From the Cover: Modeling sporadic loss of heterozygosity in mice by using mosaic analysis with double markers (MADM)

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The initiation and progression of many human cancers involve either somatic activation of protooncogenes or inactivation of tumor-suppressor genes (TSGs) in sporadic cells. Although sporadic gain-of-function of protooncogenes has been successfully modeled in mice [e.g., Johnson L, Mercier K, Greenbaum D, Bronson RT, Crowley D, Tuveson DA, Jacks T (2001) Nature 410:1111–1116], generating a similar degree of sparseness of TSG loss-of-function remains a challenge. Here, we use mosaic analysis with double markers (MADM) to achieve TSG inactivation and concurrent labeling in sporadic somatic cells of mice, closely mimicking loss of heterozygosity as occurs in human cancers. As proof of principle, we studied the consequence of sporadic loss of p27kip1, a cyclin-dependent kinase inhibitor. MADM-mediated loss of p27kip1 results in mutant cell expansion markedly greater than that observed in conventional p27kip1 knockouts. Moreover, the direct comparison of WT and mutant cells at single-cell resolution afforded by MADM reveals that p27kip1 regulates organ size in vivo by cell-autonomous control of cell cycle exit timing. These studies establish MADM as a high-resolution method for modeling sporadic loss of heterozygosity in mice, providing insights into TSG function.

**Results and Discussion**

**MADM Predictably Labels Homozygous Mutant and WT Cells in Mosaic Mice.** We focused our study on the early postnatal cerebellar granule cell lineage. During normal development, granule cells are generated through postnatal expansion of granule cell progenitors in the outer external granular layer (EGL), a process that ends at approximately postnatal day 21 (P21) (12). In P7 WT mice, immunostaining reveals the presence of Ki67-positive, dividing cells in the outer EGL and the abrupt increase of p27 protein in the inner EGL, where granule cells have exited the cell cycle (Fig. 1D). p27 expression is sustained in postmitotic granule cells of the internal granular layer (IGL), where granule cells ultimately reside after they migrate across the molecular layer. We first tested whether MADM-mediated G2-X recombination results in loss of p27 protein expression in specifically labeled cells, as predicted by Fig. 1B and C. We recombined a p27 null allele (9) with the GR transgene and introduced into the genome of heterozygous GR reporter transgene reporter transgene (e.g., GFP) (5) upon Cre-mediated recombination. However, low-frequency gene knockout is difficult to achieve with this method, and the stochastic nature of two independent recombination events (6) does not guarantee 100% correlation between labeling and knockout [Fig. 1A Left, arrow and arrowhead; other strategies of visualizing mutant cells have other caveats (7)]. In contrast, the mosaic analysis with double markers (MADM) system achieves simultaneous gene inactivation and specific labeling of mutant cells through a single Cre/LoxP-mediated interchromosomal mitotic recombination event [Fig. 1A Right and supporting information (SI) Fig. 5A] (7). Moreover, recombination occurs infrequently (ranging from <0.001% to <1% of cells depending on which Cre line is used) (7), allowing sporadic gene knockout.

The MADM cassettes have been inserted into the ROSA26 locus on mouse chromosome 6 (7). By design, if a gene of interest is distal (telomeric) to ROSA26 and its mutant allele is recombined with the GR transgene, G2-X recombination in dividing cells (SI Fig. 5A Top, left branch) generates a green homozygous mutant cell and a red homozygous WT cell (Fig. 1B). Conversely, if the mutant allele is recombined with the RG transgene, G2-X recombination generates a red mutant cell and a green WT cell (Fig. 1C). G0, G1, or G2-Z recombination events create yellow cells heterozygous for the gene of interest (SI Fig. 5A Bottom, top right branch). Until now, the ability of MADM for simultaneous gene knockout and cell labeling had not yet been experimentally established.

As proof of principle for modeling sporadic LOH, we used MADM to analyze the consequence of inactivation of p27kip1 (p27), located distal to ROSA26 on chromosome 6, p27 encodes a cyclin-dependent kinase inhibitor (CKI) that functions at the G1/S transition of the cell cycle (8, 9). Loss of p27 protein correlates with poorer prognosis in a number of human cancers (10, 11), suggesting its role in tumor suppression. Moreover, p27 knockout mice exhibit a 30% increase of body size and multi-organ hyperplasia (9). Here, we report that MADM indeed permits simultaneous knockout and labeling of sporadic cells in mice. Furthermore, sporadic knockout of p27 results in a multifold increase in cell number far exceeding that observed in conventional knockout mice. Finally, we present in vivo evidence that p27 limits cell expansion by regulating cell cycle exit timing rather than cell cycle length.
same mice the RG transgene and a ubiquitously expressed Hprt-Cre transgene (13) (p27 GR/GR;Hprt-Cre, hereafter referred to as GR-MADM; Fig. 1B and SI Fig. 5B). In parallel, we also generated p27 RG/GR;Hprt-Cre mice (hereafter referred to as RG-MADM; Fig. 1C). As predicted in GR-MADM mice (Fig. 1B), p27 protein is absent in green (homozygous mutant) cells but more abundant in red (homozygous WT) cells than the surrounding yellow and colorless cells (heterozygous; Fig. 1E and E'). Conversely, in RG-MADM mice (Fig. 1C), red cells lack p27 protein, whereas green cells express higher levels of p27 protein (Fig. 1F and F'). These observations were confirmed by careful examination of >100 MADAM-label cells (e.g., SI Fig. 6). These experiments demonstrate that MADAM can achieve gene knockout in sporadic cells and predictably label them with its engineered markers.

**Cell-Autonomous Loss of p27 Results in Greater Cell Number Expansion.** Conventional p27 knockout mice exhibit a 70% increase in the number of cerebellar granule cells (14). However, LOH naturally occurs in sporadic cells rather than throughout entire tissues. Moreover, conventional knockout precludes analysis of the cell autonomy of gene function. Given the infrequency of interchromosomal recombination, MADAM leads to simultaneous gene knockout and labeling in sporadic cells (Fig. 1A and ref. 7). Using this feature, we determined whether sporadic loss of p27 leads to a similar hyperplastic phenotype. Qualitatively, MADAM-mediated sporadic knockout results in obvious expansion of mutant granule cells (Fig. 2 B and C; SI Fig. 6) compared with WT controls (Fig. 2 A and D'). To quantify this effect, we made use of p27+/−/− sibling cells that are generated simultaneously with p27−/− cells but are labeled with different markers in the same animal (Fig. 1 B and C). We assessed the difference of cell expansion by calculating the mutant-to-WT cell number ratio in systematically sampled cerebellar sections (see Materials and Methods) at the completion of granule cell development. As shown in Fig. 2D', the ratio between green and red cells in GR/GR;Cre (WT-MADM) mice is not significantly different from 1 (green/red ratio of 1.49 ± 1.37, 95% confidence interval), revealing equal expansion potential between WT sibling cells. However, the mutant/WT ratio increases to ≈6 in GR-MADM granule cells (Fig. 2 D and D'). This experiment demonstrates that p27 negatively regulates cell expansion in vivo, consistent...
with its characterized function as a G1 cyclin-dependent kinase inhibitor (8). Importantly, the cell expansion phenotype manifested in MADM-mediated sporadic knockouts is much more drastic than the 70% increase of granule cell number in conventional knockouts (14).

To test whether this greater cell number expansion applies to other tissues, we examined the consequence of sporadic loss of p27 in hepatocytes of the liver. In both GR-MADM and RG-MADM mice, we analyzed clusters of green and red cells that are adjacent to each other (twinspots; Fig. 2E). These cells most likely represent progeny from the same recombination event, because liver cells do not undergo extensive migration later in development (15). Compared with WT-MADM clones, which have a ratio of ~1 between red and green cells (Fig. 2E′, left column), the ratio of p27−/− cells over p27+/+ cells is >2 regardless of whether mutant cells are labeled green (GR-MADM) or red (RG-MADM; Fig. 2E′, center or right columns). Moreover, the mutant/WT ratio of GR-MADM and RG-MADM mice is not significantly different (P > 0.10), suggesting that fluorescence marker expression does not affect the cell-expansion phenotype. An unbiased analysis of whole-liver sections also yields comparable mutant/WT ratios (see Materials and Methods). Our results demonstrate that the mutant hepatocyte number rises by 110–180% compared with WT cells, whereas hepatocyte number increases by only 60% in conventional knockouts (9). Therefore, sporadic p27 knockout by MADM consistently generates a more extreme cell expansion phenotype than conventional knockouts in different organs.

The difference of cell expansion between organismal and sporadic knockout can be explained by a few possible mechanisms. First, given the small proportion of mutant cells in MADM mice, sporadic expansion may evade global organ-size-control mechanisms. Observations of mice after partial hepatectomy suggest the existence of such global mechanisms in liver-size determination (16, 17). Second, whereas conventional knockouts reveal the consequence of chronic loss of gene function, MADM-mediated conditional mutagenesis permits acute knockout at a later point in development, an event that may lead to different phenotypes because of lack of compensation (18). Finally, interactions between MADM-generated sporadic mutant cells and their heterozygous neighbors may allow greater mutant cell expansion than in a situation where every cell is mutant. Regardless, the phenotypes observed in MADM knockouts should more closely recapitulate the consequence of sporadic TSG inactivation as occurs in human cancers.

Expansion of p27−/− Cells Results from a Delay in Cell Cycle Exit. We next explored how p27 controls organ size in vivo. As suggested...
previously (19), decreased apoptosis may lead to the expansion of p27−/− cells. To examine whether decreased apoptosis contributes to p27−/− granule cell expansion, we performed immunostaining for cleaved (active) caspase-3. We find that cell death occurs very infrequently during granule cell expansion (data not shown), consistent with a previous report (20). Moreover, we rarely observe the presence of active caspase-3 in MADM-labeled cells regardless of genotype (SI Fig. 7A and Fig. 7A′). These data suggest that the expansion of p27−/− cells is not because of decreased apoptosis.

Previous studies have suggested two different mechanisms that promote increased proliferation of p27−/− cells. Because p27 functions as a cyclin-dependent kinase inhibitor at the G1/S transition (8), its loss could result in a shortened G1 phase, leading to an increase in cell cycle rate (19, 21). On the other hand, a delay in cell cycle exit permits prolonged expansion and thus increased proliferation (22, 23). To assess the contributions of these two mechanisms in vivo, we performed quantitative comparisons of mutant and WT cells within the same mosaic animal using MADM. In the G1 shortening model, one would expect an increased proportion of cells in other cell cycle phases. To test this hypothesis, we determined the proportion of colored cells labeled with a pulse of bromodeoxyuridine (BrdU, S-phase marker) or phospho-Histone 3 (pH3, M-phase marker) in GR-MADM mice at P4, a time point when most granule cell progenitors are proliferating. We find that the proportion of p27−/− (green), p27+/− (yellow), and p27+/+ (red) cells in the S phase (46–49% of cells) and M phase (10–12%) is equivalent (Fig. 3), suggesting no significant G1 shortening due to p27 loss.

To test whether p27−/− cells exhibit a delay in cell cycle exit, we took advantage of the stereotypical developmental process of granule cell progenitors in which dividing cells reside in the EGL, whereas postmitotic granule cells situate in the IGL. We compared the number of p27−/− and p27+/+ cells in the EGL and IGL of GR-MADM mice at P4, when some granule cells begin to differentiate. If cell cycle exit timing is unperturbed, the

EGL-to-IGL ratio (EGL/IGL) of mutant cells should be equivalent to that of WT cells. If cell cycle exit timing is delayed because of p27 loss, mutant cells should remain longer in the EGL and undergo extra divisions, leading to a higher EGL/IGL ratio for mutant cells in comparison with WT cells (Fig. 4A). Indeed, in MADM mosaics, we observe the latter (Fig. 4B), suggesting a delay in cell cycle exit of p27−/− cells. An alternative interpretation of an increased EGL/IGL ratio could be that p27−/− cells differentiate on time but fail to migrate out of the EGL, similar to the role of p27 in cortical neuronal migration (24). Two observations argue against this possibility. First, EGL p27−/− cells express the proliferative marker Ki67 (SI Figs. 7B and 7B′), implying that they are still dividing rather than simply failing to migrate after cell cycle exit. Second, p27−/− cells ultimately migrate to the IGL and express appropriate differentiation markers (SI Figs. 7C–7D′) but outnumber p27+/+ cells in adults (Fig. 2D'), an outcome that would not result from a simple migration defect.

We also performed a quantitative analysis to determine whether p27 similarly mediates cell cycle exit in developing hepatocytes. After a recombination event in a dividing hepatocyte progenitor, each daughter cell normally undergoes n cell divisions (in a symmetric mode as suggested by the equal number of red and green cells in WT-MADM; Fig. 2E′) to produce 2n cells. In a delayed cell cycle exit model, p27−/− daughter cells would undergo X extra cell divisions to generate 2n−X cells. X should not vary with n. In a faster cell cycle rate model, p27−/− daughter cells would undergo Y extra cell divisions to generate 2n+Y cells in the same period in which their WT siblings produce 2n cells. In this case, Y should be proportional to n (Fig. 4C). An analysis of hepatocyte twinspots reveals that there is no positive correlation between X or Y and n (Fig. 4D), ruling out a faster cell cycle rate model. Thus, loss of p27 appears to result in a delay in cell cycle exit leading to overproliferation of both cerebellar granule cells and hepatocytes. The degree of mutant cell expansion of granule cells (6-fold, i.e., two to three extra cell divisions) is greater than that of hepatocytes (∼2.5-fold i.e., one to two
extra cell divisions), implying that p27 plays a more significant role in cell cycle exit timing in granule cells.

Our findings suggest that p27 controls the precise timing of cell cycle exit, an important mechanism in organ-size control. Although such a role has been suggested from previous in vitro studies of mammalian cells (14, 25) or during Drosophila embryonic development (22), our work adds clear in vivo evidence supporting this notion in mammalian neural tissue and hepatocytes. In some cases, studies of certain tissues that have synchronized developmental timing, including the organ of Corti (25), ovarian follicles (26), and the retina (27), suggest the involvement of p27 in controlling cell cycle exit in mice. However, the MADM system allows the study of all of the other cell types that do not fall into this category. Moreover, mosaic analysis demonstrates that this function of p27 is cell-autonomous. The importance of sporadic knockout for studies of cell autonomy is also supported by another observation. All conventional p27−/− mice develop pituitary adenomas of the pars intermedia by 10 weeks of age (9). However, human pituitary tumors primarily originate from the pars distalis and do not exhibit LOH of p27 (28). We examined five pituitaries of GR-MADM and RG-MADM mice (>10 weeks old) and never found clonal expansion of labeled p27−/− cells (data not shown). This observation suggests that a non-cell-autonomous mechanism might be responsible for the formation of pituitary adenomas in conventional p27−/− mice.

**Use MADM for LOH Modeling.** Our study confirms the use of the MADM system for gene knockout and predictable labeling in sporadic cells, making it a superb genetic tool for modeling sporadic LOH in mice. There are several appealing features of the MADM system. It guarantees 100% correlation between labeling and genotype, allowing unambiguous phenotypic analysis with single-cell resolution, which enables the study of cell-autonomous functions of tumor-suppressor genes and the interactions between mutant cells and their microenvironment. Furthermore, labeled WT siblings of mutant cells serve as an *in situ* control, which greatly simplifies phenotypic analysis. Using this feature, we have provided definitive evidence for the *in vivo* function of p27 in controlling cell cycle exit timing. Overall, MADM allows the study of immediately early events upon TSG loss, which should shed light on the initial early events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light on the initial events upon TSG loss, which should shed light

**Tissue Preparation and Histology.** All animal procedures were based on animal care guidelines approved by Stanford University’s Administrative Panels on Laboratory Care (A-PLAC). Brain and liver tissues were isolated from anesthetized mice perfused with 4% paraformaldehyde (PFA) in 0.1 M PBS, fixed overnight in 4% PFA at 4°C, cryoprotected in 30% sucrose, and embedded in optimal cutting temperature (OCT). For cell cycle stage analysis, 60 μg/kg BrdU was administered by i.p. injection 1–3 h before sacrifice. Tissues were sectioned at 10- to 30-μm thickness. Cryosections were treated for immunofluorescence and processed for confocal imaging as described (7). GFP was detected by anti-GFP primary antibody (chicken, 1:500, Cat. no. GFP-1020; Aves Labs, Tigard, OR). MYC-tagged DsRed2 was detected by anti-MYC primary antibody (goat, 1:200, Cat. no. 600-338; Novus Biologicals, Littleton, CO). Primary antibodies against the following proteins were also used: p27kip1 (mouse, 1:100, Cat. no. 610242; BD Transduction Laboratories, Lexington, KY), Ki67 (rabbit, 1:500, Cat. no. NCL-Ki67p; Vision Biosystems, Norwell, MA), BrdU (rat, 1:250, Cat. no. OBT0030; Accurate Chemical, Westbury, NY), phospho-Histone 3 (Ser-10; rabbit, 1:250, Cat. no. 06-570; Upstate Biotechnology, Lake Placid, NY), cleaved caspase-3 (Asp-175; rabbit, 1:50, Cat. no. 9664S; Cell Signaling Technology, Beverly, MA), NeuN (mouse, 1:250, Cat. no. MAB377; Chemicon, Temecula, CA), and GABA-A receptor α6 (rabbit, 1:500, Cat. no. AB5610; Chemicon).

**Quantification Methods.** Granule cell expansion (Fig. 2 D and D′) was quantified from P22 cerebella of p27 GR/GR;Wnt1-Cre mice. Wnt1 is expressed in an anteroposterior gradient in cerebellar progenitors (33). The Wnt1-Cre line leads to sparser labeling of granule cells than Hprt-Cre, permitting more accurate quantification. Unbiased sampling was performed by counting all green and red cells in lobule VIII from 5–10 nonadjacent 20-μm sagittal sections (150–250 μm apart) across the mediolateral span of the cerebellum (n = 9 for WT-MADM and n = 6 for GR-MADM) under fluorescence microscopy. Hepatocyte expansion (Fig. 2 E and E′) was determined by quantification of all cells in hepatocyte twospots (n = 20–30 per genotype from 2+ mice), defined as clusters of adjacent green and red cells at least 300 μm away from other green or red cells. Twospots never spanned >275 μm in diameter (average of 128 μm). To ensure that twospot quantifications were unbiased, all green and red cells were counted from >5 nonadjacent 25-μm sections (150–250 μm apart) of the left and right main liver lobes (n = 3–4). “Mutant”-to-WT ratios for WT-MADM and GR-MADM/GR-MADM were 1.15 ± 0.064 SEM and 2.73 ± 0.84 SEM, respectively, in line with twospot quantifications (Fig. 2E′). Geometric means in original scale were used throughout statistical analyses of ratios. Cell cycle stage profiling (Fig. 3) was performed by counting the proportion of 30–70 green, red, and yellow cells per P4 GR-MADM cerebellum that were labeled with BrdU or pH3 by using 1-μm optical sectioning by confocal microscopy (n = 4). Cell cycle exit timing of granule cells (Fig. 4B) was quantified by counting all green and red cells in the EGL and IGL separately in 10–12 nonadjacent 30-μm sagittal sections (150–250 μm apart) across the mediolateral span of P4 GR-MADM cerebellum (n = 10).

**Materials and Methods**

**Generation of MADM Knockout Mice.** GR/GR;Cre (WT-MADM), p27 GR/GR;Cre (GR-MADM), and p27 GR/GR;Cre (RG-MADM) mice were generated as described in SI Fig. 5B. p27 heterozygote mice were obtained from The Jackson Laboratory, Bar Harbor, ME (9). Hprt-Cre (13) was used throughout the study except in the cerebellar mutant/WT ratio quantification (Fig. 2 D and D′), where Wnt1-Cre (33) was used. Genotyping of the RG and GR transgenes, the p27 null allele, and Cre transgenes was performed by PCR as described (7, 9). The studies were performed in mixed background mice generated by crossing Cre transgene (129S1, B6, CBA), p27 heterozygote (129S4), and MADM mice (129S1, CD-1) of varying genetic backgrounds.

**Statistics.** Statistical comparison of mutant/WT ratios of WT-MADM, GR-MADM, and RG-MADM granule cells (n = 9 for WT-MADM, n = 6 for GR-MADM; Fig. 2D) and hepatocyte twospots (n = 21 for WT-MADM, n = 29 for GR-MADM, n = 20 for RG-MADM; Fig. 2E′) was performed by permutation analysis with 100,000 iterations (Matlab). Comparison of EGL/IGL of red and green granule cells from P4 GR-MADM mice (Fig. 4B) was performed by permutation analysis with 10,000 iterations (Matlab). The extent of correlation between mutant/WT ratios and hepatocyte twospot size (n = 49, Fig. 4D) was determined by linear
regression analysis (Excel) and computation of the correlation coefficient of variation and $P$ value. The standard for significance in all statistical analyses was $P < 0.05$.

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