HHS Public Access

Author manuscript

Dev Biol. Author manuscript; available in PMC 2020 December 15.

Published in final edited form as:

Dev Biol. 2019 December 15; 456(2): 154–163. doi:10.1016/j.ydbio.2019.08.012.

PRMT5 is necessary to form distinct cartilage identities in the knee and long bone

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Abstract

During skeletal development, limb progenitors become specified as chondrocytes and subsequently differentiate into specialized cartilage compartments. We previously showed that the arginine dimethyl transferase, PRMT5, is essential for regulating the specification of progenitor cells into chondrocytes within early limb buds. Here, we report that PRMT5 regulates the survival of a separate progenitor domain that gives rise to the patella. Independent of its role in knee development, PRMT5 regulates several distinct types of chondrocyte differentiation within the long bones. Chondrocytes lacking PRMT5 have a striking blockage in hypertrophic chondrocyte differentiation and are marked by abnormal gene expression. PRMT5 remains important for articular cartilage and hypertrophic cell identity during adult stages, indicating an ongoing role in homeostasis of these tissues. We conclude that PRMT5 is required for distinct steps of early and late chondrogenic specialization and is thus a critical component of multiple aspects of long bone development and maintenance.

Keywords

PRMT5; Knee; chondrogenesis; chondrocyte differentiation; joint; articular cartilage; patella; progenitors; growth plate; homeostasis

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Authors' roles: Study design: JR, ZL, RSG and SAV. Study conduct and Data collection: SAV, RSG, JR and ZL. Data analysis: JR, ZL. Data interpretation and approving final version of manuscript: JR, ZL, RSG and SAV. SAV and JR take responsibility for the integrity of the data analysis.

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INTRODUCTION

Starting with the initial specification of chondrocytes from mesodermal cells, a combination of extrinsic signals and cell-intrinsic responses drive successive steps of cartilage development. Ultimately, these result in the formation of a cartilage template for the skeleton as well as the formation of numerous types of specialized cartilage cells (reviewed in (1)). During the initial stages of development, nascent chondrocytes temporarily stop proliferating and start expressing early differentiation markers (*Sox9* and *Collagen2a1*) as they change shape and coalesce to form the bone rudiments.

Chondrocyte proliferation subsequently resumes on the ends of the bones as chondrocytes in the center progressively differentiate into hypertrophic chondrocytes in part through a signaling loop driven by Indian hedgehog and Parathyroid hormone-related protein (2). This second phase of hypertrophic chondrocyte maturation is essential for normal long bone growth and ossification and requires the establishment of new genetic programs driven by RUNX2, FOXA2, and MEF2C which activate the transcription of terminal differentiation markers *Collagen10a1* and *Mmp13* (3-7). Dysregulation of these terminal differentiation programs leads to a wide range of cartilage and bone defects including skeletal dysplasias, characterized by increased cell hypertrophy and reduced/delayed bone formation (8,9). Hypertrophic chondrocytes ultimately have two fates, to either undergo apoptosis after establishing permissive conditions for osteoblast colonization or transdifferentiate into osteoblasts (10,11).

After the formation of the initial cartilage anlagen, the knee joint is generated by the addition of several different cell types. Externally-derived GDF5-expressing cells engraft onto or within the existing bone to form the synovial joint, and additional progenitor cells marked by *Scleraxis* and *Sox9* give rise to the patella (12,13). These joint structures, along with the proliferative distal ends of the long bones subsequently differentiate and become ossified, generating a mature, multi-tissue knee joint (13-15). In contrast to the long bone itself, the origin and regulation of these additional joint progenitors are still poorly understood.

We recently found that the methyltransferase PRMT5 is important for chondrocyte specification. Using *PrxCre* to conditionally remove *Prmt5* from the limb buds, we found that forelimb morphogenesis was profoundly impaired while the hindlimbs were relatively normal (16). Because *PrxCre* is expressed at comparatively earlier stages of forelimb than hindlimb development (17), it suggested that PRMT5 has a critical role in the initial specification of chondrocytes from progenitor cells, but is dispensable for later chondrocyte development. In addition to chondrocyte specification, PRMT5 has distinct roles in the development of many other organ systems, including the lung, muscles, oligodendrocytes and osteoclasts (18-21). Several of these roles implicate PRMT5 in the maintenance of stem/progenitor cells (22,23). While we previously demonstrated that PRMT5 is not necessary for maintaining nascent chondrocytes in limb buds (16), we hypothesized that it might be required for the transition to more differentiated types of cartilage as well as for the differentiation of later cartilage populations derived from different progenitor populations.

Here, we show that loss of *Prmt5* at different developmental stages results in specific cartilage maturation defects in the knee joint and long bones. Consistent with our initial hypothesis, we find that PRMT5 is required for the survival of patella progenitor cells. PRMT5 is separately required for the differentiation of multiple cartilage domains within the long bones, including the growth plate and articular cartilage. PRMT5 continues to be required for cartilage homeostasis during adulthood, where it remains necessary for hypertrophic cartilage differentiation in the growth plate and maintains signature gene expression in the articular cartilage. We conclude that PRMT5 is essential for the transition of chondrocytes through multiple states of differentiation during chondrogenesis.

MATERIALS AND METHODS

Mice

Experiments involving mice were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin (AUP-2016-00255 and AUP-2018-00276). *Prx-Cre* (17) and *Col2-Cre* (24) mice were crossed with *Prmt5*$^{tm2c(EUCOMM)Wtsi}$ (referred to as *Prmt5*$^{c/c}$) (16) females to generate control, *PrxCre;Prmt5*$^{c/c}$, and *Col2Cre;Prmt5*$^{c/c}$ mice respectively. *ATC-Cre* (25) mice were crossed with *Prmt5*$^{c/c}$ females to generate control and *ATC;Prmt5*$^{c/c}$ mice. Doxycycline (Dox) was administered by intraperitoneal (IP) injections starting at 4 weeks of age (P28), once a week at 10 mg/kg body weight for four weeks. *OC-Cre* mice (26) were purchased from Jackson Laboratory (Stock# 019509) and crossed with *Prmt5*$^{c/c}$ females to generate control and *OC-Cre;Prmt5*$^{c/c}$ mice.

Micro-CT Analysis

Whole-mount skeletal preparation was performed as previously described (27). For Micro-CT analysis, hind limbs were harvested from control *and PrxCre;Prmt5*$^{c/c}$ mice at P21, fixed for three days in 10% buffered formalin and stored in 70% ethanol until imaging. Samples were scanned by the high-resolution X-ray CT facility at UT Austin on a Fein Focus High Power source, 140 kV, 0.16 mA, aluminum filter, with a Perkin Elmer detector.

Histology and immuno-staining

Following antigen retrieval in Tris-EDTA, pH 9 at 70°C for ten minutes, paraffin sections of adult limbs (decalcified in formic acid) were incubated with anti-PRMT5 (Abcam, ab109451, 1:100) or anti-RUNX2 (MBL International, D130-3, 1:100), processed using a Vectastain ABC HRP kit (Vector Labs, PK-4000) and developed using DAB substrate (Roche 11718096001). Visualization of PRG4 was similarly carried out using anti-PRG4 (Abcam, ab28484, 1:400) following antigen retrieval in 0.1mg/ml hyaluronic acid at 37 °C for 10 minutes. Immunofluorescent staining was performed on frozen tissues harvested and fixed for 1hour in 4% paraformaldehyde and subsequently embedded in OCT. 10μm-sections were then post fixed in 4% paraformaldehyde for 10 minutes at room temperature, permeabilized in 0.06% PBST (Triton-X) for 30 minutes, and blocked in 3% bovine serum albumin (BSA) and 5% normal goat serum (NGS)/PBST (1% Tween-20) for 1 hour at room temperature. Samples were then incubated with: anti-SOX9 (Millipore, AB5535, 1:200), anti-phospho-Histone H3 (Millipore, 06-570, 1:200), anti-GFP (Aves Labs, GFP-1020, 1:500). After overnight incubation at 4°C, the samples were visualized using the following

secondary antibodies: Alexa 568 goat anti-rabbit secondary (Life Technologies, A11036, 1:250) and Cy2 anti-chicken secondary (Jackson Immuno Research, 703-225-155, 1:200). Apoptosis was detected by TUNEL staining was performed using an In Situ Cell Death, TMR detection kit (Roche, 12156792910). Fluorescent samples were counterstained for nuclei with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, D1306, 1:5000).

Section In-Situ Hybridization

Paraffin sections (5μm) were permeabilized in Proteinase K (7.5μg/mL in PBST) for 5 minutes at room temperature, and post-fixed in 4% paraformaldehyde and 2% glutaraldehyde. Samples were incubated with Digoxygenin-labeled antisense riboprobes overnight at 68°C. Followed by incubation with a sheep anti-Dig-POD antibody (Sigma, 11207733910, 1:500) at 4°C overnight. Hybridization was detected using a tyramide-amplified fluorescent antibody (Perkin Elmer, NEL753001KT) incubated at room temperature for 10-20 minutes (incubation times varied depending on the probe).

Quantitative-RT-PCR

RNA was extracted from embryonic knee samples using Trizol Reagent (Life Technologies, 15596-026), DNase-treated, and 500ng was then used for cDNA synthesis (SuperScript II, Invitrogen 18064-014). Quantitative RT-PCR was performed using SensiFast SYBR Lo-Rox (Bioline BIO-94005) on a Via7 (ABI) platform. Gene expression was normalized to GAPDH, and fold change was calculated using the delta-CT method (28). The following qRT-PCR primers were used: *Prmt5* (F) CATGAGAGCAAGCCCACAAA, (R) CCCCACCAGCATTTTCCTAA; *Bmp4* (F) ACGTACTCCCAAGCATCACC, (R) GCACAATGGCATGGTTGGTT; *Gapdh* (F) GGTGAAGGTCGGTGTGAACG, (R) CTCGCTCCTGGAAGATGGTG.

Results

PRMT5 is required for the differentiation of distinct cartilage sub-types

We previously showed that *Prmt5* is required for the initial specification of appendicular chondrocytes but is not required to maintain them after this specification (16). To determine if *Prmt5* is subsequently important for later events in appendicular skeletal development, we examined 3 week old (P21) hindlimbs of *PrxCre;Prmt5* conditional knockouts *(PrxCKOs)* by micro computed tomography (Micro-CT) and found that *PrxCKOs* had pronounced knee defects. Specifically, we observed sharp reductions in bone volume within the patella and the epiphyseal regions of the femur and tibia (Fig. 1A-C). We additionally noted a characteristic bowing of the tibia and fibula (Fig. 1B). These defects suggest that PRMT5 is required for the normal development of the knee and long bone.

We next examined sections through the knee joint and observed severe reduction in the secondary ossification centers of *PrxCKO* mice (Fig. 1D,E), which was more pronounced in the tibia than in the femur. Cells within the most superficial layers of the epiphysis (which normally make up the articular cartilage) in *PrxCKOs* are more disorganized, forming small clusters of cells (Fig. 1I), in contrast to the stereotypic columnar organization in wild-type tissues (Fig. 1F). The articular chondrocyte marker *Proteoglycan 4 (Prg4)* which is normally

restricted to the most superficial layers of articular cartilage cells, is mis-expressed throughout multiple layers of cartilage (Fig. 1G,J). Loss of/severe reductions in the secondary ossification center in *PrxCKOs* is further marked by the presence of *Collagen2a1 (Col2a1)*-expressing cells within the deeper articular layers (Fig. 1H, K).

In the growth plate, we observed a large expansion in the size of the hypertrophic cell domain (Fig. 1S). There is a ~2-fold increase in the average size of hypertrophic chondrocytes and a 2-fold decrease in their cell density (Fig. 1R,T). There was no significant change in the number of cells within this domain between control and *PrxCKO* samples (an average of 209 cells versus 175 cells, respectively; n=5, p=0.3114 Student's t-test). The expanded domain was also marked by a striking increase in the domain of *Collagen10a1 (Col10a1)* expression and a corresponding reduction in *Collagen2a1 (Col2a1)* expression (Fig 1M-N;P-Q). *Col10a1*-expressing cells in *PrxCKOs* also expressed robust levels of *Indian hedgehog (Ihh)* (Supplemental Fig. 1A,C). Together, these findings indicate that PRMT5 is required for the development of multiple chondrogenic sub-types within the epiphysis.

PRMT5 is required for the survival of early patella progenitors

To determine at what stage PRMT5 first presents a phenotype, we examined wholemount skeletal preparations at embryonic timepoints. Compared to sibling controls, *PrxCKO* embryos have significantly reduced patellas at both E18.5 and E16.5 (Fig. 2A-F). Additionally, we examined SOX9 expression in the patella at E16.5 and observed that *PrxCKOs* contained a clearly reduced domain of SOX9-positive cells at E16.5 (Fig. 2 G,H). Although the mitotic index was unchanged in *PrxCKOs* (Supplemental Fig. 2A-C), there was a small but significant increase in cell death by TUNEL within the patella of *PrxCKO* mice, suggesting that cell death may contribute to this reduced domain (Supplemental Fig. 2D-F). It is presently unclear why the patella appears more reduced at E16.5 than at E18.5. As there is a clearly reduced domain of SOX9+ cells at this stage, as well as low levels of cell death, we speculate that the difference could be due to an initial delay in development due to lower cell numbers. To determine whether patella size reduction occurred before or after chondrocyte specification in PRMT5 knockouts, we also inactivated *Prmt5* with *Col2-Cre*, which is first expressed in chondrocytes after specification (24). In contrast to *PrxCKO, Col2-Cre;Prmt5*$^{c/c}$ (*Col2-CKO*) embryos had normally sized patellas (Fig. 2I-K), suggesting that PRMT5 is not required for the differentiation of committed chondrocytes in the patella, but instead is required earlier during patella specification. If this is the case, reduced patella size in *PrxCKOs* could either be due to a reduction in the number of chondrocyte progenitors or a delay in the differentiation of chondrocytes from mesenchymal limb bud progenitors.

The patella is initially formed by a population of SOX9^{+}SCX^{+} progenitor cells that coalesce on the articulating femur head at E13.5 and subsequently differentiate into patella-specific chondrocytes by E14.5 (29,30). Based on the reduction in patella size at E16.5, we hypothesized that PRMT5 might be similarly required for the normal commitment and survival of patella progenitor cells. To test this, we used the *Scleraxis-GFP (Scx-GFP)* allele in combination with antibodies against SOX9 to visualize SOX9^{+}SCX^{+} progenitor cells in *PrxCKOs* (31). There were comparable numbers of SOX9^{+};SCX^{+} progenitor cells in control

and mutant knees at E13.5 (72%; n=4 versus 69%; n=4 respectively; Fig. 3A-B). Strikingly, there was a 30% increase in cell death within the patella progenitors in mutants (Fig. 3C-D,I). We also noted increased TUNEL staining within the SCX-GFP$_+$ connective tissues surrounding the early long bones. Elevated levels of cell death also occurred in *PrxCKO* patellas at E14.5 (Fig. 3G-H, I), highlighting a 24-hour window for PRMT5 regulation. We then examined the expression of *Bmp4*, which is upregulated in early limb buds in *PrxCKOs* (16). As expected, *Prmt5* levels are substantially reduced. However, there were no significant differences in the expression *Bmp4* within knee at E14.5 (Fig. 3J). We conclude that an early increase in patella progenitor death in *PrxCKOs* is the primary cause for the overall reduction in patellar size observed at later developmental stages.

A postnatal requirement for PRMT5 in hypertrophic cartilage maturation in the long bones

To determine when the cartilage differentiation defects observed within the long bones of P21 *PrxCKOs* first appeared, we assayed for morphological and gene expression changes in the growth plates of *PrxCKO* tibias at E18.5 (Fig. 4). At E18.5, there was a mild increase in cell size in the hypertrophic zone (Fig. 4A,E). This was accompanied by increased cell death both within the hypertrophic zone as well as along the ossification boundary in the growth plate (Fig. 4B,F). There was also a decrease in RUNX2 expression within the pre-hypertrophic/hypertrophic chondrocytes in *PrxCKOs* compared to controls (Fig. 4C,G). However, at this stage *Col2a1* expression in the proliferating chondrocytes and *Col10a1* expression in the hypertrophic chondrocytes was largely unaltered between controls and mutants (Fig. 4D,H,I,K). Similarly, expression of *Mmp13* (a direct transcriptional target of RUNX2) is still expressed within the growth plate (Fig. 4J,L)(32), suggesting that the general organization of the growth plate into sub-type specific zones is unaffected by loss of *Prmt5* from the limb at embryonic stages.

The relatively subtle changes in the growth plate at late gestation contrast with the pronounced abnormalities present at P21 (Fig. 1), indicating that defects within the epiphysis intensify during early postnatal development. To refine the timing, we assayed tibias from P10 pups using *Col2-Cre* to delete *Prmt5* specifically within committed chondrocytes (Supplemental Fig. 3A-B). The proliferative domain of the growth plate has a reduced SOX9 expression (Fig. 5A,C). In contrast, there is persistent SOX9 expression in some hypertrophic chondrocytes in *Col2-CKOs*, a pattern which was not observed in control mice (Fig. 5A,C). We also found dramatic reductions in the SOX9 target gene *Col2a1* within the proliferative zone (Fig. 5E,G). These changes in gene expression patterns are comparable to the initial growth plate defects seen in *PrxCKO* mice at P21 (Fig. 1N-Q). As chondrocytes transition into hypertrophy, RUNX2 activates terminal differentiation programs by downregulating SOX9 and activating *Mmp13* (5,6,32,33). We observed reductions in RUNX2 expression within the growth plate (Fig. 5B, D) and a near absence of *Mmp13* expression in *Col2-CKO* mutants (Fig. 5F, H), suggesting that PRMT5 is progressively important for maintaining RUNX2 expression within differentiating growth plate cells between E18.5 and P10. We also observed reduced expression of *Mef2c* expression, another regulator of hypertrophy, in the hypertrophic chondrocytes of the growth plate (Supplemental Fig. 1B, D) (4). Together these results indicate that PRMT5 becomes

increasingly important for the terminal differentiation of hypertrophic chondrocytes during the early postnatal period.

Previous studies have implicated PRMT5 in regulating aspects of bone and osteoclast differentiation (19,34). Because the *Col2Cre* driver can directly or indirectly contribute to bone as well as chondrocytes (10,35), some aspects of the phenotypes within the epiphyseal tissues could be caused by a requirement for PRMT5 in the bone lineage. To address this, we crossed conditional *Prmt5* mutants to the osteoblast-specific *Osteocalcin-Cre* (*OC-Cre*) (26) line (Supplemental Fig. 4A-B) and observed no detectable knee or bone phenotypes (Supplemental Fig. 4C-G), indicating that PRMT5 is not required in mature osteoblasts.

Prmt5 is required for maintenance of adult chondrocytes

To determine whether PRMT5 is further required for the maintenance of chondrocytes in the adult knee, we generated *Prmt5* conditional knockout mice containing *Acan enhancer-driver Tetracycline-inducible Cre (ATC-Cre*). This Cre is active in all chondrocytes within the articular cartilage, and in pockets of growth plate chondrocytes (25). We induced recombination by administering Doxycycline (Dox) at 1 month of age and continuing treatment out to 2 months. The mice were then kept for 2 more months after the end of Dox administration prior to analysis (Fig. 6A). When examined by in situ hybridization, *Prmt5* is nearly undetectable in the articular cartilage and is absent within patches of chondrocytes in the growth plate in *ATC;Prmt5*$^{c/c}$ (*ATC-CKO*) mice at 4 months, indicating the effectiveness of this conditional knockout approach (Supplemental Fig. 5A,B). *ATC-CKO* joints had no obvious morphological change in the organization of articular and growth plate chondrocytes compared to controls (Fig. 6B-D,F,I, K). However, in contrast to the embryonic deletion where we observed increases in *Prg4*, there was a decrease in PRG4 expression in the articular cartilage of Dox-induced *ATC;Prmt5*$^{c/c}$ mice (Fig. 6 E,G,H) (see Discussion). In addition, there is a significant expansion in the number of *Col1Oa1*-posiiive cells (Fig. 6J,L,M), similar to the phenotype observed in the embryonic deletion. We conclude that PRMT5 helps regulate the homeostasis of the articular cartilage and growth plate.

DISCUSSION

Differentiated chondrocytes encompass several specialized cell sub-sets which represent a divergence of developmental pathways from a more limited set of common progenitor cells. These pathways have multiple transient states, that are regulated by diverse mechanisms as they differentiate. We previously showed that PRMT5 is required during very early development for the survival of progenitor cells during the initial specification of SOX9-expressing chondrocytes (16). Here, we find that PRMT5 has a similar role in maintaining the survival of the different progenitor cells that give rise to the patella. Distinct from these roles, PRMT5 is also required for the differentiation of chondrocytes into some specific cell types, including articular cartilage and hypertrophic cartilage. Some of these roles are ongoing in adult bones, indicating that PRMT5 is involved in homeostasis.

Interestingly, conditional deletions of *Prmt5* with *PrxCre* or *Col2Cre* resulted in different phenotypes. *PrxCKOs* have a reduced patella while the patella is not reduced in the

chondrocyte-specific *Col2-CKOs*. The patella initially forms from a population of progenitors cells expressing SOX9 and SCX that reside adjacent to the femur head (29). *PrxCre* is active in the limb bud mesenchyme several days before the onset of *Col2Cre*. Thus, a likely explanation for the difference in phenotypes between the two Cre drivers lies in the transient requirement for PRMT5 in patella progenitor cells prior to their differentiation into patella-specific chondrocytes. In support of this possibility, $SOX9^{+}$;SCX^{+} cells have high levels of apoptosis in *PrxCKOs* at E13.5-E14.5, during initial patella formation, almost certainly leading to the marked decrease in initial patella size. Although surviving patella progenitors are able to differentiate into SOX9 and *Col2a1* -expressing chondrocytes, the domain size remains small, suggesting that patella size is limited by the initial number of progenitor cells. This function is analogous to the temporally-specific cell survival requirement for PRMT5 in progenitor cells that give rise to $SOX9^{+}$ chondrocytes in the early limb bud (16).

Several previous studies have found that PRMT5 mutants have upregulated BMP signaling in different cell contexts, including the embryonic lung, early limb buds (16,36). In contrast to these findings, we do not observe elevated *Bmp4* in mutants either during embryonic or postnatal stages (Fig. 3, Supplemental Fig. 6A,B). We cannot exclude the possibility that *Bmp4* is upregulated in mutants at specific times that were missed in our analysis. Alternatively, PRMT5-dependent regulation of *Bmp4* is context-dependent.

Deletion of *Prmt5* causes a striking phenotype in the growth plate, where visibly hypertrophic cells appear to be blocked in the process of hypertrophic differentiation. Hypertrophic chondrocyte differentiation requires PRMT5 starting during late gestation and continuing into adulthood. Proliferative chondrocytes have reduced levels of SOX9. *Col2a1* is a direct transcriptional target of SOX9 and thus the reduced levels of SOX9 likely account for the reduced levels of *Col2a1* seen in these cells (37). Within the upper hypertrophic chondrocytes, there is a prominent expansion in the size of the *Col10a1*-expressing domain which is not due to an increase in cell number, but rather in size. Additionally, these cells contain persistent levels of SOX9 expression and a near absence of RUNX2. As SOX9 and MEF2C function as transcriptional activators of *Col10a1*(25), the continued presence of SOX9 and residual *Mef2c* suggests a possible mechanism for *Col1Oa1* initiation. RUNX2 and MEF2C drive further hypertrophic chondrocyte differentiation, in part by activating *Mmp13*. Therefore, the near absence of RUNX2 and reduction in *Mef2c* suggest that hypertrophic chondrocytes arrest in a partially differentiated state in which they are unable to exit development (5,6,32,33). We note that the phenotype is similar to that observed in *Mmp13* knockouts, which also have expanded hypertrophic domains expressing *Col1Oa1* (38). As RUNX2, in combination with RUNX3, is required for embryonic *Collagen lO* activation (6), the expanded domain of *Col1Oa1* was initially unexpected. However, RUNX2 becomes progressively depleted in hypertrophic chondrocytes (compare Figs 4G and 5D). Presumably, RUNX2 levels are initially sufficient to activate *Col10a1* but are not required to sustain it during later developmental timepoints. This may also explain the lack of reduction in *Ihh*, which is initially dependent on RUNX2 (6).

Following initial joint development, *Prmt5* continues to be required for ongoing chondrocyte homeostasis in the adult knee. The loss of *Prmt5* results in reduced numbers of PRG4

expressing cells (Fig. 6E,G,H), a finding that is in contrast with the upregulation of *Prg4* observed when *Prmt5* is lost during developmental stages (Fig. 1J, 6E). The difference in these roles could highlight differences in the requirement for PRMT5 during developmental versus adult stages. PRG4 is a component of the cartilage extracellular matrix and synovial joint fluid, and its expression is both necessary and sufficient to prevent osteoarthritis (39-41). The loss of *Prmt5* from adult articular cartilage tissues may result in loss of superficial cartilage identity and thus a depletion of PRG4 similar to phenotypes reported in response to cartilage degeneration (39,40,42). In future studies, it will be interesting to determine if PRMT5 activity has a pathological role in preventing degenerative bone diseases such as osteoarthritis. *Prmt5* also has an ongoing role in regulating pre-hypertrophic cell identity in adults (Fig. 6L), suggesting that *Prmt5* is broadly required for specialized chondrocyte identity throughout an organism's lifetime.

The requirement for PRMT5 in mediating step-wise chondrogenic identity at distinct developmental timepoints raises interesting questions regarding its molecular mechanism. PRMT5 is dispensable for maintaining generic chondrocytes but is required for multiple distinct lineages at later timepoints. It is possible that PRMT5 represses distinct sets of target genes in these systems through epigenetic repression via histone modifications. In this case, perhaps a conserved suite of specification/differentiation genes that are broadly required for iterative steps in cartilage specialization, could require PRMT5-mediated activation and result in context-specific differentiation. PRMT5 could additionally influence cell fate decisions by regulating the activity of one or more target proteins through direct methylation. This possibility is supported by evidence that PRMT family members (PRMT4 and PRMT5) interact directly with- and confer di-methyl marks on SOX9 to regulate its activity (43,44). In this case, we predict that additional protein targets or regulatory mechanisms will also occur as the phenotypes we observe are inconsistent with post-natal reductions in SOX9 alone (43). A final possibility is that chondrocytes lacking *Prmt5* may lack robustness to transition to different cell fates. This reduced robustness might be sufficient to allow chondrocytes to maintain the status quo but would make cell fate transitions particularly challenging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We would like to thank Dr. Mathew Hilton, Dr. Veronique Lefebvre, Dr. Fanxin Long, Dr. Susan Mackem, Dr. Francesca Mariani, Dr. John Schwarz, and Dr. Ronen Schweitzer for providing mouse strains and reagents. We thank Jann Uy for technical assistance and Rachel Lex for comments on the manuscript. This study was supported by NIH Grants R01-HD073151 (SV), R01-AR072009-01 (RG), F32-AR073648-01 (ZL), and a UT Austin Provost Graduate Excellence Fellowship (JR).

REFERENCES

1. Kozhemyakina E, Lassar AB, Zelzer E. A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation. Development. 2015;142(5):817–31. [PubMed: 25715393]

2. Kronenberg HM. Developmental regulation of the growth plate. Nature. 2003;423(6937):332–6. [PubMed: 12748651]

3. Inada M, Yasui T, Nomura S, Miyake S, Deguchi K, Himeno M, et al. Maturational disturbance of chondrocytes in Cbfal-deficient mice. Dev Dyn. 1999;214(4):279–90. [PubMed: 10213384]

4. Arnold MA, Kim Y, Czubryt MP, Phan D, McAnally J, Qi X, et al. MEF2C transcription factor controls chondrocyte hypertrophy and bone development. Dev Cell. 2007;12(3):377–89. [PubMed: 17336904]

5. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell. 1997;89(5):755–64. [PubMed: 9182763]

6. Yoshida CA, Yamamoto H, Fujita T, Furuichi T, Ito K, Inoue KI, et al. Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. Genes Dev. 2004;18(8):952–63. [PubMed: 15107406]

7. Tan Z, Niu B, Tsang KY, Melhado IG, Ohba S, He X, et al. Synergistic co-regulation and competition by a SOX9-GLI-FOXA phasic transcriptional network coordinate chondrocyte differentiation transitions. PLoS Genetics. 2018(14):1–32.

8. Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, et al. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell. 1997;89(5):773–9. [PubMed: 9182765]

9. Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, et al. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell. 1994;79(6): 1111–20. [PubMed: 8001137]

10. Yang L, Tsang KY, Tang HC, Chan D, Cheah KSE. Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. PNAS. 2016 8 19;114(33): 13. [PubMed: 28031485]

11. Zhou X, von der Mark K, Henry S, Norton W, Adams H, de Crombrugghe B. Chondrocytes Transdifferentiate into Osteoblasts in Endochondral Bone during Development, Postnatal Growth and Fracture Healing in Mice. PLoS Genet. 2014;10(12).

12. Shwartz Y, Viukov S, Krief S, Zelzer E. Joint Development Involves a Continuous Influx of Gdf5-Positive Cells. Cell Rep. 2016;15(12):2577–87. [PubMed: 27292641]

13. Eyal S, Blitz E, Shwartz Y, Akiyama H, Schweitzer R, Zelzer E. On the development of the patella. Development. 2015;142(10):1831–9. [PubMed: 25926361]

14. Decker RS, Koyama E, Enomoto-Iwamoto M, Maye P, Rowe D, Zhu S, et al. Mouse limb skeletal growth and synovial joint development are coordinately enhanced by Kartogenin. Dev Biol. 2014;395(2):255–67. [PubMed: 25238962]

15. Chen D, Hilton MJ, Keefe RJO. Cartilage-specific β-CATENIN signaling regulates chondrocyte maturation, generation of ossification centers, and perichondral bone formation during skeletal development. J Bone Miner Res. 2012; 27(8): 1680–94. [PubMed: 22508079]

16. Norrie JL, Li Q, Co S, Huang B-L, Ding D, Uy JC, et al. PRMT5 is essential for the maintenance of chondrogenic progenitor cells in the limb bud. Development. 2016;143(24):4608–19. [PubMed: 27827819]

17. Logan M, Martin JF, Nagy A, Lobe C, Olson EN, Tabin CJ. Expression of Cre Recombinase in the Developing Mouse Limb Bud Driven by a Prxl Enhancer. 2002;80:77–80.

18. Li Q, Jiao J, Li H, Wan H, Zheng C, Cai J, et al. Histone arginine methylation by Prmt5 is required for lung branching morphogenesis through repression of BMP signaling. J Cell Sci. 2018;131(14):jcs217406. [PubMed: 29950483]

19. Dong Y, Song C, Wang Y, Lei Z, Xu F, Guan H, et al. Inhibition of PRMT5 suppresses osteoclast differentiation and partially protects against ovariectomy-induced bone loss through downregulation of CXCL10 and RSAD2. Cell Signal. 2017;34:55–65. [PubMed: 28302565]

20. Chen D, Zeng S, Huang M, Xu H, Liang L, Yang X. Role of protein arginine methyltransferase 5 in inflammation and migration of fibroblast-like synoviocytes in rheumatoid arthritis. J Cell Mol Med. 2017;21(4):781–90. [PubMed: 27860244]

21. Blanc RS, Richard S. Arginine Methylation: The Coming of Age. Mol Cell. 2017;65(1):8–24. [PubMed: 28061334]

22. Tee WW, Pardo M, Theunissen TW, Yu L, Choudhary JS, Hajkova P, et al. Prmt5 is essential for early mouse development and acts in the cytoplasm to maintain ES cell pluripotency. Genes Dev. 2010;24(24):2772–7. [PubMed: 21159818]
23. Ancelin K, Lange UC, Hajkova P, Schneider R, Bannister AJ, Kouzarides T, et al. Blimpl associates with Prmt5 and directs histone arginine methylation in mouse germ cells. Nat Cell Biol. 2006;8(6):623–30. [PubMed: 16699504]
24. Long F, Zhang XM, Karp S, Yang Y, Mcmahon AP. Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. 2001;128(9):5099–108.
25. Dy P, Wang W, Bhattaram P, Wang Q, Wang L, Ballock RT, et al. Sox9 Directs Hypertrophic Maturation and Blocks Osteoblast Differentiation of Growth Plate Chondrocytes. Dev Cell. 2012;22(3):597–609. [PubMed: 22421045]
26. Zhang M, Xuan S, Bouxsein ML, Von Stechow D, Akeno N, Faugere MC, et al. Osteoblast-specific knockout of the insulin-like growth factor (IGF) receptor gene reveals an essential role of IGF signaling in bone matrix mineralization. J Biol Chem. 2002;277(46):44005–12. [PubMed: 12215457]
27. Allen BL, Song JY, Izzi L, Althaus IW, Kang J-S, Charron F, et al. Overlapping roles and collective requirement for the co-receptors Gasl, Cdo and Boc in Shh pathway function. Dev Cell. 2011;20(6):775–87. [PubMed: 21664576]
28. Lewandowski JP, Du F, Zhang S, Powell MB, Falkenstein KN, Ji H, et al. Spatiotemporal regulation of GLI target genes in the mammalian limb bud. Dev Biol. 2015;406(1):92–103. [PubMed: 26238476]
29. Eyal S, Blitz E, Shwartz Y, Akiyama H, Schweitzer R, Zelzer E. On the development of the patella. Development. 2015;142(10):1831–9. [PubMed: 25926361]
30. Eyal S, Kult S, Rubin S, Krief S, Felsenthal N, Pineault KM, et al. Bone morphology is regulated modularly by global and regional genetic programs. 2019;1:1–15.
31. Pryce BA, Watson SS, Murchison ND, Staverosky JA, Dunker N, Schweitzer R. Recruitment and maintenance of tendon progenitors by TGF signaling are essential for tendon formation. Development. 2009;136(8):1351–61. [PubMed: 19304887]
32. Jiménez MJ, Balbín M, López JM, Alvarez J, Komori T, López-Otín C. Collagenase 3 is a target of Cbfal, a transcription factor of the runt gene family involved in bone formation. Mol Cell Biol. 1999;19(6):4431–42. [PubMed: 10330183]
33. Ionescu A, Kozhemyakina E, Nicolae C, Kaestner KH, Olsen BR, Lassar AB. FoxA Family Members Are Crucial Regulators of the Hypertrophic Chondrocyte Differentiation Program. Dev Cell. 2012;22(5):927–39. [PubMed: 22595668]
34. Kota SK, Roening C, Patel N, Kota SB, Baron R. PRMT5 inhibition promotes osteogenic differentiation of mesenchymal stromal cells and represses basal interferon stimulated gene expression. Bone. 2018;117(9):37–46. [PubMed: 30189247]
35. Hilton MJ, Tu X, Long F. Tamoxifen-inducible gene deletion reveals a distinct cell type associated with trabecular bone, and direct regulation of PTHrP expression and chondrocyte morphology by Ihh in growth region cartilage. Dev Biol. 2007;308(1):93–105. [PubMed: 17560974]
36. Li Q, Jiao J, Li H, Wan H, Zheng C, Cai J, et al. Histone arginine methylation by Prmt5 is required for lung branching morphogenesis through repression of BMP signaling. J Cell Sci. 2018;131(14):jcs217406. [PubMed: 29950483]
37. Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alphal(II) collagen gene. Mol Cell Biol. 1997;17(4):2336–46. [PubMed: 9121483]
38. Inada M, Wang Y, Byrne MH, Rahman MU, Miyaura C, Lopez-Otin C, et al. Critical roles for collagenase-3 (Mmpl3) in development of growth plate cartilage and in endochondral ossification. Proc Natl Acad Sci. 2004;101(49):17192–7. [PubMed: 15563592]
39. Rhee DK, Marcelino J, Baker M, Gong Y, Smits P, Lefebvre V, et al. The secreted glycoprotein lubricin protects cartilage surfaces and inhibits synovial cell overgrowth. J Clin Invest. 2005;115(3):622–31. [PubMed: 15719068]

40. Coles JM, Zhang L, Blum JJ, Warman ML, Jay GD, Guilak F, et al. Loss of cartilage structure, stiffness, and frictional properties in mice lacking PRG4. Arthritis Rheum. 2010;62(6):1666–74. [PubMed: 20191580]

41. Ruan M ZC, Erez A, Guse K, Dawson B, Bertin T, Chen Y, Jiang MM, Yustein J, Gannon F. Proteoglycan4 expression protects against the development of osteoarthritis. Sci Transl Med. 2013;5(176).

42. Decker RS, Um H Bin, Dyment NA, Cottingham N, Usami Y, Enomoto-Iwamoto M, et al. Cell origin, volume and arrangement are drivers of articular cartilage formation, morphogenesis and response to injury in mouse limbs. Dev Biol. 2017;426(1):56–68. [PubMed: 28438606]

43. Sun M, Hussain S, Hu Y, Yan J, Min Z, Lan X, et al. Maintenance of SOX9 stability and ECM homeostasis by selenium-sensitive PRMT5 in cartilage. Osteoarthr Cartil. 2019;2.

44. Ito T, Yadav N, Lee J, Furumatsu T, Yamashita S, Yoshida K, et al. Arginine methyltransferase CARM1/PRMT4 regulates endochondral ossification. BMC Dev Biol. 2009;9(1):1–10. [PubMed: 19126199]

Highlights

- PRMT5 promotes the survival of patella progenitor cells before specification.
- Loss of *Prmt5* results in an expanded domain of hypertrophic chondrocytes.
- Mutant hypertrophic chondrocytes undergo a blockage in terminal differentiation.
- PRMT5 maintains articular cartilage gene expression in adults.

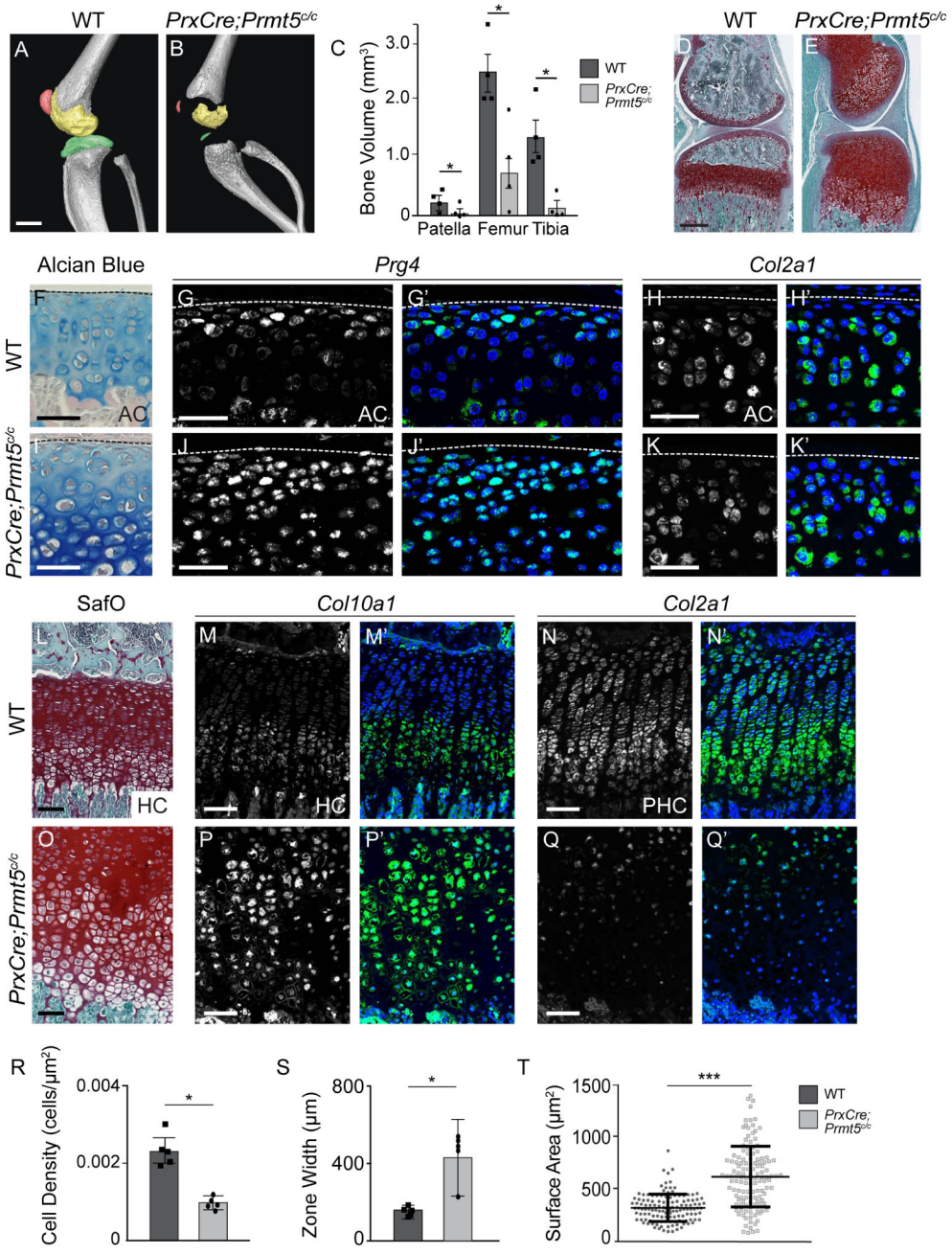

Figure 1. PRMT5 is required for normal knee and bone development.

A-C. Micro-CT analysis of the knee joint in wild type (WT) and *PrxCre;Prmt5*$^{c/c}$ mutants at P21, false colored to show the patella (red), the epiphysis of the tibia (green) and the epiphysis of the femur (yellow). Bone volume of each joint tissue was quantified in sibling control (n=4) and mutants (n=4). D-E. Histological analysis of knee joint cross-sections at P21 in WT and *PrxCre;Prmt5* $^{c/c}$ mice. Sections were stained with SafraninO and Fast green. F, I. Detailed histological analysis of articular cartilage in the tibia at P21. Sections were stained for Alcian Blue and eosin (3 control and 3 *PrxCre;Prmt5*$^{c/c}$). G,J. Section fluorescent *in situ* hybridization for *Prg4* expression (green), and nuclei visualized with DAPI (blue), in

the articular cartilage of 3 WT and 3 *PrxCre;Prmt5*$^{c/c}$ mice at P21. H,K. *Col2a1* expression (green) in the articular cartilage of the tibia in 3 WT and 3 *PrxCre;Prmt5*$^{c/c}$ mice at P21 (DAPI in blue). L,O. Histological analysis of growth plate cartilage layers in the tibia at P21. Sections from 5 control and 5 *PrxCre;Prmt5*$^{c/c}$ mice were stained with SafraninO and Fast Green. M,P. Section *in situ* hybridization for *Col10a1* expression (green) in the growth plate of 3 WT and 3 *PrxCre;Prmt5*$^{c/c}$ mice at P21 (DAPI in blue). N,Q. *Col2a1* expression in the growth plate of the tibia in 3 WT and 3 *PrxCre;Prmt5*$^{c/c}$ mice at P21 (DAPI in blue). R-T. Hypertrophic cell density (R), zone width (S) and surface area (T) measured across 5 control and 5 mutant mice. *p<0.05; **p<0.01; ***p<0.001, using an unpaired Student's t-test. AC-articular cartilage; HC-hypertrophic chondrocytes (Scale = 100μm).

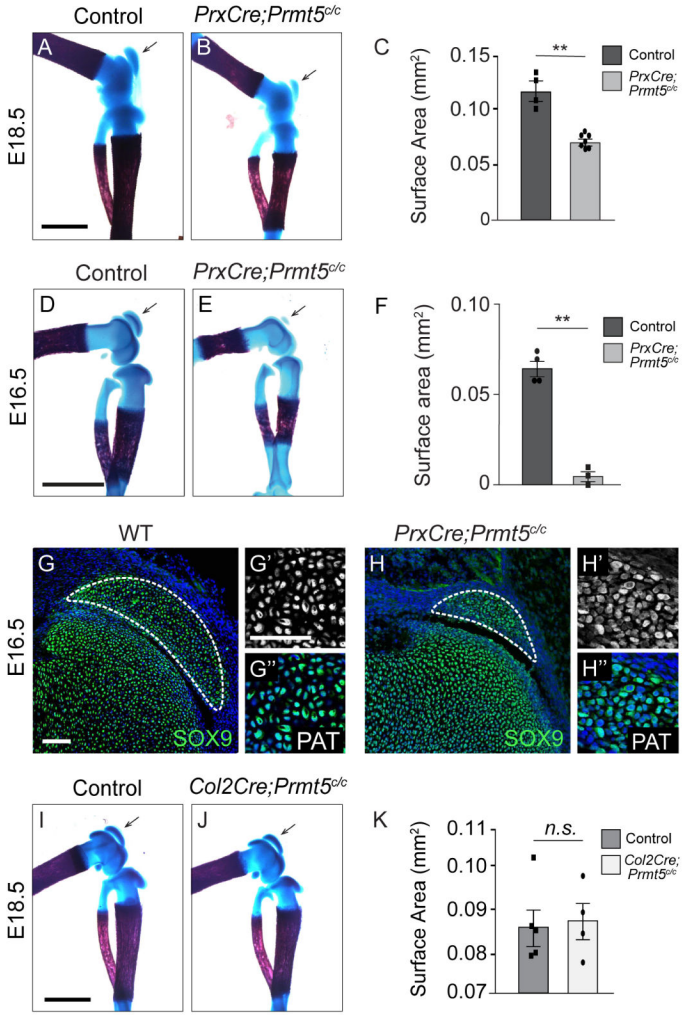

Figure 2. *PrxCre-CKO embryos* have a reduced patella.

A-C. Whole mount skeletal preparation of control and *PrxCre;Prmt5$^{c/c}$* knees at E18.5, stained for alcian blue and alizarin red. Patella surface area was quantified across 4 control and 7 mutant embryos. (Scale = 1mm) D-F. Whole mount skeletal preparations of E16.5 sibling control (n=4) and *PrxCre;Prmt5$^{c/c}$* (n=3) hindlimbs, stained for alcian blue and alizarin red. (Scale = 0.5mm) G-H. SOX9 expression (green; DAPI in blue) analysis in 3 control and 3 mutant knees at E16.5 by immunofluorescence. (Scale = 100μm) I-K. Whole mount skeletal preparation of control and *Col2Cre;Prmt5$^{c/c}$* knees at E18.5, stained for alcian blue and alizarin red. Patella surface area was quantified across 5 control and 4 mutant limbs. PAT-patella (Scale = 1mm).

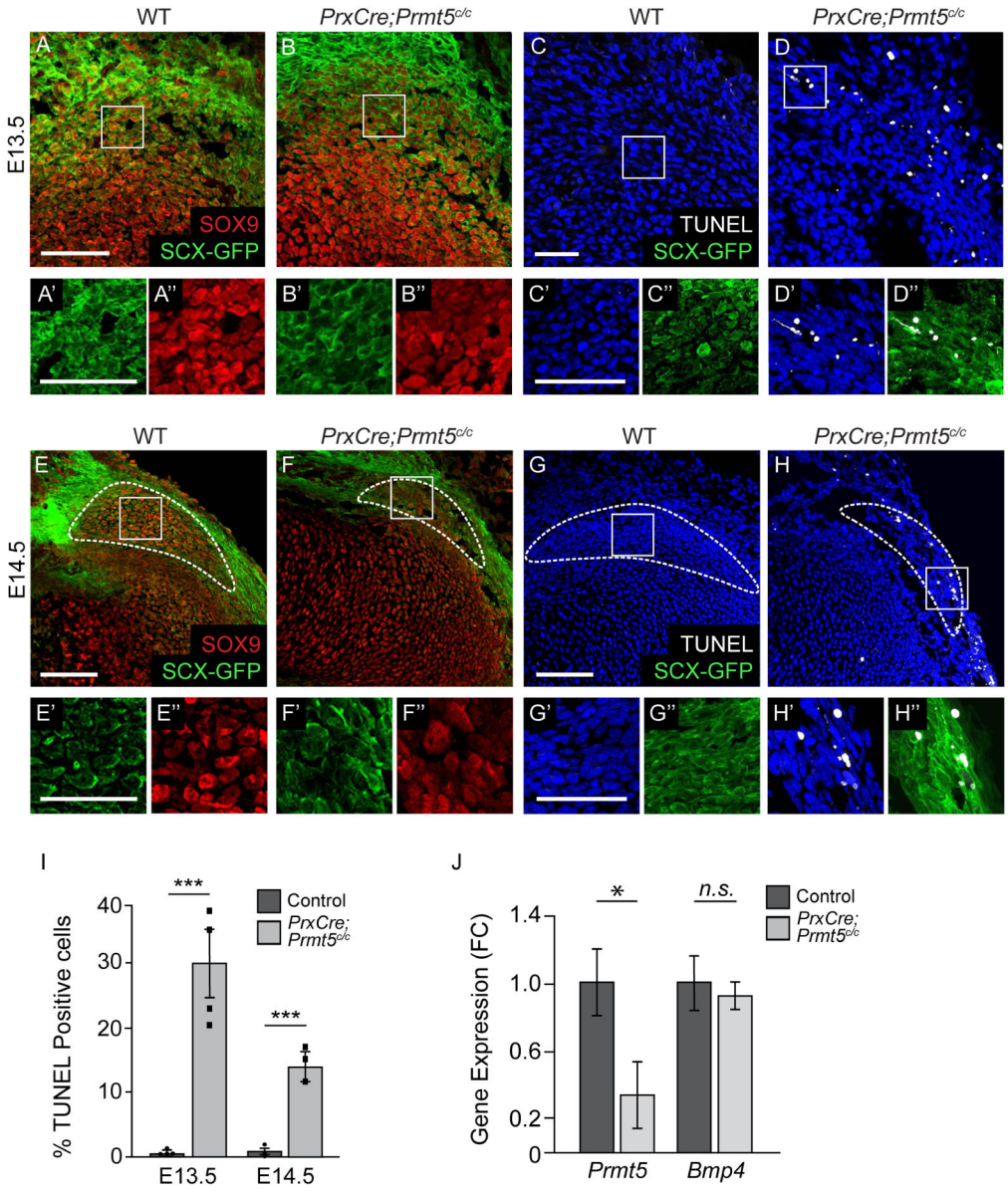

Figure 3. PRMT5 is required for the survival of early patella progenitors.

A-B. Early committed patella cells were visualized by SOX9 (red), SCX-GFP (green) expression at E13.5 in the knee joint of 4 control and 4 *PrxCre;Prmt5*$^{c/c}$ embryos. C-D. TUNEL staining (white) of apoptosing cells in E13.5 control and mutant knees. E-F. Visualization of differentiating patella progenitor cells in the E14.5 knee joint of 3 control and 3 *PrxCre;Prmt5*$^{c/c}$ embryos. G-H. TUNEL staining (white) of apoptosing cells in E14.5 knees. Nuclei stained with DAPI (blue). I. Percentage of TUNEL-positive cells in E13.5/ E14.5 was quantified across 4 control and 4 mutant knees at E13.5 and across 3 control and 3 mutant knees at E14.5. J. Gene expression of *Prmt5* and *Bmp4* were measured through quantitative-RT-PCR in 3 control and 3 mutant knee joints at E14.5. *p<0.05; **p<0.01; ***p<0.001 using an unpaired Student's t-test (Scale = 50μm).

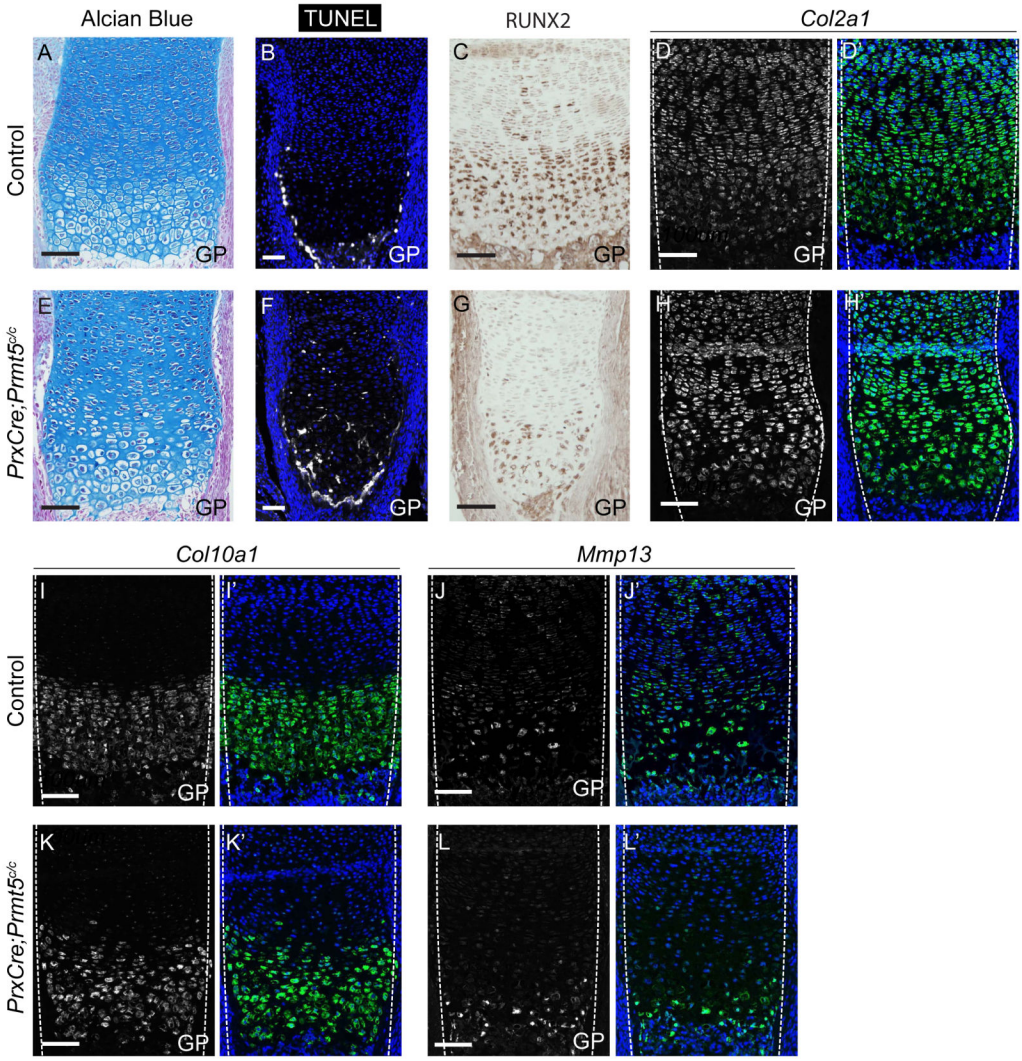

Figure 4. PRMT5 is partially required for the differentiation of embryonic cartilage.

A,E. Histological analysis of *PrxCre;Prmt5*$^{c/c}$ knees at E18.5 by alcian blue staining revealed subtle organizational defects in the growth plate cartilage layers of the long bones (n=5 controls; and 5 mutants). B,F Apoptosing cells in the tibia of 4 control and 4 *PrxCre;Prmt5*$^{c/c}$ embryos were visualized by TUNEL staining (white; DAPI in blue). C,G. Visualization of RUNX2 expression (brown) by immunohistochemical staining in the tibia of control and *PrxCre;Prmt5*$^{c/c}$ mice at E18.5. D,H. Visualization of *Col2a1* expression (green) in the tibia of control and *PrxCre;Prmt5*$^{c/c}$ mice at E18.5 by fluorescent *in situ* hybridization (DAPI in blue). I,K. Visualization of *Col10a1* expression (green) in the tibia of control and *PrxCre;Prmt5*$^{c/c}$ mice at E18.5 by fluorescent *in situ* hybridization (DAPI in blue). J,L. Visualization of *Mmp13* expression (green) in the tibia of control and mutant embryos by fluorescent *in situ* hybridization (DAPI in blue). D-L. Gene expression studies were performed on 3 control and 3 mutant knees. GP-growth plate (Scale = 50μm).

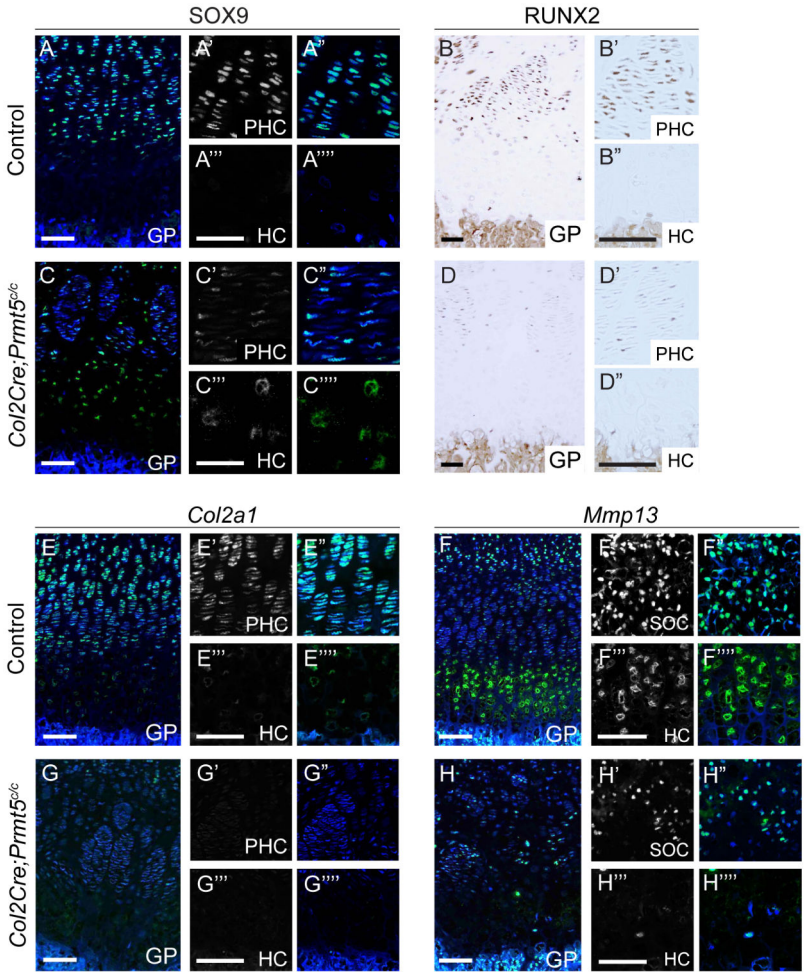

Figure 5. PRMT5 is required for later differentiation of cartilage cells.

A,C. SOX9 protein expression (green) by immunofluorescence in the tibia of control and *Col2Cre;Prmt5*$^{c/c}$ mice at P10 (DAPI in blue). B,D. Visualization of RUNX2 expression (brown) by immunohistochemical staining in the tibia of control and *Col2Cre;Prmt5*$^{c/c}$ mice at P10. E,G. Visualization of *Col2a1* expression (green) in the tibia of control and *Col2Cre;Prmt5*$^{c/c}$ mice at P10 by fluorescent *in situ* hybridization (DAPI in blue). F,H. Visualization of *Mmp13* expression (green) in the tibia of control and *Col2Cre;Prmt5*$^{c/c}$ mice at P10 (DAPI in blue). Differentiation marker studies were performed on 3 control and 3 mutant knees at E18.5. GP-growth plate; PHC-pre-hypertrophic chondrocytes; HC-hypertrophic chondrocytes (Scale = 100μm).

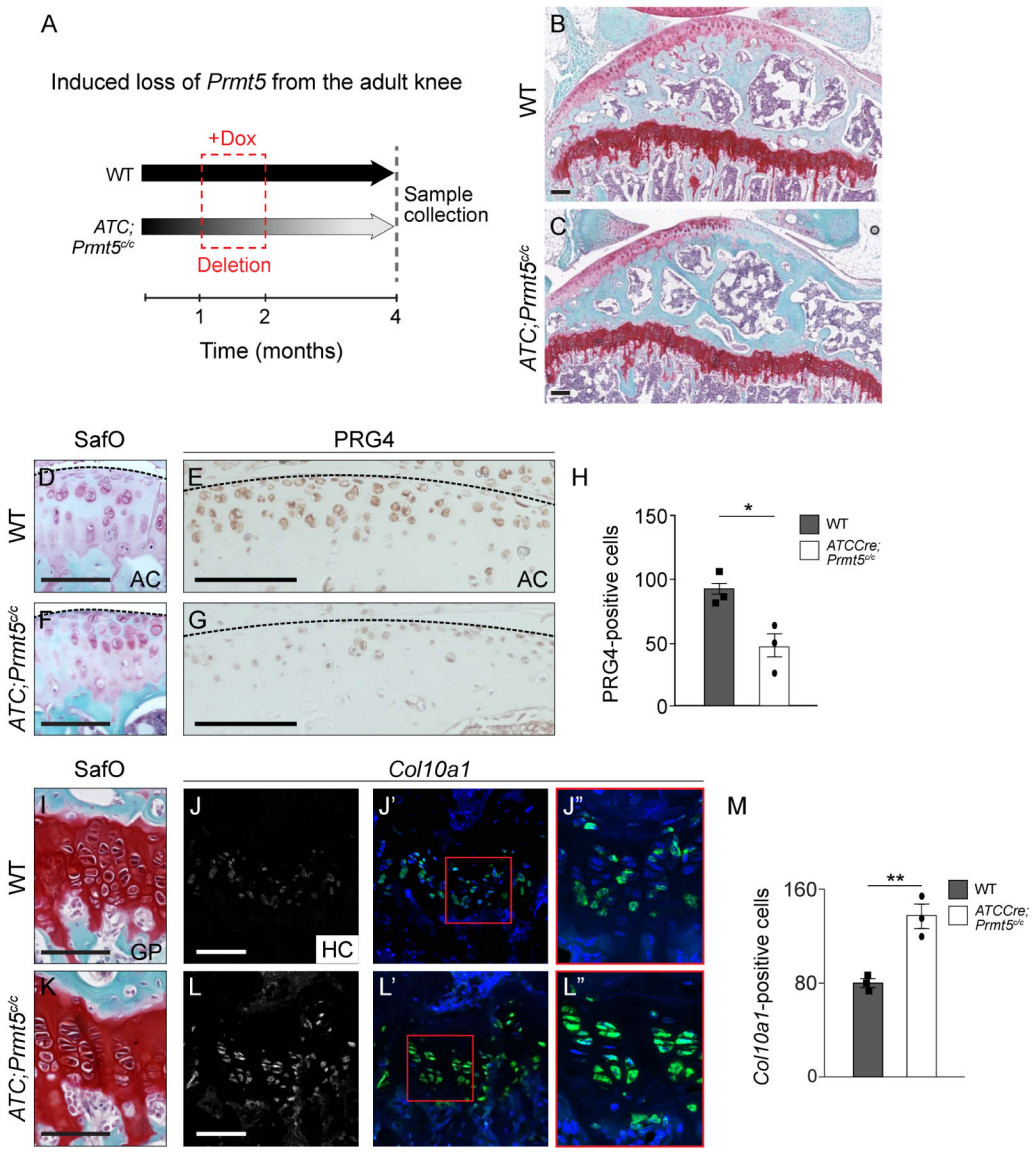

Figure 6. Conditional loss of *Prmt5* from the adult knee results in altered gene expression.

A. *ATC;Prmt5*$^{c/c}$ mice were induced with Doxycycline starting at 1month for a total of 1 month. Knees from control and *ATC;Prmt5*$^{c/c}$ mice were collected at 4 months for analysis. B,C. Histological analysis of control and *ATC;Prmt5*$^{c/c}$ mice at 4 months by Safranin O and fast green staining. D,F,I,K. The articular (D,F) and growth plate cartilage (I,K) of control and *ATC;Prmt5*$^{c/c}$ mice are indicated by Safranin O and fast green staining. E,G. PRG4 protein expression (brown) in the articular cartilage of control and *ATC;Prmt5*$^{c/c}$ mice at 4 months was visualized by immunohistochemistry and quantified across 3 controls and 3 mutants (H). J,L. *Col10a1* expression (green) in control and *ATC;Prmt5*$^{c/c}$ mice at 4 months was visualized by fluorescent *in situ* hybridization (DAPI in blue). M. The number of *Col10a1*-expressing cells was quantified across 3 controls and 3 mutants. All studies were performed on 3 control and 3 mutant mice. *p<0.05; **p<0.01 using an unpaired Student's

t-test. GP-growth plate; AC-articular cartilage; HC-hypertrophic chondrocytes (Scale = 100μm).