). To discriminate parental alleles, genomic imprinting requires that a stable epigenetic imprint is set in the gamete when the parental genomes are still physically separated (Iurlaro et al., 2017). Typically gametic imprints are concentrations of 5-cytosine DNA methylation marks in so-called CpG islands, regions with unusually high CpG dinucleotide content compared to the rest of the genome (Iurlaro et al., 2017). These regions referred to as gametic differentially methylated regions (gDMR) maintain parental allele-specific DNA methylation throughout subsequent cell divisions. A subset of these gDMRs control imprinted expression of a set of genes in their vicinity and are called imprint control regions (ICRs). These can be definitively identified by genetic deletion, which leads to a loss of imprinted expression of regulated genes (Kelsey and Feil, 2013). Genes under the control of genomic imprinting are referred to as imprinted genes, and are often clustered due to common regulation by one ICR. Some ICRs have the potential to control genes over extremely large distances, with the *Igf2r* cluster (spanning 10 million base-pairs (Mbp) in mouse) being the largest known cluster (Andergassen et al., 2017). Much is known about the nature and evolution of different types of DMRs, the molecular machinery involved in setting, maintaining and erasing germline imprints (including DNA methylation independent imprints), as well as the molecular mechanisms that read the imprint and allow the ICR to exert its function. These processes are detailed elsewhere by several excellent reviews (Barlow and Bartolomei, 2014; Chen and Zhang, 2020; Iurlaro et al., 2017; Kelsey and Feil, 2013; MacDonald and Mann, 2020; Monk et al., 2019; Ondičová et al., 2020; Tucci et al., 2019).

4. Mapping imprinted gene expression by using hybrid mouse models and RNA-sequencing

4.1. Genomic imprinting as a result of parental allele specific expression bias

Historically, the definition of imprinted expression was binary, with an imprinted gene considered to be completely repressed on one of the two parental alleles (Fig. 1A). Otherwise known as canonical imprinted expression, this definition appears appropriate for many well-known imprinted genes, although the impression of all-or-nothing imprinted expression may largely be due to technical limitations. For example, the *Igf2r* gene was initially reported to be exclusively expressed from the maternal allele using the relatively insensitive RNA hybridization technique (Barlow et al., 1991). Later using more sensitive assays it was shown that *Igf2r* is expressed at very low, yet detectable levels, from the silent paternal allele (Latos et al., 2009). Such biased imprinted expression appears to be relatively common. An analysis of 50 imprinted genes revealed that 21/27 maternally expressed genes and 5/23 paternally expressed genes show an expression bias rather than exclusive monoallelic expression (Khatib, 2007). The above finding was confirmed by a large scale analysis of imprinted expression in multiple mouse tissues at different developmental stages (discussed in detail below). In the respective study the vast majority of imprinted genes

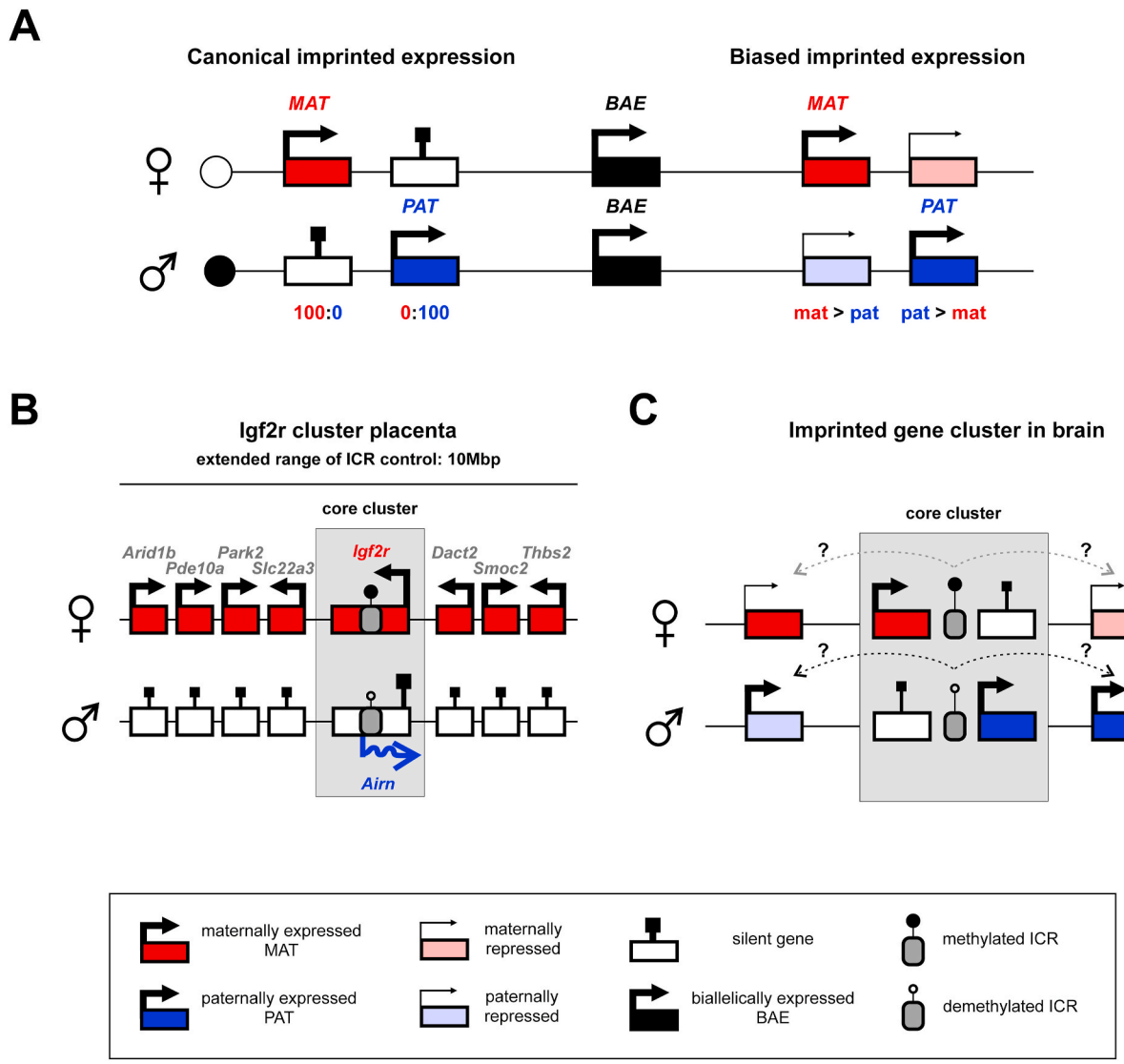


Fig. 1. Genomic imprinting in brain, placenta and extra-embryonic tissues. (A) Schematic of canonical imprinted gene expression and biased imprinted gene expression. Maternally expressed gene (*MAT*) in red, paternally expressed gene (*PAT*) in blue and biallelically expressed gene (*BAE*) in black. **(B)** Extended range of gene expression control by ICRs in extra-embryonic tissues (EET) illustrated by the largest imprinted cluster known to date, the *Igf2r* cluster in placenta. Grey shaded area indicates the core *Igf2r* cluster, consisting of the *Igf2r* and *Airn* genes, with imprinted expression in most tissues of the mouse (core cluster). The imprinting control region (ICR) controls all imprinted gene expression in this cluster. Illustration is not drawn to scale and only genes with imprinted expressions in placenta are indicated. **(C)** ICRs control imprinted genes within the core cluster in brain, similar to other tissues. It is unknown whether brain specific biased imprinted expression is controlled by ICRs and thus is mediated by expansion of imprinted gene clusters, similar to EET (dotted arrows with question marks).

showed detectable expression from the repressed allele in one or more tested tissues (Andergassen et al., 2017). In both above studies imprinted expression was analyzed at the tissue level including multiple distinct cell types. This raises the question of how biased imprinted expression, detected at the tissue level, relates to imprinted expression at the single cell level, a focus of this review.

4.2. Mapping imprinted genes using RNA-seq and hybrid mice

In order to tackle the above questions, affordable methods that allow mapping of imprinted expression at large scale seem necessary. Technical advances in RNA-seq and the availability of DNA sequences of genetically distant mouse strains, and even of individual humans, enables quantification of allelic expression at increasing scale. These technologies make use of single nucleotide polymorphisms (SNPs) that represent differences in the genomic DNA sequence between the two parental alleles. If heterozygous SNPs are located within the body of a gene, the relative abundance of each SNP in steady-state mRNA can be accurately quantified by RNA-seq to enable the calculation of an allelic expression ratio (Castel et al., 2015; DeVeale et al., 2012). In mouse studies, F1 hybrid mice from crosses between genetically distinct mouse strains, such as C57BL/6 and CAST/Ei, are commonly used for allele specific analyses. While the above is a powerful approach for identifying imprinted expression, biological and technical biases may introduce systematic errors, hence careful experimental set-up and dedicated analyses are required to avoid this.

An example of a biological feature that has to be accounted for is so-called strain bias in allelic expression that results from genetic differences between the alleles rather than an epigenetic imprint (Andergassen et al., 2017). Strain specific differences can be unambiguously distinguished from imprinted expression by analyzing F1 hybrids resulting from reciprocal crosses, called forward and reverse crosses. For example in the forward cross the mother is C57BL/6 and the father is CAST/Ei, whereas in the reverse cross the mother is CAST/Ei and the

father is C57BL/6. In other words the strain of the parental alleles is swapped in the forward and reverse crosses. Imprinted genes will show the same parental allele bias in both forward and reverse cross, regardless of strain, whereas strain-biased genes will show a bias dependent on the strain rather than parental origin (explained in detail elsewhere (Andergassen et al., 2015)).

Technical issues that may affect the analysis mainly stem from the exquisite sensitivity of RNA-seq based SNP quantification. These can be overcome by the use of restrictive thresholds and large sample sizes. In addition, extensive validation of novel imprinted genes is necessary to confirm imprinted genes identified using this technology (Bonthuis et al., 2015; Castel et al., 2015; DeVeale et al., 2012; Kelsey and Bartolomei, 2012; Perez et al., 2015). SNP-based allelic expression quantification has become a standard tool to map imprinted genes in mouse (Andergassen et al., 2017; Babak et al., 2015; Bonthuis et al., 2015; Perez et al., 2015), and in human (Babak et al., 2015; Baran et al., 2015; Metsalu et al., 2014; Morcos et al., 2011; Mozaffari et al., 2018; Zink et al., 2018). Besides the clear advantages of this technology, the increased sensitivity also created new challenges once it became possible to detect small, but statistically significant allelic biases (Bonthuis et al., 2015; Perez et al., 2015), as discussed in more detail below (Fig. 1A). Most well-known imprinted genes show a high imprinted expression ratio (expressed/silent allele ratio of at least 70/30 (Andergassen et al., 2017)). These genes have been intensely studied and the biological significance of strong allelic expression biases has been repeatedly demonstrated (Barlow and Bartolomei, 2014; Peters, 2014; Tucci et al., 2019). Although there is currently no reason for disputing their existence, the biological relevance of genes showing imprinted expression biases lower than 70/30 is to date unclear. Further work is necessary to determine the phenotypic consequences that may result from loss of such minor imprinted expression bias.

4.3. A high number of imprinted genes are expressed in brain and extra-embryonic tissues

A recent study used the power of RNA-seq analysis of hybrid mice to compare imprinted gene expression across 23 different tissues and developmental stages and provided a comprehensive picture of imprinted expression during mouse development (Andergassen et al., 2017). This study used the Allelome. PRO bioinformatic pipeline allowing high confidence identification of strongly biased imprinted expression (expressed/silent allele ratio of at least 70/30) from RNA-seq data by using samples from only 4 F1 hybrid individuals, from reciprocal crosses between genetically distinct inbred mice (Andergassen et al., 2015). In agreement with other studies (Babak et al., 2015), the greatest number of imprinted tissues was seen in the brain and in extra-embryonic tissues, namely the placenta and visceral yolk sac. The large number of imprinted genes detected in extra-embryonic tissues, including a number of novel imprinted genes, was likely due to the extended range of expression control by ICRs in these tissues (Andergassen et al., 2017). In this study, most tissues showed progressively decreasing numbers of imprinted genes from embryonic to adult time points. In contrast, a relatively large number of imprinted genes were detected throughout brain development, including only a few novel brain imprinted genes. Careful analysis of multiple single cell RNA-seq datasets independently confirmed the above finding and revealed that expression of imprinted genes was especially prevalent in certain neuronal cell types and brain regions like the hypothalamus (Higgs et al., 2020).

Other RNA-seq SNP studies of mouse brain used large numbers of replicates and more complex statistical analyses, with no allelic ratio cutoff, and identified a relatively large number of novel imprinted genes (41/142, (Bonthuis et al., 2015; Perez et al., 2015)). In these studies, the novel imprinted genes showed minor parental-specific expression bias in the brain areas under investigation, but often biallelic expression in other tissues of the mouse. Together the literature indicates that brain and extra-embryonic tissues harbor the largest number of imprinted genes, although the number of imprinted genes detected in the brain varies largely between studies due to different experimental and analytical approaches.

5. Brain and extra-embryonic tissues show unusual genome-wide DNA methylation patterns

The brain and the extra-embryonic tissues placenta and visceral yolk sac, carry out very different physiological functions. Yet both show high numbers of imprinted genes. Interestingly these two tissues also share another epigenetic feature since both have quite unusual genome-wide distributions of DNA methylation. Placenta and visceral yolk sac generally show low levels of DNA methylation (hypomethylation) when compared to embryonic and adult tissues (Kulinski et al., 2015). Conversely, brain tissue generally shows higher levels of DNA methylation than other tissues (hypermethylation), especially at non-CpG dinucleotides (Cholewa-Waclaw et al., 2016; Renuka Prasad and Jho, 2019). It is unknown whether high or low genome-wide levels of DNA methylation have a direct impact on the number of imprinted genes in a tissue. The larger number of imprinted genes observed in extra-embryonic tissues appears to originate from an extended range of gene expression control by ICRs. The most extreme example is represented by the *Igf2r* cluster. Allele-specific gene expression analysis indicated that the *Igf2r* cluster expands over 10 Mbp and includes 8 protein-coding genes in placenta (Fig. 1B). Deletion analysis of the ICR of the *Igf2r* cluster showed loss of imprinted expression of all eight genes, confirming that all these genes belong indeed to the cluster (Andergassen et al., 2017). A similar analysis is missing for brain-specific imprinted genes. Therefore, it is still unclear whether genes with brain specific expression bias also exemplify an extended range of gene expression control by ICRs (Fig. 1C), or if some other mechanism is responsible for the increased number of imprinted genes in the brain.

6. Imprinted expression at single-cell resolution

6.1. Models to explain imprinted expression bias in complex tissues

The cerebral cortex is a complex brain tissue with unparalleled cell type heterogeneity (Ecker et al., 2017; Lein et al., 2017; Zeng and Sanes, 2017). While the developmental programs controlling neocortex development are relatively well characterized (Lodato and Arlotta, 2015), the precise mechanisms that generate cortical cell-type diversity are not well understood. Mounting evidence suggests that transcriptional programs regulated by epigenetic mechanisms are at the heart of the machinery driving cortical cell fates (Amberg et al., 2019; Mayer et al., 2018; Mi et al., 2018; Nowakowski et al., 2017; Telley et al, 2016, 2019). Cell type specific imprinted expression is one epigenetic mechanism that could in principle control cell fate specification. This idea has gained momentum based on the finding that multiple brain areas show tissue-specific biased imprinted expression (Bonthuis et al., 2015; Perez et al., 2015). Perez and colleagues proposed three different models to explain how biased imprinted expression could arise (Perez et al., 2016).

The first model predicts that all cell types of a given tissue show biased expression for a certain gene. The second model predicts that there are two different types of cells present. One cell type would express the respective gene biallelically. The second cell type however exclusively expresses the gene from a single parental allele. In this case, analysis of the whole tissue will indicate a biased imprinted expression for this gene rather than canonical imprinted expression, even though canonical imprinted expression is present in a subset of the cells. This is similar to a model also proposed by others (Kulinski et al., 2013). The third model predicts that some cell types express only the paternal allele and others express only the maternal allele (allelic switching). If the cell types were present in unequal numbers, tissue-wide analysis will again indicate biased imprinted expression. Importantly, the last two models

both predict that strong imprinted expression in a subset of cells is obscured by biallelic or opposing biased expression in other cell types of the same tissue or organ. These models can be tested by performing allele-specific expression analysis of single cell types, or even at single-cell resolution.

6.2. Cell type specific imprinted expression in extra-embryonic tissues in the mouse

In order to test the above hypothetical models of biased imprinted expression it is necessary to isolate different cell types from a tissue or organ, and analyze imprinted expression for each cell type separately. One experimental approach is mechanical separation and isolation of cell types, which is possible for the extra-embryonic visceral yolk sac (Hudson et al., 2011). Extra-embryonic tissues support the developing embryo and comprise the placenta and three bilaminar membranes that interface with the placenta and surround the embryo: the amnion, visceral yolk sac and parietal yolk sac (Fig. 2A). Following enzymatic digestion, the visceral yolk sac can be mechanically separated into endoderm and mesoderm layers that appear to be composed of single cell types. Using this approach it was demonstrated that imprinted expression was restricted to the endoderm layer. Importantly, imprinted expression bias detected in the whole visceral yolk sac originated from strong imprinted expression in visceral endoderm and biallelic expression in the visceral mesoderm ((Hudson et al., 2011), Fig. 2A). These results supported the second model described above (Kulinski et al., 2013; Perez et al., 2016). Thus strong imprinted expression biases in one cell type can appear weaker due to simultaneous biallelic expression in another cell type of the same tissue.

6.3. Imprinted expression is uniform with no allelic switching at single-cell resolution in mouse neocortex

The intricate mixing of different cell types in the brain does not easily permit physical separation of single cell types using current technologies. However, fluorescence in situ hybridization of RNA (RNA-FISH) including allele specific single molecule RNA-FISH (Ginart et al., 2016; Urbanek and Krzyzosiak, 2017), and reporter fusion proteins (Judson et al., 2014; Stelzer et al., 2016) allow single-cell analysis of imprinted expression. RNA-FISH is relatively low throughput, and reporter fusions require multiple mouse lines. These limitations indicate that systematic and high throughput analysis of a large number of imprinted genes is not easily feasible with either of these methods. Both FISH and reporter fusion strategies are histology based, so distinguishing different cell types represents an additional challenge.

An alternative approach that can overcome these issues is RNA-seq of single cells combined with SNP based allelic expression quantification (Deng et al., 2014; Lin et al., 2016; Santoni et al., 2017). Single cell

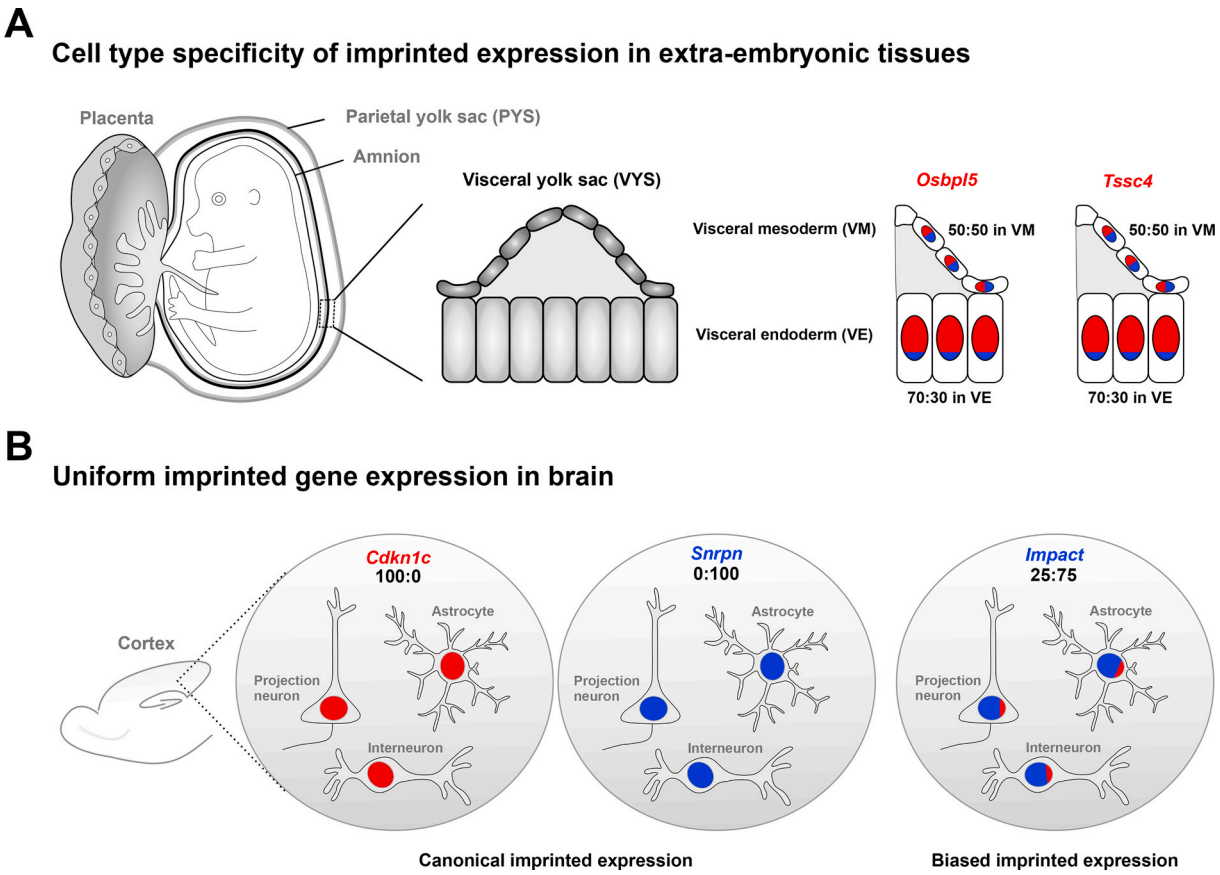


Fig. 2. Tissue-specific differences of cell type specificity of imprinted expression – examples of extra-embryonic tissues and brain. (A) Cell type specific imprinted expression in visceral yolk sac (VYS). (Left) Schematic of extra-embryonic tissues surrounding the embryo (adapted from (Hudson et al., 2011)). (Middle) The VYS is composed of two layers, the visceral mesoderm (VM) and visceral endoderm (VE) that appear to be composed of single cell types. (Right) Two genes, *Osbpl5* and *Tssc4*, show cell type specific imprinted expression bias only in visceral endoderm but not in visceral mesoderm. Note that the biallelic expression in visceral mesoderm obscures strong imprinted expression in visceral endoderm when VYS is studied as a whole. **(B)** Uniform imprinted expression in different cell types of neocortex. Imprinted gene expression analysis of excitatory projection neurons, inhibitory interneurons and astrocytes revealed that canonically imprinted genes are expressed from the same parental allele in all three cell types. *Cdkn1c* is maternally expressed and *Snrpn* is paternally expressed. For both genes, 100:0 expression patterns were observed, indicating that only one parental allele is active whereas the other parental allele is silent. For biased imprinted genes, like *Impact* (paternally expressed gene), biased expression pattern was detected across all cell types. Note that no allelic switching was detected.

RNA-seq technologies that preserve the spatial information of cells within the tissue have been reported, but further optimization of cellular resolution and gene coverage is ongoing (Rodriques et al., 2019; Vickovic et al., 2019). In a typical single cell RNA-Seq experiment, tissues are dissociated into single cells and processed in order to determine single cell transcriptome signatures. Since spatial information of the position of cells within the tissue is lost, prior knowledge of cell type specific expression of marker genes is utilized to group single cells into larger cell clusters. Such an approach is powerful and can help to determine the cell-type complexity of the nervous system (Saunders et al., 2018; Zeisel et al., 2018).

Recently we used this method to analyze imprinted expression in well-defined cell types of mouse brain (Laukoter et al., 2020c). To this end, RNA-seq of F1 hybrid mice (from crosses between the genetically distinct inbred mouse strains C57BL/6 and CAST/Ei) was utilized to characterize genetically-defined cell lineages. Specific cell types were fluorescently labelled by using Cre-LoxP reporter mice and isolated by Fluorescence Activated Cell Sorting (FACS). First population level RNA-seq analysis was carried out. Specifically, cells originating from the *Emx1* lineage or the *Nkx2.1* lineage in three different brain regions: neocortex, hippocampus and olfactory bulb were subjected to analysis. The *Emx1* lineage comprises mainly excitatory projection neurons, but also olfactory bulb neuroblast cells and glial cells such as astrocytes, oligodendrocytes (Gorski et al., 2002). The *Nkx2.1* lineage predominantly includes inhibitory interneurons, but also a low number of oligodendrocytes (Xu et al., 2008). In total, 25 imprinted genes were analyzed whereby most of them showed uniform allelic expression across all investigated cell types.

Next, the resolution was increased for the analysis of the *Emx1* lineage in neocortex by performing single-cell RNA-seq on neonatal (P0) and adult brain (P42). Bioinformatic analysis identified 5 major cell classes within the *Emx1* lineage: astrocyte intermediate progenitor cells, mature astrocytes, oligodendrocytes, early postnatal neurons and adult neurons. Interestingly, even at the individual cell level, imprinted expression was uniform in different cell types. For example, maternally-expressed *Cdkn1c* and paternally expressed *Snrpn* showed strict maternal/paternal expression in all cell types (Fig. 2B). Finally, the focus was put on genes that do not show a strict 100:0 imprinted expression, but rather show imprinted expression bias. At the population level, imprinted expression bias was maintained across genetically defined cell types, albeit with some cell type specific variation in the degree of bias. Importantly, similar imprinted expression bias was also detected at the single-cell level across all five cell types of the *Emx1* lineage. For example, the *Impact* gene showed biased paternal expression across all examined cell types (Fig. 2B). Importantly, none of the investigated genes showed signs of allelic switching.

Taken together, there appears currently no evidence for cell type specific imprinted expression in the mouse neocortex. Imprinted expression across examined cell types appears uniform at both the population and single-cell level, with no indication for parental allele specific expression switching (Laukoter et al., 2020c). These findings support the first model from Perez and colleagues (Perez et al., 2016) and suggest that for neocortex, imprinted expression is invariable between cell types. Yet, the above findings are based on genes with a relatively strong imprinted expression bias of at least 70/30 (active/-silent). In the future it will be interesting to determine the generality of these findings and to determine whether genes with more subtle imprinted expression bias in the neocortex also show a consistent bias between cell types. It will also be important to further increase the cell-type resolution. Currently the focus was put on major cell types in the neocortex and thus defined two clusters containing all neuronal cells (Laukoter et al., 2020c). It is well established that neurons in the neocortex are separated into multiple subtypes that reflect spatially distinct cortical regions and that can be distinguished based on their transcriptome (Tasic et al., 2016; Zeisel et al, 2015, 2018). In the future increasing the number of cells subject to single cell RNA-Seq, or employing alternative methods that allow neurons from the different cortical regions to be analyzed separately may reveal a higher complexity of imprinted expression in the neocortex.

Neuronal development as well as neuronal cell types are largely conserved between mouse and human brain, despite species specific differences that have been discussed in detail elsewhere (Zhao and Bhattacharyya, 2018). Given the importance of imprinted genes in neuronal development, it is of interest to understand the level of conservation of imprinted expression in mouse and human brain. Thus far, available data only allowed direct comparison at the tissue level. Three important features became evident. First, many imprinted genes in the brain appear to be conserved between human and mouse. Second, genes with imprinted expression in both mouse and human tend to show a stronger allelic bias. Third, the nervous system shows most tissue-specific imprinted expression in both human and mouse (Babak et al., 2015). To date it is unclear whether specific genes show conserved subtle imprinted expression bias between mouse and human, although it has been suggested that the phenomenon of minor imprinted expression biases *per se* is conserved in human (Kravitz and Gregg, 2019). In summary, it is likely that certain aspects of cell type specific imprinted expression in mouse brain are conserved in human. More work is needed to further refine our understanding of the conservation of cell type specific imprinted expression in the brain of different species.

7. Uniparental chromosome disomy as a tool to probe functional relevance of imprinted gene expression

Our current understanding of genomic imprinting indicates that there is a wide spectrum of tissue and cell type specific imprinted expression. Imprinted expression in brain and extra-embryonic tissues is implicated in development and disease (Hanna, 2020; Huang et al., 2018; Kravitz and Gregg, 2019). Hence the establishment of new tools to probe the functional importance of imprinted expression is necessary. In principle, the ideal method would allow both over-expression and suppression of imprinted genes. Since imprinted genes often synergize in imprinted gene networks (Patten et al., 2016), perturbation of multiple imprinted genes at the same time could also be advantageous. These requirements are fulfilled in uniparental chromosome disomy (UPD). Cells with UPD carry either two maternal and no paternal, or two paternal and no maternal copies of a particular chromosome, while all other chromosomes are unaffected. Canonical imprinted genes located within a chromosomal region affected by UPD are either increased to a double dose or are not expressed (suppressed), depending on the parental origin of the UPD. Notably, the expression of genes showing an imprinted expression bias is also affected when located within a UPD region. Since imprinted genes are highly dose-sensitive, both conditions (over-expression or suppression) lead to an abnormal gene dosage that can cause significant phenotypes (Andrews et al., 2007; Fitzpatrick et al., 2002; Lau et al., 1994; Wutz et al., 2001).

7.1. UPD and their relevance for human disease

UPDs are relatively common in humans (1:3500 live births) and can lead to a variety of diseases (Soellner et al., 2017; Yamazawa et al., 2010). Growth disorders resulting from UPD are often connected to imprinted genes. For instance, Silver-Russell Syndrome (maternal UPD with *Igf2* suppression and *Cdkn1c* over-expression), Beckwith-Wiedemann Syndrome (paternal UPD with *Igf2* over-expression and *Cdkn1c* suppression) (Chang and Bartolomei, 2020), and Temple and Kagami-Ogata Syndrome (paternal UPD with *Dlk1* over-expression) (Prasasya et al., 2020) represent such disorders. Neurodevelopmental diseases can also be linked to UPDs, including the Prader-Willi (PWS) and Angelman (AS) syndromes. PWS and AS are characterized by developmental and neurological deficits, and both involve the identical chromosomal region on human chr. 15, 15q11-13 containing the *Ube3a* imprinted cluster (Knoll et al., 1989). Suppression, mutation or deletion

of the maternally expressed *Ube3a* causes AS (Kishino et al., 1997), whereas paternally inherited deletion of a region encompassing the paternally expressed *SNORD109A* gene, the *SNORD116* cluster (also known as *HBII-85 C/D box small nucleolar RNA*) and part of the long non-coding RNA *IPW* is likely the main cause for PWS (Bieth et al., 2015).

Therefore, UPD of chr. 15 from the father (patUPD) causes AS whereas UPD of chr. 15 from the mother (matUPD) leads to PWS. Interestingly, differences in the nature of clinical symptoms, and disease severity of AS and PWS patients have been reported to correlate with the genetic origin of the disease. In very broad terms, patients suffering from AS due to gene deletion present with more severe symptoms compared to patients that carry a patUPD (Buiting et al., 2016). Similarly, PWS patients present with different severities in physical, cognitive and behavioral symptoms depending on whether the disease originates from a gene deletion or matUPD (Butler and Thompson, 2000; Proffitt et al., 2019). To date it remains unclear how the syndromic nature of AS and PWS arises, and how clinical differences due to UPD or gene deletion manifest.

7.2. Study of UPD led to major discoveries in genomic imprinting

Historically, UPDs have been used to obtain key insights into the functional relevance and mechanisms of genomic imprinting. Pronuclear transfer experiments enabled the production of embryos that carry two complete sets of chromosomes originating either from the mother (whole genome matUPD) or from the father (whole genome patUPD) (McGrath et al., 2017). Both whole genome matUPD and patUPD embryos failed to develop normally and died during embryonic development. Thus, the maternal and paternal genomes are both essential for mouse development (McGrath and Solter, 1984; Surani et al., 1984). To overcome early lethal phenotypes, chimeric embryos were created that included a limited number of embryonic stem (ES) cells with whole genome matUPD and patUPD (Barton et al., 1991; Fundele et al., 1990; Keverne et al., 1996). Chimeric whole genome UPD embryos showed improved developmental potential compared to their whole mouse UPD counterparts, indicating that at least some deleterious effects of UPD represent systemic effects. Cells with matUPD were found to mainly contribute to neuroectoderm whereas cells with patUPD showed a tendency to contribute to mesodermal tissues, indicating that genomic imprinting acts in a lineage specific fashion (Barton et al., 1991). In chimeric embryos, cells with whole genome patUPD showed enhanced growth while their matUPD counterparts were underrepresented in most embryonic lineages (Barton et al., 1991; Fundele et al., 1990). In contrast, brain-specific analysis revealed that cells with whole genome patUPD contributed relatively little to the forebrain compared to cells with whole genome matUPD, indicating a lineage-specific response to whole genome UPD (Keverne et al., 1996).

Subsequent studies revealed that the severe developmental defects in cells with whole genome matUPD could be traced to the *Igf2* and *Dlk1* imprinted clusters that have paternally methylated DMRs (Kawahara et al., 2007; Kawahara and Kono, 2010). Simultaneous deletion of these DMRs not only resulted in proper development of whole genome matUPD embryos into adulthood, but also allowed these matUPD mice to survive significantly longer than wild-type mice. The above findings indicate that specific imprinted genes are responsible for severe UPD-induced growth phenotypes, and that some chromosome specific UPDs may show milder phenotypes. Indeed, experiments using Robertsonian and reciprocal chromosome translocations allowed the creation of animals with single chromosome UPD, and showed embryonic lethal phenotypes for UPD of chr. 7 and chr. 12, but not chr. 11 (reviewed in (Cattanach et al., 2004)). These studies illustrated the utility and relevance of UPD for the study of genomic imprinting.

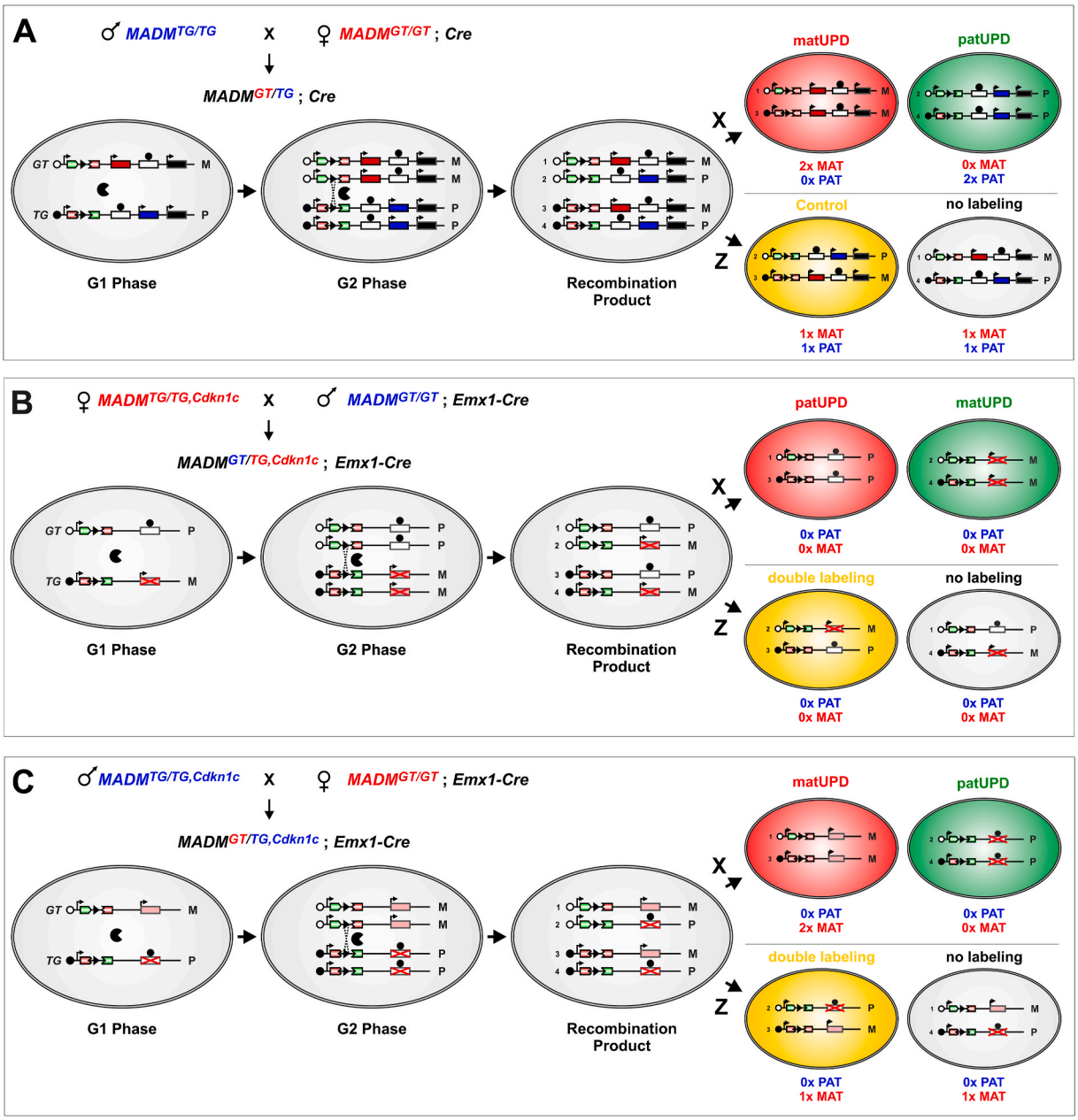
7.3. Mosaic Analysis with Double Markers (MADM) for the sparse induction of uniparental chromosme disomy with single cell resolution

Embryonic lethal phenotypes caused by whole animal UPD can be overcome by creating chimeric animals, enabling phenotypic analysis across all organs (Barton et al., 1991). Chimeric animals were typically created by using nuclear transfer experiments that need a high level of skill and dedicated equipment. Therefore, induction of UPD without micromanipulation of embryos would be advantageous for facilitating functional analysis of imprinted genes. One technology, Mosaic Analysis with Double Markers (MADM), fulfills this criterion (Beattie et al., 2020; Contreras et al., 2020; Hippenmeyer et al., 2013; Tasic et al., 2012; Zong et al., 2005). MADM is based on the use of split marker genes that contain partial coding sequences for tdTomato (tdT) and green fluorescent protein (GFP) interspersed by LoxP sites. The presence of a *Cre* recombinase induces interchromosomal recombination, which results in 1) homozygosity of the chromosomal part distal to the MADM cassette (UPD); and 2) fluorescent labeling of the two daughter cells in distinct colors upon cell division. For example, in one scenario all red-labelled cells (tdT^{+}) are strictly linked to unimaternal chromosome disomy (matUPD) and green labelled cells (GFP^{+}) associated with unipaternal chromosome disomy (patUPD) as detailed in Fig. 3A (Contreras et al., 2020; Hippenmeyer et al., 2013; Laukoter et al., 2020b, 2020c). A library of MADM mice with MADM cassettes inserted into all autosomes is now available, allowing in principle the study of nearly all imprinted genes across the entire mouse genome (Contreras et al., 2020). Tissue or lineage-specific induction of MADM is performed using transgenic mouse lines that express *Cre* recombinase under the control of a tissue or lineage-specific promoter. MADM induction is sparse with a maximum ~5% of all *Cre* recombinase expressing cells undergoing MADM recombination (Contreras et al., 2020; Zong et al., 2005). The first proof-of-principle imprinting study of MADM-induced UPD of chr. 7 revealed cell type specific *Igf2* mediated paternal growth dominance in several mouse organs (Hippenmeyer et al., 2013).

7.4. Strength and weaknesses of studying genomic imprinting using MADM-induced UPD and SNP based analysis of hybrid mice

UPDs (including MADM-induced UPD) and F1 hybrid mice have been both used to study allelic differences and imprinted expression. Both have strengths and limitations, as is summarized in Table 1. Hybrid mice originate from crosses between genetically distinct inbred mouse strains and thus the parental alleles carry genetic differences. This situation is similar to humans that also carry genetic differences between the parental alleles due to genetic variation within the human population (McVean et al., 2012). Genetic differences can be used to study allele specificity of any feature that preserves differences in DNA sequence, like DNA methylation, histone modifications, RNA and protein levels independent of the species (Andergassen et al., 2017; Gaur et al., 2013; Wingo et al., 2017; Xie et al., 2012). Altered expression of imprinted genes in hybrid mice, compared to the respective inbred strains, have been reported (Arévalo and Campbell, 2020; Gardner et al., 2019). This is however no major concern because imprinted expression as well as imprinted epigenetic features are preserved in F1 hybrid mice (Andergassen et al., 2017; Xie et al., 2012). Importantly, allelic differences can be directly quantified from the maternal and paternal allele within the same cells, independent of variation between samples. In combination with high throughput based DNA, RNA or protein sequencing technologies, F1 hybrid mice are thus an ideal tool to study relative allele specific differences with unperturbed imprinted expression.

MADM induces sparse cells with matUPD and patUPD in equal amounts within the same tissue (Fig. 3A). Cells with matUPD and patUPD have well defined, predictable changes in imprinted gene dose, which may also induce cell type specific secondary effects at the whole tissue and systemic level ((Laukoter et al., 2020c; Schulz et al., 2006)



(caption on next page)

Fig. 3. MADM-induced UPD to study the function of the *Cdkn1c* locus. **(A)** MADM uses Cre/LoxP-dependent interchromosomal recombination to reconstitute two reciprocal chimeric marker genes (GT and TG). Recombination during G1 phase of cell cycle reconstitutes green and red markers in the same cell and thus results in yellow cells without altering the genotype (not shown). If recombination happens in G2 phase two resolutions are possible upon mitosis. First, both reconstituted marker genes (and corresponding chromosomes) segregate to the same daughter cell, called Z-Segregation. This scenario does not alter the genotype and produces one yellow and one unlabeled cell (bottom). Note that imprinted expression in the yellow cells is similar to wild type and thus these cells can serve as controls. Second, recombination in G2 phase is resolved by X-Segregation the two recombinant chromosomes segregate into distinct daughter cells. This scenario results in cells expressing either GFP (green) or tdTomato (tdT, red). In addition red/green cells carry near complete uniparental chromosomal disomy (UPD, top branch). This schematic illustrates a case where the GT MADM cassette is inherited from the mother and the TG MADM cassette from the father. Thus red cells carry maternal UPD (matUPD) and green cells paternal UPD (patUPD). Note that swapping the parental inheritance of MADM cassettes also causes a swap in color connected to UPD (see B, C). As a result cells with matUPD over-express maternally expressed genes and repress paternally expressed genes. By contrast, cells with patUPD over-express paternally expressed genes and repress maternally expressed genes. In sum, genes with imprinted expression show differential expression between cells with matUPD and patUPD. Genes expressed from both parental alleles (non-imprinted genes, black) are not directly affected by matUPD/patUPD. **(B)** Maternal deletion of *Cdkn1c* in MADM-induced UPD. Schematic as in (A) but with floxed/deleted *Cdkn1c* genetically linked to the TG cassette and inherited from the mother. Note that parental origin of the TG/GT MADM cassettes and thus the colors of the resulting UPDs are swapped when compared to (A). Yellow cells: deletion of maternal *Cdkn1c* locus and silent paternal allele; Green cells: two deleted *Cdkn1c* loci; Red cells: two silent paternal *Cdkn1c* loci and no *Cdkn1c* expression. Note that in this scenario all cells show no expression of *Cdkn1c* **(C)** Paternal deletion of *Cdkn1c* in MADM-induced UPD. Schematic as in (A) but with floxed/deleted *Cdkn1c* genetically linked to the TG cassette and inherited from the father. Yellow cells: maternal *Cdkn1c* expression and paternal *Cdkn1c* deletion; Red cells: two maternal *Cdkn1c* loci and double dose of *Cdkn1c* when compared to control; Green cells: two deleted paternal *Cdkn1c* alleles. Symbols are indicated in the key. Figures in part adapted and modified under Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/) from (Laukoter et al., 2020b).

Table 1
Comparison of MADM-induced UPD and hybrid mice based allelic mapping.

	MADM-induced UPD	Hybrid mice
Detection of parental allele specific differences	Yes with absolute quantification at single cell level in combination with possible non-cell-autonomous systemic whole tissue effects	Yes with relative quantification at tissue and single cell level
Change of imprinted gene dose	Yes, predictable, sparse and with single cell resolution	Yes, whole mouse, single cell resolution difficult to obtain, depends on genetic background
Possible assays for analysis	Molecular biology and cell-based assays, including live cell imaging and phenotypic analysis	Only sequence based

and described later). Mapping of allele specific DNA methylation as well as RNA abundance has been successfully performed by comparing cells with matUPD and patUPD (Joshi et al., 2016; Laukoter et al., 2020b, 2020c; Schulz et al., 2006). Thus it should be possible to also map other allelic features using UPD. These assays will reveal both features directly controlled by genomic imprinting as well as secondary system effects due to perturbed imprinted gene dose. Some aspects of genomic imprinting itself might be influenced by UPD-induced systems effects, and therefore some allelic features might not be accurately quantifiable using UPDs.

Importantly the strength of MADM-induced UPD lies in the mapping of phenotypes related to changed imprinted gene dose (described in detail below). Permanent fluorescent labeling of cells can be exploited for a number of assays, like quantitative histological and cell-based assays, including live cell imaging (Hippenmeyer et al., 2010). Additionally MADM-labeling alone or in combination with additional fluorescent markers can be used to purify and study specific cell populations using FACS (Laukoter et al., 2020a). Therefore, MADM-induced UPD holds the potential for systematic examination of the functional relevance of imprinted expression in specific tissues and across distinct cell types.

8. Probing imprinted gene function using genetic candidate gene approaches

In general, two questions arise when studying imprinted genes. First, what is the physiological function of the gene of interest? Second, is imprinted expression relevant for its function? The cellular function of an imprinted gene can be assessed by genetic deletion experiments like for any other gene. However, since one allele of an imprinted gene is naturally silenced, only deletion of the active allele is expected to result in a phenotype. Thus, deletion of the silent allele can serve as a control to validate that the removal of the gene product rather than the genetic manipulation causes the phenotype. The functional relevance of imprinted expression can be assessed with over-expression experiments, ideally by increasing the gene dose two-fold. Technically, over-expression can be achieved by using transgenes, by deleting the ICR of an imprinted gene cluster, or by inducing UPD.

8.1. The logic of deletion and over-expression experiments to study an imprinted gene

A classic example, illustrating the logic of deletion and over-expression experiments to uncover the function of imprinted expression, is represented by the experimental manipulation of the maternally expressed *Igf2r* gene located on mouse chr. 17 (Barlow et al., 1991). Deletion of the ICR, located in intron 2 of the *Igf2r* gene, leads to re-expression of *Igf2r* from the silent paternal allele (Stöger et al., 1993; Wutz et al., 2001). Inheritance of the ICR deletion from the father, but not from the mother resulted in biallelic expression of *Igf2r* and smaller embryos (Wutz et al., 2001). Deletion of *Igf2r* exons 13–18 led to loss of the gene function, inducing an overgrowth phenotype and perinatal death only when inherited from the mother, but not from the father (Lau et al., 1994). Finally, MADM-induced UPD of chr. 17 showed a reduced ratio of cells with matUPD/patUPD in liver indicating a growth advantage for cells with patUPD and/or reduced growth of cells with matUPD of chr. 17 (Contreras et al., 2020). To date *Igf2r* is the only known imprinted gene located on chr. 17 with maternal expression and with documented function in the regulation of growth in liver (Andergassen et al., 2017). Therefore the reduced ratio of cells with matUPD/patUPD of chr. 17 in liver can likely be attributed to over-expression/suppression of *Igf2r*. Taken together, the above experiments identified the general function of *Igf2r* as a growth suppressor, and demonstrate the functional relevance of its imprinted expression status.

8.2. The conundrum of the Cdkn1c locus

The *cyclin-dependent kinase inhibitor 1c* (*Cdkn1c*, encoding the $p57^{KIP2}$ protein) was long considered to be a classical example of an imprinted growth repressor, and to act as a tumor suppressor via inhibition of cyclin-dependent kinases (CDKs) (Besson et al., 2008; Hatada and Mukai, 1995; Matsuoka et al., 1995; Zhang et al., 1997). *Cdkn1c* is a maternally expressed imprinted gene in mouse and human (Hatada and Mukai, 1995; Matsuoka et al., 1996). The ICR controlling its imprinted expression is located >250 kb upstream within an intron of the *Kcnq1* gene (Smilinich et al., 1999). Deletion of the ICR on the paternal, but not

the maternal allele, results in biallelic expression of *Cdkn1c* and smaller embryos (Fitzpatrick et al., 2002). Similar results were obtained using BAC transgenes over-expressing *Cdkn1c*, which led to growth deficiency and an embryonic lethal phenotype (Andrews et al., 2007). Finally, deletion of *Cdkn1c* from the expressed maternal, but not from the silent paternal allele, results in overgrowth and embryonic lethality (Zhang et al., 1997). Altogether, the above studies established that *Cdkn1c* acts as a dose-sensitive growth suppressor.

The *Cdkn1c* locus has an unusual genomic architecture. It consists of large exons spanning less than 3 kb of genomic sequence with relatively small introns, and with a large part of the gene body comprising a CpG island (Laukoter et al., 2020b). Due to the importance of *Cdkn1c* in Beckwith–Wiedemann syndrome, a pediatric overgrowth disorder with predisposition to tumor development, *Cdkn1c* is intensely studied (Chang and Bartolomei, 2020). At least 4 independent *Cdkn1c* deletion alleles, removing different parts of the *Cdkn1c* genomic locus, have been reported (Mademtzoglou et al., 2017; Takahashi et al., 2000; Yan et al., 1997; Zhang et al., 1997). Phenotypic analyses of these deletion alleles indicate a more complex picture of *Cdkn1c* function than initially anticipated. Two reports show a growth reduction upon maternal *Cdkn1c* deletion in either embryonic (Mademtzoglou et al., 2017) or adult stages (Takahashi et al., 2000). A growth reduction phenotype upon deletion of a growth suppressor is surprising, given that the opposite phenotype is expected. Gene deletion phenotypes can be influenced by the specific genetic makeup of the mouse strain. Thus changing the genetic background is frequently reported to have a profound impact on the resulting phenotypes in both neurodevelopmental and non brain-related contexts (Doetschman, 2009; Sittig et al., 2016). It is therefore possible that differences in *Cdkn1c* deletion phenotypes are related to different mouse strains used in the various studies. Indeed part of the *Cdkn1c* conundrum was resolved for one deletion allele (Zhang et al., 1997) by careful investigation of strain background, and developmental time of *Cdkn1c* deletion analysis. By studying the *Cdkn1c* deletion in a pure 129S2/SvHsd background and at a defined developmental window, E15.5 until prior to birth, Tunster and colleagues revealed an embryonic overgrowth phenotype that was not detectable anymore after birth. Several placental abnormalities, including compromised integrity of the trilaminar trophoblast layer, loss of giant cells of the labyrinth layer and reduced glycogen storage provide possible explanations for the phenotype (Tunster et al., 2011).

As described above, the *Cdkn1c* gene is CpG rich. Conditional deletion alleles typically remove the complete *Cdkn1c* gene and thus ~3 kb of genomic sequence (Mademtzoglou et al., 2017; Matsumoto et al., 2011b). Non-conditional alleles replace the *Cdkn1c* gene with a transcriptionally active cassette expressing an antibiotic resistance gene (Takahashi et al., 2000; Yan et al., 1997; Zhang et al., 1997). In all of these deletion alleles the genomic region of the *Cdkn1c* gene is rearranged, which could have an influence on the phenotype. In other words, genetic engineering of the *Cdkn1c* genomic locus may cause a phenotype independent of the *Cdkn1c* gene product (mRNA or protein). The paternal allele of *Cdkn1c* could be used to test this hypothesis since modifications of the naturally silent paternal allele have no influence on *Cdkn1c* levels. Three studies investigated the paternal *Cdkn1c* deletion and found no differences in overall survival and gross morphology (Takahashi et al., 2000; Yan et al., 1997; Zhang et al., 1997). However, detailed analyses of *Cdkn1c* deletion phenotypes focused exclusively on the maternally inherited *Cdkn1c* deletion. It remains unclear whether and how the paternal *Cdkn1c* allele may be involved in more subtle phenotypes such as increased apoptosis, which appears to be confined to specific tissues like the lens (Zhang et al., 1997) and intestine (Takahashi et al., 2000).

Interestingly, brain-specific deletion of *Cdkn1c*, using *Nestin-Cre* in combination with a conditional *Cdkn1c* deletion allele resulted in thinning of the neocortex (Matsumoto et al., 2011a). This finding was not compatible with a growth suppressor function of *Cdkn1c*. However, it is likely that this phenotype results from a secondary effect originating from severe hydrocephalus due to a defect in the subcommissural organ (Matsumoto et al., 2011a). It is of note that the hydrocephalus phenotype and the changes in subcommissural organ might not be strictly connected to *Cdkn1c* deletion (Imaizumi et al., 2020). Nevertheless, these data indicate that loss of *Cdkn1c* in whole organism or entire organ leads to systemic effects that might influence the phenotype non-cell-autonomously.

9. MADM analysis revealed a cell autonomous function for the *Cdkn1c* genomic locus rather than *Cdkn1c* gene product

MADM was recently utilized to determine whether over-expression or suppression of *Cdkn1c* had a cell-autonomous phenotypic effect on cell growth (Laukoter et al., 2020b). Since *Cdkn1c* is located on mouse chr. 7, MADM-induced UPD of chr. 7 during early cortical neurogenesis was analyzed. The focus was put on the *Emx1* lineage giving rise to the majority of excitatory neurons in the mouse neocortex (Gorski et al., 2002). It was confirmed that *Cdkn1c* was expressed 2-fold in cells with MADM-induced matUPD, compared to control cells (red, yellow cells Fig. 3A). No *Cdkn1c* expression was detectable in cells with MADM-induced patUPD (green cells Fig. 3A). In such experimental paradigms the *Cdkn1c* genomic locus remained unmodified and thus wild-type in all investigated cells. Surprisingly, neurons with MADM-induced matUPD and patUPD of chr. 7 were present in equal amounts, despite largely different *Cdkn1c* levels. There were also no obvious differences in cell cycle progression between MADM-induced matUPD and patUPD cells (Laukoter et al., 2020b). The *Cdkn1c* gene product is therefore unlikely to cell-autonomously control cell cycle and/or growth in the *Emx1* lineage of the mouse neocortex. These findings are in line with earlier experiments. Studies in cultured fibroblasts did not find a central role for *Cdkn1c* in regulating CDK activity or cell cycle progression (Takahashi et al., 2000). Knockdown of *Cdkn1c* mRNA in neuroepithelial cells of mouse neocortex at E14 using shRNA had no influence on the number of HuC/D$^+$ or TuJ1$^+$ neurons (Itoh et al., 2007). Finally, over-expression of *Cdkn1c* using Bacterial Artificial Chromosome (BAC) transgenes in whole mouse showed no increase in NeuN$^+$ cortical neurons despite a profound impact on behaviour (McNamara et al., 2018).

The induction of MADM-based UPD of chr. 7 changed *Cdkn1c* levels in early neocortical progenitors. Thus the lack of a growth phenotype was surprising considering previous findings. Deletion of *Cdkn1c* in the whole mouse resulted in over-proliferation of pancreatic and retinal progenitor cells, indicating a role in cell cycle control (Dyer and Cepko, 2000; Georgia et al., 2006). Furthermore, conditional deletion of *Cdkn1c* caused loss of quiescence and increased proliferation in hematopoetic stem cells (Matsumoto et al., 2011b). Interestingly deletion of *Cdkn1c* in the whole mouse caused an over-proliferation of muscle stem cells but conditional deletion of *Cdkn1c* specifically in muscle stem cells rather caused the loss of muscle stem cell compartment (Mademtzoglou et al., 2018). This indicates that systemic effects associated with *Cdkn1c* deletion influence the phenotype, at least for certain cell types. It is important to note that in none of the described examples deletion of the paternal allele was investigated. Therefore the discrepancy between phenotypes originating from changing *Cdkn1c* levels via MADM-induced UPD of chr. 7 and *Cdkn1c* deletions could originate for 2 reasons: 1) MADM-induced UPD of chr. 7 is sparse and *Cdkn1c* deletions only show a phenotype if the whole mouse or the whole organ lacks *Cdkn1c*, 2) deletion of the *Cdkn1c* locus, independent of the *Cdkn1c* gene product, causes the phenotype.

To test both possibilities, a mouse line where a *Cdkn1c* floxed allele was genetically linked to one of the MADM cassette (*Cdkn1c*-MADM) was created. Combination of *Cdkn1c*-MADM with *Emx1-Cre* created a heterozygous deletion of *Cdkn1c* in all cells of the *Emx1* lineage in the neocortex (yellow and unlabeled cells, Fig. 3 B, C). Histological analysis of mice with a heterozygous *Cdkn1c* deletion revealed an unexpected result. Deletion of *Cdkn1c* from either the maternal or the paternal allele

caused severe microcephaly due to massive apoptosis (Laukoter et al., 2020b). In other words, deletion of either the active or the silent *Cdkn1c* allele resulted in an identical phenotype.

On top of the heterozygous *Cdkn1c* deletion, *Emx1-Cre* activity in *Cdkn1c*-MADM cells also creates sparse cells with MADM-induced chr. 7 matUPD and patUPD, respectively. Due to the linkage of the *Cdkn1c* floxed allele with one MADM cassette, cells with MADM-induced matUPD and patUPD carried either two *Cdkn1c* deletion alleles or two wild-type *Cdkn1c* alleles in the same tissue (red and green cells, Fig. 3 B, C). Quantitative assessment of cells with MADM-induced matUPD and patUPD revealed that cells with two intact *Cdkn1c* alleles were more abundant than cells with two deleted *Cdkn1c* alleles. Importantly, the parental origin of the two intact *Cdkn1c* alleles did not influence the phenotype because cells with two active maternal or two silent paternal *Cdkn1c* alleles were equally protected from apoptosis (Laukoter et al., 2020b).

Taken together, analysis of neurons with MADM-induced UPD of chr. 7 and concomitant *Cdkn1c* over-expression or suppression revealed no defect in the generation of neurons in the neocortex. Due to the sparsity of cells with MADM-induced UPD it may be concluded that the *Cdkn1c* gene product is not required cell autonomously to control cell cycle or neuron production. Conversely, growth defects seen in *Cdkn1c* deficient mice are likely due to systemic effects of the whole mouse or organ being deficient for *Cdkn1c*. Combining MADM-induced UPD of chr. 7 with a *Cdkn1c* floxed deletion allele allowed the study of cellular phenotypes originating from the heterozygous deletion of the *Cdkn1c* genomic locus. Deletion of either the maternal or the paternal *Cdkn1c* locus induced apoptosis. Conversely, presence of two intact maternal or paternal *Cdkn1c* loci rescued the phenotype and protected cells from apoptosis. Thus it may be conluded that the *Cdkn1c* locus, independent of the *Cdkn1c* gene product, harbors a dosage-sensitive survival element, the nature of which requires further investigation. It is possible that this survival element is only necessary during early neurogenesis. Deletion of *Cdkn1c* using a Tamoxifen-inducible *Nestin-Cre* late in neuronal development revealed no increase in apoptosis, but rather an increase in the numbers of neurons in the hippocampus (Furutachi et al., 2013). Regardless of the exact molecular mechanism underlying the apoptosis phenotype, the above studies demonstrate the utility of the MADM system for the investigation of the cell-autonomous function of imprinted gene expression in specific cell types.

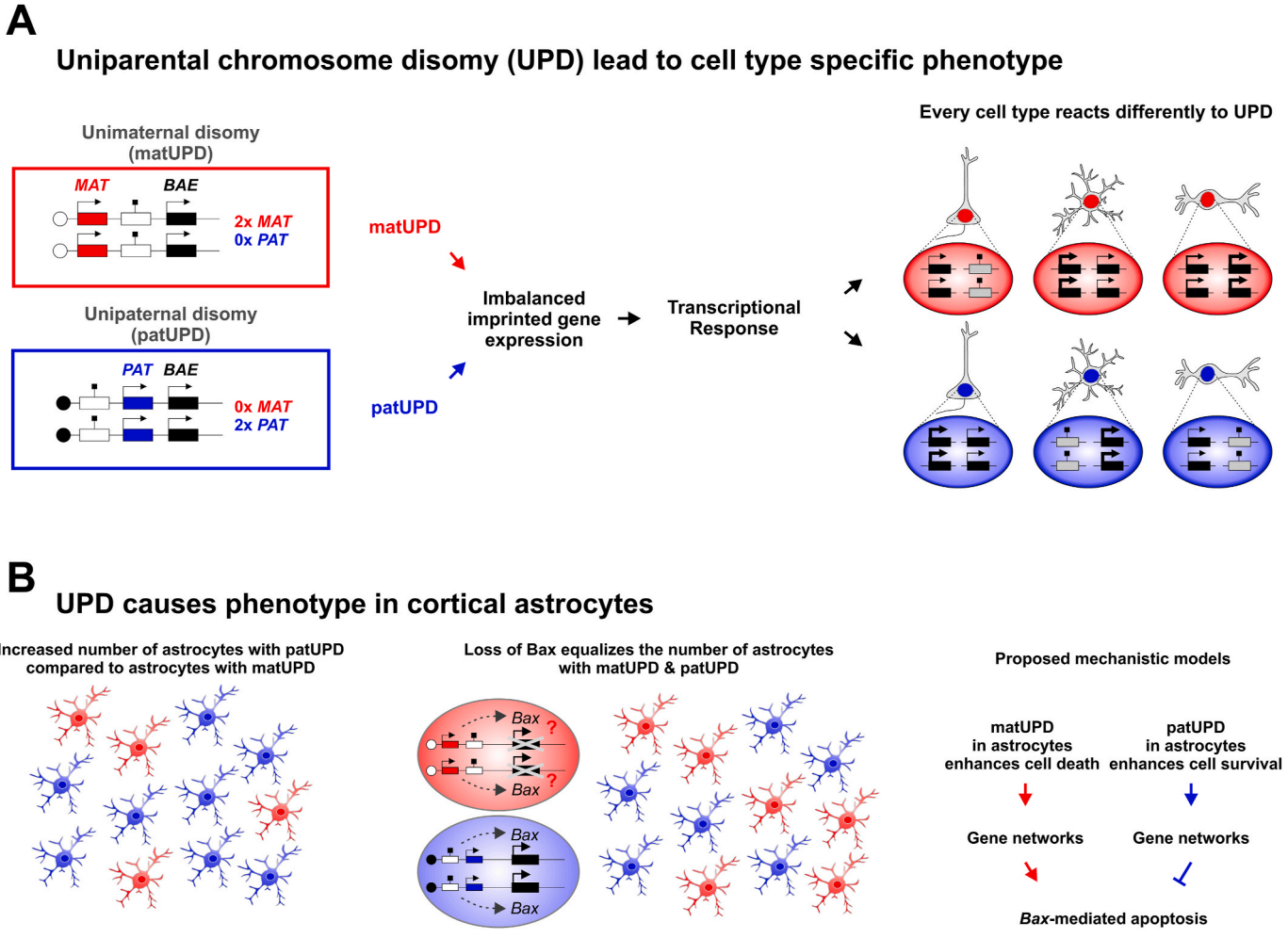


Fig. 4. Cell type specific function of genomic imprinting in neocortex revealed by MADM-induced UPD. (A) Uniparental chromosome disomy (UPD) enables the modification of imprinted gene dosage. Unimaternal chromosome disomy (matUPD) express the double dose of maternally expressed genes with no expression of paternally expressed genes. Unipaternal chromosome disomy (patUPD) express the double dose of paternally expressed genes with no expression from maternal allele. In neocortex, UPD induces strong transcriptional response that is highly cell type specific. Cell symbols as in Fig. 2. **(B)** (Left) Astrocytes with MADM-induced UPD of chr. 7 show overgrowth phenotype. PatUPD astrocytes are more abundant compared to matUPD astrocytes. (Middle) Loss of *Bax* in astrocytes with matUPD equalized the number of matUPD and patUPD astrocytes in the neocortex. (Right) Models explaining the observed phenotypes: (1) matUPD perturbs gene networks rendering astrocytes more prone for cell death. (2) patUPD perturbs gene networks rendering astrocytes more likely to survive. (3) Both models could act in parallel, as they are not mutually exclusive. Figures are in part adapted and modified with permission from the publisher (Laukoter et al., 2020c).

10. Expanding MADM-induced UPD analysis to the whole genome

10.1. Cell type specific response to MADM-UPD

Mouse chr. 7, 11 and 12 contain a number of prominent imprinted genes including *Igf2, Cdkn1c, Ube3a* (chr. 7)*, Grb10* (chr. 11) and *Dlk1* (chr. 12) whose human homologs are centrally involved in human imprinting diseases. Thus the study of UPDs, particularly on these chromosomes, holds the promise for important insights not only into mouse development, but also into the underlying causes of human imprinting disorders. To systematically investigate the consequence of UPDs in different cell types in the mouse brain the power of fluorescent labeling of MADM-induced UPDs was combined with genome-wide gene expression analysis by RNA-seq (Laukoter et al., 2020c). Genetically defined *Emx1* and *Nkx2.1* cell lineages with MADM-induced UPD of chr. 7, 11 and 12 were purified from the neocortex, hippocampus and olfactory bulb followed by transcriptome analysis. Imprinted genes were over-expressed/suppressed in cells with matUPD or patUPD as predicted based on their imprinted expression status. No change in cell identity could be detected in cells with MADM-induced UPD. Interestingly, responses to matUPD and patUPD induced in the same cell type were more similar to each other than to the responses induced by the same UPD in different cell types (Fig. 4A). The overall transcriptional response included changes of genes that were commonly deregulated in matUPD and patUPD, and other genes that responded in a UPD and/or cell type specific manner. Gene ontology (GO) enrichment analysis from these data revealed that genomic imprinting might be involved in different biological processes, including regulation of neuronal circuits.

To further delineate the origin of gene expression changes resulting from MADM-induced UPD of chr. 7 single-cell RNA-seq was perfomed in cells from the *Emx1* lineage. More than 1000 transcriptomes of single cells with MADM-induced matUPD or patUPD of chr. 7 were generated at different embryonic and postnatal time points. Analysis of these transcriptomes allowed the classification of cells into neurons, astrocytes and oligodendrocytes. The cells could also be immersed into distinct developmental trajectories. Such analysis enabled the study of MADM-induced UPD-related expression changes during neurogenesis, oligodendrogenesis and astrogliogenesis. Altogether, the above analysis revealed cell type specific transcriptional changes in response to MADM-induced UPD in all investigated cell types and developmental stages. Interestingly, mature astrocytes showed the most dramatic response, indicating the importance of imprinted expression of genes on chr. 7 in the development of this cell type (Laukoter et al., 2020c).

10.2. Imprinted genes on chr. 7 control cortical astrocyte development

In cells with MADM-induced UPD of chr. 7, the most deregulated genes were detected in mature cortical astrocytes. GO term analysis indicated the involvement of deregulated genes in growth and apoptosis. Indeed, the relative number of astrocytes with patUPD compared to astrocytes with matUPD was increased from progenitor level to adult stages (Fig. 4B left). More in-depth transcriptional analysis of purified cortical astrocytes with MADM-induced UPD of chr. 7 uncovered a connection between five imprinted genes (*Snrpn*, *Peg12*, *Peg3*, *Ndn* and *Cdkn1c*) and gene networks affecting apoptosis and cell cycle/growth. Two candidate genes were investigated further by loss of function approach: 1) paternally expressed *Igf2,* known to cause an overgrowth phenotype of MADM-induced patUPD hepatocytes in liver (Hippenmeyer et al., 2013); and 2) biallelically expressed *Bax,* which is implicated in neuronal cell death (Wong and Marín, 2019). No obvious involvement of *Igf2* in astrocyte production was detectable. In contrast, when *Bax* was deleted in cortical astrocytes with matUPD, the number of cortical astrocytes with matUPD and patUPD was equalized (Fig. 4B middle). The same *Bax* deletion in cortical astrocytes with patUPD had no effect on the astrocyte overabundance phenotype (Laukoter et al., 2020c). In summary, the study of MADM-induced UPD revealed a highly specific transcriptional response to MADM-induced UPD of chr. 7 translating into a highly cell type specific phenotype in cortical astrocytes. There are two possible explanations for the different response of astrocytes to MADM-induced chr. 7 patUPD and matUPD. Astrocytes with a patUPD, but not a matUPD, may be protected from a cell intrinsic apoptosis pathway. Alternatively, matUPD in astrocytes could cause deregulation of genes regulating apoptosis. Both scenarios could also act in parallel since they are not mutually exclusive (Fig. 4B right).

An intriguing question is the evolutionary benefit of the survival advantage of astrocytes with patUPD. One theory explaining evolution of genomic imprinting is the coadaptation theory. This theory argues that maternal expression of genes is selected in order to enhance the adaptive integration of the maternal and the infants' genomes. Ultimately this process leads to an increased fitness of the offspring (Wolf and Hager, 2006). This theory provides a theoretical framework to explain the results of cross fostering experiments using F1 pups from crosses of genetically distinct inbred strains. In these experiments F1 pups showed higher resource allocation from foster mothers of the same strain as their biological mother. This was contrasted by lower resource allocation of F1 pups from foster mothers of the same strain as their father (Hager and Johnstone, 2003). Insights into the mechanisms underlying these observations are largely missing. Astrocytes are a diverse group of cells serving multiple functions including the regulation of the architecture and activity of neuronal circuits that directly influence animal behavior (Farhy-Tselnicker and Allen, 2018; Khakh and Sofroniew, 2015). It is therefore tempting to speculate that imprinted genes influence behavior related to mother offspring interaction by regulating astrocyte abundance and function.

Finally, it will be interesting to investigate whether imprinted genes are involved in apoptosis of astrocytes in human. Several neurodevelopmental diseases are connected to changes in astrocyte abundance (Molofsky et al., 2012; Sloan and Barres, 2014). MADM-induced UPD of chr. 7 might model some aspects of AS/PWS, as homologs of imprinted genes involved in AS and PWS in human are located on mouse chr. 7. Astrocytes are not a major focus of research in AS/PWS. Currently, few studies have investigated astrocyte abundance in AS/PWS, and these did not report any significant changes (Fink et al., 2017; Mardirossian et al., 2009). It would thus be interesting in the future to screen the abundance of astrocytes in larger AS or PWS patient cohorts, especially where the underlying cause of the disease is a UPD. Chromosomal linkage of imprinted clusters is usually not conserved between mouse and human. For example the imprinted clusters of mouse chr. 7 are located on multiple different human chromosomes: chr. 19 (*USP29/PEG3*), chr. 15 (*UBE3A/SNRPN*), chr.10 (*INPP5F)*, and chr. 11 (*IGF2/H19* and *KCNQ1/CDKN1C*). It is therefore unlikely that a single human UPD will recapitulate the mouse chr. 7 UPD phenotype. However once we deepen our understanding of the molecular and physiological properties of astrocytes with chr. 7 UPD in mouse this might serve as a platform for new discoveries in a human context.

11. MADM-induced UPD to study imprinted expression in extra-embryonic tissues and the placenta-brain axis

MADM-induced UPD has been shown to be a useful tool to probe the cell type specific functional relevance of imprinted expression in the brain and peripheral organs (Hippenmeyer et al., 2013; Laukoter et al., 2020c). Based on these initial results, the MADM approach can also be extended to extra-embryonic tissues like the placenta and visceral yolk sac. Besides the brain, extra-embryonic tissues are thought to be the organs where imprinted expression may be functionally most relevant, discussed in detail elsewhere (Ivanova and Kelsey, 2011; Perez et al., 2016; Pulix and Plagge, 2020; Tucci et al., 2019). Interestingly, there is increasing recognition of a "placenta-brain axis" where the placenta and the maternal as well as the embryonic brain interact. This becomes most obvious when disruptions in the placenta lead to abnormalities in brain

development (Rosenfeld, 2020). Given the sensitivity of neural development, it is possible that part of the phenotypes observed in imprinting disorders originate early in development due to placental defects that in a broader context also affect brain development. On the other hand hormones, like progesterone and placental lactogens, produced by the placenta influence the maternal brain, more specifically the hypothalamus, to cause behavioral changes in anticipation of future needs of the offspring (Creeth and John, 2020; Keverne, 2013).

Important for this discussion, accumulated evidence indicates a role for imprinted genes in the placenta influencing embryonic brain development as well as the behavior of the mother (Fig. 5). Perhaps the best studied example for such a gene function is maternally expressed *Phlda2* gene in mouse. Functionally *Phlda2* is an indirect negative regulator of placental hormone levels. Increased *Phlda2* expression causes a loss of cells from the placental spongiotrophoblast lineage, a major site of placental lactogen production (Creeth and John, 2020). When embryos that either over-express *Phlda2* on BAC transgenes or do not express *Phlda2,* due to gene deletion, are transferred into wildtype mothers two changes occur in the mother. First, several hundred genes change expression in maternal hypothalamus and hippocampus that might be related to altered behaviour. Second, maternal behavior related to nest building and pup care is altered after birth (Creeth et al., 2018). Interestingly, a connection between human placental *PHLDA2* levels, placental hormone levels, and postnatal depression symptoms in mothers has been suggested (Creeth and John, 2020). Thus, there is a possibility that some aspects of mouse placental *Phlda2* functions could be conserved in human. A similar influence of placental expression levels on maternal behavior has been suggested in the mouse and human for *Peg3* and mouse *Grb10*, but evidence for such connection is weaker in these cases (Creeth et al., 2019; Gardner et al., 2019; Janssen et al., 2016).

Altered levels of imprinted genes in placenta can also directly influence behavior in the offspring in mouse and human. In mouse, deletion of a placenta specific transcript of *Igf2*, termed *Igf2-P0,* caused a shift in the balance of placental supply of nutrients and fetal nutrient demand. Interestingly, deletion of this placenta specific transcript correlated with a behavioral phenotype later in life manifesting in an increased response to anxiety-provoking stimuli (Mikaelsson et al., 2013). In human infants a correlation between placental expression of a set of imprinted genes, including *MEG3*, *MEST*, and *PHLDA2* was significantly correlated with the infant's behaviour (Marsit et al., 2012). Overall levels of imprinted genes in the placenta appear to be involved in the well-documented function of the placenta in shaping embryonic and maternal behavior (Keverne, 2013). The precise molecular mechanisms connecting imprinted genes with cellular properties in extra embryonic tissues, including placenta, is however largely unknown.

The MADM-UPD approach may represent an attractive strategy for the investigation of the function of imprinted expression in extra-embryonic tissues for two reasons. Imprinted genes in placenta and yolk sac often show imprinted expression restricted to specific cell types, particularly the visceral endoderm layer in the yolk sac, and different trophectoderm lineages in the placenta (Hudson et al., 2011). Hence, UPD targeted to the appropriate cell type could allow the relevance of imprinted expression of the targeted genes to be investigated. In extra-embryonic tissues, gene expression control by ICRs can be greatly expanded to cover a much larger genomic region and results in a larger number of imprinted genes associated with imprinted gene clusters (Andergassen et al., 2017). Importantly, MADM induced UPDs enables gene expression of entire enlarged clusters to be over-expressed or suppressed in specific cell types. The knowledge of cell type specific UPD induced effects in placenta can then be used to study the relevance of such effects on brain development and behavior, increasing the understanding of the influence of genomic imprinting on the placenta-brain axis.

12. Summary

In this review we explain and advocate the utility of UPDs for the study of a broad range of imprinting effects. The MADM system represents an experimental tool to induce UPDs in a genetically-defined cell type and in temporally defined manner. Mouse lines with MADM cassettes on every autosome are readily available, allowing the study of almost all imprinted genes known to date (Contreras et al., 2020). The number of different cell types that can be studied using MADM-induced UPDs is only limited by the availability of appropriate *Cre* lines. Initial work has investigated the effect of MADM-induced UPD on cell number (Contreras et al., 2020; Hippenmeyer et al., 2013; Laukoter et al., 2020c) and the transcriptome (Laukoter et al., 2020c). However, analysis of MADM-induced UPD is not limited to these assays because the fluorescently marked cells can be purified or analyzed in situ by any cellular, molecular or physiological assay. Collectively, the MADM approach can enable detailed cell biological analysis to dissect the role of imprinted genes in relevant cell types throughout any organ in the mouse.

MADM-induced UPD allows the investigation of all possible imprinting effects present on a single chromosome. Such an approach also enables the identification of cell types most affected by UPD-induced allelic expression changes. As shown for the neocortex (Laukoter et al., 2020b, 2020c), this approach can reveal cell type specific phenotypes, and is a useful tool for further investigations of the underlying molecular mechanism. It is highly likely that well-known canonically imprinted genes will be centrally involved in any cell type specific phenotypes detected by MADM-induced UPD. However, the MADM

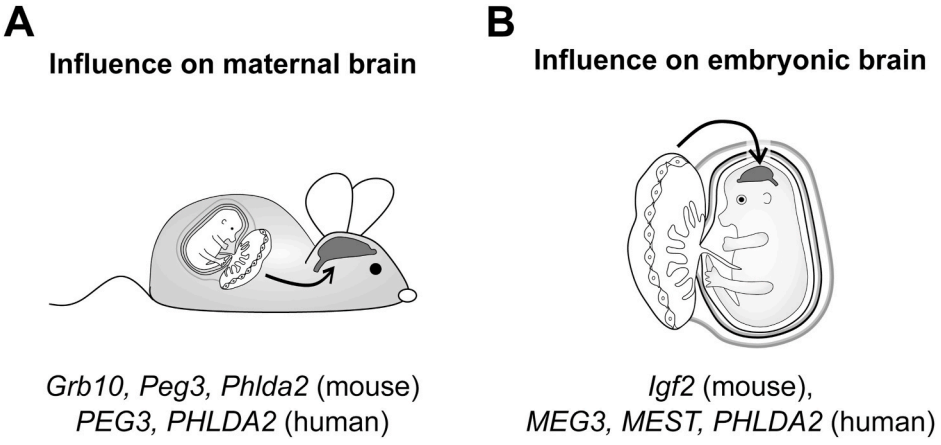


Fig. 5. Brain – placenta interaction. Selected mouse and human genes with imprinted expression in the placenta that influence maternal brain and behavior (A) as well as fetal brain development (B) are shown. Details and citations see text.

approach may also be used to determine the biological relevance of more subtle imprinted expression biases in specific cell types. A key feature of the MADM approach is that all imprinted genes on a particular chromosome are affected at once. While this allows relatively quick perturbation and analysis of all imprinted expression on one chromosome, in some cases deregulation of multiple imprinted genes may complicate interpretation of the resulting phenotypes. Thus, upon initial phenotype identification, follow-up studies may be necessary to clarify which imprinted gene or genes are responsible for the observed phenotype. However, due to cell type specific expression, and prior knowledge of well-characterized imprinted genes, we anticipate that in most cases this will not be a major issue. In summary, the MADM technology is a valuable tool for investigating the origin of diseases caused by UPD, and for understanding the effect of imprinted gene expression on phenotype at the single-cell level.

CRediT authorship contribution statement

Florian M. Pauler: Conceptualization, Project administration, Writing - original draft. **Quanah J. Hudson:** Conceptualization, Writing - original draft. **Susanne Laukoter:** Conceptualization, Visualization, Writing - review & editing. **Simon Hippenmeyer:** Conceptualization, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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