Gain-of-function Mouse Models to Investigate Biological Roles of PRMT6

Alessandra Di Lorenzo

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GAIN OF FUNCTION MOUSE MODELS TO INVESTIGATE BIOLOGICAL

ROLES OF PRMT6

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Dedications

I would like to dedicate this thesis to my two children, Nicholas and Lucas, who have made the graduate school adventure very interesting, giving me the motivation to keep going regardless the failures. I want them to take this path as an example not to give up when things will become difficult in their life. I love you both more than anything, my little bundles of joy!

I would also like to dedicate this to my father, who passed away for a horrendous cancer many years ago, when still very young. I know he would be very proud of what I have accomplished.
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Gain-of-function Mouse Models to Investigate Biological Roles of PRMT6

Alessandra Di Lorenzo, Ph.D. Candidate
Mentor: Dr. Mark T. Bedford

Protein Arginine Methyltransferase 6 (PRMT6) is the histone tail writer that methylates the H3R2 (arginine 2 of histone H3) residue, which counteracts the activating H3K4me3 mark. PRMT6 has been shown to behave both as transcriptional co-repressor (i.e. thrombospondin-1, p21, p53), and co-activator (nuclear receptors). The co-repressor function of PRMT6 is likely the result of H3K4me3 antagonism, while the mechanism by which PRMT6 exerts its co-activator function has yet to be elucidated. PRMT6 is over-expressed in several types of tumors including small cell lung cancer, lymphoma, cervical cancer, bladder cancer, oligodendroglioma, breast cancer, and osteosarcoma, suggesting an oncogenic role for this enzyme. Importantly, we have found a positive correlation between PRMT6 over-expression and H3R2me2a elevation in small cell-lung cancer tissue arrays.

The aim of this project was to study the consequence of PRMT6 over-expression and the effects of elevated histone arginine methylation at the organismal level in inducible PRMT6 gain-of-function mouse models. To this purpose, I generated two different systems. One mouse model is based on an ER\(^*\)-Flag-PRMT6 fusion (under a beta-actin promoter), while the other model is based on a Cre-inducible system, generated by cloning a floxed transcriptional Stop cassette upstream a Flag-PRMT6 (under a beta-actin promoter). Upon PRMT6
induction, both mouse models show elevated asymmetric dimethylation of H3R2 compared to wild type mice, demonstrating for the first time that PRMT6 methylates this residue \textit{in vivo}. The ER*-PRMT6 mice die upon stabilization of the chimera and serum analyses have revealed elevation of Interleukin 6 (IL-6) levels, a key interleukin regulated by the transcription factor NF-κB. This finding has led us to the discovery that PRMT6 functions as a co-activator for NF-κB. I have found that PRMT6 is in the NF-κB complex, and that its overexpression enhances NF-κB transcriptional activity in luciferase assays and quantitative Real-Time PCR experiments. The activity of PRMT6 is clearly necessary for the coactivator function. ChIP analysis demonstrated that PRMT6 was recruited to the IL-6 promoter upon TNF-alpha stimulation. Moreover overexpression of PRMT6 caused RelA shuttling into the nucleus in our system, which could justify, at least in part, the mechanism underlying the coactivator function. I have also found that PRMT6 can deposit the H4R3me2a activating mark \textit{in vivo}, which could explain the co-activator role of this arginine methyltransferase.

The Cre-inducible PRMT6 transgenic mice have been crossed with a mouse line expressing a Cre recombinase under a Keratin 5 (K5) promoter, as well as with a MMTV-Cre mouse line. I have conducted carcinogenesis studies with these mice. The K5-Cre crossed mice have been subjected to skin carcinogenesis study using the DMBA/TPA system and to a p53+/- genetic cross. The MMTV-cre crossed mice have been subjected to ageing studies. I have also performed RNA-seq experiments on primary mammary epithelial cells from the MMTV-Cre crossed mice.
In conclusion, this work identifies PRMT6 as a novel regulator of the inflammatory process, indeed we found that it cooperates with the transcription factor NF-κB in activating early response inflammatory genes such as IL-6 and TNF-alpha.
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<th>Description</th>
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<tbody>
<tr>
<td>CARM1</td>
<td>Coactivator Associated Arginine Methyltransferase 1</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immuno-Precipitation</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co- Immuno-Precipitation</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-Dimethylbenz(a)anthracene</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethyl Alcohol</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>hPRMT6</td>
<td>human Protein Arginine Methyltransferase 6</td>
</tr>
<tr>
<td>IF</td>
<td>Immuno-Fluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immuno-Histo-Chemistry</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IP</td>
<td>Immuno-Precipitation</td>
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<tr>
<td>K5</td>
<td>Keratin 5</td>
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<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblasts</td>
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<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>OHT</td>
<td>(Z)-4-Hydroxytamoxifen</td>
</tr>
<tr>
<td>PRMT6</td>
<td>Protein Arginine Methyltransferase 6</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>SCT</td>
<td>Spindle Cell Carcinoma</td>
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<tr>
<td>TAD</td>
<td>Transactivation Domain</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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</tr>
<tr>
<td>Tamox</td>
<td>Tamoxifen</td>
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<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TPA</td>
<td>(12-O-Tetradecanoylphorbol-13-Acetate)</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blotting</td>
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Chapter 1

Introduction

1.1 Arginine methylation

Arginine methylation is an important post-translational modification catalyzed by the protein arginine N-methyltransferase (PRMT) family of enzymes, composed by nine members. Arginine methylation is involved in many cellular processes, like transcription, RNA metabolism, and DNA damage repair (1). Arginine residues can be methylated in three different modes on the guanidino group: monomethylated (MMA), symmetrically dimethylated (sDMA) and asymmetrically dimethylated (aDMA), and each of these methylated states can result in different biological outcomes (Figure 1). There are nine PRMTs – type I and type II enzymes (PRMT1, 2, 3, 4, 6 and 8) catalyze the formation of a MMA, type I PRMTs further catalyze aDMA formation, while type II PRMTs (PRMT5) catalyze the formation of sDMA. PRMT7 is a type III enzyme that predominantly catalyzes MMA, and the PRMT9 has yet to be characterized (Figure 2). Arginine methylation constitutes a signal to recall readers of the histone code. This recognition event is fundamental in order for specific biological outcomes to happen. For example, methylation of SmB by PRMT5 is required for an interaction with the Tudor domains of SMN and TDRD3 (2), methylation of CA150 by CARM1 also provides a docking site for SMN (3), and H3R17 methylation by CARM1 promotes TDRD3 binding (4). Thus, motifs harboring either aDMA or sDMA residues bind a subset of Tudor domain-containing proteins. An aromatic “cage” in the Tudor domain forms the methyl-binding pocket (5). Arginine methylation can also block protein-protein interactions. For example, the
methylation of arginine residues in the vicinity of proline-rich motif can block the binding to Src homology 3 (SH3) domains (6). Another example of arginine methylation blocking a protein-protein interaction is the CARM1-modified-GRIP1 binding domain of p300 (5). Finally, the methylation of histone H3R2 (by PRMT6) antagonizes the binding of effector proteins to the N-terminal tail of H3, thus serving as a repressive mark that counteracts the activa H3K4me3 mark (7-9).
Figure 1. Types of Methylation on Arginine Residues.
*From Bedford and Clarke, 2009 (1).*
Copyright License number 3324800889013 (Molecular Cell).
Figure 2. The Protein Arginine Methyltransferase Family. The mammalian PRMT family currently contains nine highly related members; all harbor signature motifs I, post-I, II, and III and the conserved THW loop (in black bars). Protein domains that might assist in substrate recognition are marked in gray boxes.

From Bedford and Clarke, 2009 (1).

Copyright License number 3324800889013 (Molecular Cell).
1.2 PRMT6

PRMT6 is an enzyme with auto-methylation capabilities that localized strongly in the cell nucleus (10). Substrates for this PRMT include the nuclear scaffold protein HMGA1a/b (11,12), DNA polymerase beta, and the HIV Tat protein (13,14). PRMT6 has also shown to impact the histone code, indeed it is the primary enzyme responsible for H3R2 methylation in mammalian cells (7-9). PRMT6 can strongly methylate H3K4me1 and H3K4me2 peptides, and weakly methylate a H3K4me3 peptide (7). However, prior methylation of the H3R2 site prevents the MLL1 complex from methylating H3K4 (9). H3K4 methylation is found in promoters of active genes and recalls chromatin-remodeling enzymes that open chromatin for transcription. The effector molecules that recognize H3K4me3 possess methyl-specific binding domains, like PHD, Chromo and Tudor domains. H3R2 methylation blocks the capability of many specific domains to bind to H3K4me3 in in vitro assays (7). Therefore PRMT6, by generating the H3R2me2a mark, functions as a transcriptional repressor because this mark impedes recruitment of remodelers to the H3K4me3 mark (Figure 3). In a massive qChIP study to identify the histone marks that are present on chromatin prior to Myc binding, it was found that H3R2me2a (the PRMT6 mark) is associated with the repressive H3K27me3 mark (deposited by EZH2) and low-affinity Myc binding site (15). Related studies by the same group found a negative correlation between H3R2me2a and H3K4me3, and a negative correlation between H3R2me2a promoter methylation and transcription (8). This finding supports the proposed role of PRMT6 in transcriptional repression and implicated this PRMT in the regulation of Myc recruitment and binding to chromatin. In yeast, the H3R2me2a mark has also been associated with transcriptional
repression, although the yeast enzyme that deposits this mark has not yet been identified (16,17). The tumor suppressors p21, p27 and p53 have been recently reported to be directly repressed by PRMT6 through deposition of the repressive H3R2me2a mark in their promoters (18-21). PRMT6 also deposits the H2AR29me2a mark, which is enriched at the promoters of genes that are transcriptionally repressed by PRMT6 (22).

The assumption that PRMT6 is a transcriptional repressor has recently been challenged by finding it functioning as SHR coactivator: Its mode of action is similar to that of PRMT1 and CARM1, indeed it functions as a secondary coactivator to p160/SRC proteins. PRMT6 binds to SRC-1, and acts synergistically with SRC-1 to coactivate ERα (23), but the mechanism for this coactivator function is yet to be elucidated. PRMT6 also methylates the N-terminal tails of histones H4 and H2A (their first 5 residues are the same) at R3 in in vitro reactions (9); however the methylation of these sites is yet to be shown in vivo. The H4R3me2a is known to be associated with actively transcribed gene promoters, and it is deposited primarily by PRMT1 in vivo (24). In the coactivation context, methylation of H4R3 and/or other active sites may be important. Indeed, PRMT6 has recently been reported to methylate H3R42 and that this non-tail histone methylation event positively affects transcription, likely by disrupting the histone-DNA interaction and making the nucleosome slidable (25).
Figure 3. The site of PRMT6-mediated methylation on the H3 histone tail is noted. The well-characterized CARM1 methylation sites, which are associated with transcriptional activation, are also indicated. H3R2 methylation structurally impedes the binding of H3K4me3 recognition domains and also prevents H3K4 methylation by the MLL1 complex. H3R2 methylation antagonizes the docking of a number of H3K4me3 effector proteins. PRMT6 thus functions as a general transcriptional repressor by blocking the activator functions H3K4me3. From Bedford and Clarke, 2009 (1). Copyright License number 3324800889013 (Molecular Cell).
1.3 PRMTs and Nuclear Factor Kappa B (NF-κB)

Nuclear factor Kappa B (NF-κB) is an inducible transcriptional factor that plays a pivotal role in immune system regulation, cell survival, differentiation and proliferation (26). Dis-regulation of NF-κB has been associated with numerous diseases, including malignancies in which it is intrinsically activated, resulting in tumor survival by enhancing transcription of proliferative and anti-apoptotic genes (27). Five NF-κB family members in mammals have been described: RelA/p65, RelB, c-Rel, p50 (NF-κB 1), and p52 (NF-κB 2). The N-termini of NF-κB factors harbor a conserved region of 300 amino-acids, called Rel homology domain (RHD). Of the five members described, only p65, c-Rel, and RelB have C-terminal transactivation domains (TADs), which function to activate transcription (28). NF-κB proteins bind either as homodimers or heterodimers to the target DNA - RelA(p65)/p50 dimer is the most abundant and well studied NF-κB form. NF-κB dimers are retained in the cytoplasm in normal conditions by association with the inhibitory IκB proteins. Extracellular stimuli such as cytokines (i.e. tumor necrosis factor alpha (TNF-alpha)), bacterial lipopolysaccharide (LPS), and others activate phosphorylation cascades that result in phosphorylation, ubiquitination, and proteasomal degradation of IκB proteins, with consequent translocation of NF-κB dimer into the nucleus and transcription of target genes (29,30). Intracellular responses to DNA damage and reactive oxygen species can also activate NF-κB (26). NF-κB-dependent gene transcription requires multiple co-activators that function by bridging it to the basal transcription machinery and altering chromatin structure (31). The co-activators histone acetyl-tansferases p300 and its homolog,
the CREB-1-Binding Protein (CBP), are known to impact NF-κB-dependent gene expression and have been shown to interact directly with RelA. Moreover NF-κB-dependent gene expression involves another class of transcriptional coactivators, such as steroid receptor-coactivator-1 (SRC-1), which interacts with the p50 subunit of NF-κB to potentiate transactivation (32). In Figure 4 is shown the complex (Enhanceosome) that forms at IL-6 promoter one of the NF-κB target genes (33). Members of the protein arginine methyltransferase family (PRMTs), namely PRMT1 and PRMT4/CARM1, well known as steroid hormone receptor (SHR) coactivators (34,35), have also been shown to cooperate in NF-κB-dependent gene activation. CARM1 has been shown to function as promoter-specific coactivator for NF-κB in concert with p300/CBP and SRC-2/TIF2/GRIP1 through deposition of the H3R17me2a active mark at NF-κB-regulated promoters (36,37). PRMT1 has been reported to coactivate NF-κB-dependent gene expression synergistically with CARM1 and PC1/poly(ADP-ribose) polymerase 1 (PARP1) (38).
Figure 4. Overview of the phosphorylation pathways leading to activation of the IL-6 enhanceosome. The expression of IL-6 needs to be very strictly regulated and the IL-6 gene promoter contains a plethora of sequence elements, which can be triggered via different activation schemes. Induction of IL-6 by the inflammatory cytokine TNF is exclusively mediated by the stress-responsive transcription factor NF-kB. However, other DNA-bound transcription factors also contribute to the general level of expression and help to establish a multi-protein complex, a so-called ‘enhanceosome’, at the promoter DNA. Herein, NF-kB serves as the ultimate trigger to turn this enhanceosome into a fully transcriptionally competent complex, which is capable of gene induction. (Ac, acetyl; AP-1, activator protein-1; BTM, basal transcription machinery; C/EBP, CCAAT enhancer-binding protein; CREB, cAMP-responsive element binding protein; p/CAF, p300/CBP-associated factor; Pol, polymerase; SRC, steroid receptor activator. Source: (33). Copyright License number 3324800111184 (the EMBO Journal).
1.4 PRMTs, PRMT6 and Cancer

PRMTs have been shown to impact numerous cellular processes, including proliferation, transformation and the anti-apoptotic pathway, therefore they may play an important role in tumorigenesis (39). PRMT1 mRNA levels have been reported to be higher in breast cancer cell lines than in controls (40). In addition, PRMT1 plays a role in the transforming abilities of the MLL-EEN gene fusion product (41). CARM1 levels are elevated in castration-resistant prostate cancer, as well as in aggressive breast tumors (42,43). Importantly, CARM1 methylates AIB1, thereby regulating its activity and stability (44,45). CARM1 is recruited to ERα regulated promoters by AIB1, and it is essential for estrogen-induced proliferation of the MCF-7 breast cancer cell line (46). Moreover, while this thesis was being written, a publication showed that CARM1 methylates the chromatin remodeling factor BAF155 to enhance tumor progression and metastasis (47). PRMT5 is recruited by SNAIL to actively repress E-cadherin expression (48). A hallmark of epithelial-mesenchymal transition (EMT) is the loss of E-cadherin expression, which plays an important role in tumor progression. PRMT5 overexpression promotes anchorage-independent cell growth (49), supporting the notion that PRMT5 might be an oncoprotein. Consistent with this idea, PRMT5 levels are elevated in gastric cancer (50) and in leukemia cells (51). Microarray analysis of PRMT6 knockdown cells revealed that thrombospondin-1 (TSP-1) is transcriptionally repressed by this PRMT (52). TSP-1 is a potent natural inhibitor of angiogenesis, and reduction of its levels have been shown to promote tumor progression. The HOXA10 gene is strongly regulated (repressed) by PRMT6 activity (9). HOXA10 is a positive regulator of the p53 tumor
suppressor gene (53). Thus, increased PRMT6 levels will result in decreased HOXA10 levels and consequently attenuated p53 function. Moreover, as already mentioned, the tumor suppressors p21, p27 and p53 have been recently reported to be directly repressed by PRMT6 through deposition of the repressive H3R2me2a mark in their promoters (18-21).

As already mentioned, PRMT6 generates the H3R2me2a mark, and the majority of H3K4me3 effector molecules are sensitive to H3R2me2a methylation (7). A key group of these H3K4me3 readers that are blocked from binding are the ING family of PHD domain-containing proteins. ING proteins are down-regulated in a broad variety of cancer types and are regarded as tumor suppressors (54). Elevated PRMT6 levels result in increased H3R2me2a methylation, which in turn will effectively incapacitate ING protein effector functions. A search of the Oncomine transcriptome database (www.oncomine.org) confirmed that indeed PRMT6 is over-expressed in a number of tumors, including SCL, cervical cancer (Figure 5A), as well as acute lymphoblastic leukemia and others (data not shown). In addition, PRMT6 expression levels correlate with poor prognosis in oligodendroglioma patients (Figure 5B). To confirm this observation at the protein level, we obtained small cell lung carcinoma tissue microarrays from Folio Biosciences, which had 33 carcinomas and 5 normal samples arrayed. These arrays were probed for both PRMT6 and for the mark that it deposits, H3R2me2a (Figure 5C). In the normal lung tissue, PRMT6 levels were mostly below the level of detection (although there was strong non-specific cytoplasmic staining in a subpopulation of cells, apparently of secretory material). All tumors had nuclear PRMT6 staining not seen in normal bronchial epithelium. The average % positive nuclei was 12.6% (range, 2.8-36.3%)
for PRMT6. In control bronchial epithelium, 5.9% cells had nuclear staining for H3R2me2a. Ninety-four percent (31/33) of tumors analyzed had higher proportions of H3R2me2a-positive nuclei than control tissue. On average, the percentage of positive nuclei in tumor cells (mean, 35.5%; range, 0.6-82.6%) was six-fold the percentage in control tissue and, overall, the H3R2me2a staining intensity was greater in tumor nuclei compared to non-neoplastic cell nuclei. Nuclear staining for H3R2me2a and PRMT6 was evaluated on slides scanned by an Aperio ScanScope using ImageMate software. An average of 14,000 tumor cells per sample were evaluated. Control tissue was bronchial epithelium from adjacent normal tissue, where small cell carcinomas of the lung are believed to arise. Altogether, 2,600 control cells were evaluated.

The H3R2me2a mark is deposited by PRMT6. Thus, tumor samples that overexpress PRMT6 should also display elevated levels of H3R2me2a. To establish if there is such a positive correlation between PRMT6 and H3R2me2a levels, paired analyses were obtained for 33 tumor samples and results were expressed as percentage immunopositive nuclei. Linear regression analysis was performed to determine if there was a relationship between PRMT6 and H3R2me2a staining (Figure 5D). A least squares rho value of 0.806 was obtained, with an F-test value of 9.2994. This indicates that PRMT6 values can explain approximately 80% of the variance in H3R2me2a values. An F value of greater than 4 is considered statistically significant. Thus, there is a positive correlation between PRMT6 and H3R2me2a staining.

A detailed analysis of PRMT6 (and PRMT1) deregulated expression in different human cancers was performed. This study reported that PRMT6 is overexpressed.
in a number of human cancers, including bladder, small cell lung carcinomas, lymphomas, breast cancer, and cervical cancer (55) (Figure 5E). Recently, PRMT6 has also been found to be dramatically elevated in prostate carcinomas (56).

Thus, three separate lines of evidence support the fact that PRMT6 levels are significantly elevated in tumors: 1) Oncomine data, 2) tissue microarray analysis performed by us, and 3) a thorough analysis of PRMT6 levels in a large number of different tumors performed by the group of Hamamoto and a recent paper from the group of Jeronimo (55,56). Importantly, although there are numerous in vitro studies linking aberrant PRMT function to cancer, no mouse models have been developed to address this issue.
Figure 5. PRMT6 levels are elevated in tumors. (A) Analysis of the Oncomine database reveals that PRMT6 is significantly overexpressed in SCLC and cervical cancer. (B) PRMT6 expression levels correlate with poor prognosis in oligodendrogloma patients. (C) Tissue microarray analysis of PRMT6 and H3R2me2a in small cell lung carcinomas. Top two panels represent adjacent normal tissue. The other six panels represent three of the 33 carcinoma cores, stained for both PRMT6 and H3R2me2a. Pictures were taken by Donna Kusewitt. (D) Linear regression analysis was performed on the levels of PRMT6 and H3R2me2a in each sample; their expression levels strongly correlate. E, PRMT6 levels are elevated in a number of different tumors. The data is presented as a signal intensity ratio (Tumor/Normal). The data in (E) was published data adapted from Yoshimatsu et al. 2011 (55).
1.5 DMBA/TPA skin carcinogenesis

The two stage skin carcinogenesis system that employs the tumor initiator agent DMBA (7,12-dimethylbenz[a]anthracene) and the tumor promoter TPA (O-tetradecanoylphorbol-13-acetate) has been largely employed to address a number of questions about the fundamental biology of epithelial cancers (57). In this protocol, by administering a single dose of DMBA followed by repeated applications of TPA, mice develop initially primarily papillomas, which progress to Squamous Cell Carcinomas (SCCs) that are histologically very similar to human SCCs. In accordance with the cancer stem cells hypothesis for cancer development, the mechanism for tumor initiation in DMBA/TPA two-stage carcinogenesis protocols involves mutation in the critical target Ha-ras gene of stem cells. These cells are found in the bulge region of hair follicles or basal compartment of interfollicular epidermis in mice (57). The gene targets for initiation by chemicals in mouse skin resemble those found in other human epithelial cancers (e.g., lung, colon, and pancreatic cancers) (58). The tumor promoter TPA will affect gene expression, and stimulate epidermal cell proliferation. The progression of papillomas to SCC does not depend on the tumor promoter agent TPA, it rather occur stochastically following numerous gene expression changes (59,60).

1.6 Innovation of this study

The innovation of this project lies in the novel and sophisticated mouse models, which allow for the conditional and inducible modulation of PRMT6 activity to study the role of this PRMT in biological processes, such as cancer. There is evidence gleaned from in vitro experiments that PRMT6 functions as both a transcriptional
repressor and activator. In this study I sought to determine the biological relevance of these findings in an \textit{in vivo} setting by investigating the transcriptional repressor/activator functions of PRMT6 in gain-of-function mouse models. This study is highly relevant due to the recent observations that PRMT6 is up-regulated in a number of tumors, including SCLC, lymphomas and cervical cancer (Figure 5). I have exploited novel mouse models to understand the role of PRMT6 in cancer development using state-of-the-art approaches.

Epimutations, unlike genetic mutations, can be reversed by chemotherapeutic intervention, which makes epigenetic therapy conceptually extremely appealing. Drug therapies that target chromatin have been touted as the next emerging frontier for the treatment of cancer. Epigenetic drugs are currently being used in the clinic. Clearly, dramatic changes in epigenetic patterns and gene transcription are major signatures of cancer and PRMT6 may play a significant role in this reprogramming. The reversal of these aberrant patterns is emerging as a therapeutic strategy for the treatment of cancer.
Chapter 2
Rationale, Hypothesis and Specific aims

Rationale

PRMT6 is an arginine methyltransferase that was first identified and described by our group. PRMT6 has been reported to be the predominant enzyme to methylate the H3R2 (arginine 2 of histone H3) residue. The proximity of this arginine residue to the H3K4me3 activation mark raises the possibility of crosstalk between these two methylation events. PRMT6 likely functions as a transcriptional repressor in two ways: 1) by blocking the recruitment of transcriptional activators to the methylated H3K4 mark and 2) by preventing H3K4 methylation by MLL1. On the other hand, PRMT6 has recently been described as co-activator for nuclear receptors by the Dowhan’s group, although the mechanism for this coactivation was not described in the manuscript. A likely mechanism by which PRMT6 will function as a coactivator has recently come to light with the finding that it methylates a site in the core of histone H3, H3R42me2a and that this non-tail histone methylation event positively affects transcription, possibly by disrupting the histone-DNA interaction and making the nucleosome “slidable”. Moreover, PRMT6 has been shown to generate the active H4R3me2a mark in in vitro reactions, however this methylation event is yet to be shown in vivo. The ability of transcription factors and transcriptional regulators to function as both activators and repressors in a context dependent manner is not novel, and well-characterized examples include p53, myc, E2Fs, LSD1 and TRIM24.

Importantly, PRMT6 is over-expressed in several types of tumors including small cell lung cancer, lymphoma, cervical cancer, bladder cancer,
oligodendroglioma, breast cancer, and osteosarcoma, suggesting an oncogenic role for this enzyme. Notably, we have found a positive correlation between PRMT6 over-expression and H3R2me2a elevation in small cell-lung cancer tissue arrays. We would like to understand the consequence of altered PRMT6 expression at the organismal level.

Hypothesis

_We hypothesized that PRMT6 is a major transcriptional regulator may contribute to cancer susceptibility. To explore the potential roles of PRMT6 in cancer development, we generated two PRMT6 gain-of-function mouse models._

Specific Aim 1 - Generation and characterization of two types of PRMT6 gain-of-function mouse models.

Transgenic mouse lines over-expressing an ER*-Flag-PRMT6 chimera were generated. In order to produce these mice an _ER*-Flag-PRMT6_ sequence was cloned in the pCAGGS vector and the construct was employed for pronuclear injection, generating _ER*-PRMT6 lines. As an alternative approach, we generated a transgenic mouse model based on a Cre-inducible system. For this second model, a transcriptional stop cassette was cloned upstream a Flag-PRMT6 in a pCAGGS vector and the construct was employed to produce transgenic mouse lines. These lines (called STOP-PRMT6) were crossed with mice expressing a Cre recombinase under a Keratin 5 promoter (K5-Cre mice) as well as with mice expressing a Cre recombinase under a MMTV promoter (MMTV-Cre mice).
Specific Aim 2 - To determine the impact of PRMT6 over-expression on skin carcinogenesis.

A chemical skin carcinogenesis experiment was set-up with STOP-PRMT6 mice (crossed to the K5-Cre mouse line) employing the tumor initiator agent DMBA and the promoter TPA. A number of endpoints were compared between the control and experimental group, including tumor incidence, latency, multiplicity, and malignancy.

Specific Aim 3 - To determine if increased PRMT6 activity cooperates with p53 deficiency in tumor development.

Mice heterozygous for p53 are prone to develop tumors by loss of the intact p53 allele. The STOP-PRMT6/K5-Cre mice were crossed onto a p53+/− background to determine if increased PRMT6 activity cooperates with tumor development through epigenetic silencing of the p53 WT allele.

Specific Aim 4 - To determine the impact of PRMT6 overexpression on the mammary gland.

In order to investigate the impact of PRMT6 over-expression on the mammary gland we crossed the STOP-PRMT6 mice with a MMTV-Cre mouse line and characterized them. The bigenic mice STOP-PRMT6/MMTV-Cre showed increased proliferation in the mammary gland epithelium, therefore we set-up ageing studies in order to explore the possibility of increased mammary tumorigenicity. We also performed a RNA-Seq experiment using primary mammary epithelial cells (organoids) isolated from the bigenic STOP-PRMT6/MMTV-Cre mice, along with 2 control groups.
(STOP-PRMT6 and MMTV-Cre), in order to investigate the impact of PRMT6 overexpression on the mammary transcriptome.
Chapter 3

Materials and Methods

Antibodies, cells and reagents

HEK 293 and HeLa cells were purchased from ATCC and cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing pen strep, non-essential amino-acids and FBS 10%. Tamoxifen (cat. # T5648), OHT (cat. # H7904) and TNF-alpha (cat. # T6674) were purchased from Sigma. Anti-RelA antibody for WB and IF was from Abcam (cat. # ab16502). Anti-hPRMT6 antibody was from Imgenex (cat. # IMG-506). Anti-PRMT6 atibody (for mouse and human) was from Bethyl Laboratories (cat. # A300-929A). Anti-Flag antibody (cat. # F3165) and anti-Flag agarose (cat. # A2220) were obtained from Sigma. Anti-H3R2me2a antibody was from Millipore (cat. # 05-808). Anti-GFP antibody was from Life Technologies (cat. # A6455). Anti-Lamin A/C antibody was from Santa Cruz (cat. # sc-20681). Anti- H4R3me2a was from Active Motif (cat. # 39705). Protein A/G Ultra Link resin was from Thermo Scientific (cat. # 53132). DMBA was purchase from Sigma, TPA was from LC Laboratories (cat # P-1680).

Transgene construction and mouse generation

Estrogen receptor hormone-binding domain variant (ER*) that bind Tamox, but not estrogen, was a gift from Martin McMahon (61). ER* was generated by PCR (from the pBP3hbER* vector) and cloned into a pCAGGS construct containing flagged human PRMT6 cDNA. For the Cre-inducible construct, a Stop cassette was
amplified by PCR from the RAGE vector, previously described (62) and cloned into a pCAGGS construct containing flagged human PRMT6 cDNA. In both cases, an aliquot of the construct produced by Midi Prep using Qiagen kit (Qiagen Scientifics, Maryland) was linearized overnight using Scal, then column purified using PCR purification kit from Qiagen (Qiagen Scientifics, Maryland). This transgene was injected into the male pronucleus of day 1–fertilized (FVB) embryos. Injected embryos were transferred into day 1–plugged pseudo-pregnant foster mice, and litters were screened for the presence of the transgene using Southern analysis to identify heterozygous founders. Founder mice were backcrossed to establish lines of animals.

**Southern genotyping**

Approximately 1000 bp of PRMT6 cDNA sequence was cloned out from a pCAGGS-Flag-PRMT6 construct to use as a template for probe generation by PCR using the primers Forward 5’-ATGGACTACAAGGACGACGATGACAA-3’ and Reverse 5’-GATAGGCAGCTGCACCTGAGGAGTG-3’. The radiolabeled hybridization probe was prepared with 32P-dCTP using the Random Primer Labeling kit from Agilent Technologies (cat. # 300385), following manufacturer’s instructions. Genomic DNA was isolated from the tails of 3-week old mice and digested EcoRI enzyme. Digested DNA was run on 1% agarose gel overnight and transferred to a nitrocellulose membrane. The membrane was then cross-linked and incubated in 10 mL Southern hybridization buffer containing SDS, the radioactive probe and salmon sperm DNA overnight at 42° C, then washed with SSC buffer (3.0 mol /L NaCl, 0.3 mol /L sodium citrate) and exposed to film for 1–3 days at −80° C.
Administration of tamoxifen to mice

To activate ER*-PRMT6 in adult transgenic mice by Tamox topical administration, Tamox was dissolved in ethanol (1 mg/0.2 mL) and applied topically to a shaved area of dorsal skin. As control, WT littermates treated with Tamox or transgenic littermates treated with ethanol, were included in these studies. For intraperitoneal injections, Tamox (1mg/0.1 mL) was dissolved in sunflower oil.

Stable cell line generation

HEK 293 cells were transfected with the pCAGGS-ER*-PRMT6 construct (10 μg) along with an empty plasmid carrying neomycin resistance (1μg). Selection of stable transfected cells was performed using neomycin (G418).

Immuno-staining of mouse tissue and immuno-fluorescence HeLa cells

Tissue samples were obtained from WT or transgenic mice, Tamox or vehicle treated. Tissues were fixed overnight in neutral buffered formalin (24 hrs), transferred to 70% ethanol, then embedded in paraffin and sectioned (5 μm). Slides were de-paraffinized with standard procedure. For immunohistochemistry (IHC), non-specific antibody binding was blocked by incubating slides with Biocare Blocking Reagent (cat# BS966M) for 10 minutes. Slides were drained and incubated with primary anti-Flag antibody for 30 minutes at room temperature, then washed with PBS and incubated with biotinylated rabbit-anti-mouse secondary antibody for 15 minutes at room temperature. After washing, slides were incubated with SA-
HRP (Biocare) for 30 minutes at room temperature, washed again and incubated with BioGenex DAB monitoring staining development. Slides were then washed, counterstained, dehydrated and mounted. For immunofluorescence (IF), after deparaffinization slides were blocked with 20% FBS 30 minutes, washed and incubated with primary antibody for 1hr, then secondary antibody Texas Red-conjugated, followed by DAPI staining. In the case of HeLa cells, cells were grown on a coverslip to 70% confluency and transfected with GFP or GFP-PRMT6 for 24 hrs. Cells were then rinsed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were washed with washing solution (0.1% NP-40 in PBS), blocked in 20% FBS, incubated with primary antibody anti-RelA (in washing solution) 1 hr at room temperature, washed 3 times incubated with secondary antibody Alexa Fluor 555-conjugated 30 minutes, and DAPI stained afterwards.

**Isolation of mouse embryonic fibroblasts**

Briefly, a 12.5-day plugged mouse was sacrificed following standard procedure. Intact embryos were collected and placed in 6-well plates containing 3 mL DMEM 10% FBS and pen-strep. Embryos were mechanically disrupted passing them through a 5 mL syringe with a 18G needle 5 times, then plated in 10 cm dish containing 10 mL of media. Media was changed every day for 3 days.

**Western Blotting (WB) and co-immunoprecipitation (co-IP)**

For total cell lysates, cultured cells (80% confluency) were scraped from a tissue culture dish, lysed in RIPA buffer containing protease inhibitor cocktail and
denatured by boiling. In the case of cell fractionation, the NE-PER kit from Thermo Scientific (cat. # 78835) was employed. For tissue lysates, tissues were harvested, snap frozen in liquid nitrogen, grounded using a mortar, transferred in RIPA buffer with protease inhibitors and sonicated 3 times using Fisher Scientific Sonicator Model 500 (25% power, 10 sec each cycle). Protein lysates (30-50 μg) were resolved on SDS-PAGE gel. For the co-IPs cells were lysed in a mild co-IP buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 15 mM MgCl2] containing protease inhibitor cocktail and incubated with primary antibody anti-GFP or with anti-flag agarose O/N at 4 °C. In the case of anti-GFP IP, lysates were incubated with protein A/G agarose the next day for 3hrs at 4°C, then the agarose was washed in co-IP buffer 3 times, denatured by boiling and resolved on SDS-PAGE gel. In the case of anti-Flag agarose, we proceeded with washing the beads extensively, boiling and resolution on SDS-PAGE gel. The resolved proteins were transferred on PVDF membrane, incubated in 5% non-fat milk 30 min at RT, and subsequently incubated with specific primary antibody in non-fat milk overnight at 4 °C. The s were washed three times in PBS containing 0.2% Tween-20 and incubated for 1 hr in peroxidase-conjugated IgG diluted 1/5000 in non-fat milk at RT. s were washed and immune complexes detected with ECL.

**Acid extraction of histones**

For histone purification, cells were first fractionated using NE-PER kit from Thermo Scientific (cat. # 78835). After isolating the nuclear fraction, 150 μL of 0.8 M HCl was added to the chromatin pellet and sonication at 30% power using Fisher
Scientific Sonicator Model 500 (15 seconds) was performed. Samples were then centrifuged, and the supernatant neutralized adding Tris–HCl 1.5M, pH 8.5 (90 μL) and loaded on SDS-PAGE gel for quantification by coomassie staining.

**Chromatin immunoprecipitation (ChIP)**

HeLa cells at 80% confluency were transfected with 10 μg of a FLAG-PRMT6 vector. 24 h after transfection, cells were treated with TNF-alpha or PBS (vehicle). ChIP analysis was performed using the Millipore Magna ChIP assay kit protocol (cat# 17-610). For the immunoprecipitation, 2 μg of PRMT6 antibody was used for each condition and incubated overnight at 4°C. Quantitative Real time-PCR was performed with the Applied Biosystems 7900HT real-time PCR instrument using the iTaq Universal SYBR Green Supermix form Bio-Rad (cat. # 172-5121).

The primer sequences were as follows:

**8000 upstream TSS:**

For. 5’- GCTCCTCCATCTGGTGCAT-3’, Rev. 5’-AAATTGGGGGTAGGGTTGTC-3’

**IL-6 TSS:**

For. 5’-AATGTGGGATTTTCCCATGA-3’, Rev. 5’-AGTTCATAGCTGGGCTCCTG -3’

**RNA isolation and quantitative real-time PCR**

RNA was isolated from 80% confluent cell plates using TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA was prepared from total RNA (1 μg) using the Superscript III First Strand Synthesis System for RT-PCR from Invitrogen (cat. # 18080-051) following the manufacturer’s protocol. Quantitative Real time-PCR was performed
with the Applied Biosystems 7900HT real-time PCR instrument using the iTaq Universal SYBR Green Supermix form Bio-Rad (cat. # 172-5121) with primers for the indicated genes. Primers were designed across exons.

The primer sequences were as follows:

**IL-6:**
For. 5’-ACTCACCTCTTCAAGAAGGTTG-3’, Rev. 5’-CATTTTTGGAAGGTTCAGTGGT-3’

**TNF-alpha:**
For. 5’-CCCCAGGGACCTCTCTCTAA-3’, Rev. 5’-TGAGGTACAGGCCCTCTGAT-3’

**MCP1/CCL2:**
For. 5’-TCTGTGCTGCTGCTCAG-3’, Rev. 5’-GCTTCTTGGGACACTTGCT-3’

**COX-2:**
For. 5’-TGAACCTCCAACACA-3’, Rev. 5’-GAGAAGGCTTCCCAGCCTTTT-3’

**IKB-alpha:**
For. 5’-AGACCTGGCTCCATCACT-3’, Rev. 5’-TGTCACAGGCAAGGTGAG-3’

**GAPDH:**
For. 5’-AGCCACATCGCTCAGACAC-3’, Rev. 5’-GCCCAATACGACCAAATCC-3’

**Luciferase reporter assay**

HEK 293 cells were cultured in 10% FBS supplemented DMEM in 24-well plates to 70% confluency. For reporter gene assays, cells were co-transfected with 200 ng of the NF-κB luciferase reporter plasmid, 25 ng Renilla control luciferase plasmid (pRL) and the indicated constructs (400 ng). The total plasmid content was balanced up to
625 ng with empty vectors when necessary. Cells were treated with TNF-alpha (10 ng/mL) or PBS for 6 hrs before luciferase assay was done using Dual-Luciferase Reporter Gene Assay from Promega (Madison, WI).

**In-vitro binding assay**

Glutathione S-transferase (GST), GST-RelA (1-431), His-Tudor (UHRF1) and His-PRMT6 were isolated from *Escherichia coli* BL21 cells following 5 h induction with IPTG. For GST fusion proteins, cells were harvested in PBS buffer plus protease inhibitors, sonicated and centrifuged to remove cell debris. GST-fusion proteins were purified by incubation with glutathione beads (Amersham Biosciences) overnight with rotation at 4°C. Beads were washed five times and bound proteins were eluted from the beads using freshly prepared reduced glutathione (33 mM). For His-tagged proteins, cells were lysed in appropriate lysis buffer (containing 1mM EDTA, 1mM EGTA, 5mM DTT and protease inhibitors), incubated with Ni-NTA agarose (Qiagen Scientifics, Maryland) overnight with rotation at 4°C, then eluted with elution buffer (containing 250mM imidazole). 10 ug of eluted GST fusion proteins and His-PRMT6 were incubated in co-IP buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 15 mM MgCl₂] overnight at 4°C. Complexes were then pulled down with glutathione beads for 2 hrs 4°C, washed extensively in co-IP buffer and resolved on SDS-PAGE gel followed by WB analysis.
**In vitro methylation assay**

*In vitro* methylation reactions were carried out in 30 μl of phosphate-buffered saline (pH = 7.4.), containing 0.5–1.0 μg of substrate, 3 μg of recombinant GST-PRMT6 and 0.42 μm [\(^{3}\)H]S-adenosyl-l-[methyl\(^{3}\)H]methionine (79 Ci/mmole from a 7.5 μm stock solution; PerkinElmer Life Sciences). The reaction was incubated at 30 °C for 1 h and then subjected to fluorography by separation on SDS-PAGE, transferred to a PVDF membrane, treated with En\(^{3}\)Hance™ (PerkinElmer Life Sciences), and exposed to film for 1–3 days at –80 °C.

**Isolation and culture of mouse primary keratinocytes**

Briefly, 1-2 day old pups were sacrificed according to standard procedures and skin was removed. Skins were placed on Trypsin-EDTA O/N 4 °C, then epidermis was carefully removed from the dermis, chopped with sterile blades and transferred in 50-mL falcon tube containing Super media (with pen-strep, 10% FBS). Cells were then strained through a 70 μm strainer after mixing 5 times with a 10 mL sterile pipette. Cells were left to adhere to a tissue culture dish for 3 hrs in Super media, then the media was replaced with keratinocyte basal media (Lonza, cat.# CC-3158) containing FBS 10%, pen-strep and additioned with components of the Lonza Kit KGM-2 SingleQuots (Cat.# CC-4152).

**Electroporation of primary MEFs**

About one million cells were trypsinized and washed extensively with PBS, then re-suspended in 800 μL of PBS and pulsed using Bio-Rad Gene Pulser Xcell
Voltage=500 Volts, Capacitance=600 farad and Resistance= ∞. Cells were immediately placed in complete media after electroporation.

**Mammary organoid isolation**

For mammary organoid isolation, we modified a protocol following Matthew J. Smalley. 8-12 week-old female mouse was euthanized and dipped a few seconds in 70% EtOH. Using sterile material, the second, third and fourth pair of mammary glands were removed, rinsed quickly in sterile PBS and placed in L15/10%FCS in a tissue culture dish. Tissue was transferred into a clean, sterile tissue culture hood and minced manually with sterile razor blades, then transferred to a 50-mL conical polypropylene tube filled with 35 ml of working collagenase solution (prepared at the moment) and incubated at 37 °C with vigorous agitation (1.5 hrs). Tissue was then centrifuged 5 min, 1400 rpm and the supernatant transferred in a clean tube and the centrifugation repeated. The two pellets were combined in 10mL L15/10%FCS medium, transferred in a new 15-cm tube and centrifuged again. The pellet from this centrifugation was re-suspended in 5 mL of red blood Lysis Buffer and left at room temperature 5 min, then centrifuged (5 min 1400 rpm). This step was repeated one more time. Pellet was then re-suspended in 10mL L15/10%FCS media and centrifuged again. To remove most of mammary fibroblasts, the pellet was re-suspend in DMEM/10% FCS and transferred to a T-80 tissue culture flask and incubated in a regular cell incubator for 1.5 hr. Organoids were gently recollected, transferred in 15-mL tube, centrifuged 5 min at 1400 rpm and re-suspended in final media (DMEM/F12 (500 mL bottle) added with: 50 µL FCS (10% final); 7µL Pen-
Strep; 500 μL L-glutamine (4 M); 110 μL (human or porcine) Insulin (10 mg/mL); 55 μL EGF (Epidermal Growth Factor) (100 μg/mL).

**Two-stage skin carcinogenesis**

Dorsal skin from 6-8 week old mice was shaved 2 days before initiation. Initiation was accomplished with a single application of 25 μg DMBA and promotion by applying 5 μg TPA/week twice a week for 23 weeks, starting two weeks after initiation. Both DMBA and TPA were dissolved in acetone (200 μL/ one application). The number and size of tumors where monitored weekly. Mann-Whitney U and X2 tests were run for statistical significance.

**Evaluation of Ki-67 staining in the mammary gland**

Mammary glands were stained for Ki67 and scanned using the Aperio slide scanner. A GENIE morphometry algorithm was created that identified ductal epithelial tissue in the glands and quantified the Ki67-positive nuclei within the epithelium. For each slide, an area of mammary gland was selected in which staining artifacts were minimal and no lymph nodes or other non-mammary tissue was present; the edges of the tissues were excluded from the area selected to eliminate problems caused by "edge effect".

**Statistical analysis**

Where not specified, statistical analysis was performed using Student’s t-test.

* indicates $p <0.05$; ** indicates $p <0.01$; *** indicates $p <0.001$. 
4.1 Characterization of tamoxifen-inducible ER*-PRMT6 chimera in cell lines

The hormone-binding domain of steroid receptors can be used as a regulatory system to probe protein function (63). This approach has been used successfully to generate conditional forms of transcription factors (c-Myc, Stat3, p53), kinases (c-Abl & Raf1), DNA methyltransferase (MGMT) and Cre recombinase (63,64). The development of a mutant estrogen receptor HBD (ER*) that is unable to bind estrogen, yet retains normal affinity for the synthetic ligand, tamoxifen (Tamox) or 4-Hydroxytamoxifen (OHT), has enhanced this approach (65). Human PRMT6 was flag-tagged and fused to ER*, and then cloned into the pCAGGS expression vector (Figure 6A). In this system, ER*-PRMT6 expression is driven by the ubiquitous β-actin promoter (66). To test the approach, this expression vector was stably transfected into HEK 293 cells. In the absence of synthetic ligand, ER*-PRMT6 is localized to the cytoplasm, upon Tamox- or OHT-treatment the chimeric protein no longer interacts with the hsp90 complex, and is released for translocation into the nucleus where it is stabilized and active (Figure 6B). This is indeed what we observed (Figure 6C). ER*-PRMT6 stable HEK 293 cells were fractionated into nuclear and cytoplasmic parts and subjected to WB analysis using an αFlag antibody. Prior to OHT-treatment, ER*-PRMT6 is restricted to the cytoplasmic fraction. After OHT-treatment, ER*-PRMT6 translocates to the nucleus and steadily accumulates there. Since PRMT6 is known to deposit the H3R2me2a mark (7-9),
we isolated core histones from the same cells used for the fractionation study in Figure 6C, and performed a WB analysis with an αH3R2me2a antibody. Within two days, the H3R2 site becomes heavily modified (Figure 6D).
Figure 6. Characterization of an inducible ER*-PRMT6 fusion. (A) Human PRMT6 cDNA was cloned into the pCAGGS vector, downstream an ER* (a truncated version of the estrogen receptor that binds tamoxifen). A Flag-tag was introduced between the two proteins. The ubiquitous β-actin promoter drives the expression of the chimeric ER*-PRMT6 protein. (B) Graphic depiction of this approach. ER*-PRMT6 is localized in the cytoplasm. Upon Tamox or OHT treatment the chimera protein becomes stabilized and translocates into the nucleus. C, HEK293 cells stably transfected with pCAGGS ER*-PRMT6 were treatment with OHT (2 μM) and then separated into nuclear [N] and cytoplasmic [C] fractions. WB analysis was performed using an αFlag antibody to detect ER*-PRMT6. An αLamin A/C WB was performed to confirm the quality of the nuclear/cytoplasmic fractionation. Time points after OHT-treatment are indicated. (D) Core histones were isolated from the same ER*-PRMT6 HEK 293 cells shown in (C). The core histones were subjected to WB analysis with an αH3R2me2a antibody to monitor accumulation of this mark. Equal loading was confirmed by Ponceau staining and αH3 WB analysis.
4.2 Characterization of tamoxifen-inducible ER*-PRMT6 transgenic mouse lines

The pCAGGS-ER*-PRMT6 construct described above was used to generate three founder transgenic mouse lines – A, B & C (Figure 7A). Lines A and C underwent germ-line transmission, but Line C displayed low levels of transgene expression. Subsequent studies were thus focused on transgenic Line A. Tamox was administered to Line A mice by daily intra-peritoneal (IP) injections, for five days, as previously described (67). At this point, we analyzed the expression levels of the ER*-PRMT6 chimera in lysates generated from a number of organs (Figure 7B). In addition, immunohistochemical analysis of ER*-PRMT6 localization in the liver shows that IP administration of Tamox causes nuclear translocation and accumulation of this chimeric protein in the nucleus (Figure 7C). Apart from IP injection, Tamox can also be administered to the surface of the skin from where it is absorbed for the activation of ER* fusion proteins in the epidermis (68), but this delivery method also goes systemic. To determine whether ER*-PRMT6 could be induced with topical Tamox-treatment, Line A transgenic mice were shaved dorsally and painted with Tamox (1 mg) or vehicle (ethanol) daily for 5 days. We then performed immunofluorescence (IF) using the αFlag antibody on paraffin-embedded skin sections and found that ER*-PRMT6 is strongly induced and localized to the nucleus of epidermal cells in these mice (Figure 7D). Epidermal scrapes were also collected from the Tamox-treated transgenic mice along with controls, and divided in equal amounts for tissue lysates and core histone extraction in order to perform αPRMT6 and αH3R2me2a WB analysis, respectively (Figure 7E). This experiment
revealed that topical Tamox-treatment of transgenic Line A mice results in a marked increase in H3R2me2a levels on the histone tail as a consequence of ER*-PRMT6 stabilization in the epidermis.
Figure 7. The inducible ER*–PRMT6 fusion is functional in vivo. (A) Three transgenic lines were established using the pCAGGS ER*–PRMT6 vector. Southern analysis was performed with a PRMT6 cDNA probe. The arrowhead indicates the transgene. (B) IP injection of 1 mg Tamox, for five consecutive days, induced stabilization of ER*–PRMT6 in lung, brain and liver of Line A mice. The vehicle (sunflower oil) was injected as a control. (C) Immunohistochemistry (IHC) using an αFlag antibody shows that upon IP injection of 1 mg Tamox (5 days) nuclear translocation of ER*–PRMT6 is induced in the liver of Line A mice. The vehicle (sunflower oil) was injected as a control. (D) Immunofluorescence for the Flag-tag performed on skin sections from transgenic Line A mice shows ER*–PRMT6 stabilization in the nucleus when Tamox was applied topically on shaved dorsal skin (1 mg/day) for 3 days. (E) Core histones were isolated from epidermal scrapes of topically Tamox-treated mice (1 mg every other day for 10 weeks) and subjected to WB with an αH3R2me2a antibody.
4.3 ER*-PRMT6 mice: An unexpected phenotype

Unexpectedly, ER*-PRMT6 transgenic mice that were subjected to daily topical Tamox treatment all died within a 3-week period (Figure 8A). Death occurred more rapidly (within 12 days) if Tamox (1 mg) was administered via IP injections (data not shown). Lethality could be avoided by applying Tamox (1 mg) topically every second day, although we did not observe a strong stabilization of the chimera in internal organs with this treatment (data not shown). In an effort to determine the cause of death of these mice, we performed several serum analyses, including evaluation of inflammatory cytokines such as Interleukin 6 (IL-6), Tumor Necrosis Factor alpha (TNF-alpha) and Interleukin 1 beta (IL-1β), after topical application of Tamox (1 mg) for 12 consecutive days. IL-6 serum levels were significantly elevated in the transgenic Tamox-treated group (Figure 8B). The high variability observed in this assay may be due to the anti-inflammatory effects of tamoxifen, which has been shown to specifically interfere with NF-κB activation (69-71). No significant difference in TNF-alpha or IL-1β was detected (data not shown). Since mouse keratinocytes are known to be a good source of IL-6 (72), we sought to confirm that ER*-PRMT6 stabilization increased IL-6 transcription in the Tg Line A primary keratinocytes. Therefore, we isolated these cells from newborn WT and Tg mice (Figure 8C), cultured for one day, treated with OHT for 2 days (without replacing the media), and stimulated with TNF-alpha 60 min prior to extracting RNA. As shown in Figure 8D, the keratinocytes derived from transgenic mice also show a significant increase in IL-6 transcription.
Figure 8. ER*-PRMT6 mice die upon stabilization of the chimera and show increased IL-6. (A) The ER*-PRMT6 Line A mice die upon prolonged Tamox administration. A survival curve generated after topical Tamox administration (1 mg/day) until death of all transgenic mice occurred (n=14). WT mice were not impacted by this treatment. (B) IL-6 serum levels after 12 topical application of Tamox (1 mg/day) were measured by ELISA. IL-6 serum levels (pg/mL) in four groups of animals (WT, WT Tamox treated, Tg Line A, Tg Line A Tamox-treated) are shown. Wilcoxon and Student t-tests were run and a p value of 0.004 was obtained for the Tg Tamox-treated group (n=32) when compared with the WT Tamox-treated group (n=20) for both tests. All the mice in this study were healthy at the time of serum harvest. All mice were between 8 and 9 week-old, and females and males were equally represented. Primary keratinocytes isolation from 12 newborn mice (6 WTs and 6 Tgs) was performed (mice were from two litters). All cells were cultured for two days, treated with OHT (1 µM) for 48 hrs, and then treated with TNF-alpha for 60 min, at which point and RNA isolation and cell lysates were performed. (C) WB for the flag tag on cell lysates shows stabilization of the ER*-PRMT6 in the Tg mice. A WB using αkeratin-5 antibody shows that these are indeed keratinocytes (as negative control we loaded a cytoplasmic extract of Line A MEFs). (D) RNA was analyzed by quantitative RT-PCR for the expression of IL-6 and normalized for GAPDH. The RQ difference between the two groups of mice is statistically significant (p = 0.0335).
4.4 PRMT6 interacts directly with the RelA subunit of NF-κB

The Nuclear Factor-kappa B is known to regulate the expression of inflammatory cytokines (28). PRMT family members CARM1 and PRMT1 have been shown to co-activate NF-κB-dependent gene expression (36,38). Thus, we asked if PRMT6 might exhibit this function as well, which would explain the elevated levels of IL-6 observed in the Line A transgenic mice. We first asked if PRMT6 is in the NF-κB complex, by performing an immunoprecipitation (IP) using lysates of primary Mouse Embryonic Fibroblasts (MEFs) from WT and Tg Line A mice using an anti-Flag agarose. With this system we could co-IP endogenous RelA from the transgenic but not the WT cells (Figure 9A). In order to exclude the possibility that the ER* portion of the fusion protein could mediate this interaction, we transiently transfected HEK 293 cells with constructs expressing GFP, GFP-CARM1 or GFP-PRMT6 along with a pCDNA-RelA vector for 48 hrs, then treated the cells with TNF-alpha for 30 min. IP using αGFP or αIgG antibodies was performed on cell lysates. WB using αRelA antibody shows that RelA binds to GFP-CARM1 and GFP-PRMT6, but not to GFP alone (Figure 9C). CARM1 has previously been reported to interact with RelA, and it serves as a positive control in this experiment (36). A strong interaction was also confirmed by transiently transfecting a Flag-PRMT6 (not fused to ER*) construct in HEK 293 cells followed by co-IP experiments (Figure 9B). We were also able to co-IP endogenous RelA by pulling down endogenous PRMT6 in HEK 293 cells (data not shown).

The co-activators histone acetyltransferases p300 and its homolog, the CREB-1-Binding Protein (CBP) are known to impact NF-κB-dependent gene
expression and have been shown to interact directly with RelA (31). Moreover, NF-
κB-dependent gene expression involves another class of transcriptional
coactivators, the steroid receptor-coactivators (SRCs), which interacts with the p50
subunit of NF-κB (32). PRMT6 has recently been shown to function as co-activator
of several nuclear receptors (i.e. progesterone, glucocorticoid, and estrogen
receptors) and to bind to SRC-1 in a mammalian two-hybrid assay (23). Therefore,
we questioned if PRMT6 interacts directly with RelA or if the binding is bridged by
other factors, such as SRC-1. For this purpose, GST and GST-RelA (1-431) were
incubated with His-PRMT6, and a GST pull-down experiment was performed. After
extensive washes, bound proteins were resolved on SDS-PAGE, followed by WB
analysis using αHis antibody. As a negative control, a GST-Tudor domain (UHRF1)
was incubated with His-PRMT6. As shown in Figure 9D, PRMT6 and RelA interact
directly in this in vitro assay.
Figure 9. RelA co-immunoprecipitates with PRMT6 and they directly interact in vitro. (A) Primary MEFs were isolated from WT and ER*PRMT6 Line A mice, cultured for 7 days and treated with OHT (2 μM) for 24 hrs. Cells were then lysed and an immunoprecipitation (IP) was performed using αFlag agarose. A WB using αRelA antibody reveals an interaction between the ER*PRMT6 and the endogenous RelA in the lysate from the transgenic cells. (B) HEK 293 cells were transiently co-transfected with a pCDNA-RelA vector and a pCAGGS-Flag-PRMT6 construct for 48 hrs, then treated with TNF-alpha for 30 min. IP using αFlag agarose was performed on the cell lysates. A WB using αRelA antibody shows that RelA is pulled down from the cells transfected with Flag-PRMT6, but not from the Mock (cells transfected with pCDNA-RelA vector and empty pCAGGS vector). (C) HEK 293 cells were transiently co-transfected with a pcDNA-RelA vector and constructs expressing GFP, GFP-CARM1 or GFP-PRMT6 for 48 hrs, then treated with TNF-alpha for 30 min. Immunoprecipitation using αGFP or αIgG antibodies was performed on the cell lysates. A WB using αRelA antibody shows that RelA binds to GFP-CARM1 and GFP-PRMT6, but not to GFP alone. (D) Direct binding between PRMT6 and RelA. GST and GST-RelA (1-431) were incubated with His-PRMT6 and a GST pull-down was performed. For an additional negative control, GST-RelA was also incubated with a His-Tudor-UHRF1. A Western blotting using αHis antibody reveals direct binding between GST-RelA and His-PRMT6.
4.5 PRMT6 is a coactivator of NF-κB and its enzymatic activity is required for this function

In order to test if PRMT6 functions as a co-activator of NF-κB, we first performed a luciferase assay using HEK 293 cells. PRMT6 overexpression increases the luciferase activity of a NFκB-Luc luciferase reporter plasmid (Figure 10A). We used CARM1, a known co-activator for NF-κB (36), as positive control for this experiment. Furthermore, the enzymatic activity of PRMT6 is required for its co-activator function (Figure 10B). This was established by transfecting HEK 293 cells with catalytic active and inactive forms of PRMT6 (PRMT6 dead), and performing a NFκB-Luc luciferase assay. The inactive form of PRMT6 (VLD-KLA) has previously been used in a similar assay (14).

To further confirm that PRMT6 functions as a coactivator of NFκB, we performed quantitative real-time PCR experiments, in which we asked if, by overexpressing PRMT6 in HeLa cells, the transcription of IL-6 increases upon TNF-alpha stimulation. We transfected HeLa cells with constructs expressing GFP, GFP-PRMT6 or GFP-CARM1 (as positive control), and we observed enhanced transcription of IL-6 when either PRMT6 or CARM1 were overexpressed (Figure 10C). We also explored the expression levels of several other genes known to be activated in HeLa cells upon TNF-alpha stimulation. These included TNF-alpha, Monocyte chemotactic protein 1 (MCP-1/CCL-2), Cyclooxygenase 2 (COX-2) and IκB-alpha (73). Among these genes, we found that PRMT6 co-activates TNF-alpha, MCP-1 and COX-2 (Figure 10C).
We then sought to understand if PRMT6 is essential for kB-dependent transactivation function. To this scope, we took advantage of PRMT6 KO MEFs availability in our laboratory (the PRMT6 KO mice were a gift from Dr. Stephane Richard) and performed Real-Time experiments with them. In this experiment, we treated WT, PRMT6 KO and CARM1 KO cells with TNF-alpha for 2 hrs and then extracted the RNA. As shown in **Figure 10D**, the PRMT6 and CARM1 cells display a significant reduced transactivation at the IL-6 locus compared to the WT cells. Therefore, although both PRMT6 and CARM1 enhance the transactivation of NF-kB at the IL-6 locus, neither of them is essential for this function. These data are in agreement with the observations from the Hottiger’s group, who also did not see a defect in IL-6 activation in these CARM1 KO MEFs (36).
Figure 10. PRMT6 is a NF-κB coactivator. (A) HEK 293 cells were transiently co-transfected with a NF-κB firefly luciferase reporter plasmid and a renilla luciferase construct along with a GFP vector (Mock), a GFP-CARM1, or a GFP-PRMT6 construct. Cells were left un-stimulated or stimulated 20 hrs after transfection with TNF-alpha (10 ng/mL, 6 hrs). Error bars represent standard deviation calculated from quadruplicate luciferase assays. Mean values are expressed as fold changes in luciferase activity with the un-stimulated Mock arbitrarily set as 1. The $p$ values obtained for PRMT6 and CARM1 transfections compared to the Mock are 0.00016 and 0.0012, respectively, when a two sample independent t-test is run. These data are representative of four independent experiments. (B) HEK 293 cells were transiently co-transfected with a NF-κB luciferase reporter plasmid and a renilla luciferase construct along with an empty vector or pMyc-PRMT6 or a PRMT6 catalytic inactive form (dead). Cells were un-stimulated or stimulated 20 hrs after transfection with TNF-alpha (10 ng/mL, 6 hrs). Error bars represent standard deviation calculated from quadruplicate luciferase assays. The $p$ value obtained for the WT PRMT6 transfection is 0.0006, when a two sample independent t-test is run. (C) HeLa cells were transfected with GFP (Mock), GFP-PRMT6 or GFP-CARM1 constructs for 24 hrs and then stimulated with TNF-alpha (10 ng/mL) for 60 min. Total RNA was analyzed by quantitative RT-PCR for the expression of the indicated genes and normalized against GAPDH. For each gene, the TNF-alpha stimulated Mock group mean was arbitrarily set as 1. Error bars represent standard deviation calculated from triplicates. The asterisk indicates statistically significant difference compared to the Mock when a two sample independent t-test is run. For IL-6, the $p$ values for GFP-PRMT6 and GFP-CARM1 versus Mock are 0.005 and 0.0004, respectively. For TNF-alpha the $p$ values for GFP-PRMT6 and GFP-CARM1 versus Mock are 0.005 and 0.0004, respectively. For MCP1 the $p$ values for GFP-PRMT6 and GFP-CARM1 versus Mock are 0.3 and 0.07, respectively. For COX-2 the $p$ values for GFP-PRMT6 and GFP-CARM1 versus Mock are 5.8E-05 and 0.64, respectively. (D) WT, PRMT6 KO and CARM1 KO MEFs were left untreated or stimulated with TNF-alpha for 2 hrs before RNA was extracted. Total RNA was analyzed by quantitative RT-PCR for the expression of IL-6 gene and normalized against GAPDH. The mean value for the unstimulated groups was arbitrarily set as 1. Error bars represent standard deviation calculated from triplicates. The asterisks indicate significant difference of these groups compared to the WT. The $p$ values for TNF-alpha stimulated PRMT6 and CARM1 KO versus the WT cells are 0.0014 and 0.0038, respectively.
4.6 PRMT6 causes nuclear shuttling of RelA

Having established that PRMT6 coactivates NF-κB, we investigated the possible mechanism for this function. First, we explored the possibility that, when over-expressed, PRMT6 may promote the movement of NF-κB into the nucleus. For this purpose, we isolated primary MEFs from WT and ER*-PRMT6 embryos and treated them with OHT for 2 weeks. Cells were then fractionated and subjected to WB analysis using a αRelA antibody. We observed an increase of nuclear RelA upon the Tamox-induced stabilization of ER*-PRMT6 in the nuclear fraction (Figure 11A). This effect on RelA nuclear shuttling was seen in MEFs isolated from additional embryos (data not shown). No change in global levels of RelA was observed in primary MEFs (Figure 11B). In order to confirm this phenomenon we performed IF in HeLa cells upon transient transfection with a GFP-PRMT6 construct. A GFP-expressing construct was transfected in parallel for negative control. As shown in Figure 11C, cells overexpressing PRMT6 (which localizes strongly in the nucleus) show increased RelA in the nucleus. Thus, the overexpression of both GFP-PRMT6 and ER*-PRMT6 causes increased nuclear RelA. This established that the ER* component of the ER*-PRMT6 fusion is not responsible for this translocation of RelA, but rather it is the PRMT6 protein itself.
Figure 11. PRMT6 promotes RelA shuttling into the nucleus (A) Primary MEFs were isolated from WT and ER*-PRMT6 mice, cultured for 3 days and treated with OHT for 2 weeks. Cell fractionation was performed. WB analysis for the Flag-tag shows stabilization of ER-PRMT6 in the nuclear fraction, corresponding to increased nuclear RelA. (B) Primary MEFs were isolated from 2 WT and 2 ER*-PRMT6 mice, cultured for 3 days and treated with OHT for 3 days. Total cell lysates were analyzed by WB using αRelA and αFlag antibodies. Cells in which the ER*-PRMT6 is overexpressed do not show increased total levels of RelA. (C) Immuno-fluorescence using a αRelA antibody following transient transfection of GFP-PRMT6 in HeLa cells shows increased nuclear RelA in transfected cells. A GFP-expressing construct was used as negative control.
4.7 A methylation-dependent mechanism for PRMT6 coactivator function

The mechanism for PRMT6 co-activation is clearly methylation-dependent, as shown in Figure 10B. Posttranslational modification of RelA by PRMT6 could account for its increased nuclear localization. We thus tested the possibility that PRMT6 may methylate RelA by performing \textit{in vitro} methylation assays. In this \textit{in vitro} methylation assay, we observed robust methylation of histone H3, as well as automethylation of PRMT6 itself, but no methylation of recombinant RelA (Figure 12 A,B). We then hypothesized that the coactivation is associated with the PRMT6 action on the histone code. As mentioned above, PRMT6 has been shown to methylate the R3 position of the N-terminal tails of histones H4 in \textit{in-vitro} reactions (9). Thus, we questioned if the ability of PRMT6 to function as a co-activator may be linked to its capacity to also methylate the R3 position of the N-terminal tails of histones H4 \textit{in vivo}, generating the active H4R3me2a mark. To test this hypothesis, we utilized a specific $\alpha$H4R3me2a antibody and performed WB analysis on core histones isolated from primary MEFs of WT and Line A mice. As shown in Figure 13A, ER*-PRMT6 Line A MEF core histones show a global increase in H4R3me2a when PRMT6 is stabilized in the nucleus by Tamox treatment for two weeks. To further confirm this, we transiently transfected HEK 293 cells with a pCAGGS-Flag-PRMT6 construct and looked by WB. In this experiment, we saw a more dramatic global elevation of the H4R3me2a activating mark (Figure 13B). As PRMT1 is known to be the primary enzyme generating the H4R3me2a mark, we sought to test if PRMT6 could rescue this mark when PRMT1 is knocked out (KO). We took advantage of the availability of an OHT-inducible PRMT1 KO stable line, previously described (74), which we stably transfected with our ER*-PRMT6 construct. As
shown in Figure 13C, while the PRMT1 KO cells have a dramatic reduction in the H4R3me2a mark upon OHT treatment, the stable line we generated shows rescue of this mark upon concomitant loss of PRMT1 and overexpression of PRMT6 (ER*-PRMT6), therefore PRMT6 is able to maintain the H4R3me2a modification in absence of PRMT1.

We then asked if PRMT6, like CARM1 (36), might be recruited to the IL-6 promoter upon TNF-alpha stimulation and if the H4R3me2a mark concomitantly increases at this locus, therefore we performed chromatin immunoprecipitation (ChIP) using αPRMT6, H4R3me2a and H3R2me2a antibodies. We clearly observe enrichment of PRMT6 and H4R3me2a at the transcriptional start site (TSS) of IL-6 upon TNF-alpha stimulation (Figure 14). These data strongly suggest that PRMT6 is recruited to chromatin with RelA, where it impacts the histone code and/or possibly methylates other chromatin-associated proteins to facilitate transcription at the IL-6 locus.
Figure 12. PRMT6 does not methylate RelA in vitro. (A) GST-PRMT6 was incubated with recombinant histone H3, GST, or GST-RelA (1-431) and the methyl-donor tritiated S-adenosylmethionine (SAM) for 30 min. The reactions were then resolved by SDS-PAGE and transferred to a PVDF membrane. A fluorography revealed strong activity of PRMT6 on recombinant histone H3, while no activity was detected on RelA (1-431). (B) Flag-PRMT6 was pulled down from HEK 293 cells upon TNF-alpha stimulation (10ug/mL, 30 min.) and incubated with histone H4 or GST-RelA (1-431).
Figure 13. PRMT6 generates the H4R3me2a active mark in vivo. **A**, Primary MEFs were isolated from WT and ER$^\ast$-PRMT6 mice, cultured for 3 days and treated with OHT for 2 weeks. Cell fractionation and core histone isolation was performed. A WB for Flag shows stabilization of the ER-PRMT6 in the nuclear fraction from the Tg OHT-treated cells. A WB on core histones shows increased H3R2me2a as well as H4R3me2a global levels in the Tg OHT-treated cells. **B**, HEK 293 cells were transiently transfected with a pCAGGS-Flag-PRMT6 vector or an empty vector (Mock) and a WB for PRMT6 showed a highly efficient expression of the exogenous flag-PRMT6 compared to the endogenous, while PRMT1 levels were unchanged. A WB for Actin was used for loading control. Core histones from these cells were extracted and subjected to WB using $\alpha$H4R3me2a and $\alpha$H3R2me2a antibodies, which revealed an increase of these marks in the cells over-expressing Flag-PRMT6, while H4R3me2s or H3K4me3 levels did not change. **C**, An OHT-inducible PRMT1 knockout MEF line (PRMT1$^{+/+}$;ER-Cre), previously described, was stably transfectecd with our ER-PRMT6 construct. Cells were left untreated or treated with 2$\mu$M OHT along with the MEF inducible PRMT1 knock-out cells and a MEF control line (PRMT1$^{+/+}$;ER-Cre) for 7 consecutive days. WB for PRMT1 and PRMT6 on cell lysates were performed. A WB for beta-Actin is shown for loading control. Core histones from the cells were extracted and a WB for H4R3me2a was performed. A WB for Histone H4 showed equal histone loading.
Figure 14. PRMT6 ChIPs at the TSS of IL-6, with concomitant increase in the H4R3me2a mark upon TNF-alpha stimulation. HeLa cells were transiently transfected with a Flag-PRMT6 construct, and a ChIP assay was performed using αFlag, αH3R2me2a and αH4R3me2a antibodies. Upon TNF-alpha stimulation (60 min), PRMT6 is enriched at the transcriptional start site (TSS), corresponding to the consensus region for NF-kB. The H3R2me2a repressive mark is unchanged, while the H4R3me2a active mark is significantly enriched. Error bars represent standard deviation calculated from triplicates. Mean values are fold changes compared to the IgG and normalized to a control intergenic region about 8000bp upstream the TSS. The asterisk indicates statistically significant difference compared to the unstimulated when a two sample independent t-test is run.
Chapter 5

The Cre-inducible mouse model

5.1 The Cre-inducible system

A Cre-inducible hPRMT6 transgene was cloned using the pCAGGS vector, which harbors a beta-actin promoter. Between the promoter and PRMT6 we placed a Stop cassette, which is flanked by directly repeated loxP sites (Figure 15A). This Stop cassette terminates transcripts, as previously described (62), and is excised by the action of a Cre recombinase. The excision capability of a Cre recombinase was first tested in vitro, by transforming the stop-cassette containing vector in a Cre-expressing E. coli strain. With this system, the linearized construct isolated from this strain is shorter than the linearized construct used to transform the cells, due to the stop-cassette excision, therefore a gel shift of about 1,400bp (the stop cassette length) can be appreciated (Figure 15B).

Before attempting to generate mice, the inducible expression of the Flag-PRMT6 was also tested in cultured cells by performing WB analyses with an αFlag antibody upon co-transfection of HEK 293 cells with the pCAGGS-stop-PRMT6 construct and a GFP-Cre expressing vector (Figure 15C). For positive control (last lane on the right) we transfected cells with the construct isolated from Cre-expressing E. coli (Figure 15B, lane 2), which, as expected, gives a very efficient expression of the Flag-PRMT6. To ascertain that the stop cassette was efficient in blocking transcription, we also transfected cells with the pCAGGS-stop-PRMT6 construct, which, as expected, does not express Flag-PRMT6.
Figure 15. PRMT6 is re-expressed from a pCAGGS-stop-PRMT6 construct upon Cre recombination. (A) Depiction of the Cre-inducible PRMT6 construct. A Flag-hPRMT6 cDNA was cloned into the pCAGGS vector, downstream a loxP-flanked stop cassette. The ubiquitous Beta-actin promoter drives the expression of the Flag-PRMT6 transgene. Upon Cre recombination, the stop cassette is excised and a loxp-Flag-PRMT6 is left. (B). The system was tested for Cre recombination by using a Cre-expressing E. coli strain. In lane 1 is the pCAGGS-stop-PRMT6 linearized construct, while in lane 2 is the same construct after transformation and isolation from the Cre-expressing E. coli. A gel shift is indicative of proper excision of the stop cassette. (C) HEK 293 cells were transfected with an empty pCAGGS vector (Mock) or a pCAGGS-stop-PRMT6 construct along with a GFP-Cre vector, or a pCAGGS-stop-PRMT6 construct or a pCAGGS-loxp-PRMT6 construct. After 24 hrs, cells were harvested and a WB for Flag was performed on the cell lysates.
5.2 Ex vivo characterization of Cre-inducible mouse lines

The construct described above was employed to generate transgenic mouse lines, which we call STOP-PRMT6 mice. From a first pronuclear injection we obtained 2 lines (Line 1 and 2) (Figure 16A, left), but neither line re-expressed the transgene upon Cre recombination. From a second pronuclear injection we obtained three additional lines (Line 3, 4, 5) (Figure 16A, right), all of which showed re-expression of the transgene upon Cre recombination. To test the re-expression of Flag-PRMT6 we isolated primary MEFs from these mouse lines, transfected them with a GFP-Cre vector, and tested the expression of the transgene by WB using a specific anti-hPRMT6 antibody. As seen in Figure 16B, Line 1 and 2 do not re-express exogenous PRMT6 upon Cre recombination, while Line 3, 4, 5 re-express the Flag-PRMT6. The double band present in all the lanes is a non-specific doublet recognized by the specific anti-hPRMT6 antibody in use. To further confirm expression of Flag-PRMT6 we also performed IF analyses on all the lines using anti-Flag antibody. For Line 1 and 2 we did not see re-expression of Flag-PRMT6 (data not shown), while for Line 3, 4, 5 we confirmed the re-expression (Figure 16C). In this IF, the green nuclei (which are the ones transfected with GFP-Cre) are indeed positive for Flag staining (in purple).
Figure 16. Characterization of STOP-PRMT6 MEFs. (A) Five transgenic lines were established using the pCAGGS-stop-PRMT6 vector. Southern analysis was performed with a PRMT6 cDNA probe. The arrowhead indicates the transgene. (B) Primary MEFs were isolated from Line 3, 4 and 5 and cultured for 7 days, then an electroporation using a pCAGGS-GFP-Cre vector was performed. Cells were harvested 24 hrs later and a WB using a specific anti-hPRMT6 antibody was performed. The double band that appears under the specific human PRMT6 and s present in the negative control cells is an a-specific band. (C) An aliquot of primary MEFs from the electroporation described in (B) was plated on coverslip and an IF using anti-Flag antibody was performed.
5.3 Crossing the STOP-PRMT6 lines with K5-Cre mice

We crossed Line 3, 4 and 5 to keratin 5 (K5)-Cre transgenic mice (75). The K5-Cre mouse line was chosen because PRMT6 is overexpressed in a number of epithelial cancers (lung, breast, cervical and bladder cancer – Figure 5E), where the K5 promoter is active. This line is on a FVB background and was available in Science Park from Dr. David Johnson’s laboratory. When crossed with K5-Cre, all three lines generated bigenic pups that were slightly smaller than their single transgene (or WT) littermates and displayed a delay in hair development (Figure 17A), although these differences were not appreciable once the mice reached adulthood. At histological level, a hair follicle phenotype was observed. Indeed, hair follicle were somewhat disorganized, with the appearance of transversally oriented hair follicles which embedded in the fat layer (Figure 17B, arrowhead). The bigenic mice of Line 4 displayed the most severe phenotype at both gross and histological levels. Epidermal scrapes were performed on the skin from the bigenic mice of the three lines, and these lysates were subjected to WB analysis using anti-PRMT6 antibody, which revealed expression of the exogenous form of PRMT6 in all the lines (Figure 18A). Although the expression of Flag-PRMT6 seemed to be good in WB analyses, IF analyses revealed a very focal expression in both epidermis and hair follicles in all the lines. In Figure 18B is shown the IF for the Flag-tag on paraffin embedded skin sections of STOP-PRMT6/K5-Cre (Line 4) mice.

Since STOP-PRMT6 bigenic mice of Line 4 harbor the least transgene copy number (Figure 16A), show the strongest Flag-PRMT6 expression in the epidermis and the most obvious hair follicle phenotype (Figure 17), we decided to focus on this line for further characterization and experiments. We will refer to the Line 4 as
STOP-PRMT6 from here on. In addition to the epidermis, the K5-Cre transgene is also expressed in a number of other stratified and pseudo-stratified epithelial tissues, including the cervix, vagina, oral and nasal cavities, esophagus, stomach, bladder, lung, thymic epithelial, prostate, and myoepithelial cells of the mammary gland (76), therefore we performed a WB analysis on lysates from several organs of the STOP-PRMT6/ k5-Cre bigenic mice using a anti-h-PRMT6 antibody. As seen in Figure 19A, the expression of exogenous PRMT6 is predominant in epidermis, mammary gland and thymus. A residual expression was seen in brain and liver. Next, we analyzed the core histones isolated from STOP-PRMT6/K5-Cre epidermal scrapes. We observed a clear elevation of H3R2me2a levels in the bigenic mice (Figure 19B) by WB. Finally, we performed double immuno-fluorescent staining using anti-H3R2me2a (green) and anti-Flag (red) antibodies on paraffin-embedded sections from the skin of STOP-PRMT6/K5-Cre bigenic mice. We observed a dramatic elevation of H3R2me2a levels in cells expressing Flag-PRMT6 (Figure 19C). These data show that the Tg4 line has an altered histone code and provides in vivo evidence for PRMT6 impacting epigenetic signatures.
Figure 17. Bigenic mice of the K5-Cre cross display a hair phenotype. (A) Pups of each STOP-PRMT6 line that we obtained (Line 3, 4, 5) display delayed hair onset upon Cre recombination. (B) H&E sections of 3-week-old STOP-PRMT6/ K5-Cre mice showing the hair follicle phenotype.
Figure 18. The expression of Flag-PRMT6 is extremely focal in the skin of bigenic mice. (A) Epidermal scrapes were collected from bigenic mice of the three lines of the STOP-PRMT6 mice that we generated and subjected to WB analyses using a specific anti-hPRMT6 antibody. (B) Skin sections from bigenic STOP-PRMT6/K5-Cre (Line 4) mice were paraffin embedded and subjected to IF staining using anti-Flag antibody.
Figure 19. STOP-PRMT6/ K5-Cre mice display elevated H3R2me2a mark. (A) Organ lysates from a STOP-PRMT6 /K5-Cre mouse were subjected to WB analyses using a specific anti-hPRMT6 antibody. (B) Epidermal scrapes from a bigenic and a control mouse were collected and divided in two parts to perform WB analyses for PRMT6 and H3R2me2a on lysates and core histones, respectively. (C) Sections from skin of bigenic mice were paraffin embedded and a co-IF was performed using mouse anti-Flag (red) and rabbit anti-H3R2me2a (green) antibodies.
5.4 A chemical skin carcinogenesis study on STOP-PRMT6/K5-Cre mice

Having observed a gross skin phenotype in live K5-Cre/STOP-PRMT6 bigenic mice and demonstrated overexpression of PRMT6 and increased levels of the PRMT6-mediated H3R2me2a mark in bigenic epidermis, we hypothesized that pathological effects of PRMT6 overexpression in the epidermis and possibly other K5-expressing tissues may be detectable. The gross skin phenotype that is observed in our bigenic lines, including the alopecia, is similar to the phenotype observed for a number of other K5 transgenic models that have hyperplastic and hyperproliferative epidemis, including K5 models that overexpress Myc (77) and E2F1 (78). These models also develop spontaneous tumors in the skin and other K5-expressing tissues. Moreover, deregulated expression of Myc in the epidermis of transgenic mice resulted in an enhanced response to two-stage skin carcinogenesis (77). We hypothesized that STOP-PRMT6/K5-Cre bigenic mice may have increased susceptibility to skin carcinogenesis as well. This is consistent with the strong correlative data demonstrating that PRMT6 is overexpressed in several different human cancers. In order to test our hypothesis we carried out a two-stage DMBA/TPA skin carcinogenesis study on STOP-PRMT6/K5-Cre mice. Mice were initiated by applying 25μg DMBA once and promotion was performed applying 5μg TPA/week twice a week for 23 weeks, following the protocol from the group of Yuspa (79). As control group the K5-Cre mice were included in this study. We compared a number of endpoints, including tumor incidence, burden, multiplicity, and malignancy. A two group Chisq test with a 0.050 two-sided significance level will have 80% power to detect the difference between a Group 1 proportion of 0.3 and a Group 2 proportion of 0.7 when the sample size in each group is 25. Therefore, we included 27 bigenic
mice in this study. When we examined papilloma incidence, number, and burden we did not observe significant difference between the STOP-PRMT6/K5-Cre bigenic group and the control group (Figure 20). When we examined the number of squamous cell carcinomas (SCC) and spindle cell carcinomas (SCT) we also did not detect significant difference between the two groups. However, spindle cell carcinomas, which are believed to represent a more advanced form of SCC, were absent in the control group while 5 total were detected in the bigenic group.
Figure 20. Results of the chemical skin carcinogenesis study. (A) Papilloma incidence during the 23-week TPA treatment. Tumors were counted every second week. (B) Tumor burden (cumulative for all the mice), expressed in cm. (C) Tumor multiplicity, expressed in number of papillomas/mouse. (D) Analysis of the malignancies at the endpoint of the carcinogenesis study (mice were sacrificed at 23 weeks of TPA administration). MiSCC, Microinvasive Squamous Cell Carcinomas; SCC, Squamous Cell Carcinomas; SCT, Spindle Cell Carcinomas.
5.5 A cancer study on STOP-PRMT6/K5-Cre;p53+-/- mice to investigate the role of PRMT6 in tumorigenesis

The p53+-/- mice exhibit a wide array of tumors, including soft tissue sarcomas, osteosarcomas and carcinomas of various types starting at about 9 months of age (80). In order to determine if increased PRMT6 activity cooperates with p53 deficiency in tumor development we generated STOP-PRMT6/K5-Cre;p53+-/- mice. These mice were all 100% FVB. The breeding scheme to generate these mice is shown in Figure 21. As shown in Figure 19A, the expression of the exogenous hPRMT6 is quite extensive in the bigenic STOP-PRMT6/K5-Cre mice, therefore the impact of PRMT6 overexpression on tissue homeostasis and malignancy could be examined in a variety of organs using this model system. Importantly, PRMT6 overexpression is observed in tumors from several of the tissues that are targeted by the K5 transgene, including the cervix, bladder, lung and breast (Figure 5E). In performing this cancer study, we hypothesized that increased PRMT6 expression and activity would cooperate with p53 inactivation to accelerate tumor development, and we were especially interested in potential effects of PRMT6 overexpression on mammary tumor development. We calculated that 20 double transgenic mice should be enough to detect a statistically significant difference between the control and the experimental groups. [If PRMT6 over-expression increases tumor incidence to 80% by one year, then 20 mice per group will be enough to detect a statistically significant difference in tumor incidence (a two group chi-square test with a 0.05 one sided significance level will have 80% power to detect the difference between 40% and 80% when the sample size is each group is 18)]. We obtained total of 34 bigenic mice (females and males were equally represented in the group) and
monitored them for tumor formation over a 10-month period along with the K5-Cre control group (Table 1). Complete necropsy was performed by Dr. Donna Kusewitt and any lesion or abnormal looking organ was taken for histopathological analysis when mice were about 9 months old. It has to be noted that we experienced a loss of about 25-30% of mice from each group during the time period of this study (Table 1). Deaths were sudden and started around 5 months of age. We did not investigate on the cause of deaths, especially considering that this was a phenomenon happening for all the groups. Mice that died before the endpoint of this study were therefore discarded. Overall, in this study there was not a significant difference in tumorigenicity between the two groups, but the bigenic mice showed signs of increased inflammation.

Consistent with our findings about PRMT6 role in inflammatory response described in Chapter 4, overall there appeared to be increased inflammation (perivasculitis in the lung and kidney and microabscess in the liver) in the STOP-PRMT6/K5-Cre females compared to the K5-Cre control females (Figure 22, arrowheads) although the difference was not statistically significant, probably due to the inadequate number of females left at necropsy. This phenomenon was not seen in the males.
Breeding bigenic STOP-PRMT6/K5-Cre mice onto a p53+/− background

Step 1. Establishing a p53+/− colony

2 males p53+/− will be bred with 4 WT females
Considering 8 pups/litter, = 15 p53+/− mice will be generated (7 females and 7 males)

Step 2. Breeding p53+/− mice with K5-Cre mice

Three p53 +/− males will be crossed with 6 K5-cre females (1 male will be placed with 2 females)
Six p53+/− females will be crossed to three K5-cre males (2 females and 1 male per cage)
Considering that 80% of the females will get pregnant and will have 8 pups/litter,
= 20 p53+/−;K5-cre mice will be generated (10 males and 10 females)

Step 3. Breeding p53+/−;K5-cre X p53+/− mice (to generate p53−/−; K5-cre mice)

4 males p53+/−; k5-cre will be bred with 8 females p53+/− (1 male will be placed with 2 females)
8 females p53+/−; k5-cre will be bred with 4 males p53+/− (1 male will be placed with 2 females)
Considering that 80% of the females will get pregnant and will have 8 pups/litter,
= 12 p53−/−;K5-cre mice will be generated (6 males and 6 females)

Step 4. Breeding p53−/−;K5-Cre males with STOP-PRMT6 transgenic females

6 males p53−/−; k5-cre will be bred with 12 females STOP-PRMT6 (1 male will be placed with 2 females)
Considering that 80% of the females will get pregnant and will have 8 pups/litter,
= 20 STOP-PRMT6/K5-cre; p53+/− mice will be generated

Mice from this cross (along with the STOP-PRMT6) will be monitored for tumor formation over one year period. We expect that increased PRMT6 expression and activity will cooperate with p53 inactivation to accelerate tumor development. This would be consistent with the observation that PRMT6 is over-expressed in human cancer. If PRMT6 over-expression increases tumor incidence to 80% by one year, then 20 mice per group will be enough to detect a statistically significant difference in tumor incidence (a two group chi-square test with a 0.05 one sided significance level will have 80% power to detect the difference between 40% and 80% when the sample size is each group is 16).

Timeline to obtain the wanted mice: 10 months
Excess mice from each breeding will be euthanized according to the standard procedures

Figure 21. Breeding scheme to generate STOP-PRMT6/K5-Cre;p53+/− mice.
Table 1. Control and transgenic mice employed in the cancer susceptibility study.

<table>
<thead>
<tr>
<th></th>
<th>K5-Cre (p53&lt;sup&gt;+/−&lt;/sup&gt;)</th>
<th>STOP-PRMT6/K5-Cre (p53&lt;sup&gt;+/−&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At start</td>
<td>(15f; 17m)</td>
<td>(18f; 16m)</td>
</tr>
<tr>
<td>At necropsy</td>
<td>(9f; 11m)</td>
<td>(11f; 10m)</td>
</tr>
</tbody>
</table>

Figure 22. STOP-PRMT6/K5-Cre females from the tumor study (p53<sup>+/−</sup> cross) display perivasculitis in the lungs and microabscess in the liver. Representative H&E staining of liver and lungs of K5-Cre control and STOP-PRMT6/K5-Cre bigenic females. Arrowheads indicate microabscess and perivasculitis. (Pictures taken by Dr. Donna Kusewitt).
5.6 Crossing the STOP-PRMT6 mice with a MMTV-Cre mouse line

Transgenic mouse lines carrying a Cre recombinase under the control of the MMTV LTR (MMTV-Cre, Line A and Line D) have been generated by the group of Hennighausen (81,82). We crossed our transgenic STOP-PRMT6 with the MMTV-Cre Line D, which was available in Science Park from Dr. Marcelo Aldaz’ group. This strain originated on an FVB background, but has been backcrossed to B6129F1 and is now only available in this background from the Jackson’s repository. MMTV-Cre Line D mice display high levels of recombination in the virgin and lactating mammary gland, salivary gland, seminal vesicle, skin, erythrocytes, B cells and T cells when crossed to ROSA26-lox-Stop-lox-LacZ reporter strain, while little background recombination is observed in the lung, kidney, liver and brain tissues (less than 10%) (81). In crossing STOP-PRMT6 mice with MMTV-Cre mice we kept the background at 50% FVB component.

We first sought to compare the expression level of the exogenous PRMT6 in the bigenic STOP-PRMT6/MMTV-Cre mice versus the bigenic STOP-PRMT6/ K5-Cre mice, therefore we harvested mammary gland tissue and performed WB analyses using anti-hPRMT6 antibody - which revealed an equal level of expression in these two crosses (Figure 23A) - as well as immunohistochemistry (IHC) staining on paraffin embedded tissue using anti-Flag antibody (Figure 23B). The IHC shows extensive (although mosaic) expression of the exogenous PRMT6 in mice from both crosses, but there seems to be a difference in the compartment of cells that express the exogenous Flag-PRMT6: the K5-Cre cross appears to be mostly luminal, while the MMTV-Cre cross appears to be both myoepithelial and luminal. In order to evaluate the level of over-expression of the exogenous PRMT6 in comparison to the
endogenous protein and the histone arginine methylation status in the epithelial compartment of the mammary gland, we isolated primary mammary epithelial cells (organoids) from the bigenic STOP-PRMT6/MMTV-Cre mice along with the controls (single transgenic STOP-PRMT6 mice). As shown in Figure 24A, WB analysis on the cell lysates using an anti-PRMT6 antibody (which recognizes human and mouse PRMT6) reveals a strong over-expression of the exogenous Flag-PRMT6 compared to the endogenous protein. WB analyses were performed on core histones isolated from these cells to investigate levels of asymmetric dimethylation on H3R2 as well as H4R3. As shown in Figure 24A the global H3R2me2a and H4R3me2a (although to a lesser extent) levels are elevated in the mammary gland epithelium from the double transgenic mice compared to the single transgenic controls. We then took advantage of the mosaic expression displayed by the Tgs STOP-PRMT6/MMTV-Cre mice to perform double immuno-fluorescence for the Flag-tag and the histone marks of our interest. The epithelial mammary gland cells from the double transgenic mice that do not express the exogenous Flag-PRMT6 (red) display lower H3R2me2a and H4R3me2a, shown in green (Figure 24B). Therefore, we were able to confirm that PRMT6 methylates the H4R3 site in vivo with these mice.

Since PRMT6 impacts epigenetic signatures in vivo in our mouse model, we questioned if PRMT6 may induce increased proliferation in the mammary gland of the bigenic mice. For this evaluation (done by Dr. Donna Kusewitt) we examined the proliferative marker Ki-67, as described in the Materials and Methods Chapter. For STOP-PRMT6/K5-Cre mice, we utilized the females from the tumor study described in Section 5.5, but we did not detect any difference between the K5-Cre control group and the bigenic STOP-PRMT6/K5-Cre group (data not shown), however we
did see a difference in Ki-67 staining for the bigenic STOP-PRMT6/MMTV-Cre mice compared to the control groups (WT, STOP-PRMT6, and MMTV-Cre) (Figure 25A). We also examined the percentage of the area that was composed by ducts in the four groups. Interestingly, the percentage of the area examined that was composed of ducts in the MMTV-Cre mice was higher, although not statistically significant, than the WT and STOP-PRMT6. The percentage of the area occupied by ducts was higher in the bigenic mice compared to control groups MMTV-Cre, WT and STOP-PRMT6, although the $p$ value was significant only when the comparison was done with the WT and STOP-PRMT6 groups (Figure 25B). In more simple terms we can summarize these data by stating that the MMTV-Cre mice per se display more epithelialization than WT mice, but the overexpression of PRMT6 in these mice seems to increase this phenotype.

Having ascertained a hyper-proliferative phenotype in the mammary gland of the bigenic STOP-PRMT6/MMTV-Cre mice, we set-up an ageing study to investigate the possibility of increased spontaneous mammary carcinogenesis in these mice compared to the controls. These mice have been aged until 12 months and analyses on the mammary tissue are currently underway. Some of the bigenic mice have developed large mammary tumors, which are being examined by our pathologist.
Figure 23. Flag-PRMT6 expression analysis in the mammary gland of bigenic STOP-PRMT6/MMTV-Cre mice. (A) STOP-PRMT6 mice were crossed with a K5-Cre mouse line and a MMTV-Cre mouse line. WB analysis on the mammary gland extracts using an anti-hPRMT6 antibody shows expression of the exogenous PRMT6 in the bigenic females from each cross. (B) Immuno-histochemistry using an anti-Flag antibody on paraffin embedded mammary gland tissue is shown here.
Figure 24. Bigenic STOP-PRMT6/MMTV-Cre mice display elevated histone arginine methylation in the mammary gland epithelium. Primary mammary epithelial cells were isolated from bigenic STOP-PRMT6/ MMTV-Cre and control (single transgenic) females and cultured for 3 days. (A) Cell lysates were subjected to WB analysis using an αPRMT6 antibody which recognizes human and mouse PRMT6. Core histones were isolated and subjected to WB analysis using αH3R2me2a and αH4R3me2a specific antibodies. (B) Double immuno-fluorescence using αFlag/αH3R2me2a or αFlag/αH4R3me2a antibody combinations was also performed.
Figure 25. STOP-PRMT6 /MMTV-Cre female mice display increased proliferation in the mammary gland. The fourth mammary glands from 13-14 week-old females were paraffin-embedded and stained for the proliferative marker Ki-67. The groups of mice under evaluation were WT (n=7), MMTV-Cre (n=6), STOP-PRMT6 (n=7) and bigenic STOP-PRMT6/MMTV-Cre (n=7). (A) Evaluation of number of Ki-67 positive nuclei per mm² duct tissue. The p value is 0.015 when the bigenic group is compared to the MMTV-Cre group. (B) Percentage of the selected area that was identified as being occupied by ducts. For statistical analysis, the Student t test was used. Values are for a 2-tailed t-test assuming unequal variance. p values are 0.014, 0.046 and 0.38 when the bigenic group is compared to the WT, STOP-PRMT6 and MMTV-Cre group, respectively.
5.7 A RNA-Seq experiment to investigate the impact of PRMT6 overexpression on mammary gland transcriptome

Since two regulatory histone marks, namely H3R2me2a and H4R3me2a, are aberrantly methylated in the mammary gland of the bigenic STOP-PRMT6/ MMTV-Cre mice, we sought to investigate the impact of PRMT6 over-expression on the transcriptome by performing a RNA-Seq experiment in this system. In order to perform this experiment, we isolated organoids from the bigenic STOP-PRMT6/MMTV-Cre, along with 2 control groups (STOP-PRMT6 and MMTV-Cre). After 3 days in culture, we isolated the RNA from these cells and carried out the RNA-Seq experiment (done by the Molecular Biology core of Science Park). In this experiment, by applying the EdgeR analysis, we found a list of 127 differentially regulated genes (mostly repressed in the bigenic mammary cells). With a more stringent analysis (DESeq) the list of genes differentially regulated consists of 17 genes (Table 2), which have yet to be confirmed by qPCR. This experiment suggests that PRMT6 indeed has a significant impact on gene expression.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold difference between bigenic and control</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
<td><strong>Fold difference (log2ratio) (fold difference)</strong></td>
<td><strong>p value</strong></td>
</tr>
<tr>
<td>Fxyd6</td>
<td>(-1.50402426) (0.352568561)</td>
<td>3.70E-06</td>
</tr>
<tr>
<td>Anpep</td>
<td>(-1.149596896) (0.450751158)</td>
<td>3.58E-05</td>
</tr>
<tr>
<td>Serpine2</td>
<td>(-0.875114644) (0.545210539)</td>
<td>6.77E-06</td>
</tr>
<tr>
<td>Ucp2</td>
<td>(-0.867534022) (0.548082881)</td>
<td>6.06E-06</td>
</tr>
<tr>
<td>Mgst1</td>
<td>(-0.814857227) (0.568464741)</td>
<td>2.97E-05</td>
</tr>
<tr>
<td>Adamts3</td>
<td>(-0.813866665) (0.568855186)</td>
<td>1.98E-05</td>
</tr>
<tr>
<td>4931406C07Rik</td>
<td>(-0.807541437) (0.571354701)</td>
<td>5.96E-07</td>
</tr>
<tr>
<td>Add3</td>
<td>(-0.688727492) (0.620400824)</td>
<td>2.01E-05</td>
</tr>
<tr>
<td>Prom1</td>
<td>(-0.678246736) (0.624924265)</td>
<td>8.21E-07</td>
</tr>
<tr>
<td>Mansc1</td>
<td>(-0.652710633) (0.636084073)</td>
<td>6.20E-06</td>
</tr>
<tr>
<td>Cdon</td>
<td>(-0.641127291) (0.641211724)</td>
<td>4.68E-05</td>
</tr>
<tr>
<td>Man1c1</td>
<td>(-0.62168415) (0.649911801)</td>
<td>4.67E-05</td>
</tr>
<tr>
<td>Tmem176a</td>
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<td>4.58E-05</td>
</tr>
<tr>
<td>Sort1</td>
<td>(-0.593026224) (0.66295083)</td>
<td>3.18E-05</td>
</tr>
<tr>
<td>Lama3</td>
<td>(0.86487064) (1.821176374)</td>
<td>4.88E-05</td>
</tr>
<tr>
<td>Abcc4</td>
<td>(0.886930746) (1.849237784)</td>
<td>5.41E-05</td>
</tr>
<tr>
<td>Nup210l</td>
<td>(1.058591443) (2.08289692)</td>
<td>3.61E-05</td>
</tr>
</tbody>
</table>

Table 2. List of genes differentially regulated in the mammary gland epithelial compartment of bigenic STOP-PRMT6/MMTV-Cre mice. RNA was isolated from primary mammary epithelial cells of STOP-PRMT6/MMTV-Cre, STOP-PRMT6 and MMTV-Cre mice. An RNA-Seq experiment was carried out and data were analyzed by DESeq. In red are the repressed genes, in green the up-regulated genes when the bigenic group was compared to the MMTV-Cre group. Data were reproducible when the bigenic group was compared to the STOP-PRMT6 group.
Chapter 6
Discussion and Future Directions

In this thesis I describe gain-of-function PRMT6 mouse models, which, to our knowledge, are the first PRMT6 transgenic models to be generated. The phenotype of the mouse model with ubiquitous PRMT6 stabilization (the ER*-PRMT6 model) is complex, with activated PRMT6 likely impacting the methylation levels of many substrates. The impacted substrates include dramatic changes in the histone code (which we have observed), and possibly elevated methylation levels on non-histone substrates. Chromatin is an active platform that mediates external and internal cellular signals into dynamic changes in gene expression. Studies on high-order complexes during activation of NF-κB-regulated genes and the consequent impact on chromatin remodeling through histone post-translational modification (PTM) have increased enormously in the past decade and it has become clear that the NF-κB transactivation is far more complex than anticipated (26). A number of coactivators and mediators have been discovered to regulate its transcriptional activity, indeed NF-κB recruits a coactivator complex that has striking similarities to that recruited by steroid nuclear receptors and the role of factors that impact histone tail posttranslational modifications has become evident (31). Histone arginine methylation by protein arginine methyltransferases has elicited a great deal of interest due to increasing evidence that it regulates chromatin remodeling and gene expression by providing docking sites for specific readers or effector molecules (4,24,83). Herein I have shown that PRMT6 functions as a coactivator for NF-κB-mediated gene transcriptional activity. I arrived at this finding by observing an activated inflammatory response in the transgenic ER*-PRMT6 mice. The specific involvement of PRMT6 in up-
regulating inflammatory genes was confirmed by demonstrating that overexpression of PRMT6 enhances NF-κB transcriptional activity in cultured cell-based luciferase assays and quantitative real-time PCR experiments. The activity of PRMT6 was clearly necessary for the coactivator function. ChIP analysis demonstrated that PRMT6 was recruited to the IL-6 promoter upon TNF-alpha stimulation. Moreover, overexpression of PRMT6 caused RelA shuttling into the nucleus, which could justify, at least in part, the mechanism underlying its coactivator function. Notably, PRMT6 has been found overexpressed in many types of human cancers (39,55,56), therefore the facilitation of NF-κB nuclear shuttling could be an important mechanism contributing to NF-κB-dependent gene activation in cancer (84). It remains to be addressed if the increased nuclear RelA is the result a direct shuttling due to PRMT6 binding to it or an indirect consequence of PRMT6 overexpression. Moreover, the increased PRMT6 localization at the IL-6 promoter upon stimulation is indicative of regulatory signaling on PRMT6, possibly by post-translational modification. One question that I sought to answer is: what is the role of arginine methylation in PRMT6 coactivation of NF-κB transcription? A number of PTMs on NF-κB subunits have been shown to affect its stability, function, subcellular localization and binding to DNA (85). It has recently been published that PRMT5 (a type-II arginine methyltransferase) methylates p65/RelA (86). This methylation event appears to increase the ability of NF-κB to bind to κB elements and to drive gene expression. I investigated the possibility of PRMT6 methylating RelA using in vitro methylation assays, but found that this is not the case. Other modes of PRMT6 regulation could be through the methylation of non-histone proteins that are associated with the NF-κB complex or through its ability to methylate histones. A recent publication has shown that PRMT6
behaves like PRMT1 and CARM1, indeed it works as a secondary coactivator to p160/SRC proteins (23). PRMT6 binds to the AD2 domain of SRC-1, and synergistically coactivates ERα together with SRC-1. Therefore, PRMT6 can be considered a steroid hormone receptor coactivator which interacts with SRC-1 to facilitate transcription. A likely mechanism by which PRMT6 will function as a coactivator has recently come to light with the finding that it methylates a site in the core of histone H3, H3R42me2a (25). Importantly, the side chain of this arginine residue interacts with the DNA minor groove and methylation of this site is proposed to sterically interfere with this interaction, as well as remove a potential hydrogen bond donor, thus destabilizing the histone:DNA interaction. Indeed, the incorporation of semisynthetic H3R42me2a into core histone octamers, and use of these octamers in reconstituted chromatin for the generation of chromatinized transcription template revealed this to be the case. Unfortunately, there are currently no antibodies available to the H3R42me2a mark, so we are unable to test the hypothesis that this histone methylation site is responsible for the coactivator functions of PRMT6 in the context of nuclear receptor and NF-κB signaling. PRMT6 has also been reported to generate the active site H4R3me2a in vitro (9), therefore I sought to investigate if this mark is also generated in vivo, which could account for a mechanism of coactivation. I found that indeed not only PRMT6 overexpression causes global increase of this mark in vivo, but also that this mark enriches at the IL-6 promoter upon TNF-alpha stimulation in ChIP assays, concomitantly with PRMT6 recruitment, although the influence of PRMT1 in this assays cannot be excluded. The impact of PRMT6 on H4R3 could also explain the coactivator function towards nuclear receptors, but this hypothesis remains to be tested.
Due to lethality of the ER*-PRMT6 mouse model, I could not carry out cancer studies with it, therefore a Cre-inducible mouse model was generated, allowing for tissue-specific overexpression of PRMT6. These mice were crossed to K5-Cre and MMTV-Cre mice. The first experiment I set up was a skin carcinogenesis study utilizing the bigenic STOP-PRMT6/K5-Cre mice, but did not observe a difference in tumorigenicity between the experimental and control groups. This is likely due to the extreme focal nature of the Flag-PRMT6 expression in the epidermis and hair follicles of the bigenic mice. According to the cancer stem cells hypothesis for cancer development, the mechanism for tumor initiation in two-stage carcinogenesis protocols that employ DMBA/TPA involves mutation in the critical target Ha-ras gene of stem cells in the bulge region of hair follicles or basal compartment of interfollicular epidermis (57). It is thus likely that none of the cells that were targeted for Ha-ras mutation by the initiating agent DMBA was in fact over-expressing the exogenous Flag-PRMT6, therefore no difference in tumor susceptibility was detected.

From the second cancer study I carried out (by performing a cross with p53+/-mice) I did not detect increase tumorigenicity in the bigenic STOP-PRMT6/K5-Cre;p53+/-mice compared to the controls, although an interesting phenomenon occurred, indeed the females of the bigenic group showed signs of ongoing inflammatory response. This result is in line with our observations of the ER*-PRMT6 model, which also showed an inflammatory response upon PRMT6 stabilization. One question that arises is: why do only the females showed this phenotype? Although there are numerous reports showing negative cross-talk between ER and NF-κB (70,87-90), these transcription factors have also been shown to act synergistically (91-95) by forming a complex at the estrogen
responsive elements (EREs) or NF-κB responsive elements found in gene promoters. In particular, the group of Stanculescu (95) has shown that E2 enhances TNF- alpha activity on ≈15 % of TNF-alpha up-regulated genes. With this in mind, I can speculate that the increased inflammatory response observed in the female mice in our study might be the result of this synergism, given that PRMT6 is a coactivator for both these transcription factors.

With the MMTV-Cre cross I found an interesting phenotype, indeed the bigenic STOP-PRMT6/MMTV-Cre mice displayed a hyper-proliferative phenotype in the mammary gland, which was detected by Ki-67 analysis (see Figure 25). Upon this observation, I set-up an ageing study to investigate the possibility of increased spontaneous mammary carcinogenesis in the bigenic STOP-PRMT6/ MMTV-Cre mice compared to the controls. It is possible that the MMTV-Cre transgene per se is creating the ideal conditions for PRMT6 to enhance cancer susceptibility. Peculiar phenotypes in Cre-expressing mouse lines have been previously described. For example one of the MMTV-Cre lines (the Line A) that were generated by the group of Hennighausen (82) displays a lactation defect (96). For this reason, this line has been cryopreserved and is not currently being used by any laboratory. The mice from the MMTV-Cre cross have been aged until 12 months and analyses on the mammary tissue along with the controls are underway. Some of the bigenic mice have developed large mammary tumors that are currently being examined. If this study turns out to be inconclusive, it might be worth to cross the STOP-PRMT6 mice with an oncogenic mouse model (i.e. MMTV-Myc mouse), considering the hypothesis that PRMT6 may have a role in tumor promotion rather than initiation. In the future, this type of mouse model could be valuable for the in vivo analysis
of small molecule PRMT inhibitors that are currently being developed both in academic and pharma sets (97).

From the last experiment I carried out (RNA-Seq on primary mammary epithelial cells) we found a list of genes that are potentially - directly or indirectly- regulated by PRMT6. One gene that is up-regulated is **miR-1943**. MicroRNAs (miRNAs) have shown to play important roles in physiological and malignant processes, including acute myeloid leukemia (AML). The group of Humphries (98) applied the Illumina massively parallel sequencing platform to carry out an in-depth analysis of the miRNA transcriptome in a murine leukemia progression model and found **miR-1943** among the most significantly up-regulated miRs. It would be interesting to investigate if there is direct regulation of this miR by PRMT6 at chromatin level, since PRMT6 is up-regulated in leukemia.
References


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Alessandra Di Lorenzo was born in Castellammare di Stabia, Italy, in 1975 to Raffaela Cuomo and Salvatore Di Lorenzo. She attended the University of Naples Federico II, Italy, where she obtained a Bachelors and Masters of Science in 2000. She then worked as Research Assistant at the University of Naples Federico II for 5 years and as a scientific translator and proofreader for 2 years before joining the Graduate School of Biomedical Sciences at The University of Texas M.D. Anderson Cancer Center, in August 2007.