1. Introduction

Methylation is one of the major controlling posttranscriptional modification in protein function, however the cellular consequences are not well understood yet. Among others its role was implyed in gene expression regulation during cellular differentiation, such as nerve growth factor (NGF) mediated neurite outgrowth of PC12 rat pheochromocytoma cell line (Cimato, Ettinger et al. 1997). Arginine methylation was found to be more prevalent than carboxylmethylation in this process (Cimato, Tang et al. 2002).

Arginine methylation is catalyzed by protein N-arginine methyltransferases (PRMTs). Two different classes of protein arginine methyltransferase enzymes are known. Type I enzymes (PRMT1, 3, 4, 6 and 8) catalyze the formation of asymmetric NG,NG-dimethylarginine (aDMA) residues while the Type II (PRMT5) enzymes catalyze the formation of symmetric NG,N'G-dimethylarginine (sDMA) residues. Both enzyme types generate NG-monomethylarginine (MMA) intermediates. The activity of PRMT2, PRMT7 and PRMT9 has not yet been identified (reviewed in (Bedford and Clarke 2009) .

PRMT1 is the major type of protein arginine methyltransferase. PRMT1 null mice are lethal at a very early embryonic stage, indicating its essential role in embryonic development (Pawlak, Scherer et al. 2000). The same study also revealed the high expression of PRMT1 *in vivo* in the developing nervous system. Futhermore, a recent study already implicated PRMT1 in neurite outgrowth of Neuro2a neuroblastoma cells (Miyata, Mori et al. 2008). Homodimerization or homooligomerization of the PRMT1 subunit is important for its enzymatic activity (Zhang and Cheng 2003). Interestingly, the novel arginine methyltransferase PRMT8 is able to form heterodimer with the PRMT1 (Lee, Sayegh et al. 2005). Substrate specificity, cooperation and redundancy between the two proteins has not yet been investigated. PRMT8 share more than 80% homology with PRMT1, and importantly its expression is primarily limited to the brain. Until now, PRMT8 localization has been studied only in mouse central nervous system (Kousaka, Mori et al. 2009), but not in the context of developing neurons.

Importantly, the widely used PC12 cell line and Neuro2a cells resemble sympathetic neurons of the peripheral nervous system (PNS), but not of neurons of the central nervous system (CNS). Furthermore, such transformed cell lines offer limited possibility to investigate the role of PRMTs in progenitors.

Mouse embryonic stem (ES) cells are pluripotent cell lines derived from the inner cell mass of the blastocyst. ESCs can give rise to neuroectodermal derivatives in culture when treated with all-trans retinoic acid (ATRA). ATRA and its derivatives are the natural ligand for a class of nuclear receptors comprised of two subfamilies: the RARs and the retinoid X receptors (RXRs).

In the recent study we used first time embryonic stem cell to determine the role of Type I arginine methyltransferases in neural differentiation. We identified PRMT1 and PRMT8 as two member of Type I arginine methyltransferase showing elevated expression during neurogenesis. We have generated PRMT1 and 8 knockdowns Role of PRMT1 was analyzed in details in ES cells and early differentiated embryoid bodies using genome-wide analysis. We revealed the role of PRMT1 and 8 in retinoic acid induced neural differentiation.

2. Results

PRMT1 and PRMT8 expressions are increased during neural differentiation

Retinoic acid driven neural differentiation of ES cells has been set up as a model system (Figure 1A). As a first step we performed western blot analysis using Asym24 antibody that recognize asymmetrically arginine methylated proteins. A clear difference can be detected between undifferentiated ES cells and neural progenitors (Figure 1B). Proteins with size around 70 kDa, presumably the known PRMT methylated Sam68, show increased methylation in ATRA-treated samples. In contrast, a protein with size 34 kDa show downregultion during both spontaneous and neural differentiation (Figure 1B, 1E). CARM1 (also known as PRMT4) has been shown that play crucial role in maintainance of pluripotent phenotype, while a PRMT inhibitor, called AMI-5 has been recently demonstrated that enables Oct4-induced reprogramming of mouse embryonic fibroblasts, suggesting important role of arginine methylation in pluripotent phenotype. To unravel which PRMTs are liable for the detected methylation in ES cells we collected data from our genome-wide expression analysis that proved the presence of CARM1, PRMT1 and PRMT3. As a next step we identified the expressional pattern of Type I PRMTs in neurogenesis. As Figure 1D and 1E show PRMT1 and PRMT8 are upregulated during neural differentiation. While PRMT1 is already expressed in ES cells, PRMT8 can be detected only in late timepoint of the differentiation. Appearance of PRMT8 is in correlation with elevated Tuj1 expression.

PRMT1 depletion does not effect the ES pluripotency

To elucidate the role of PRMT1 and PRMT8 in neural differentiation we established PRMT1 and PRMT8 knockdown ES cells. As PRMT8 is not expressed in ES cells we could only validate the PRMT1 knockdown in ESC. Interestingly, loss of PRMT1 leads to significant decrease in total asymmetric arginine methylation, with the exception of the ~34 kDa protein. Comparison of knockdown and wild-type ES cells showed no alteration in typical ES cell markers, suggesting that PRMT1 is not responsible for maintaining pluripotency. The fact, that the ~34 kDa protein show stepped decrease during both spontaneous or neural differentiation (Figure 1E) and it is not influenced by PRMT1 downregulation may suggest that this protein is methylated by CARM1 and participate in regulation of pluripotent phenotype.

PRMT1 show cytoplasmic localization, which raise the hypothesis that in this cell type PRMT1 is not participate directly in transcriptional regulation via its coactivator function.

To further study the role of PRMT1 in undifferentiated cells we performed a genome-wide comparison of siPRMT1 and siSCR cells. GO classification of significantly different genes suggest the importance of PRMT1 in extracellular matrix and small nucleolar RNAs.

However we could detect alteration in several retinoid signaling related genes (Cyp26a1, RXRg, Tgm2, etc.) our results excluded the coregulator role of PRMT1 in the retinoid signaling both in ES and EB.

We have also tested the effect of PRMT1 depletion in early spontaneous differentiation (PRMT8 at this stage is still not expressed). As Figure 2D show, there is no complete block in any main lineage differentiation, however the mesoderm differentiation seems more pronounced.

PRMT1 and PRMT8 does not influence significantly ATRA-induced neurite outgrowth

We have used the generated knockdown cells to compare the differentiation ability of these cells. As Figure 3A show the knockdown effect is stable in case of PRMT1 during the 12 day and the expression is decreased to half in case of PRMT8. We have also measured the efficiency of knockdowns at day 10 on protein level (Figure 3B).

In contrast with our expectation we could see neurite outgrowth in each case. To exclude that it is due to the residual expression of PRMTs, we repeated the experiment using PRMT1 -/- ES cells (supplementary). Independently we also tested the effect of AMI-1, a PRMT1 specific inhibitor, that resulted similar phenomena (Supplementary). In case of PRMT8 we had no available knockout ES cell as a control. However the fact, that PRMT8 full body knockout mice are viable

without any significant detectable neurological problem may certify our results. AdOX, a non specific arginine methyltransferase inhibitor were toxic for the cells before the cells started to differentiate.

Real-time quantitative PCR (RT-qPCR) indicates that Pax6 (neuronal precursor marker), is rapidly induced afer ATRA-treatment in both wild-type, siSCR , siPRMT1 and siPRMT8 cells, then show similar decrease in each cell type when neurons differentiate. Similar expression level can be detected in case of other neural markers, such as Tuj1 (Neuron-specific class III beta-tubulin), Neurog2 (neurogenin 2) and Lhx1 (LIM homeobox 1). These data has been further confirmed using AMI-1 treated cells (Supplementary). These results suggest that PRMT1 is not essential for neurite outgrowth. PRMT8 is appear 2 days later than Pax6, in the same time as Neurog2, Lhx1 and Tuj1 are appearing.

Based on these results we can conclude that loss of PRMT1 is not blocking any of the differentiation pathways.

References

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Cimato, T. R., M. J. Ettinger, et al. (1997). "Nerve growth factor-specific regulation of protein methylation during neuronal differentiation of PC12 cells." J Cell Biol **138**(5): 1089-103.

Cimato, T. R., J. Tang, et al. (2002). "Nerve growth factor-mediated increases in protein methylation occur predominantly at type I arginine methylation sites and involve protein arginine methyltransferase 1." J Neurosci Res **67**(4): 435-42.

Kousaka, A., Y. Mori, et al. (2009). "The distribution and characterization of endogenous protein arginine N-methyltransferase 8 in mouse CNS." Neuroscience **163**(4): 1146-57.

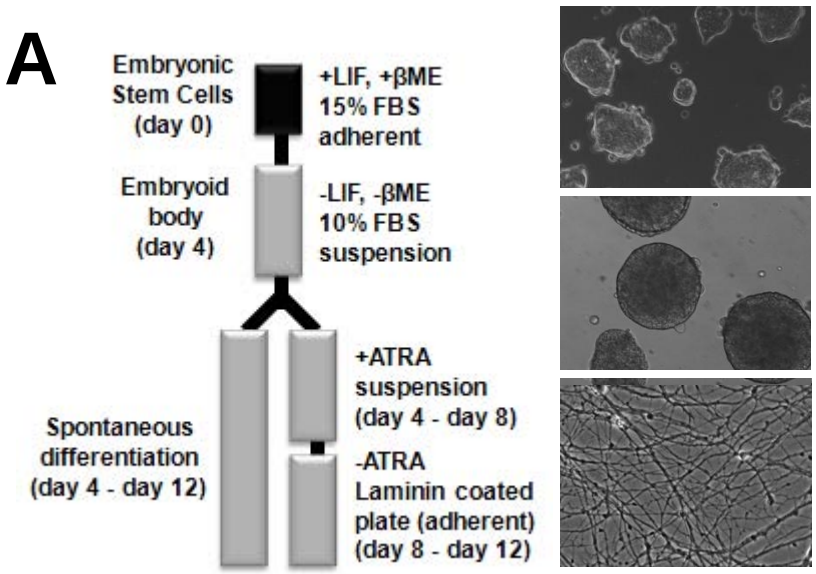
Lee, J., J. Sayegh, et al. (2005). "PRMT8, a new membrane-bound tissue-specific member of the protein arginine methyltransferase family." J Biol Chem **280**(38): 32890-6.

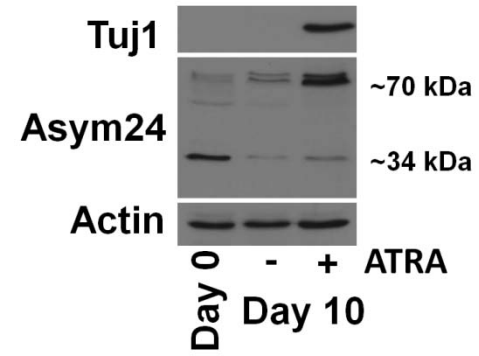
Miyata, S., Y. Mori, et al. (2008). "PRMT1 and Btg2 regulates neurite outgrowth of Neuro2a cells." Neurosci Lett **445**(2): 162-5.

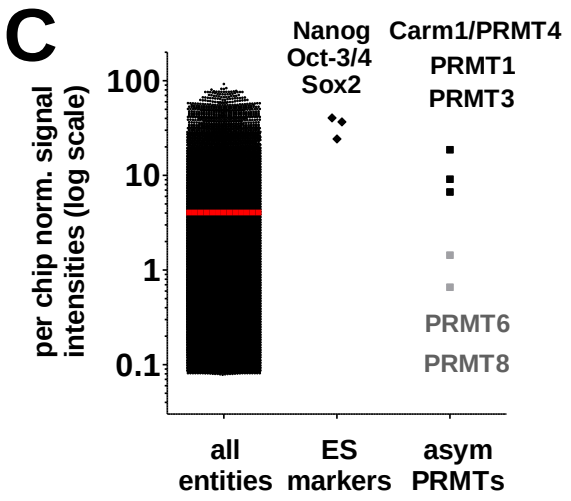
Pawlak, M. R., C. A. Scherer, et al. (2000). "Arginine N-methyltransferase 1 is required for early postimplantation mouse development, but cells deficient in the enzyme are viable." Mol Cell Biol **20**(13): 4859-69.

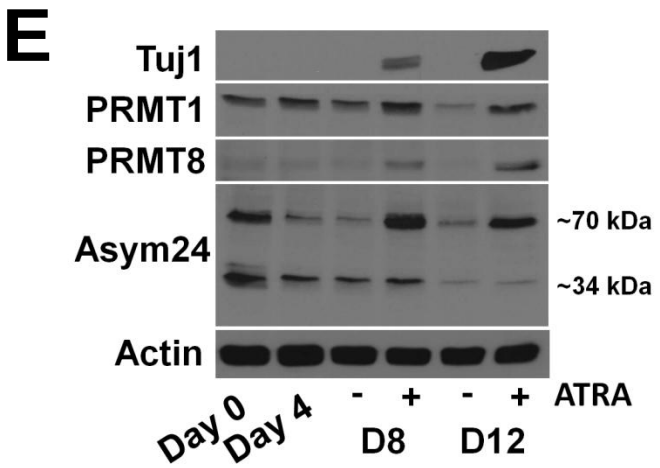
Zhang, X. and X. Cheng (2003). "Structure of the predominant protein arginine methyltransferase PRMT1 and analysis of its binding to substrate peptides." Structure **11**(5): 509-20.

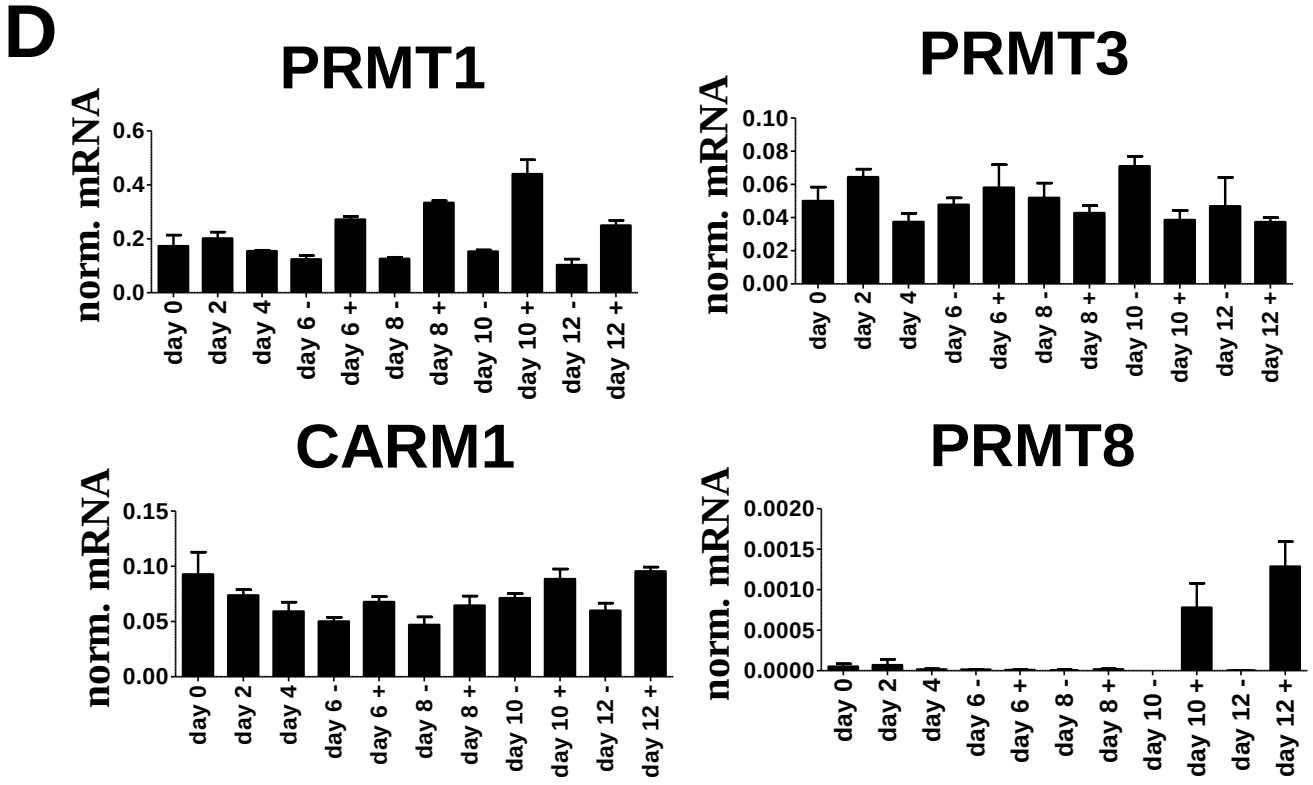
Figure 1.


A
Embryonic Stem Cells (day 0)
+LIF, +βME 15% FBS adherent
Embryoid body (day 4)
-LIF, -βME 10% FBS suspension
+ATRA suspension (day 4 - day 8)
-ATRA Laminin coated plate (adherent) (day 8 - day 12)
Spontaneous differentiation (day 4 - day 12)


B
Tuj1
Asym24
Actin
~70 kDa
~34 kDa
Day 0
-
+
ATRA
Day 10


C
per chip norm. signal intensities (log scale)
100
10
1
0.1
Nanog
Oct-3/4
Sox2
Carm1/PRMT4
PRMT1
PRMT3
PRMT6
PRMT8
all entities
ES markers
asym PRMTs


E
Tuj1
PRMT1
PRMT8
Asym24
Actin
~70 kDa
~34 kDa
Day 0
Day 4
-
+
-
+
ATRA
D8
D12


D
PRMT1
norm. mRNA
0.6
0.4
0.2
0.0
day 0
day 2
day 4
day 6 -
day 6 +
day 8 -
day 8 +
day 10 -
day 10 +
day 12 -
day 12 +
PRMT3
norm. mRNA
0.10
0.08
0.06
0.04
0.02
0.00
CARM1
norm. mRNA
0.15
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0.05
0.00
PRMT8
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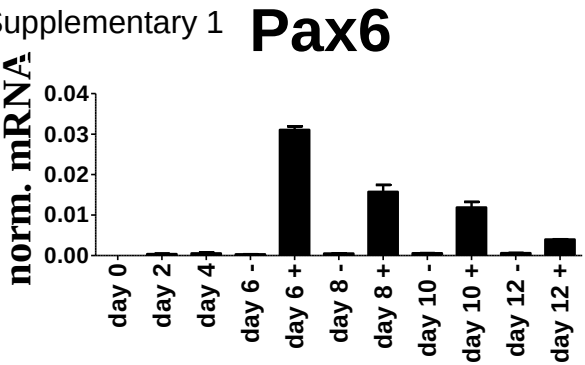
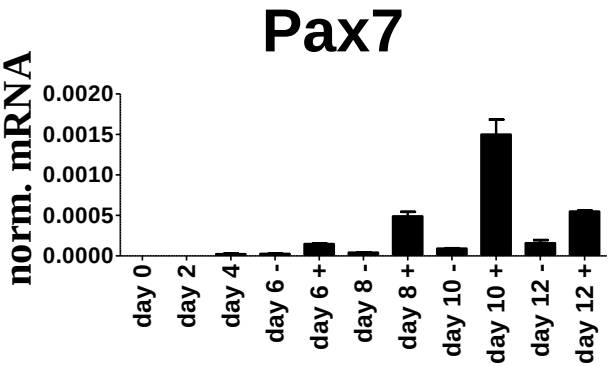
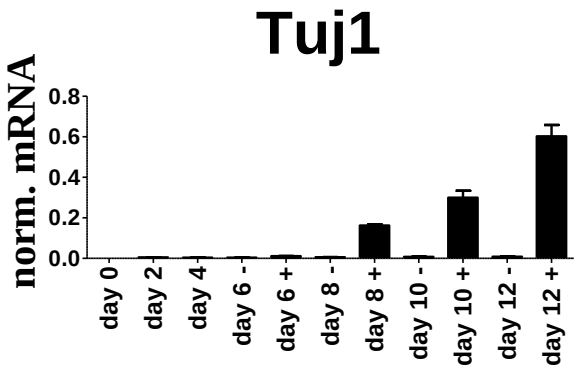
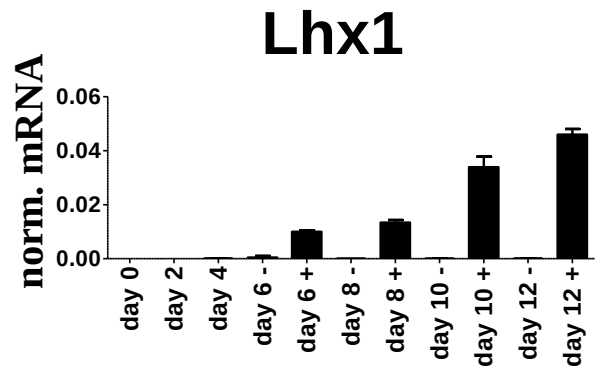
0.0010
0.0005
0.0000

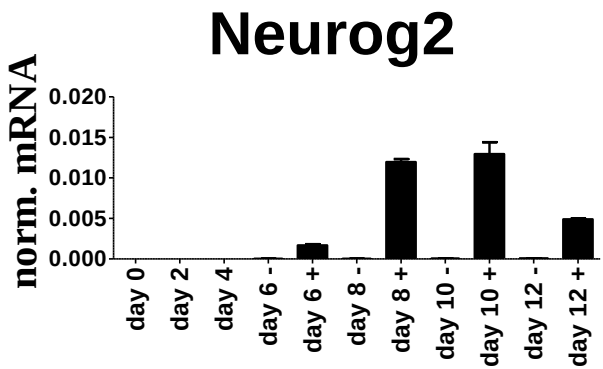
Supplementary 1

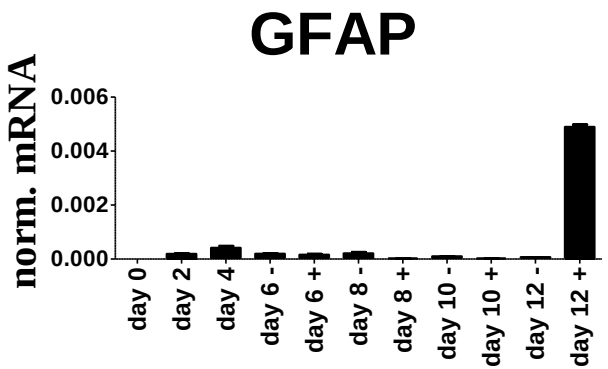












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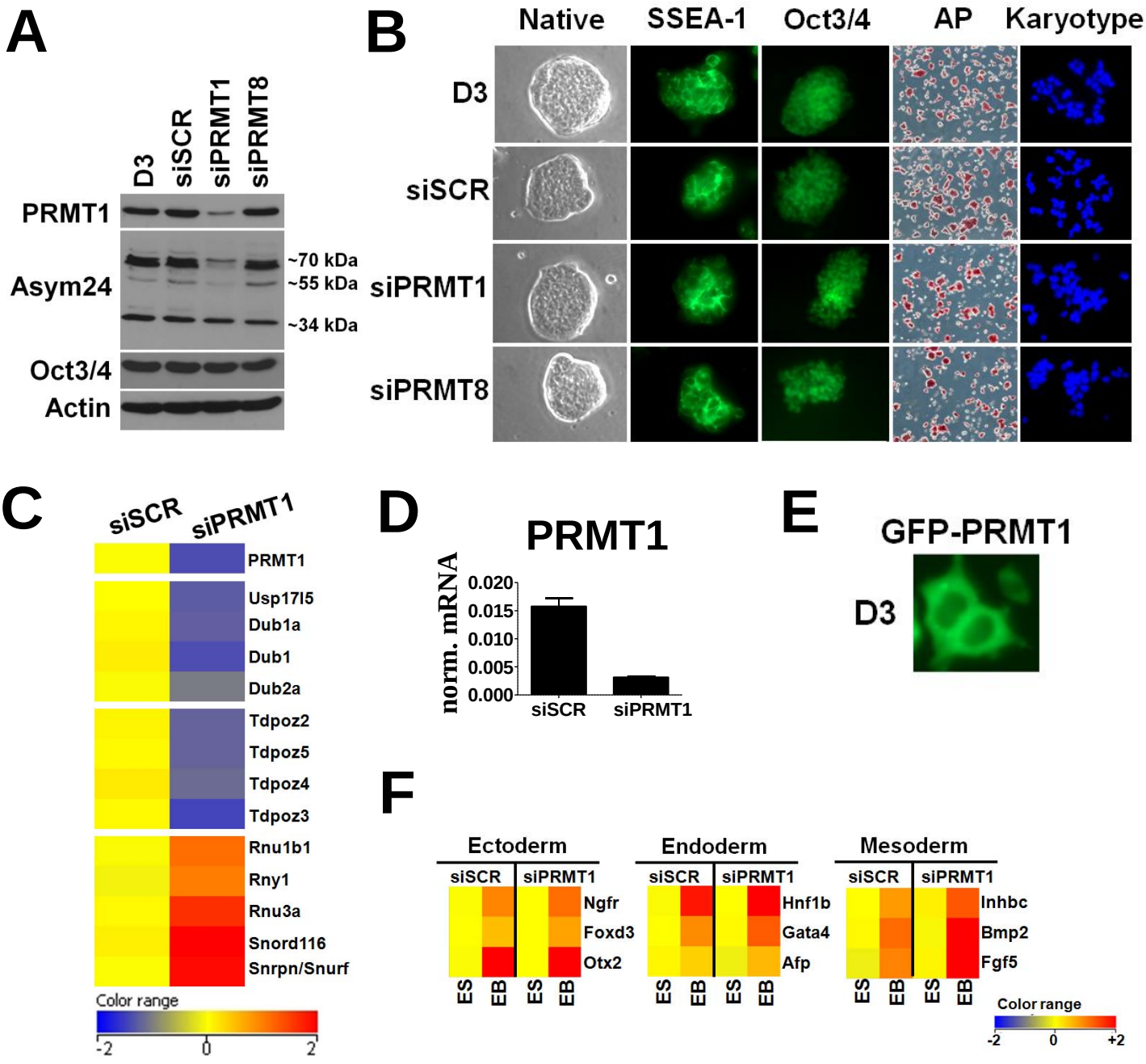
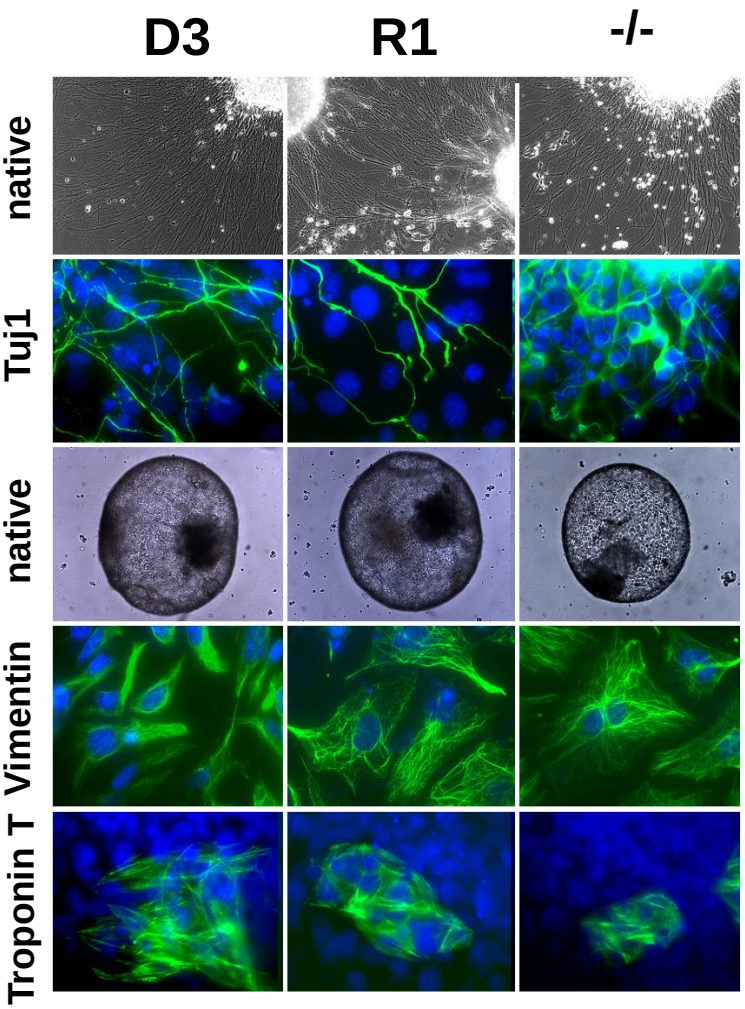

A
D3
siSCR
siPRMT1
siPRMT8
PRMT1
Asym24
~70 kDa
~55 kDa
~34 kDa
Oct3/4
Actin
B
Native
SSEA-1
Oct3/4
AP
Karyotype
D3
siSCR
siPRMT1
siPRMT8
C
siSCR
siPRMT1
PRMT1
Usp17l5
Dub1a
Dub1
Dub2a
Tdpoz2
Tdpoz5
Tdpoz4
Tdpoz3
Rnu1b1
Rny1
Rnu3a
Snord116
Snrpn/Snurf
Color range
-2
0
2
D
PRMT1
norm. mRNA
0.020
0.015
0.010
0.005
0.000
siSCR
siPRMT1
E
GFP-PRMT1
D3
F
Ectoderm
Endoderm
Mesoderm
siSCR
siPRMT1
Ngfr
Foxd3
Otx2
Hnf1b
Gata4
Afp
Inhbc
Bmp2
Fgf5
ES
EB
Color range
-2
0
+2

A

Supplementary 2A

Supplementary 2B



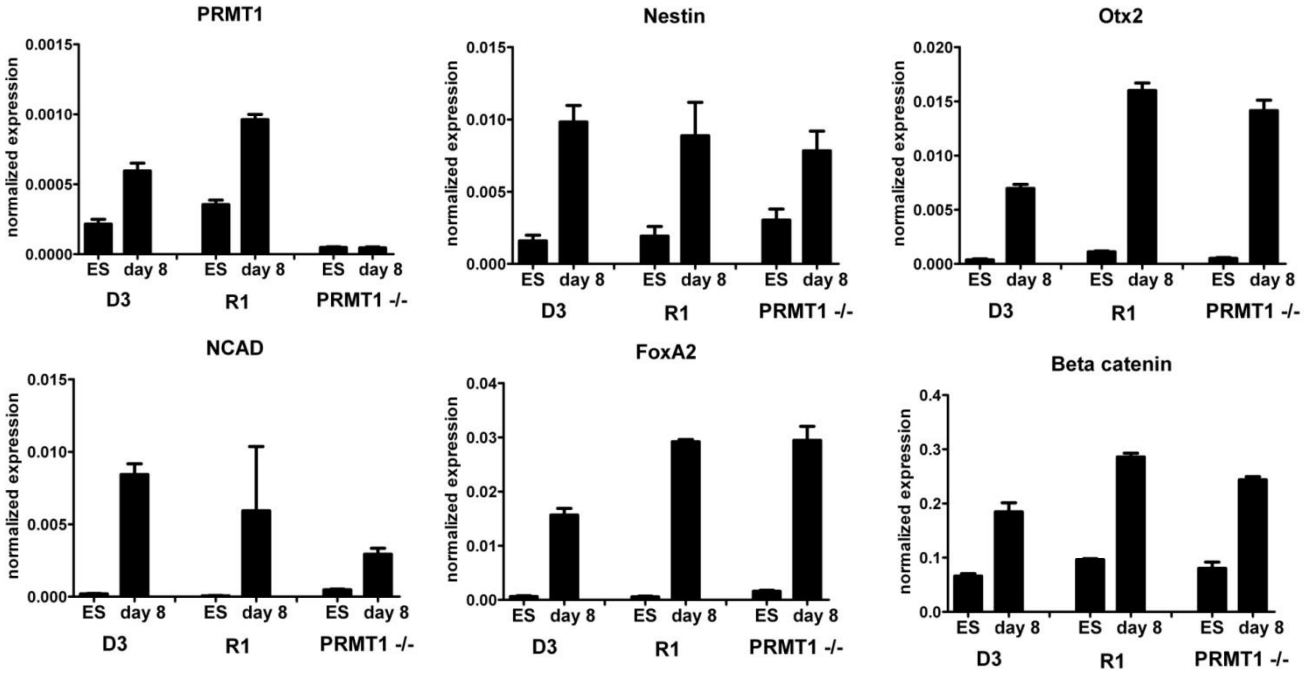
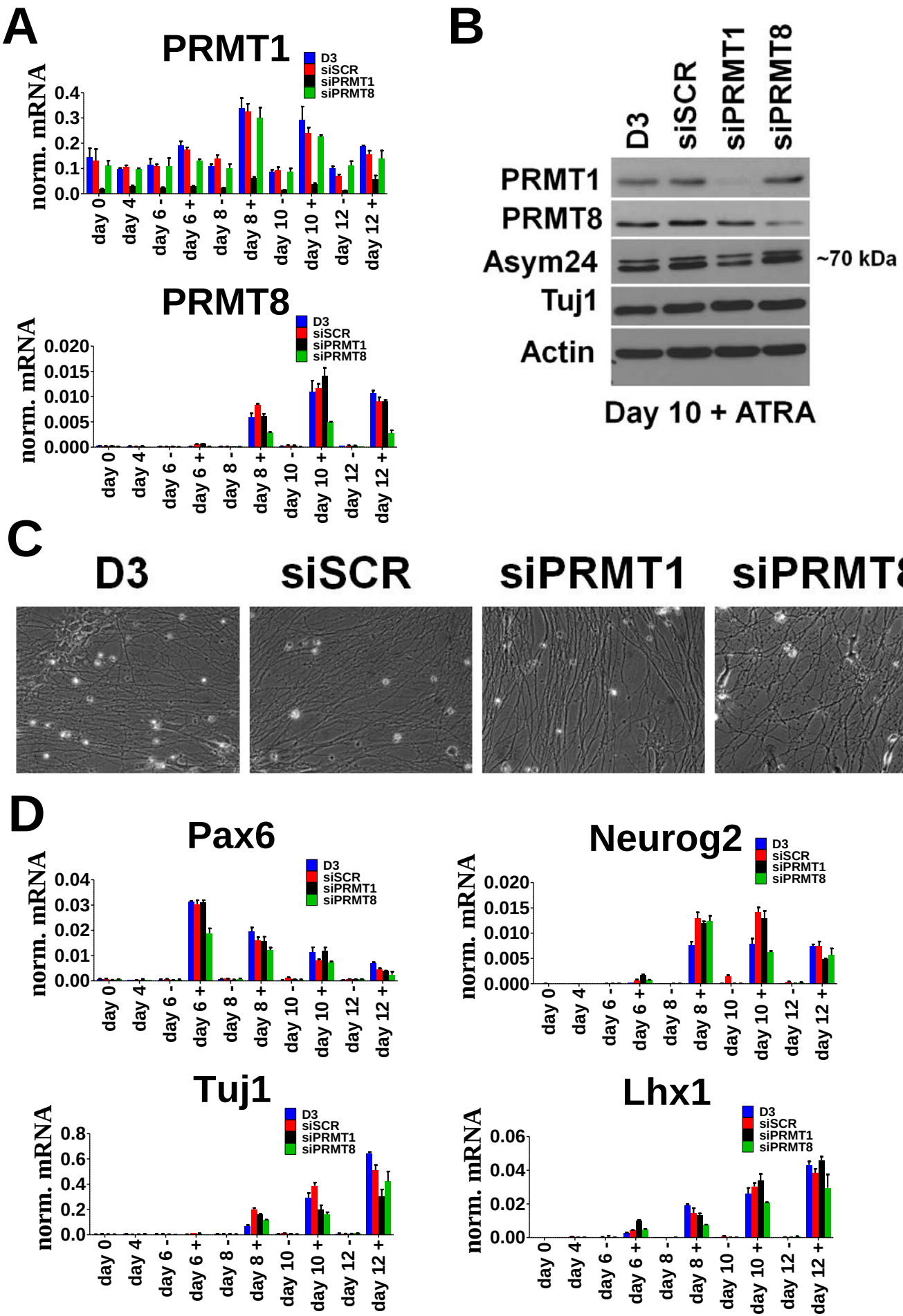
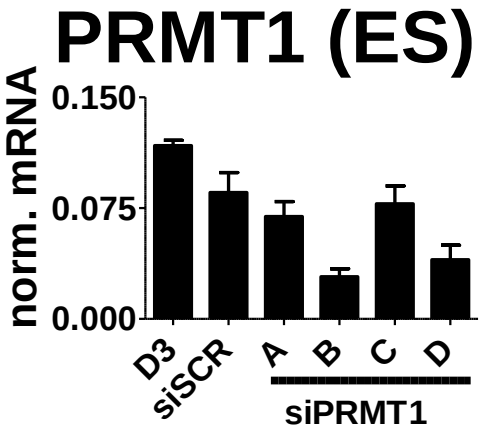


Figure 3.


A
PRMT1
norm. mRNA
D3
siSCR
siPRMT1
siPRMT8
0.4
0.3
0.2
0.1
0.0
day 0
day 4
day 6 -
day 6 +
day 8 -
day 8 +
day 10 -
day 10 +
day 12 -
day 12 +
PRMT8
norm. mRNA
0.020
0.015
0.010
0.005
0.000
B
D3
siSCR
siPRMT1
siPRMT8
PRMT1
PRMT8
Asym24
Tuj1
Actin
~70 kDa
Day 10 + ATRA
C
D3
siSCR
siPRMT1
siPRMT8
D
Pax6
norm. mRNA
0.04
0.03
0.02
0.01
0.00
Neurog2
0.020
0.015
0.010
0.005
0.000
Tuj1
0.8
0.6
0.4
0.2
0.0
Lhx1
0.06
0.04
0.02
0.00

Supplementary 3

A



Constr.	Sequence
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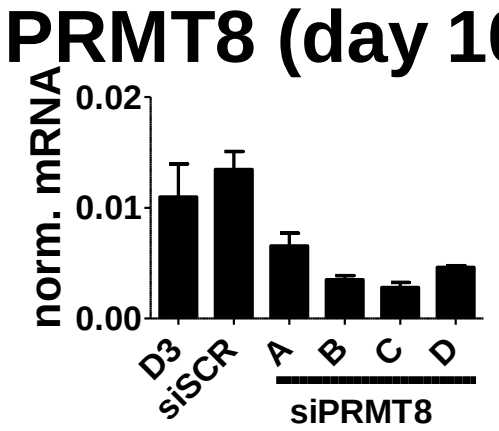
B	**GCTGAGGACATGACATCCAAA**
C	CGCAACTCCATGTTTCACAAT
D	CGCAACTCCATGTTTCACAAT

B



Constr.	Sequence
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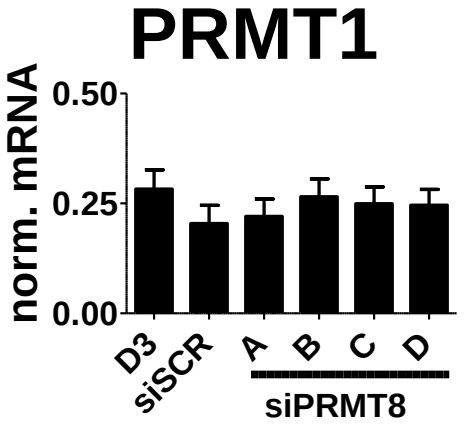
D	GATTTCACAGTAGACTTGGAT

C



Supplementary 4

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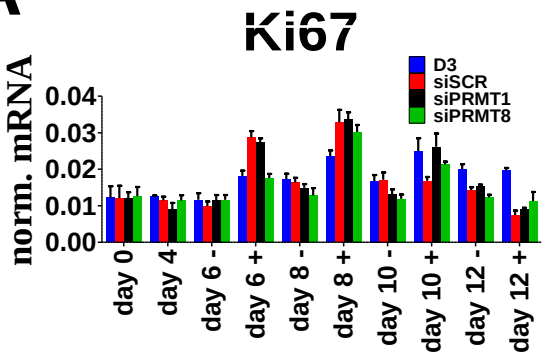
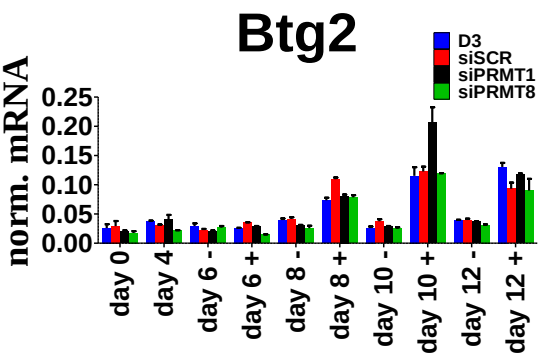
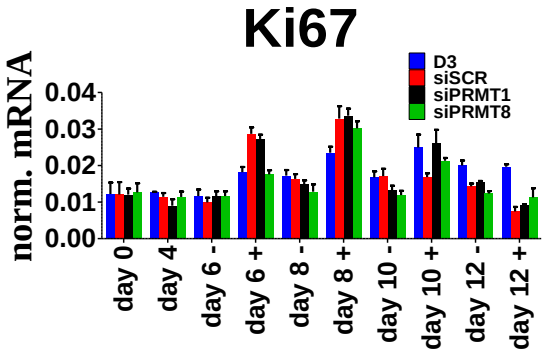
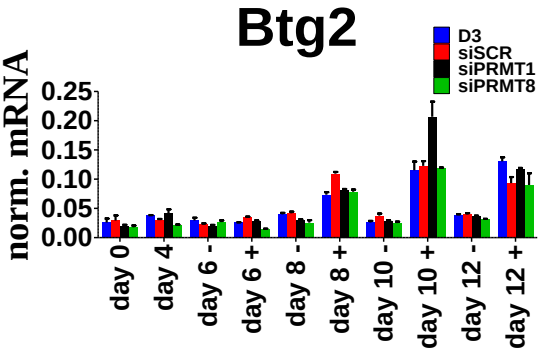


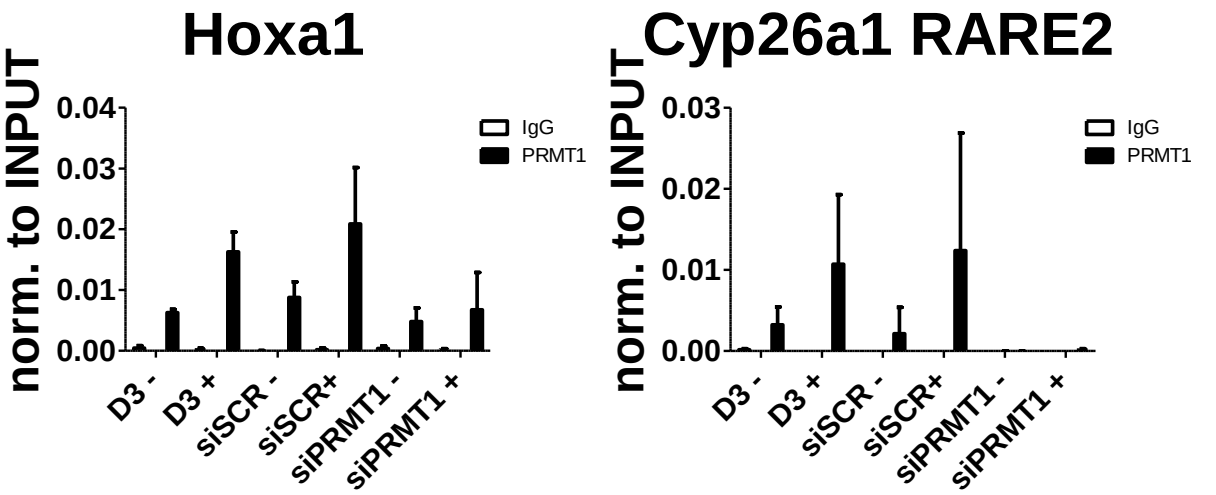






Supplementary 5

A



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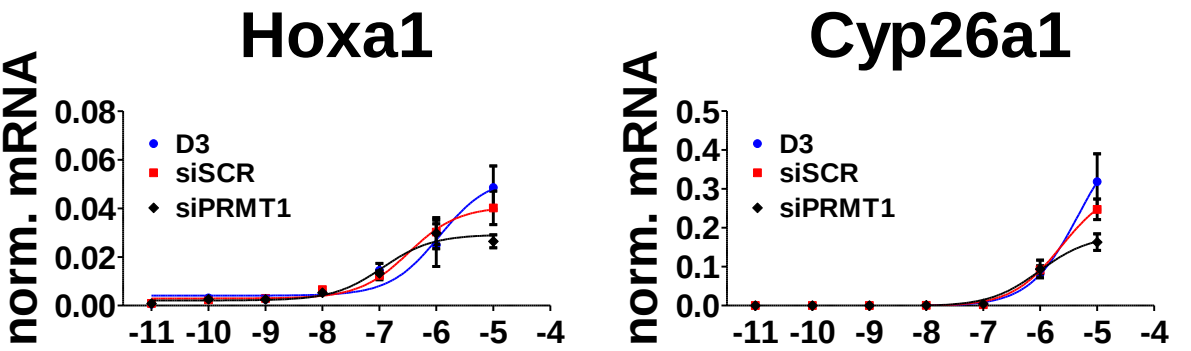

C