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REVIEW

# Dissection of gene function at clonal level using mosaic analysis with double markers

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**Abstract** **MADM (*M*osaic *A*nalysis with *D*ouble *M*arkers) technology offers a genetic approach in mice to visualize and concomitantly manipulate genetically defined cells at clonal level and single cell resolution. MADM employs Cre recombinase/loxP-dependent interchromosomal mitotic recombination to reconstitute two split marker genes—green GFP and red tdTomato — and can label sparse clones of homozygous mutant cells in one color and wild-type cells in the other color in an otherwise unlabeled background. At present, major MADM applications include lineage tracing, single cell labeling, conditional knockouts in small populations of cells and induction of uniparental chromosome disomy to assess effects of genomic imprinting. MADM can be applied universally in the mouse with the sole limitation being the specificity of the promoter controlling Cre recombinase expression. Here I review recent developments and extensions of the MADM technique and give an overview of the major discoveries and progresses enabled by the implementation of the novel genetic MADM tools.**

**Keywords** MADM, genetic mosaic, clonal analysis, lineage tracing, neural development, genomic imprinting

## Introduction

The analysis of gene function in biological processes by using genetic mosaic animals has a long tradition. In genetic mosaic individuals, two or more subpopulations of cells contain dissimilar genotypes. For the study of gene function in genetic mosaics, it is most useful if cells of a particular type with a certain genotype could be easily distinguished through a tag or marker from another class of cells with a different genotype. In an optimal assay, the manipulation and marking of cells with distinct genotypes is sparse enough to allow high resolution phenotypic analysis at the individual cell level. Scarce mosaics can also allow the study of essential genes that are critical for very early and/or multiple sequential developmental events, and permit the determination of whether a gene function is cell-autonomous (i.e. solely acts on the cell where it is expressed) or also affects neighboring cells. Experimental paradigms that would grant control over temporal and spatial induction of gene manipulation and concomitant labeling can even have the added advantage to possibly link defined cellular lineages to gene function. Historically and up to date, the generation and analysis of genetic mosaics in *Drosophila* has a long tradition and represents an unparalleled powerful tool to address fundamental questions in cell and developmental biology *in vivo* (Morgan, 1914; Stern, 1936; Xu and Rubin, 1993; Lee and Luo, 1999; Blair, 2003). Genetic mosaics can also be useful in animal models for human disease such as for example cancer which is a ‘clonal’ malady originating from a single cell with mutations driving uncontrolled proliferation. In the last decades a rich array of experimental approaches and genetic engineering methods have been established with the goal to intentionally and conditionally manipulate genes in small populations of cells or individual clones and mark these for phenotypic analysis and lineage tracing (Lee and Luo, 1999; Branda and Dymecki, 2004; Zong et al., 2005; Luo, 2007; Young et al., 2008; Legué and Joyner, 2010; Buckingham and Meilhac, 2011; Jefferis and Livet, 2012; Lao et al., 2012). This review focuses on the genetic MADM (Mosaic Analysis with Double Markers) technology in the mouse. MADM exploits site-specific recombination to induce precise single cell labeling and concomitant genetic manipulation in *one* experimental step, thereby generating genetically mosaic

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mice. I will first introduce and discuss recent further developments of the MADM technology; then highlight major advances that emerged from the application of the MADM method in the context of neuronal circuit assembly during brain development, cell proliferation and cancer, and genomic imprinting; and finally give an outlook on how the method can be further improved and expanded in the future.

## The MADM principle

MADM employs Cre recombinase/loxP-dependent interchromosomal recombination, to generate uniquely labeled homozygous mutant cells in an otherwise heterozygous background in mice (Fig. 1) (Zong et al., 2005). For MADM, two reciprocal chimeric marker genes are targeted separately to identical loci on homologous chromosomes. The chimeric *GT* and *TG* marker gene alleles (Hippenmeyer et al., 2010) consist of partial coding sequences for green eGFP[G] (Zong et al., 2005) and red tdT[T; tandem dimer Tomato] (Shaner et al., 2004) fluorescent proteins separated by an intron containing the loxP site. Following Cre recombinase-mediated interchromosomal recombination during mitosis, functional green and red fluorescent proteins are reconstituted resulting in two daughter cells each expressing one of the two fluorescent proteins upon G2-X events (recombination in G2 of the cell cycle followed by X segregation). Introduction of a mutation distal to one MADM cassette allows the generation of genetic mosaics with wild-type daughter cells labeled with one color (red in Fig. 1) and homozygous mutant siblings with the other (green in Fig. 1) in an unlabeled heterozygous environment. G2-Z, G1, or G0 (postmitotic) events do not alter the heterozygote genotype and functional red and green fluorescent proteins are restored simultaneously, resulting in double-labeled (yellow) cells (Fig. 1). Thus, recombination/segregation events that alter the genotype (G2-X) can be unambiguously distinguished from other recombination events that do not alter the genotype. In other words, labeling and gene manipulation occur in a single step and are therefore 100% correlated.

Originally, MADM cassettes were integrated into the genomic *Rosa26* locus (Soriano, 1999) on Chr. 6 (MADM-6) and MADM analysis of gene function was restricted to genes located distal to the *Rosa26* locus (Zong et al., 2005; Tasic et al., 2012). To overcome this limitation, expand MADM-based gene analyses and establish MADM with optimized recombination fidelity, we have recently generated mice with novel MADM cassettes knocked in close to the centromeres of Chr. 7 (MADM-7), Chr. 11 (MADM-11) and Chr.12 (MADM-12) (Hippenmeyer et al., 2010; Hippenmeyer et al., 2013). It is noteworthy that Chr. 7 and Chr. 11 are among the 19 telocentric (centromere located at terminal end of chromosome) mouse autosomes that display the highest gene density, and show synteny to significant stretches of human chromosomes that harbor well-characterized disease genes (Nicholls and Knepper, 2001; Bi et al., 2002; Yingling et al., 2003). Altogether, $>6000$ genes can currently be subjected to functional MADM analysis by using MADM-6, -7, -11 and -12 (Zong et al., 2005; Hippenmeyer et al., 2010, 2013; Tasic et al., 2012). For the remainder of this review I will first discuss general MADM features and applications and then concentrate on MADM-based functional gene analysis.

## MADM lineage analysis

MADM can serve as an ideal tool for lineage tracing (analysis of cell division patterns and distribution of clonally related cells that give rise to different parts of an organ) in wild-type or in combination with mutant gene analysis. In particular, if a temporally inducible CreER transgene is combined with MADM cassettes (rather than constitutive active Cre), analysis of individual G2-X MADM events can provide exact information on birth dates of clones and their cell division patterns. For CreER transgenes, the ligand binding domain of the estrogen receptor (ER) is fused to Cre recombinase (Feil et al., 1996). In the absence of a ligand such as tamoxifen (TM), the CreER is localized in the cytoplasm and incapable of catalyzing recombination in the nucleus. Upon application of TM however, CreER transiently (within 6-24h) translocates to the nucleus and Cre-mediated recombination is induced (Indra et al., 1999; Metzger and Chambon, 2001; Hayashi and McMahon, 2002). Thus, by controlling the dose, time of TM application and time point of clone analysis, this paradigm allows the tracking of individual stem cell division patterns and analysis of the distribution pattern of clonally-related cells in a variety of biological contexts including developmental and disease processes such as cancer (see also below).

Compared to other lineage tracing methods (McConnell, 1988; Sanes, 1989; Hallonet and Le Douarin, 1993; Cepko et al., 1995; Wingate and Hatten, 1999; Badea et al., 2003; Dymecki and Kim, 2007; Merkle et al., 2007; Legué and Joyner, 2010; Miyoshi et al., 2010) including transplantation, chimera generation, retrovirus infection or recombination-based fate mapping, MADM requires minimal or no embryonic manipulation but provides significant and sometimes more information about clone birth dates and cell division patterns (Buckingham and Meilhac, 2011). An important limitation of MADM-based lineage tracing, with clone induction in the embryonic stage, is however the dependence of embryonic TM injection. Since TM is an estrogen analog, the maximal dose of TM application in pregnant mice is limited in order to avoid abortion and maternal infanticide. Nevertheless, MADM cannot only provide cell labeling to track the lineal progeny originating from a progenitor cell but also enables the high resolution tracing of cellular morphology. This feature is in particular useful when analyzing clones of neurons with their elaborate

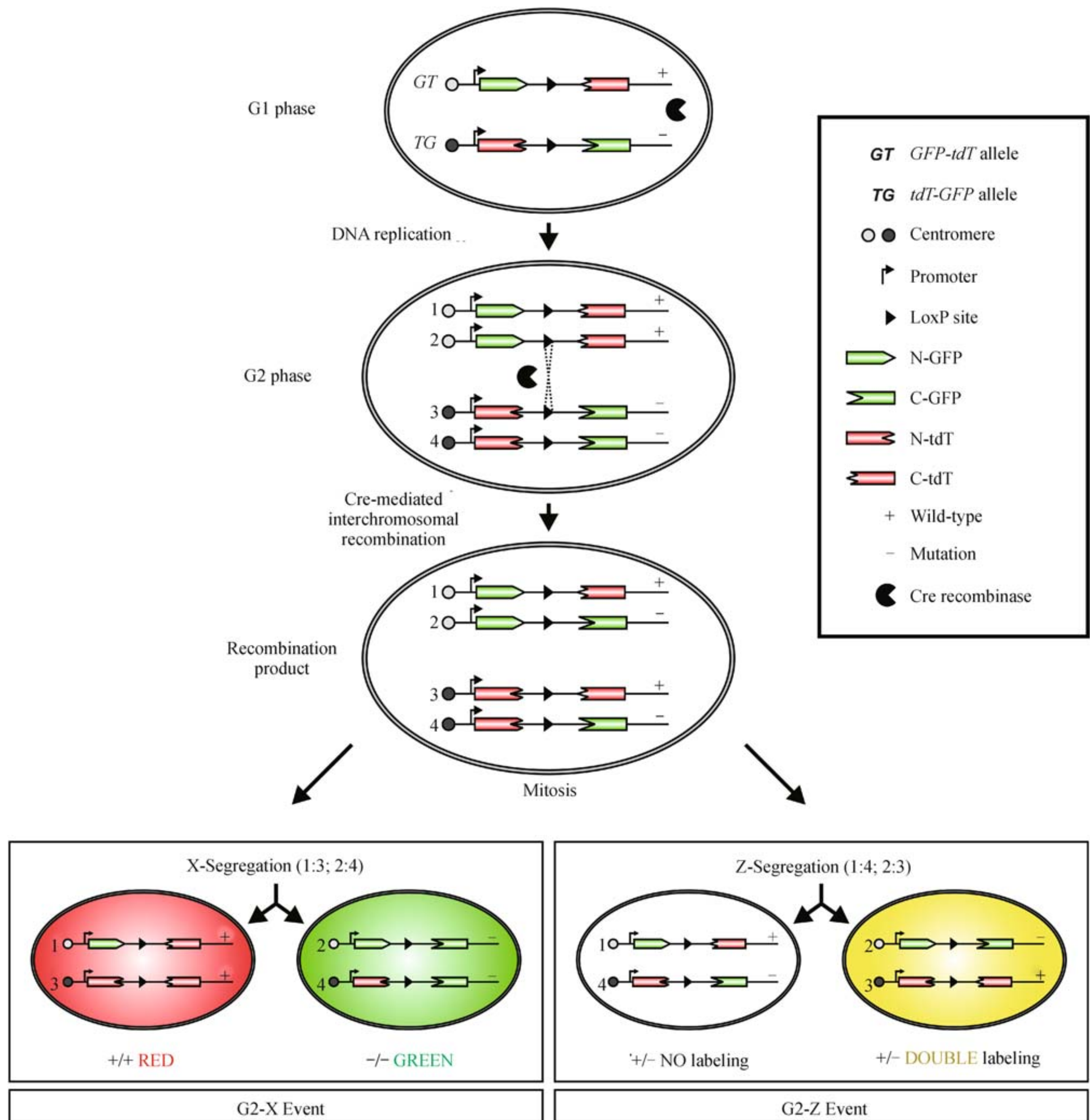


**Figure 1** The MADM principle. MADM utilizes Cre/loxP-dependent interchromosomal recombination to generate distinctly labeled homozygous mutant cells in an otherwise heterozygous background in mice. For MADM, two reciprocal chimeric marker genes – GT and TG– are targeted separately to identical loci on homologous chromosomes. Following recombinase-mediated interchromosomal recombination, functional green and red fluorescent proteins are reconstituted. If recombination occurred at the G2 phase and the two recombinant chromosomes were segregated into different daughter cells (X-Segregation, G2-X events), each daughter cell would express a single fluorescent protein. When a mutation of interest is introduced distal to one MADM cassette, then one of the daughter cells would be homozygous mutant (here the green cell) for the gene of interest, whereas its sibling, labeled by a different color (red), would be homozygous wild-type. In addition to G2-X events, recombination in G2 followed by Z-Segregation (G2-Z events, right branch), G1, or postmitotic recombinations (not shown) do not alter the heterozygote genotype, but can produce double-labeled (yellow) cells. Adapted with permission from (Hippenmeyer et al., 2010).

axonal and dendrite structures in the brain. As such, MADM has recently been used to determine the relationship between lineage and the wiring pattern of cerebellar granule cells (Zong et al., 2005; Espinosa and Luo, 2008). Given the possibility to trace axonal projection patterns of nascent cerebellar granule cells, MADM revealed that cerebellar

granule cell progenitors are 'pre-fated' with regard to their progeny's characteristic stacked axonal projections already at very early embryonic stages, despite that postmitotic granule cells are only born within the first 3 postnatal weeks (Zong et al., 2005). Subsequent experiments showed that cerebellar granule cell precursors undergo predominantly symmetric division during postnatal development and expand their clonal granule cell progeny exponentially. Nevertheless, all clonally-related cerebellar granule cells exit the cell cycle within a narrow time window and stack their axons in the molecular layer in chronological order from deep to superficial layers (Espinosa and Luo, 2008). Still, relatively little is known about the *in vivo* properties of neuronal stem cells in general during embryonic brain development and in particular in adult neurogenesis (Lehtinen and Walsh, 2011; Lui et al., 2011; Ming and Song, 2011; Franco and Müller, 2013; Ninkovic and Gotz, 2013). By taking advantage of the sparse labeling, clonal MADM analyses led to the identification of distinct division modes – symmetric and asymmetric self-renewal – of quiescent radial glia-like precursors during adult neurogenesis in the dentate gyrus of the hippocampus (Bonaguidi et al., 2011). However, in this study the authors used yellow MADM clones and since these clones only consist of one color (red plus green marker) there remains a certain probability that a single cluster might actually consist of two separate lineages. Conceptually similar clonal analyses, using yellow MADM clones, were carried out in the cerebellum and a probability of ~5% was estimated for yellow clones of cerebellar granule cells to consist of separate lineages (Zong et al., 2005). Lineage tracing studies using retrovirus-mediated transfer of a marker could allow to draw firmer conclusions when a library of genetically distinct viruses is used, which enables the delineation of clonal boundaries (Walsh and Cepko, 1992). Alternatively, clonal analyses using G2-X MADM clones, that label the progeny of a neuronal progenitor cell in two distinct colors, can also provide unambiguous lineage tracing results. Thus, MADM-based clonal analysis can offer insights into the general principles that govern neuronal precursor proliferation and differentiation of postmitotic neurons within a defined lineage. The MADM technology can also be applied outside the nervous system for lineage tracing and/or clonal analysis, for example during pancreatic (Brennand et al., 2007; Desgraz and Herrera, 2009) or liver development (Hippenmeyer et al., 2013).

In summary, analysis of MADM-based G2-X events in conjunction with temporally controlled CreER provides exact information on birth dates of clones and their cell division patterns. MADM results in an unambiguous quantitative optical readout of the proliferation mode (symmetric versus asymmetric) of progenitors at the single cell level and thus permit the determination of the developmental progenitor potential in virtually all murine tissues and organs provided the availability of an appropriate inducible CreER driver. Given the possibility to combine lineage tracing and genetic manipulation at the single cell/clonal level, future functional MADM analysis of candidate genes controlling the balance between expansive symmetric and postmitotic asymmetric (neurogenic in the brain) progenitor cell division promise the systematic dissection of molecular pathways regulating the division mode of stem cells in time and space.

## Single neuron MADM-labeling and circuit tracing in the nervous system

The identity of the distinct neuronal classes that constitute specific microcircuits within defined brain regions is to a large extent genetically hard-wired but a remarkable degree of heterogeneity in neuron types has been postulated (Nelson et al., 2006). The extent of this heterogeneity is not clear since the distinction and classification of the various neuronal types is a daunting task. The MADM technique holds the potential for systematic sparse labeling, and morphological characterization and cataloguing of genetically defined single neurons in different brain regions (Fig. 2). Diverse MADM cassettes can be combined with various cell-type specific Cre (constitutive) or CreER (temporally controllable) transgenic driver lines, which are being continuously generated by the mouse genetics community. At present, *Nestin-Cre/ER* (Petersen et al., 2002; Imayoshi et al., 2006), *Emx1-Cre* (Gorski et al., 2002), *Nkx2.1-Cre* (Xu et al., 2008), *Olig2-Cre* (Dessaud et al., 2007), *NG2-Cre* (Zhu et al., 2008), and *hGFAP-Cre* (Zhuo et al., 2001) have enabled selective and sparse labeling of a large variety of projection and interneurons, and glia (astrocytes and oligodendrocytes) in many central brain areas including the olfactory bulb, cortex, striatum, hippocampus, thalamus, brainstem, cerebellum, spinal cord and the peripheral nervous system (Zong et al., 2005; Woodruff et al., 2009; Hippenmeyer et al., 2010; Foo et al., 2011; Liu et al., 2011) (and unpublished observations).

Besides MADM, a very rich repertoire of contemporary genetic methods has been developed for sparse and combinatorial neuron labeling [reviewed comprehensively in (Jefferis and Livet, 2012). Here, I will compare MADM to some traditional and alternative genetic methods that allow the sparse labeling of neurons and their morphology in the brain. Provided cell-type specific Cre recombinase expression, the application of the MADM method is straight forward and requires minimal downstream experimental sample processing. In contrast, traditional methods like Golgi staining (Cajal, 1911) or dye filling (Cowan, 1998) are non-specific or quite laborious, and although *Thy1*promoter based transgenes can create Golgi-like labeling of restricted populations of neurons, their labeling patterns, while inheritable are mostly unpredictable. *Thy1-GFP* transgenes however do show very strong but somewhat biased expression of the marker in projection neurons and *Thy1*-driven expression often occurs only at postnatal stages (Feng et al., 2000; De Paola et al., 2003; Young et al., 2008). Neuronal

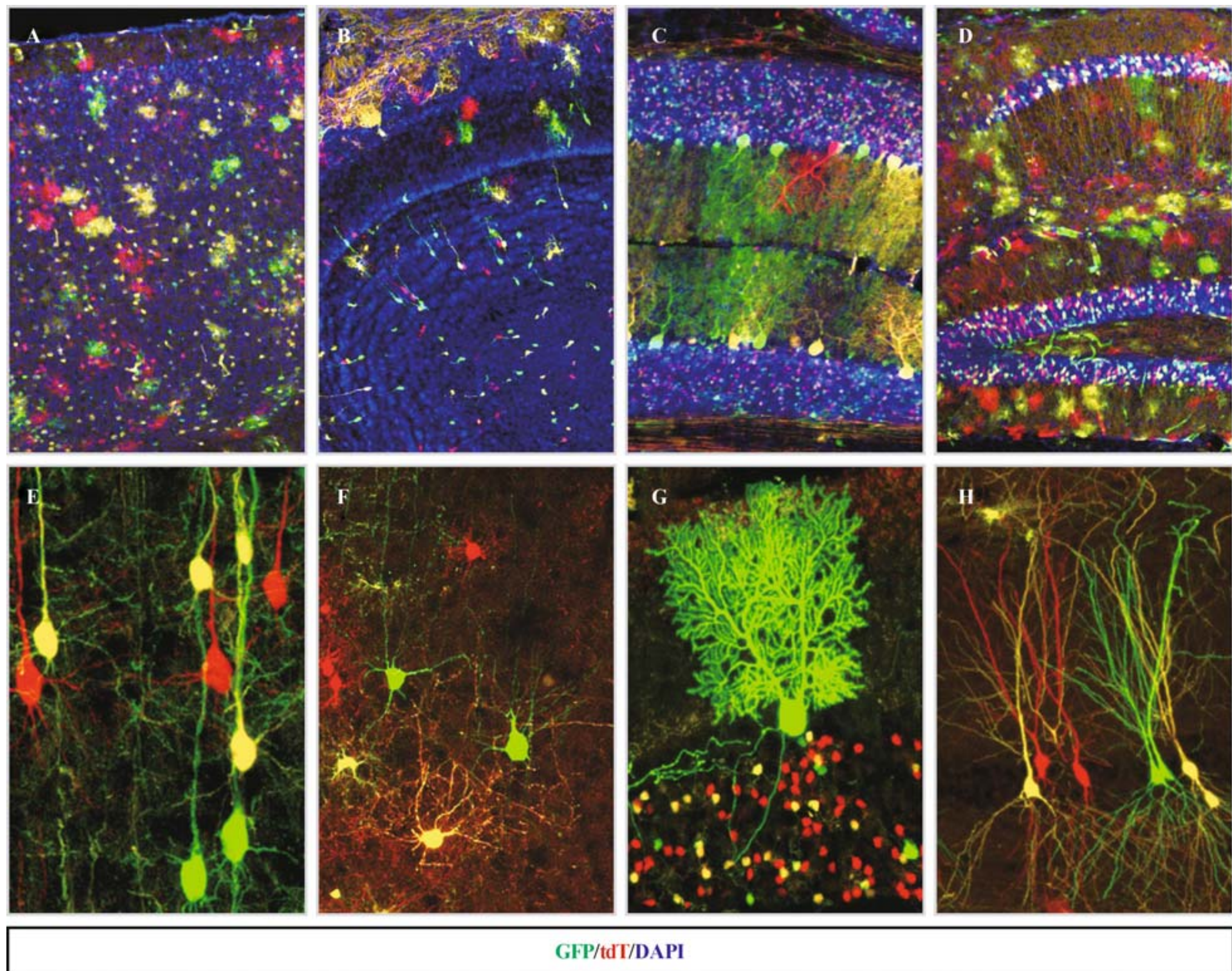


**Figure 2** MADM-labeled neurons in different brain regions. **(**A–D**)** Overview of MADM-labeled cells in cortex (A), olfactory bulb (B), cerebellum (C) and hippocampus (D). **(**E–H**)** Examples of excitatory pyramidal cells in cortex (E; MADM/*Emx1-Cre*), inhibitory interneurons in cortex (F; MADM/*Olig2-Cre*), Purkinje and granule cells in cerebellum (G; MADM/*Nestin-Cre*) and CA1 pyramidal cells in hippocampus (H; MADM/*Nestin-Cre*). E and G are adapted and modified with permission after (Hippenmeyer et al., 2010).

tracing using specific CreER drivers and 'general' reporter lines such as *R26R* (Soriano, 1999), *Ai9* (Madisen et al., 2010), *Z/EG* (Novak et al., 2000) or *Tau-mGFP* (Hippenmeyer et al., 2005) can provide more control and fidelity in labeling specificity but suffer in certain circumstances from low expression levels of the reporter when compared to expression form the *Thy-1* promoter. On the other hand, MADM promotes cell-type specific and strong targeted expression of fluorescent proteins, but it has also the added advantage of providing unambiguous information about lineage (see above) and possibility of genetic manipulation of labeled progeny (see below). Newly generated MADM-6, -7, -11, and -12 also permit visualization of both green (GFP) and red (tdT) markers without antibody amplification and allow live imaging over prolonged periods and thus the assessment of morphodynamic processes at 4D resolution (Hippenmeyer et al., 2010).

## Conditional MADM-mediated gene knockouts in single cells

The opportunity to create conditional gene knockouts in small populations or even single cells is probably the most important and unique characteristic of MADM. Provided a gene of interest is located distal to the MADM knockin site on the same chromosome, MADM could be used for conditional knockout of this particular gene. MADM labeled wild-type and mutant cells in mosaic mice can be directly compared by histological means, electrophysiological recording and optical imaging of cultured neurons and in brain slice preparations but also using *in vivo* imaging protocols. Compared to traditional conditional (Lewandoski, 2001) or recently developed FLEx (Schnütgen et al., 2003), SLICK (Young et al., 2008) and MASTR (Lao et al., 2012) methods, MADM has the following unique features: MADM (I) permits sparse

knockout of potentially every gene in the entire mouse genome; (II) allows in most if not all cases the study of essential or early embryonic lethal genes during development but also in the adult; (III) couples labeling and generation of homozygous cells through a *single* chromosomal exchange event and ensures 100% correlation between labeling and genetic alteration; (IV) labels homozygous wild-type sibling cells with a different color in the same experimental sample, providing unambiguous internal controls; (V) for MADM, the gene of interest can but does not need to be 'floxed', constitutive mutations are also sufficient; (VI) MADM-based conditional knockout is not restricted to any cell-type, tissue or organ with the promoter driving Cre recombinase being the only specificity criteria. However, MADM cassettes have currently not yet been inserted on all mouse autosomes. Thus MADM-based functional gene analysis is still limited although efforts in several laboratories are ongoing with the goal to create a MADM library for all mouse chromosomes to enable genome-wide MADM (see also section 'Future Perspectives'). Below, I highlight recent key examples of MADM-based functional gene analysis in the context of neurogenesis and neuronal migration during brain development, cell proliferation, and tumor progression in mouse models for cancer.

**Genetic mosaic dissection of neuronal development and circuit assembly**

During development of the brain, neurons of different classes and subtypes coalesce into defined nuclei or laminae with characteristic properties. The mature cerebral cortex is one of the largest structures in the brain and consists of distinct layers with different cellular composition and function as a result of eminent developmental processes including neurogenesis, neuronal migration, axon and dendrite patterning, synaptogenesis and circuit formation, refinement and consolidation. The cortical projection neurons are generated in the developing embryo within the ventricular zones (VZ, SVZ) in a defined temporal sequence by neuronal stem cell (NSC) progenitors (Lui et al., 2011); and concerted radial migration of newly born cortical neurons, from their birthplace in the VZ/SVZ to their final target lamina, is a key step in the assembly of the cerebral cortex (Ayala et al., 2007; Marín et al., 2010). The importance of most accurate neuronal migration for correct brain development is highlighted in patients that suffer from isolated lissencephaly sequence (ILS) or Miller-Diecker Syndrome (MDS) (Ross and Walsh, 2001; Wynshaw-Boris et al., 2010). Lissencephaly is characterized by smooth brain surface, abnormal brain morphology and function. About 40% of ILS and virtually 100% of MDS cases occur due to the loss of one copy of the gene called *Lissencephaly-1* (*LIS1*) (Reiner et al., 1993; Wynshaw-Boris et al., 2010). By using newly established MADM approaches we have recently revealed novel insights into how *Lis1* and its 'partner' gene *Ndel1* regulate neuronal migration: *Lis1* promotes migration efficiency in a dose-dependent manner while *Ndel1* is essential for a specific, so far uncharacterized, late step of neuronal migration: entry into the target lamina (Hippenmeyer et al., 2010). Comparisons with previous genetic perturbations of *Lis1* and *Ndel1* also suggest a perhaps surprising degree of cell-nonautonomous function for these proteins in regulating neuronal migration. Most indicative of cell-nonautonomous effects is the fact that the phenotypes in brains with sparse MADM-based *Ndel1* knockout are distinct from those observed in whole cortex *Ndel1* knockout (Youn et al., 2009; Hippenmeyer et al., 2010). In brief, $Ndel1^{-/-}$ mutant neurons in the MADM context are surrounded by $Ndel1^{+/-}$ or $Ndel1^{+/+}$ neurons, and $Ndel1^{-/-}$ show 'only' a very specific defect (entry into target lamina) in radial neuron migration (while most aspects of the migration behavior are normal). In contrast, in the whole cortex *Ndel1* knockout situation, all cells are $Ndel1^{-/-}$ and blocked in their migration at all stages of the migration journey. In principle, the proposed cell-nonautonomous effects could be 'positive' or 'negative' depending on the context. In whole cortex *Ndel1* knockout, $Ndel1^{-/-}$ mutant neurons are side by side and contact each other. Thus the lack of NDEL1 may provoke negative interactions among the mutant $Ndel1^{-/-}$ cells resulting in complete inhibition of migration. In contrast, normal $Ndel1^{+/-}$ or $Ndel1^{+/+}$ neurons surround $Ndel1^{-/-}$ cells in the mosaic MADM context and could positively support their migration at certain stages. The precise nature and mechanism of the above described cell-nonautonomous effects are currently unknown. Future MADM studies hold the potential to systematically dissect the cell-autonomous and cell-nonautonomous *Ndel1* (and *Lis1*) gene functions and such new advances likely can have implications for the understanding of the detrimental neurodevelopmental disorder Lissencephaly. More generally, by comparing the phenotype of mutant cells in the sparse mosaic MADM paradigm with the phenotype of mutant cells in full or conditional (for essential genes) knockout contexts, it will be possible to assay the functional cell-autonomy versus non-autonomy (at the single cell level) of any gene located on available MADM chromosomes.

The MADM method can be applied in a variety of neurodevelopmental contexts and has been used to analyze the cell-autonomous function of *NR2B*, encoding the predominant NR2 subunit of NMDAR (N-methyl-D-apartate receptor), during brain development (Espinosa et al., 2009). Functional MADM analyses of *NR2B* in barrel cortex layer 4 spiny stellate cells (bSCs) revealed that while overall dendritic growth was not affected, dendrite patterning was abnormal in *NR2B* mutant bSCs. While morphological analyses revealed in great detail the cellular dendrite patterning phenotype of *NR2B* mutant neurons, physiologic $Ca^{2+}$ responses in mutant, heterozygote and wild-type neurons could also be measured in cultured hippocampal neurons with Fura-2 (a ratiometric fluorescent dye that binds to free intracellular calcium) as part of the phenotypic analysis

(Espinosa et al., 2009). These experiments showed that the MADM technique can also be used to assay physiologic properties of single live cells in culture besides tracing the neuronal morphology at high resolution. The above study also illustrated that MADM represents a powerful tool to delineate the assembly of neuronal circuits. MADM-labeled cells can be subjected to detailed analysis of physiologic parameters and intracellular recordings. Such experimental paradigms could in principle be used to systematically trace and functionally assay the maturation of individual clonally-related cells and their integration into neuronal circuits during development and in adult neurogenesis. Furthermore, systematic paired recordings of clonally-related neurons within individual clones may reveal the extent of the role of lineage in determination of specificity of connectivity in the assembly of neuronal circuits (Smith and Fitzpatrick, 2012; Gao et al., 2013). Lastly, MADM-labeling may also enable to trace and assay circuit disintegration during neuronal degeneration.

### MADM for genes controlling cell proliferation and mouse models of human cancer

Careful examination of individual G2-X MADM events (Fig. 1) provides exact information on cell division patterns and proliferation potential of progenitor stem cells. MADM in combination with temporally inducible CreER can afford unambiguous quantitative optical readout of the proliferation mode of progenitors at the single cell and clonal level (see also above). Since G2-X events result in two daughter cells (with distinct red and green colors), originating from a single mitosis, the ratio of the absolute numbers of red and green cells should be 1 in an individual clone if cell division were symmetric. If the cell division is asymmetric, red and green progeny numbers are different and thus the green/red ratio distinct from 1. In the case where cell proliferation is assessed using MADM in combination with constitutive Cre drivers (stochastic induction of interchromosomal recombination), the random distribution of colors in asymmetric clones would still ensure a green/red ratio of 1 as long as a large number of independent G2-X events is sampled. By using this experimental MADM paradigm we assayed the cell-autonomous requirement for *Lis1* in neurogenesis and the production of astrocytes by using the constitutive *Emx1-Cre* driver (Gorski et al., 2002) expressed in neuronal progenitors. We found that *Lis1* is essential and cell-autonomously required not only for the production of cortical projection neurons (Tsai et al., 2005; Yingling et al., 2008) and astrocytes in cortex but for most, if not all, classes of neurons (Hippenmeyer et al., 2010). A similar MADM approach using constitutive *Nestin-Cre* drivers (Tronche et al., 1999; Petersen et al., 2002), to direct interchromomal recombination in neuronal progenitors was also applied in order to mechanistically demonstrate that 1) the zinc-finger transcription factor specificity protein 2 (Sp2) functions cell-autonomously to promote cell cycle progression during neurogenesis *in vivo* (Liang et al., 2013); and 2) the mosaic ablation of the Kv1.1 voltage-gated potassium channel in hippocampal neurons (but not astrocytes) results in their overproduction, which partly explains the megencephaly (enlarged brain) phenotype in *mceph* mutant mice (Yang et al., 2012).

One important application of MADM is the tracing of cell proliferation and possible tumorigenic growth upon ablation of tumor suppressor genes in a small subset of cells within a particular tissue (Muzumdar et al., 2007). Cells that are homozygous mutant for a candidate tumor suppressor gene are uniquely labeled and can be followed dynamically *in vivo* to study for example tumor progression and metastasis or to potentially assay for the effects of therapeutic agents in certain experimental paradigms. To this end, MADM was exploited to track tumor growth in a mouse model for glioma (Liu et al., 2011). It was found that the cell of mutation in glioma is not necessarily identical with the cell (or cell-type) driving tumor development (cell of origin). While mutagenic events were induced by MADM in neural stem cells (NSCs), which give rise to different neuronal and glial cell types, only oligodendrocyte precursor cells (OPCs) showed hyperproliferation and developed eventually into malignant tumors (Liu et al., 2011). Since MADM enabled the very sporadic induction of mutation in NSCs in combination with differential coloring of mutant cells, this paradigm allowed rigorous tracing of the entire tumorigenic process by quantifying the ratio of mutant/wild-type cell numbers at different stages. Most importantly, in combination with specific marker staining, MADM analysis demonstrated that mutant OPCs showed massive expansion (but not NSCs) already at pretransforming stages of gliomagenesis. By using OPC lineage-specific *NG2-Cre* driver it was further demonstrated that OPCs can be transformed directly by MADM-induced mutagenesis. In general, by analyzing and tracing the growth and/or aberrant expansion at pretransforming stages and of all cell lineages that derive from particular mutant stem cells, it should be possible in the future to pinpoint the cell of origin also in distinct cancer types in the brain and peripheral organs.

## Mosaic analysis of genomic imprinting

Genomic imprinting is a unique genetic phenomenon and results in preferential expression of either the maternal or paternal inherited parental allele of certain genes (Barlow, 2011; Bartolomei and Ferguson-Smith, 2011). Imprinting is essential for mammalian development and its deregulation causes many diseases (Feinberg, 2007; Mabb et al., 2011). Recently, we have used MADM to create uniparental disomies (UPD, somatic cells with two copies of either the maternal or paternal chromosome) and visualize imprinting effects with single cell resolution in mice (Hippenmeyer et al., 2013). As described in Fig. 1, MADM can produce mitotic

recombination at G2 phase, followed by X-segregation of recombined chromosomes. These G2-X events produce near complete UPD of a particular chromosome carrying the MADM cassettes (Fig. 3). Thus imprinted genes located on such a chromosome will be homozygosed and either over-expressed or not expressed depending on their imprinting

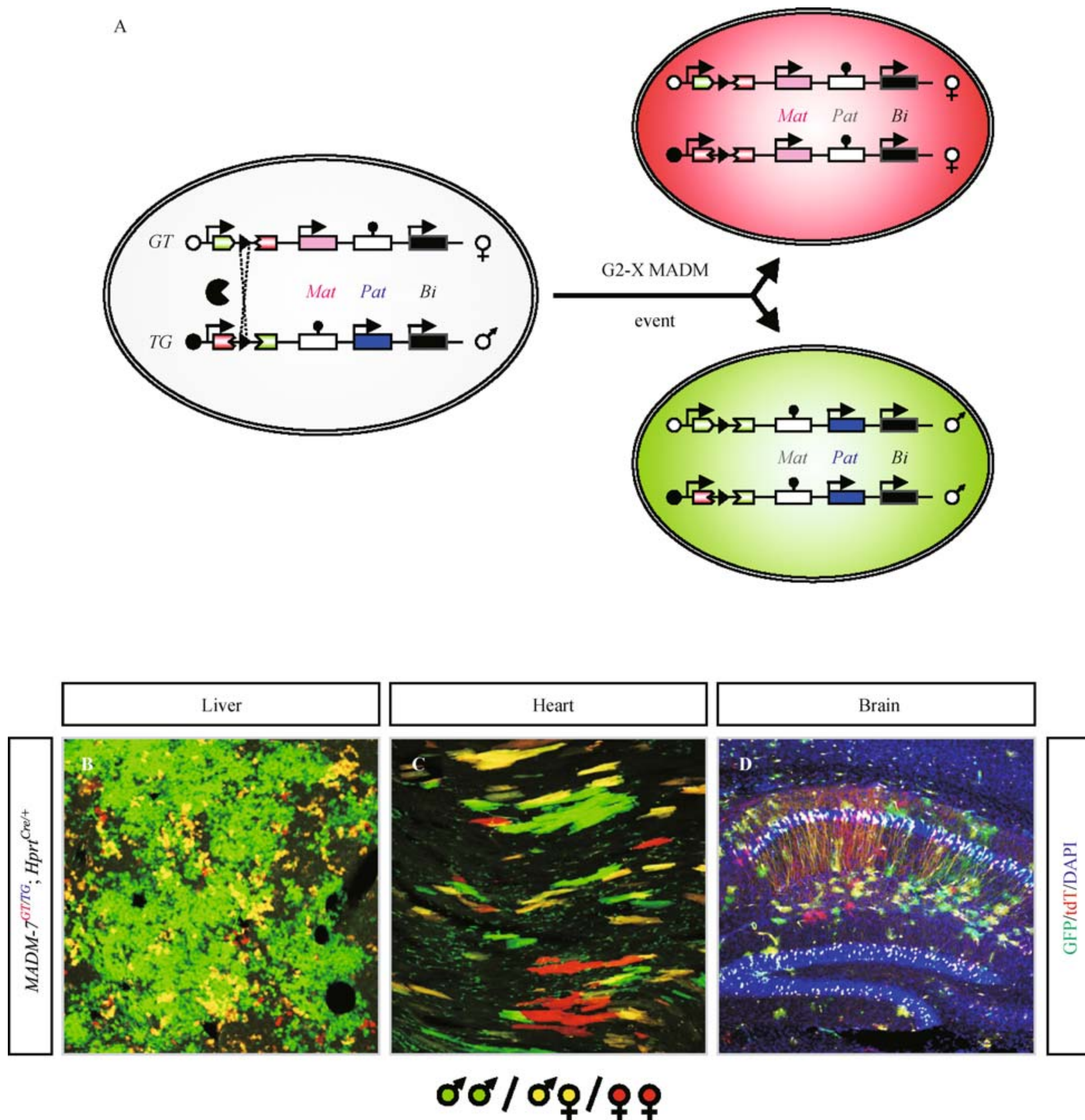


**Figure 3** Mosaic generation of chromosomal disomy to probe genomic imprinting. **(**A**)** G2-X MADM events result in near complete uniparental chromosomal disomy labeled in green (GFP) and red (tdT) fluorescent colors, respectively. The *GT* MADM cassette is inherited from the mother (♀) and the *TG* MADM cassette from the father (♂), as shown in this schematic, red cells harbor unimaternal chromosomal disomy (♀♀) and green cells unipaternal chromosomal disomy (♂♂). Consequently, maternally expressed genes (*Mat*, pink) are expressed at twice the normal dose and paternally expressed genes (*Pat*, blue) are not expressed in red cells with unimaternaldisomy. In contrast, paternally expressed genes are expressed at twice the normal dose and maternally expressed genes are not expressed in green, unipaternal disomy cells. Thus, genes subject to imprinting are differentially expressed depending on the uniparental chromosomal disomy. Biallelically expressed genes (*Bi*, black) are not affected. **(**B-D**)** MADM labeling of Chr.7 UPDs in the liver (B), heart (C) and brain (hippocampus) (D) at P21. The *GT* allele was introduced from the mother (pink), and the *TG* allele from the father (blue), consequently, the ♂♂ cells are green and ♀♀ cells are red. Note the increased number of green ♂♂ cells in the liver (B) but equal number of green ♂♂ and red ♀♀ cells in heart (C) and brain (D). DAPI staining (blue) outline the general organization of the hippocampus. Adapted with permission from (Hippenmeyer et al., 2013).

status whereas biallelically expressed genes are not affected (Fig. 3A). In an experimental breeding, where the female carries the GT-MADM cassette and the male the TG-cassette, cells with unipaternal disomy are fluorescently labeled with green GFP and sister cells with unimaternal disomy with red tdT (Fig. 3A), or vica versa (color will reverse if the sex of the parents is reversed). Thus, MADM provides a unique platform to systematically assay the consequences of genomic imprinting at the whole chromosome level by visualizing defined UPDs at the single cell level in genetic mosaic animals. Perhaps surprisingly, Chr.12 UPD did not produce detectable phenotypes while Chr.7 UPD caused highly significant paternal growth dominance in the liver and lung but not the brain or heart (Fig. 3B–3D). A single gene on Chr.7, encoding the secreted Insulin-like growth factor 2 (Igf2) appears to account for most of the paternal dominance. These findings ultimately lead to the question how IGF2, as a secreted factor can account for paternal Chr.7 UPD cell expansion. In principle three models may explain this finding. First, IGF2 acts in a dose-dependent pure autocrine fashion and only on paternal UPD cells although such a mechanism is not readily explained in molecular terms. Second, additional 'paternal' (P) factors act in concert and in a dose dependent fashion with IGF2. Third, the lack of a 'maternal' (M) factor in paternal UPD which normally counteracts IGF2 may account for overexpansion of cells with paternal Chr.7 UPD. Since the third model requires the least assumptions it may be an attractive model to explore and experimentally test in the future. Altogether, the above study revealed chromosomal and cell-type specificity of imprinting effects and established an unprecedented strategy to probe for genomic imprinting effects, on a whole chromosome level, in a cell-type specific manner (Hippenmeyer et al., 2013).

## Future perspectives

The MADM technology can label and at the same time manipulate genetically defined cells at low density and clonal level in the mouse with major *in vivo* applications for lineage tracing, single cell morphology analysis and phenotype assessment in conditional knockout or uniparental chromosome disomy.

The MADM principle requires a single interchromosomal recombination/segregation event to reconstitute the red and green marker genes and accordingly render the genotype in case functional gene analysis is pursued. The interchromosomal (trans) recombination rate between two homologous chromosomes is much lower than for intrachromosmal (cis) recombination providing the ground for sparse MADM labeling and gene manipulation (Zong et al., 2005). For inducible CreER-mediated lineage analysis however, potent CreER drivers and MADM chromosomes with a relatively high capacity for interchromosomal recombination events is desirable, especially if the lineage of progenitor cells present in small populations, shall be traced. In general, interchromosomal recombination efficiency for distinct homologous chromosomes is subject to enormous variation (Liu et al., 2002). In MADM paradigms, interchromosomal recombination is dependent on the chromosome and genomic location of the loxP sites contained in the MADM cassettes, and the cell-type specificity and dosage level of Cre/ER recombinase expression (unpublished observations). While the interchromosomal recombination efficiency in different MADM chromosomes is determined empirically, it is also clear that some MADM chromosomes and/or cassettes afford a higher rate of G2-X versus G2-Z events (unpublished observation) although the mechanisms and parameters that could affect this rate are unclear (Liu et al., 2002; Armakolas and Klar, 2006). There is evidence that a higher number of back-to-back loxP sites can lead to an increase in the interchromosomal recombination frequency (Liu et al., 2002) and some MADM chromosomes now contain multiple loxP sites (Liu et al., 2011; Hippenmeyer et al., 2013). The direct confirmation that multiple loxP sites in the MADM cassettes indeed increase the efficiency of MADM recombination events (when compared to single-loxP MADM cassettes) is still missing but experiments are currently under way to address this issue. On the other hand multi-loxP cassettes for interchromosomal recombination have also been proposed to skew the segregation pattern more toward G2-Z (Liu et al., 2002) (in MADM resulting in yellow color and no change in genotype) and may therefore not always be useful in MADM experiments if a high rate of G2-X events (producing red and green labeled cells with distinct genotypes) is required. These considerations should be kept in mind for the future construction of new MADM chromosomes. In any case, MADM-based conditional gene knockout requires a pair of MADM knockin cassettes between the gene of interest and the centromere on a particular chromosome. Thus, MADM cassettes inserted close to the centromere of all 19 telocentric mouse autosomes could enable virtually every gene of the whole mouse genome to be subjected to MADM analysis. Such efforts are currently ongoing and first require the identification of appropriate genomic loci (Hippenmeyer et al., 2010, 2013) on all homologs chromosomes for knockin of the MADM cassettes within regions as close as possible to the centromere. Besides generating a future library of MADM chromosomes, the MADM cassettes may also be adjusted to enable even more widespread applications such as for example overexpression of genes (Tasic et al., 2012) or manipulation of neuronal activity by for example reciprocal splitting of channelrhodopsin and/or halorhodopsin (Zhang et al., 2007; Hegemann and Möglich, 2011; Chow et al., 2012) for knockin cassettes. Altogether, the knockin of current and future, possibly more versatile, MADM cassettes into genomic loci close to the centromere of all 19 mouse autosomes will precipitate into a continuously developing resource for the study of progenitor stem cell lineages and functional gene analysis at clonal level and single cell resolution.

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## Compliance with ethics guidelines

Simon Hippenmeyer declares that he has no conflict of interest and that all IST Austria institutional and national guidelines for the care and use of laboratory animals were followed.

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