THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Transcriptional and epigenetic program of embryonic stem cell differentiation into neurons by retinoic acid

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I. ABBREVIATIONS

aDMA	asymmetric arginine dimethylation
BSA	bovine serum albumin
ChIP	chromatin immunoprecipitation
CNS	central nervous system
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DR	direct repeat
ESC	embryonic stem cell
FBS	fetal bovine serum
HDAC	histone deacetylase
kDa	kilodalton
LBD	ligand binding domain
NR	nuclear receptor
RT-qPCR	real-time quantitative polymerase chain reaction
PRMT	protein arginine methyltransferase
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RXR	retinoid x receptor
sDMA	symmetric dimethylarginine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
SMRT	silencing mediator of retinoid or thyroid receptor
TF	transcription factor
TSS	transcription start site

II. INTRODUCTION

In molecular terms, the DNAs of all differentiated cells are identical. The same genome is responsible for making all the cell types of the body, each of which has its own function: the information transmitting neurons, red blood cells that exchange oxygen, muscle cells that are able to expand and contract, and cells in the immune system which recognize pathogens. How a cell fate is determined and how such tremendously diverse cells are generated during development are intriguing questions in biology and has great importance in regenerative medicine.

Specific functions of different cell types are generated through differential gene regulation. It is estimated that the human genome encodes approximately 25,000 protein coding genes (Consortium 2004). Only a small percentage of the genome is expressed in each cell. Almost all cells express, in any given tissue or in any given organism, housekeeping genes, such as genes for transcription, translation, metabolic activity or transportation. This accounts for more than 50-60% of the total number of genes. However, the rest of the genes are expressed in a tissue-specific manner. These genes, that regulate cell identity, are turned on under very specific temporal, spatial, and environmental conditions to ensure that a cell is able to perform its designated function.

How does this differential gene expression occur? Understanding the tissue-specific pattern of gene expression is critical in elucidating the molecular mechanisms of tissue development, gene function, and transcriptional regulations of biological processes (Song, Ahn et al. 2013). Among others, the Encyclopedia of DNA Elements (ENCODE) is one of the largest worldwide efforts which aimed to extend our recent knowledge about which regions in a particular cell type are transcribed into RNA, which regions are likely to control the genes that are used in a specific cell, and which regions are associated with a

wide variety of proteins (Ho, Jung et al. 2014). These large-scale studies highly influenced our understanding about the regulation of transcription in eukaryotes.

According to our recent knowledge, regulation of transcription in eukaryotes is a result of the combined effects of the (1) chromatin structure (how DNA is "packaged") and (2) the interactions of proteins called transcription factors and co-regulators.

III. LEVELS OF GENE EXPRESSION REGULATION

1. Chromatin structure

The packaging of genomic DNA into chromatin regulates processes that use DNA as template such as DNA replication, cell cycle progression and gene expression. In the eukaryotic nucleus, DNA is complexed with histones and non-histone proteins in a dynamic polymer called chromatin.

Epigenetic regulation of chromatin structure is fundamental to the activation or repression of genes during embryonic development. From a chromatin point of view, eukaryotic genomes can generally be divided into two geographically distinct environments. The first is a relatively relaxed, "open" state, containing most of the active genes, referred to as euchromatin. Other genomic regions, such as centromers, telomers and most of the inactive genes are in a compact environment that is called heterochromatin (Bannister and Kouzarides 2011).

The fundamental structural unit of chromatin is the nucleosome which consists of 146 bp of DNA wrapped around an octamer of histones containing two copies each of four core histones (H2A, H2B, H3 and H4). Histones are consists of two separate functional domains: 1) a histone fold motif for both histone-histone and histone-DNA contacts within the nucleosome and 2) an NH-terminal and COOH-terminal tail domain that contain sites for posttranslational modifications, such as acetylation, phosphorylation,

methylation and ubiquitination. Euchromatin and heterochromatin are both enriched or depleted for certain histone modifications. A histone protein can undergo distinct posttranslational modifications. There are over 60 different residues on histones where modifications have been detected either by mass spectrometry or specific antibodies (Kouzarides 2007) (Figure 1).

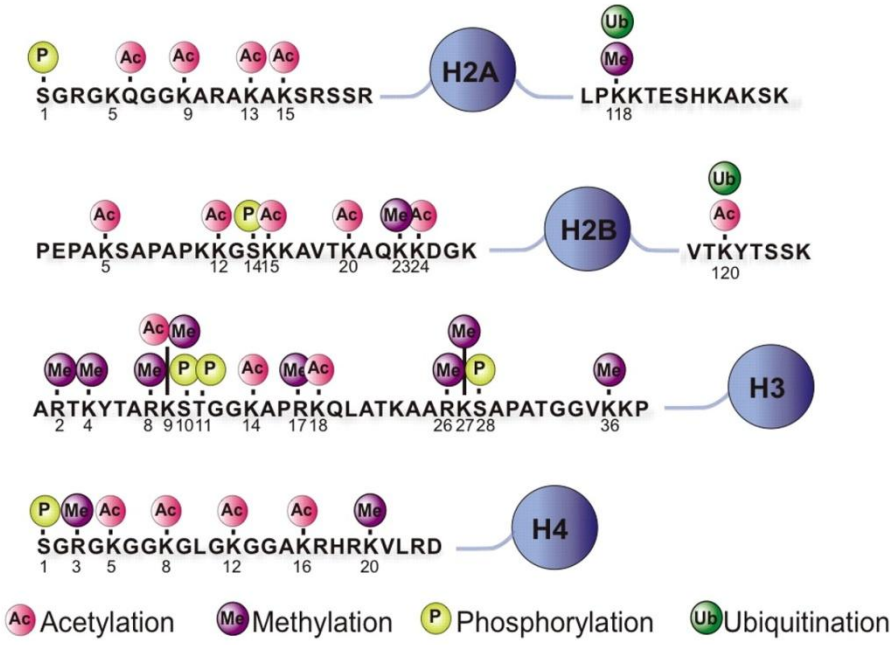

Figure 1. Overview of histone tail modifications. *Taken from* (Kato, Inoue et al. 2010).

The histone modifications altering the higher-order chromatin structure by affecting the interaction between histones and DNA (Mersfelder and Parthun 2006). For the purposes of transcription, modifications can be divided into those that correlate with active transcription and those which correlate with repression. Of all the known modifications, acetylation has the highest potential to unfold chromatin structure via neutralization of the basic charge of lysine (Allfrey, Faulkner et al. 1964; Kouzarides 2007). Phosphorylation is another modification which has important consequences for chromatin compaction.

Extra complexity comes from the fact that methylation at lysine residues may result in three different forms: mono-, di- or trimethyl-lysine (Zhang, Bernatavichute et al. 2009).

Arginine methylation also results in three forms: monomethylation and asymmetric or symmetric-dimethylation (Gayatri and Bedford 2014). These distinct forms of methylated lysine or arginine residues are signatures of functionally different regions. Table 1 summarize examples of functional associations most typically observed with certain histone marks.

Histone modification	Functional association
H3K4me1	Active enhancer
H3K4me3	Active promoter
H3K9ac	Transcriptional activation
H3K9me	Transcriptional repression
H3K27ac	Active enhancer and promoter
H3K27me3	Inactive chromatin
H4R3me2a	Transcriptional activation

Table 1. Histone modifications and their functional annotations

However, there are several indications that this type of classification is oversimplified. Even the same modification might fulfill different functions. For example, methylation at H3K36 has a positive effect on transcription when found in the coding region and a negative effect when it is in the promoter (Wagner and Carpenter 2012).

In addition, in mouse embryonic stem cells bivalent DNA domains have been identified that possess histones with both activating and repressive modifications. The use of novel technologies based on chromatin immunprecipitation (ChIP) revealed that H3K27me3 (repressive) and H3K4me3 (active) marks co-existing in these bivalent domains (Bernstein, Mikkelsen et al. 2006). The enrichment of these opposing modifications within bivalent domains correlate with low-level expression. When the stem cells are differentiated, the bivalent domains tend to preserve either the repressive or active modifications, but not both.

These examples nicely demonstrate that the more we look in to modifications, the more it will become clear that everything is context-dependent.

2. Histone-modifying enzymes

The transition between euchromatin and heterochromatin is dynamic during cellular differentiation. The targeted recruitment of proteins and complexes with specific enzymatic activities is now an accepted dogma of how histone modifications are established. Enzymes have been identified for the vast majority of histone modifications, including acetylation, deacetylation and methylation, discussed in the next sections.

Acetylation

In general, histone acetylation increases gene expression. Histone acetyltransferases (HATs), such as GNAT, MYST and CBP/P300 are enzymes that acetylate conserved lysine amino acids on histone proteins by transferring an acetyl group from acetyl-CoA to form ε-*N*-acetyl-lysine. Different HATs, usually in the context of multisubunit complexes, have been shown to acetylate specific lysine residues in histones.

CREB-binding protein (CBP) and its paralog P300 are fundamentally important in various signal-modulated transcriptional events. P300 and CBP both have a potent histone acetyltransferase activity (Bannister and Kouzarides 1996; Ogryzko, Schiltz et al. 1996), mainly targeting H3K18 and H3K27 *in vivo*. Importantly, over 70 other, non-histone proteins were also identified as target (Holmqvist and Mannervik 2012).

Despite their high levels of homology, P300 and CBP are both indispensable during embryogenesis. Animals null mutants for P300 die between days 9 and 11.5 of gestation, exhibiting defects in neurulation, cell proliferation and heart development (Yao, Oh et al. 1998).

Deacetylation

The reversal of acetylation is believed to correlate with transcriptional repression. Histone deacetylases (HDACs) form a class of enzymes that remove acetyl groups from an ε-N-acetyl-lysine amino acid on a histone, allowing the histones to wrap the DNA more tightly. There are 11 HDACs in the HDAC superfamily. These proteins can be classified into four families (class I, IIa, IIb and IV), which differ in structure, enzymatic function, subcellular localization and expression patterns (Haberland, Montgomery et al. 2009). In addition to these classical HDACs, mammalian genomes encode another group of deacetylases, the sirtuins, which are sometimes referred to as class III HDACs.

Importantly, deletion of any member of the class I HDAC family in mice is embryonic lethal, demonstrating the unique and non-redundant roles of each HDAC in the control of specific gene expression programmes (Haberland, Montgomery et al. 2009).

As it was discussed regarding the function of CBP/P300, a large number of non-histone proteins can also undergo reversible acetylation. Proper acetylation/deacetylation of non-histone proteins are essential in a wide range of cellular processes including transcription, translation, DNA repair, metabolism, and cell structure. Alterations in HDAC-mediated deacetylation of non-histone proteins is implicated in many human diseases, including cancer or Alzheimer's disease (Peng and Seto 2011; Bahari-Javan, Sananbenesi et al. 2014).

Methylation

Methylation is a well-known mechanism of epigenetic regulation, since both DNA methylation and histone modifications are involved in establishing patterns of gene regulation during development (Cedar and Bergman 2009). Histone methylation and demethylation influences the availability of DNA for transcription. The protein

methyltransferases (PMTs) constitute a large and important class of enzymes that catalyze site-specific methylation of lysine or arginine residues on histones and other proteins. Proteins are methylated by the transfer of methyl group from the methyl donor S-adenosyl methyonin (SAM). The site and degree of lysine methylation is dynamically modulated through the enzymatic activities of protein lysine methyltransferases and protein lysine demethylases (Del Rizzo and Trievel 2014).

Arginine methylation is also a common posttranslational modification that alters roughly 0.5% of all arginine residues in the cells (Gayatri and Bedford 2014). Arginine methylation is catalyzed by a family of Protein aRginine MethylTransferases (PRMTs). PRMTs can methylate different arginine residues on histone H2A, H3 and H4 tails that are linked to distinct functions: they can be associated with both active and repressed chromatin states, depending on the residue involved and the configuration of the deposited methyl groups (Molina-Serrano, Schiza et al. 2013). To date, eleven mammalian PRMTs have been identified (PRMT1-11). Except for PRMT3, all PRMTs that possess detectable methyltransferase activity are able to methylate a histone substrate (Litt, Qiu et al. 2009).

Based on their enzyme activities PRMTs form three groups. Type I enzymes (PRMT1, 2, 3, 4, 6 and 8) catalyze the formation of asymmetric dimethylarginine (aDMA) residues while the Type II (PRMT5) enzyme catalyze the formation of symmetric dimethylarginine (sDMA) residues. Both Type I and II enzymes generate monomethylarginine (MMA) intermediates. The Type III enzyme (PRMT7) only generates a MMA mark (Gayatri and Bedford 2014) (Figure 2).

Histone modifications catalyzed by members of the family are summarized in Table 2. It is important to note that specificity of the arginine methyltransferases are not restricted to histones and members of the family show a wide spectrum of substrate specificity for

other proteins as well, many of which have been linked to the development, progression and aggressiveness of different types of cancer (Baldwin, Morettin et al. 2014).

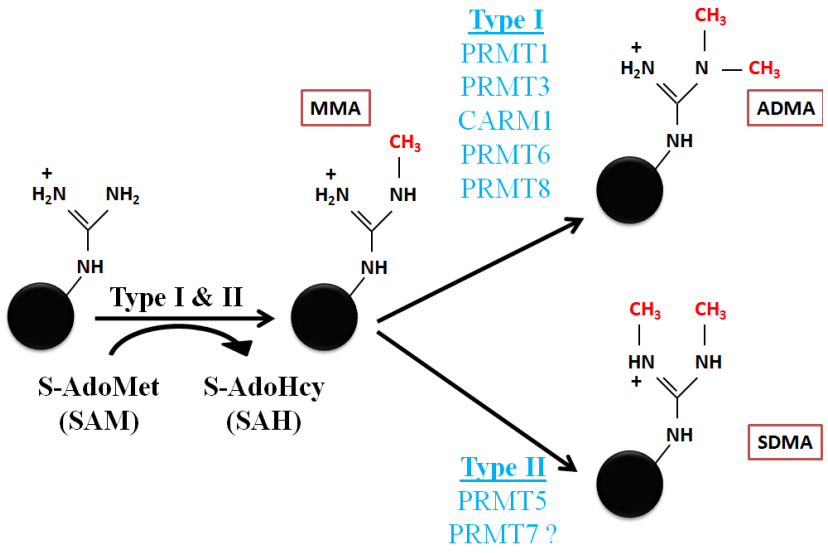

Figure 2. Family of Protein Arginine Methyltransferases (PRMTs). Both Type I and Type II PRMTs catalyze the formation of the monomethylarginine (MMA) intermediate. The Type I enzymes (PRMT1, PRMT3, CARM1, PRMT6 and PRMT8) convert the intermediate to asymmetrical dimethylarginine (aDMA), whereas the Type II enzymes (PRMT5, PRMT7 and PRMT9) transfer methyl groups to the ω-nitrogen of the arginine residue, resulting in symmetrical dimethylarginine (sDMA).

Arginine methyltransferase	Histone mark
PRMT1	H4R3me2a (asymmetric)
PRMT4 / CARM1	H3R17, H3R26
PRMT5	H4R3me2s (symmetric)
PRMT6	H3R2
PRMT8	H4R3me2a (asymmetric)

Table 2. Examples of histone modifications mediated by arginine methyltrasferases (based on (Bedford and Clarke 2009).

3. Transcription factors

Mammalian transcription is controlled by a multitude of regulatory elements dispersed throughout the genome. These sequence elements can be located in the vicinity of transcription start sites (TSS) to function as promoters or can be positioned up- or downstream distally of initiation sites to function as enhancers/silencers. A typical mammalian cell contains thousands of active enhancers, and it has been estimated that there may be ~1 million enhancers active in any of human cells (Hnisz, Abraham et al. 2013).

Promoters and enhancers harbor binding sites for transcription factors (TFs). TFs are regulatory proteins whose function is to activate or inhibit transcription through binding to their specific DNA sequences. TFs have defined DNA-binding domains (DBDs) with higher affinity for their target sequences than for the remainder of the DNA strand. There are approximately ~2600 proteins in the human genome that contain DNA-binding domains, and most of these are presumed to function as transcription factors (Babu, Luscombe et al. 2004; Vaquerizas, Kummerfeld et al. 2009).

TFs are key components of the cell that control gene expression. We distinguish the class of general transcription factors required for proper gene expression in each cell and cell-type specific transcription factors important for lineage specific transcription.

The general transcription factors

Transcriptional regulation by RNA polymerase II is a multifaceted process requiring the concerted action of large number of transcription factors. An important class of transcription factors called general transcription factors (GTFs) are necessary for transcription to occur. Many of these GTFs don't actually bind DNA but are part of the large transcription preinitiation complex that interacts with RNA polymerase directly.

The most common GTFs are TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH. Transcription by RNA polymerase II is divided into four stages: initiation, promoter clearance, elongation and termination (Lin, Garrett et al. 2011). The initiation stage of transcription requires nucleosomal remodeling followed by the recognition of the promoter elements by the basal transcription machinery and RNA PolII. TFIID binds to the promoter at a short distance from the transcriptional start site. TFIID functions as a multiprotein complex composed of TATA-binding protein (TBP) and the highly conserved TBP-associated factors (TAFs). This is followed by the binding of TFIIB, TFIIF and RNA PolII. While this classical description implies a stepwise accretion of TFs, recent evidence suggests the existence of stable, preformed basal transcription complexes (Koleske and Young 1994).

Cell-type specific transcription factors

The precise, temporal order of gene expression during cellular differentiation is critical to ensure proper lineage commitment, cell fate determination, and ultimately, organogenesis (Ladewig, Koch et al. 2013). Although many cell-type specific TFs have been identified, surprisingly little is known about the total number of these regulators and their cell-type specific distribution and function. As stated above, an estimation suggest that ~2600 DNA-binding transcription factors are encoded by the human genome, with ~250 being expressed in each cell type at any given time. During differentiation, chromatin accessibility for lineage instructive transcription factors becomes progressively limited to tissue specific genes and also to subsets of metabolic, cell cycle and housekeeping genes (van Oevelen, Kallin et al. 2013).

A number of studies have provided evidence regarding the existence of cell-type specific key transcription factors. According to the recent model, as cellular differentiation

progress, cis-regulatory elements bound by primary TFs may facilitate the binding of a secondary set of TFs, which then leads to chromatin remodeling and full activation of the enhancer (van Oevelen, Kallin et al. 2013).

The so-called transcription factor-mediated cell reprogramming clearly demonstrates the power of TFs in cell fate determination. There are many striking examples of transcription factor-induced reprogramming of specialized cells into other cell types. The most dramatic example was the famous "Yamanaka-experiment" in 2006 in which he and his colleagues demonstrated that somatic cells can be reprogrammed to a pluripotent state by the expression of a transcription factor mixture (Oct3/4, Sox2, c-Myc, and Klf4), generating the so called induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). The instructive role of other transcription factors in lineage specification was already demonstrated in the 1980s, when Lassar's lab discovered that forced expression of MyoD can induce the myotube formation in fibroblasts (Tapscott, Davis et al. 1988). Later studies of the hematopoietic system have shown that the order in which transcription factors are switched on is responsible for lineage outcome: sustained expression of C/ebpα generates granulocytes-macrophages, while sustained expression of Gata2 generates mast cells. Eosinophils and basophils are generated in a sequential order (see (Graf and Enver 2009) for details).

Direct conversion from one lineage to another different lineage holds a great promise for translational medicine. A remarkable example for potential therapeutic application is that delivery of three transcription factors (Pdx1, Ngn3, MafA) to an adult mouse pancreas caused the transdifferentiation of acinar cells to beta cells (Graf and Enver 2009). Transdifferentiation of fibroblasts into neural lineage cells by the introduction of defined transcription factors (Ascl1, Brn2, and Myt1l) has also proven the cell's plasticity (Shi and Jiao 2012) (Figure 3).

These experiments have revolutionized our thinking about cell plasticity, differentiation, and stem cells and underpinned the importance of transcription factors in cell fate determination.

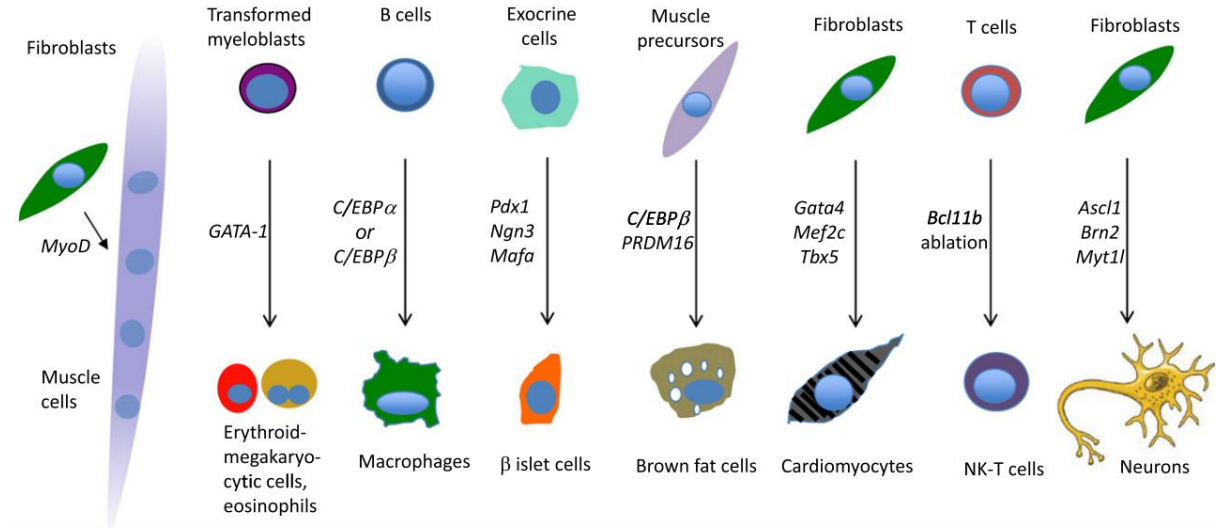

Figure 3. Transdifferentiation induced by transcription factors. *Taken from* (Graf 2011)

The nuclear receptor family

Nuclear receptors (NRs) are among a few ligand sensitive receptors that interact directly with chromatin by binding to their hormone response element of the target genes on the DNA. To date, 48 and 49 nuclear receptors were identified in human and mouse genomes, respectively. They have major roles in cellular signaling and differentiation (Tsai and O'Malley 1994; McKenna, Lanz et al. 1999). With a few exceptions, such as Dax-1 and Shp1, all NRs expressed in vertebrates share a common domain structure. Broadly, the receptor structure is comprised of an amino-terminal activation function (AF-1), a DNA-binding domain (DBD), a hinge region, and a carboxy-terminal ligand binding domain (LBD). The DBD is required for promoter recognition and contains structural features allowing the nuclear receptors to bind differentially to target genes (Khorasanizadeh and Rastinejad 2001). The LBD plays a crucial role in ligand-mediated

activity of the nuclear receptor and also contains an activation function-2 (AF-2) domain, whose conformation is highly dependent on the bound ligand (Kumar and Thompson 2003) (Figure 4).

Based on their ligands and mechanistic action NRs are divided into four groups: 1) steroid receptors 2) retinoid X receptor heterodimers 3) dimeric orphan receptors 4) monomeric orphan receptors.

Upon ligand activation, type I steroid receptors (SRs) bind to inverted repeat DNA response elements as homodimers. This class includes estrogen receptor (ER), progesteron receptor (PR), glucocorticoid receptor (GR), androgen receptor (AR) and mineralocorticoid receptor (MR). Type II nuclear receptors form heterodimers with retinoid X receptor (RXR). These include the receptors for thyroid hormone (TR), vitamin D (VDR), all-trans retinoic acid receptors (RARs) and proliferator-activated receptors (PPARs).

The third class of nuclear receptors termed "orphan receptors" have no known cognate ligands and include receptors such as estrogen related receptors (ERRs), testis receptors (TR2 and TR4) and chicken ovalbumin upstream promoter transcription factor (COUP-TF) among others (see also www.nursa.org).

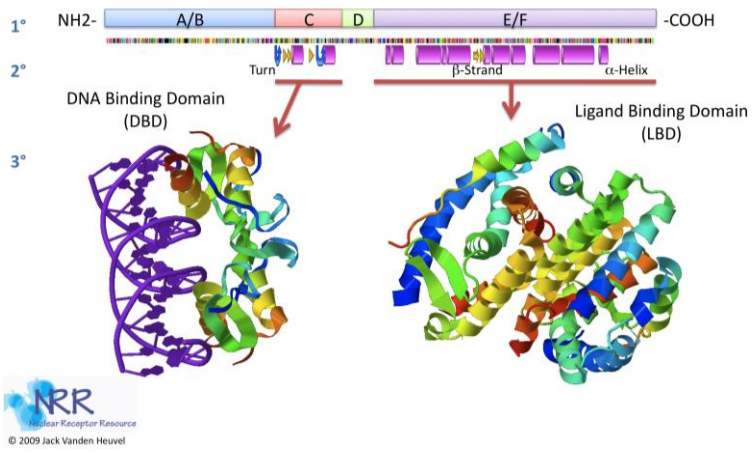

Figure 4. Structural organization of nuclear receptors. *Taken from* http://nrresource.org/

Retinoic Acid Receptors: from Vitamin A to transcription

The retinoic acid signaling pathway is one of the best understood example for ligand-mediated direct transcriptional regulation. The pathway is activated by the endogenous active metabolite of Vitamin A. Since the discovery of Vitamin A in 1913, numerous studies have come to light documenting its indispensable effect in embryogenesis and adult health (Sommer 2008). There are two sources of dietary vitamin A. Retinoids are immediately available to the body from intracellular stores. Precursors (such as carotenoids, mainly found in plants) first must be converted to active forms. Figure 5 summarize the major steps of the retinoic acid synthesis and action. The dominant form of retinoid present in the mammalian fasting circulation is the retinol. In aqueous environments retinol is bound to the 21 kDa retinol-binding protein 4 (Rbp4) (Noy 1992). Two active derivatives, all-*trans* retinoic acid (atRA, referred as RA later) and 13-*cis*-retinoic acid (13-cis-RA) can be also found in the plasma in the fasting state (Horst, Reinhardt et al. 1995), although at much lower concentration (Cullum and Zile 1985). Retinol is transported into the cytoplasm of the target cell through Stra6 (Kawaguchi, Yu et al. 2007), where cellular retinol binding proteins (Crbp1 and Crbp2) are able to bind this in many cell types, which provide storage of retinol (Molotkov, Ghyselinck et al. 2004). For biological effects retinol must be converted into its active forms in two steps. In the first step, alcohol dehydrogenases (ADHs) and retinol dehydrogenases (RDHs) are catalyzing the oxidation of retinol to retinaldehyde (retinal). The second step is the oxidation of retinaldehyde to retinoic acid that is catalyzed by retinaldehyde dehydrogenases (Aldh1a1, Aldh1a2 and Aldh1a3). Importantly, retinoic acid is not produced by all the cells of the body (Duester 2008), however cells without active RA synthesis still may have access to it from the environment. The intracellular level of RA is controlled through its metabolism (Ross and Zolfaghari 2011).

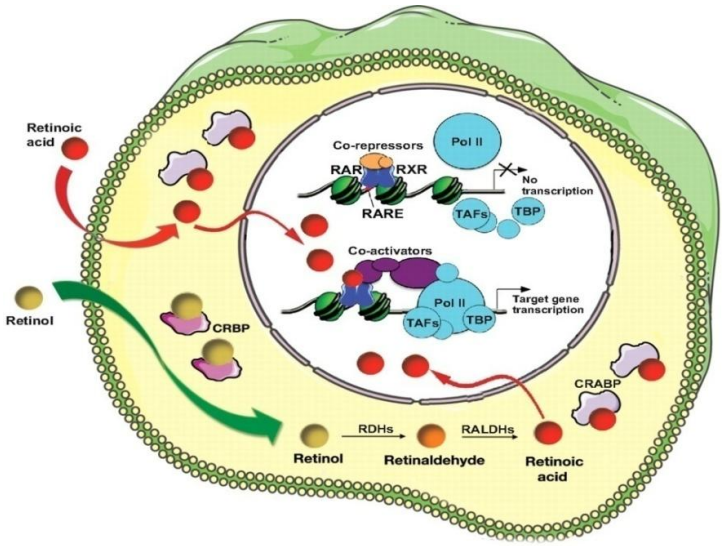

Figure 5. Summary of the RA signalling pathway. CRBP, Cellular retinol binding protein; RDH, retinol dehydrogenase; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid response element. *Taken from* (Rhinn and Dolle 2012).

Enzymes of the cytochrome P450 subfamily (Cyp26a1, b1 and c1) catalyze reactions that convert RA into metabolites, primarily 4-hydroxy-RA (White, Beckett-Jones et al. 1997). In the cytoplasm, RA binds to intracellular retinoic acid binding protein 2 (Crabp2) and delivered into the nucleus (reviews in (Blomhoff and Blomhoff 2006; Duester 2008; D'Ambrosio, Clugston et al. 2011).

In the nucleus, RA acts via the activation of retinoic acid receptor (RAR) that forms an obligate heterodimer with the retinoid X receptor (RXR). RARs (including 3 isoforms: RARα, RARβ and RARγ) and RXRs (including RXRα, RXRβ and RXRγ) are two families of nuclear receptors that bind DNA and directly regulate transcription (review in (Chambon 2005)). RARs and RXRs exhibit the conserved structure of nuclear receptors, including DBD and LBD. RARs bind RA, and the heterodimer RXR partner bind 9-*cis*-RA. However, 9-*cis*-RA is normally undetectable except when retinol is present in excess (Mic, Molotkov et al. 2003). Liganded RAR:RXR heterodimers bind to specific regulatory DNA element, called RA response element (RARE) (Umesono, Giguere et al.

1988; Glass 1994). *In vitro* binding and transactivation studies demonstrated that RAR:RXR preferentially bind to direct repeat (DR) sequences of the hexameric motif (A/G)G(G/T)TCA, often spaced by 5 or 2 nucleotides (DR5 and DR2) (Mahony, Mazzoni et al. 2011; Moutier, Ye et al. 2012).

4. Transcriptional co-regulators

The broadest definition of co-regulators is that these proteins participate in a complex that is recruited to the genome by DNA-binding transcription factors and the complex up- or down-regulates the rate of transcription of given genes (Millard, Watson et al. 2013).

Recent data have indicated multiple modes of action of co-regulators, including direct interactions with basal transcription factors (eg. nuclear receptors), and covalent modification of histones and other proteins. Reflecting this functional diversity, many co-regulators exist in distinct complexes, which are thought to associate in promoter-specific configurations (McKenna, Lanz et al. 1999).

Transcription co-regulators that result in activation of gene transcription are referred to as co-activators while those that repress are known as co-repressors. At the molecular level, co-repressors and co-activators are involved in the fine-tuning of transcriptional activity.

In general, co-regulator complexes can be directed to many transcription factors. Similarly, each transcription factor may recruit different co-regulator complexes under different circumstances. Taken together, this means the specificity of action of co-regulator complexes relies upon a complicated network (Millard, Watson et al. 2013).

Complexity of the role of co-activators and co-repressors in transcriptional regulation is supported by recent observations. Strikingly, a recent paper provide evidence that both HATs and HDACs are targeted to transcribed regions of active genes (Wang, Zang et al. 2009). These result suggest that the dynamic cycle of acetylation and deacetylation by the

transient binding of HATs and HDACs may poise the primed genes for further activation. It was also recently noted that HDAC inhibition caused transcriptional repression by blocking RNA polymerase II elongation (Kim, Greer et al. 2013). HDAC1 and 2 have been linked to stem cell self-renewal due their importance in maintaining expression of key pluripotent transcription factors (Jamaladdin, Kelly et al. 2014). Better understanding of how HDACs contribute to the regulation of gene expression will require further studies.

Co-regulators of nuclear receptors

The concept that combinatorial control of gene expression by NRs requires recruitment of functionally distinct co-regulator complexes has become well established by now (McKenna and O'Malley 2002). An early indication of the interaction of activated nuclear receptors with co-factors was the phenomenon of squelching. Squelching means the reduction in transactivation of a promoter positively regulated by one transcription factor in the presence of a distinct, activated transcription factor (Meyer, Gronemeyer et al. 1989). These observations indicated that such common co-factors available in limited quantities and might be an important link between the nuclear receptor and transcriptional initiation and suggested the existence of a level of control at enhancer.

The first NR co-regulators were discovered through efforts to identify proteins that interact with ligand-bound or unliganded NRs. These experiments led to the identification of SRC1 and NCoR/SMRT (Halachmi, Marden et al. 1994; Chen and Evans 1995; Onate, Tsai et al. 1995). To date, more than 350 co-regulators (both co-activators and co-repressors) have been identified (Millard, Watson et al. 2013).

The molecular mechanisms through which NRs recruit co-regulator complexes has been extensively studied. A common feature of NR co-activators is that they contain one or

more LXXLL binding motifs (a contiguous sequence of 5 amino acids where L = leucine and X = any amino acid) referred to as NR boxes. The LXXLL binding motifs have been shown by X-ray crystallography to bind to a groove on the surface of ligand binding domain of nuclear receptors. Upon the binding of an agonist, nuclear receptors use a charge clamp pocket, in part composed of the C-terminal AF-2 helix, to form a hydrophobic groove for binding of the LXXLL motif of the co-activators (Nagy and Schwabe 2004).

According to the recent model, in the absence of ligands Type II nuclear receptors (eg. RAR, TR, VDR) are located in the nucleus, bind to DNA response element of target gene and recruit co-repressor complexes that contain histone deacetylases, which in turn induce a repressive chromatin state at the target locus. Importantly, Type I steroid hormone receptors are either located in the cytoplasm or kept inactive by chaperons in the absence of specific ligands. Our knowledge about the role and importance of transcriptional co-repressors is still in infancy, despite the fact that several co-repressor have been already implicated in a wide range of biological processes and diseases (Chuang, Leng et al. 2009). Critical insights into the potential mechanisms of transcriptional repression by nuclear receptors were provided by the discovery of NCoR, SMRT and Sin3, which form one complex (Nagy, Kao et al. 1997; Nagy, Kao et al. 1999).

Ligand binding triggers an allosteric change that lead to the exposure of NR surface for interaction with co-activators (NCoA, CBP/P300, etc.) (Glass and Rosenfeld 2000; Rochette-Egly 2005). Alternatively, ligand dependent repression of target genes has also been reported (Horlein, Naar et al. 1995).

A combination of genetic and functional data suggest that the SWI/SNF complex, CBP/P300, CARM1 and PRMT1, p160 factors, P/CAF and the TRIP/DRIP/ARC complexes are likely to be critical regulators for some subsets of nuclear receptor

regulated genes. Figure 6 summarizes the major co-regulator complexes involved in the ligand-mediated co-activator - co-repressor cycle.

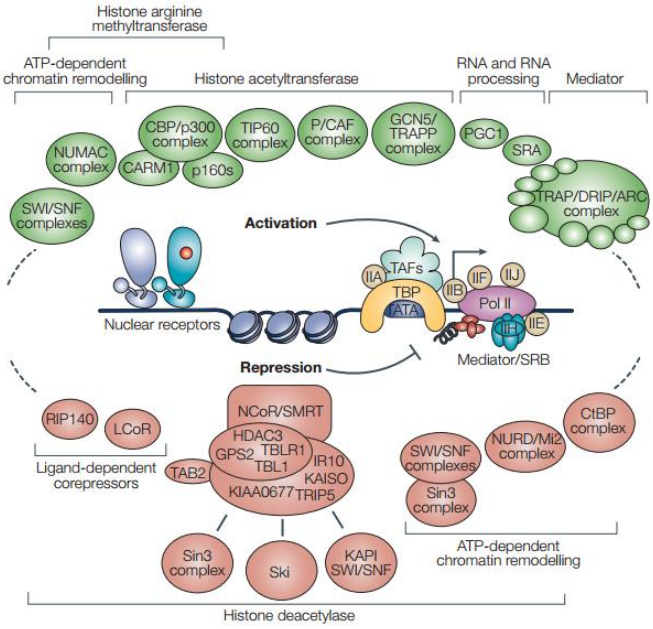

Figure 6. The circular logic of co-factor cycles. *Taken from* (Perissi and Rosenfeld 2005).

Considerable experimental data have established that agonist-bound steroid receptors directly recruit the SRC/p160 family co-activators, which subsequently recruit secondary co-activators (Koh, Chen et al. 2001). Many of these secondary co-activators contain intrinsic enzymatic activities and have been discussed in the previous section of histone modifying enzymes. The CBP/P300 complex has potent histone acetyltransferase activity, whereas SRC-1 and SRC-3/ACTR exhibit relatively weak acetyltransferase activity. CARM1 and PRMT1 have histone methyltransferase activity, and SWI/SNF complexes possess ATP-dependent nucleosome remodeling activity (Martens and Winston 2003). It has been generally accepted that following recruitment to the promoter, these co-activators can modify local chromatin structure and increase DNA accessibility to the basal transcription machinery (Xu and Li 2003; Feng, Yi et al. 2006).

Protein arginine methyltransferases

PRMTs (protein arginine methyltransferases) specifically modify the arginine residues of key cellular and nuclear proteins as well as histone substrates (Litt, Qiu et al. 2009). So far, the best characterized effect of arginine methylation is its positive or negative influence on protein-protein interactions (Boisvert, Chenard et al. 2005), and it has been implicated in various cellular processes. Compared to other post-translational modification (eg. phosphorylation), arginine methylation seems to have a milder effect on protein function and is responsible more for the modulation of certain processes.

PRMT4, which is referred to as CARM1 (co-activator-associated arginine methyltransferase 1), was the first member of the family identified as a binding partner for the p160 transcriptional co-activator GRIP1 (glucocorticoid receptor-interacting protein 1) (Koh, Chen et al. 2001). Later studies identified PRMT1, another member of the family that can act as co-regulator for various nuclear receptors (Balint, Szanto et al. 2005; Rizzo, Renga et al. 2005; Barrero and Malik 2006) (Figure 7).

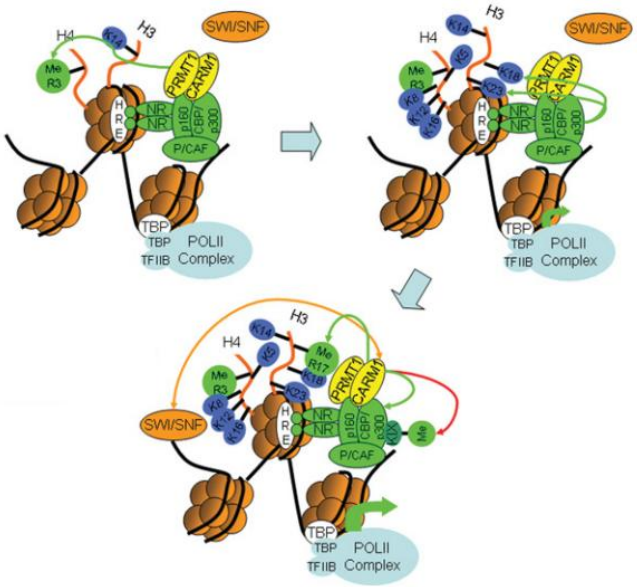

Figure 7. Transcriptional regulation of hormone response genes via arginine methylation. *Taken from* (Litt, Qiu et al. 2009).

The concept of context-dependent co-regulation

Composition of co-activator and co-repressor complexes is cell-type and context dependent and contributes to cell specification and potentially gene-specific transcription (Lonard and O'Malley B 2007). Tissue-specific expression profiles of co-regulators also allow diverse interpretation of the same ligand treatment.

As Lonard et al. suggest in (Lonard and O'Malley B 2007), co-regulators are like actors, resistant to being typecast as solely positive acting or negative acting. For instance, HDAC1 that is normally considered as a transcriptional repressor due to its histone deacetylase activity can function as a co-activator of glucocorticoid-mediated transcription (Qiu, Zhao et al. 2006). Well-established co-repressors such as SMRT and NCoR have also been shown recently to function as co-activators in the context of certain promoters (Berghagen, Ragnhildstveit et al. 2002).

Similarly, despite the plethora of information demonstrating that PRMT1 functions as a co-activator of NRs, increasing evidence suggests that it can also act as a transcriptional repressor by modifying a diverse set of substrates (Kleinschmidt, Streubel et al. 2008; Lafleur, Richard et al. 2014).

Based on these examples, it is likely that the definition of a co-regulator that restricts its role to amplifying or silencing the transcriptional output of NR-regulated promoters is insufficient to account for its elaborate functionality (McKenna and O'Malley 2002).

Crosstalk between co-regulators

A spectrum of post-translational modifications is known to regulate the functional relationships between nuclear receptors and their co-regulator complexes and contribute to their target gene networks. Enzymatic modifications, such as acetylation, methylation or phosphorylation have been shown to have a variety of effects on co-regulator function.

Histone acetyltransferase activity of CBP or P300 is not only regulated by substrate availability but also by genomic context. Importantly, CBP and P300 transcriptional activity can be regulated by post-translational modifications, such as phosphorylation (Ait-Si-Ali, Ramirez et al. 1998). Arginine methylation of co-regulators have been also shown to modulate the assembly or disassembly of co-regulator complexes. CBP is specifically methylated by CARM1 on conserved arginine residues and this is required for GRIP1-dependent transcriptional activation and hormone induced gene activation (Chevillard-Briet, Trouche et al. 2002). CARM1 has been also indicated in the methylation of P300 within the KIX domain, which results in the abrogation of P300 - CREBP interaction (Xu, Chen et al. 2001). Studies with isolated histones have shown that arginine methylation of Histone 4 by PRMT1 stimulates its subsequent acetylation by P300 (Wang, Huang et al. 2001).

An intriguing scenario is that co-activators and co-repressors are programmable and through specific posttranslational modifications they can serve as "memory cards" to sensitize cells for subsequent transcriptional responses (McKenna and O'Malley 2002).

However, how these above discussed co-regulator complexes exert their cell type and developing stage-specific activity is remain largely unknown. Co-regulators may be viewed as control interfaces for integrating multiple afferent stimuli into an appropriate cellular response. Formation of tissue-specific combinations of co-regulator complexes is a critical mechanism for tissue-specific transcriptional regulation (Yokoyama, Igarashi et al. 2014).

5. Theory of cell-type specific transcriptional "hot-spots"

A typical differentiated mammalian cell contains thousands of active enhancers, but considering all the human cells, it has been estimated that there may be in total ~1 million enhancers.

Likely, cell-type specificity emerges from the interplay of the above discussed levels of transcriptional regulation. Not only co-regulators, but the DNA, histones, transcription factors and signaling enzymes all communicate with each other via reversible covalent modifications, providing a complicated but critical harmony of voices that direct cell differentiation (Lonard and O'Malley B 2007).

Previous mass spectrometry analysis have already revealed that co-regulators do not work alone but instead as multiprotein complexes. Detailed analysis of how trans-bound RAR on estrogen receptor responsive enhancers regulate the ER enhancer function, recently led to the concept that units of transcriptional regulation form MegaTranscription Factor complexes, which have cell-type and enhancer specific composition (Liu, Merkurjev et al. 2014).

Recently, the concept of cell-type specific enhancers has been expanded by the discovery of "super-enhancers" (Whyte, Orlando et al. 2013). Super-enhancers are domains that are highly enriched for cell-type specific transcription factors and are involved in looping between enhancers and promoters.

Recognition of these super-enhancers was obtained from the analysis of genome-wide chromatin immunprecipitation data. Mapping 13 sequence-specific transcription factors in embryonic stem cells revealed the existence of highly dense binding loci, showing the frequent co-binding of various transcription factors at given genomic regions. Those regions showing high enrichment to various TFs have been called as transcriptional "hot-spots" or high occupancy target regions, which also bind a large number of co-regulators

(Chen, Xu et al. 2008). Since P300 is among the most widely used transcriptional co-activators in mammalians, it is expected to occupy the genome at many cis-regulatory DNA sequences together with numerous different transcription factors (Holmqvist and Mannervik 2012). These "hot-spots" seems to function as tissue-specific developmental enhancers driving gene expression patterns in selected cells. Indeed, studies in mammalian cells suggest that P300 occupancy depends on cell-type specific transcription factors (Visel, Blow et al. 2009).

IV. RETINOIC ACID IN EMBRYOGENESIS AND STEM CELL DIFFERENTIATION

Cell fate specifications in mammals permit the formation of ~200 different cell types (Christophersen and Helin 2010). Our understanding of how different regulatory networks are activated to guide this complex process is still limited.

1. The morphogenic retinoic acid in neurogenesis

Retinoic acid is the first described morphogen that is involved in patterning the anterior-posterior axis of structures, including the hindbrain. Recently, endogenous RA gradient has been visualized across embryonic development (Shimozono, Iimura et al. 2013). Earlier studies demonstrated that during embryonic development RA acts as a morpho-regulator, forming an anterior-posterior (A–P) concentration gradient (Casci 2008) and cross-talks with other key embryonic signals, especially FGF (fibroblast growth factor), BMP (Bone morphogenetic protein), Shh (sonic hedgehog), Wnt or Nodal signaling pathways (Niederreither and Dolle 2008).

Possible functions of Vitamin A and RA were first inferred by studying its excess and absence during embryogenesis. First reports of symptoms caused by vitamin A-deficiency

(VAD) suggested a wide variety of effects on vertebrate development, including the malformation of the ocular, cardiac, respiratory and urogenital systems (reviewed in (Mark, Ghyselinck et al. 2009)).

Elimination of each Aldh1a2 and Cyp26a1, the two key enzymes responsible for determining the local RA concentration, lead to early morphogenetic defects and embryonic lethality, indicating the importance of the precise regulation of RA concentration along the body (Niederreither, Subbarayan et al. 1999). Genetic ablation of other enzymes and receptors involved in the retinoic acid signaling pathway resulted in tremendous progress in characterizing the numerous developmental events regulated by the retinoid pathway. Table 3, taken from (Rhinn and Dolle 2012) summarize how targeted inactivation of retinoid signaling pathway genes affected the mouse phenotipically.

These studies indicate the essential role of RA in neural development, although there has been some disagreement as to where along the central nervous system RA is required (Duester 2013). The wide distribution of RARs and RXRs throughout the mammalian brain strongly suggest that retinoid signaling is necessary for neurogenesis and proper brain function. Several studies have provided evidence that retinoic acid plays a role in sleep, learning, and memory, but the precise mechanisms through which it influences these processes remain unclear (Drager 2006). During motor neuron specification, RA is also known to be needed to induce Pax6 (MuhChyi, Juliandi et al. 2013). In the developing neural tube, retinoid signaling initiates neural differentiation, controls patterning and differentiation of spinal motor neurons, and specifies caudal hindbrain and rostral cervical spinal identity (Sockanathan and Jessell 1998; Niederreither, Vermot et al. 2000; Novitch, Wichterle et al. 2003). In contrast, RA activity in the early forebrain is not essential, but there are several evidence suggesting the importance of RA-dependent

induction of Hoxb1 and other Homeobox genes in the posterior hindbrain (Begemann and Meyer 2001; Glover, Renaud et al. 2006).

Like other developmental molecules, RA continues to play a role after development has been completed (Maden 2007). It has been reported that vitamin A may impact neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) (Takeda, Nyssen et al. 2013). Neuroprotective effect of *9-cis*-RA has also been shown in CNS injury in ischemia model.

Despite the valuable information obtained in these *in vivo* models, our understanding are still limited in terms of the mechanistic details and molecular events of retinoid signaling pathway. Cellular model systems, such as embryonic stem cells can help us to better understand the mechanistic details how RA coordinate the cell fate decisions.

2. Stem cells as a model system of cellular differentiation

Since their discovery, embryonic stem cells became one of the most important model systems in cell differentiation studies. Stem cells are unspecialized cells that have the ability to self-reproduce and differentiate into functional mature cell-types. They can be originated from embryonic, fetal or adult tissues.

Embryonic stem cells (ESCs) are pluripotent cells isolated from the inner cell mass (ICM) of preimplantation embryos (day 3.5) (Evans and Kaufman 1981). As long as the embryonic stem cells in culture are grown under appropriate conditions, they can remain undifferentiated and retain the ability to generate all of the cell types of an organism. Differentiation of ESCs resemble normal embryonic development much closer than previously thought, and provide a valuable *in vitro* model for studying the effects of various factors on cell lineage decisions during early stages of mammalian embryonic development (ten Berge, Koole et al. 2008).

Table 3.

Gene(s)*	Stage of lethality	Loss-of-function phenotype	Main references
Adh1	Viable	Postnatal increase in vitamin A toxicity	Deltour et al., 1999b; Molotkov et al., 2002b
Adh5	Postnatal	Growth deficiency, vitamin A toxicity	Deltour et al., 1999b; Molotkov et al., 2002a; Molotkov et al., 2002b
Adh7	Viable	Sensitivity to vitamin A deficiency	Deltour et al., 1999a; Deltour et al., 1999b
Rdh5	Viable	Vision: delay in dark adaptation	Driessen et al., 2000
Rdh10	E10.5-14.5	Small optic vesicles/eyes; abnormal hindbrain and posterior branchial arches; abnormal heart tube; small forelimb buds; defects in organogenesis (lung, gut, pancreas, kidney)	Cunningham et al., 2011; Sandell et al., 2007; Rhinn et al., 2011
Raldh1	Viable	No abnormality reported	Fan et al., 2003; Matt et al., 2005
Raldh2	E9.5-10.5	Hypoplastic optic vesicles; abnormal hindbrain ('anteriorisation'), lack of posterior branchial arches; impaired heart looping and chamber differentiation; truncation of body axis, asymmetry in somite formation; absence of limb buds; defects in organogenesis (lung, gut, pancreas, kidney)	Mic et al., 2002; Niederreither et al., 1999; Niederreither et al., 2001; Niederreither et al., 2000; Ribes et al., 2009; Ribes et al., 2006; Sirbu and Duester, 2006; Vermot et al., 2005
Raldh3	Neonatal	Shortening of ventral retina; nasal abnormality (choanal atresia); altered GABAergic neuronal differentiation in forebrain basal ganglia	Chatzi et al., 2011; Dupé et al., 2003; Matt et al., 2005; Molotkov et al., 2006
Cyp26a1	Neonatal	Abnormal hindbrain ('posteriorisation'); truncation of posterior body, sometimes with sirenomelia ('mermaid-like tail'); vertebral transformations	Abu-Abed et al., 2001; Sakai et al., 2001
Cyp26b1	Neonatal	Limb defects (abnormal distal skeleton and cartilage maturation); craniofacial abnormalities (reduced maxilla and mandible, cleft palate); gonadal abnormalities (premature meiosis, apoptosis of male germ cells)	Bowles et al., 2006; MacLean et al., 2009; MacLean et al., 2007; Yashiro et al., 2004
Cyp26c1	Viable	No abnormality reported	Uehara et al., 2007
Cyp26a1;Cyp26c1	E9.5-10.5	Reduced forebrain and midbrain, hindbrain expansion; deficiency in cranial neural crest	Uehara et al., 2007
Rara	Postnatal (variable)	Growth deficiency; vertebral transformations/abnormalities; malformed laryngeal cartilages; webbed digits (variable); male sterility (degeneration of testis germinal epithelium)	Ghyselinck et al., 1997; Lufkin et al., 1993
Rarb	Viable	Growth deficiency; vertebral transformations/abnormalities; ocular abnormality (retrolenticular membrane); locomotor behavioural defects	Ghyselinck et al., 1997; Krezel et al., 1998
Rarg	Postnatal (variable)	Growth deficiency; vertebral transformations/abnormalities; malformed laryngeal and tracheal cartilages Webbed digits (variable); abnormal differentiation of keratinocytes; male sterility (abnormal seminal vesicle and prostate epithelia)	Chapellier et al., 2002; Ghyselinck et al., 1997; Lohnes et al., 1993
Rara;Rarb	Neonatal	Abnormal hindbrain patterning (abnormal r5-r7); absence/abnormality of posterior branchial arch derivatives (thymus, parathyroids) and salivary glands; heart outflow tract and large vessel abnormalities; severe laryngeal/tracheal abnormalities; lung hypoplasia, lack of oesophagotracheal separation; kidney and female genital tract abnormalities	Batourina et al., 2001; Dupé et al., 1999; Ghyselinck et al., 1997; Lohnes et al., 1994; Mendelsohn et al., 1994
Rara;Rarg	E12.5 to neonatal	Abnormal hindbrain patterning ('anteriorisation'); absence/abnormality of posterior branchial arch derivatives (thymus, parathyroids) and salivary glands; eye defects (retinal coloboma, absence of lens); heart outflow tract and large vessel abnormalities, myocardial hypoplasia; severe laryngeal/tracheal abnormalities; craniofacial and limb skeletal defects; kidney, male and female genital tract abnormalities	Ghyselinck et al., 1997; Lohnes et al., 1994; Mendelsohn et al., 1994; Wendling et al., 2001
Rxra	E13.5-16.5	Heart outflow tract and large vessel abnormalities, myocardial hypoplasia; eye defects (shortening of ventral retinal abnormal cornea); placental defect (disorganisation of labyrinthine zone)	Gruber et al., 1996; Kastner et al., 1994; Merki et al., 2005; Sapin et al., 1997; Sucov et al., 1994
Rxrb	Partial perinatal lethality	Male sterility (abnormal Sertoli cells, impaired spermatozoid production)	Kastner et al., 1996
Rxrg	Viable	Behavioural and depression-like defects	Krezel et al., 1998; Krzyzosiak et al., 2010
Rxra;Rxrb	E9.5-10.5	Truncation of posterior body; abnormal nasal region and posterior branchial arches; abnormal heart tube; placental defect (absence of labyrinthine zone)	Wendling et al., 1999

*All phenotypes refer to homozygous germline mutants with gene disruptions generated in embryonic stem (ES) cells. Some examples of compound (double homozygous null) mutations leading to severe embryonic abnormalities are also given. The double mutations were generated through mouse intercrosses, except for *Cyp26a1;Cyp26c1*, for which the two neighbouring genes were deleted in ES cells.

Table 3. Genetic models of retinoic acid signaling. *Taken from* (Rhinn and Dolle 2012).

Still, there are many empirically determined factors in ESCs differentiation, which play a role that is not sufficiently understood. For instance we and others identified Vitamin C as a promoting factor in cell fate decisions (Cuaranta-Monroy, Simandi et al. 2014), but mechanistically there is no clear explanation to those observations. Progress in the past few years has greatly enhanced our understanding of epigenetic control of ESCs. Epigenetic factors appear to be essential for regulating cell fate decisions and maintaining the cellular state of differentiated cells (Christophersen and Helin 2010). Identification of these key regulators and the molecular mechanisms will be a great help for directing the differentiation of ESCs toward particular lineages.

The research to understand the role of ESCs in neurogenesis has been particularly active, due to the great promise of treating variety of neurological diseases and central nervous system (CNS) injuries. Fate-specific differentiation of ESCs to neural progenitors and specific terminally differentiated mature neurons attracts keen interest in modern medicine (Germain, Banda et al. 2010) (Fan, Sun et al. 2014).

Importantly, during ESC neurogenesis, morphological structures comparable to those arising during embryogenesis have been observed *in vitro*. Generally, during differentiation, ESCs are allowed to form aggregates (EBs), that are capable of generating all the three lineage-specified cells. Spontaneous differentiation of EBs will generate a mixture of cell types representing all three germ layers with very few neural phenotypes.

Several examples confirmed that reproducing *in vivo* inductive and patterning events *in vitro* efficiently directs the differentiation of ESCs into selected neural derivatives (Germain, Banda et al. 2010). Signals, such as retinoic acid, FGFs, Wnt and Dkk are known to be expressed in a concentration gradient from the posterior of the embryo to the anterior and play indispensable role in the patterning of vertebrate embryonic CNS.

Application of these inductors in a temporal manner resulted in various neural cell type specification from ESCs. Treating mouse ESCs with Dkk1 inhibit the Wnt and Nodal signaling and results in increased generation of cells representing the early neuroectoderm stage (Watanabe, Kamiya et al. 2005). Forebrain specific neural progenitors could be enriched by addition of SHH and Wnt. GABAergic neurons can be efficiently differentiated with a serum-free, retinoic acid-free selection protocol and co-treatment with SHH and FGF8 (Barberi, Klivenyi et al. 2003). Differentiation of cortical neurons has markedly been improved by treatment of ESCs with an SHH antagonist (Gaspard, Bouschet et al. 2008). Spinal motor neurons can be specified by first caudalizing the neural progenitors using RA and then ventralizing the cultures with SHH (Figure 8).

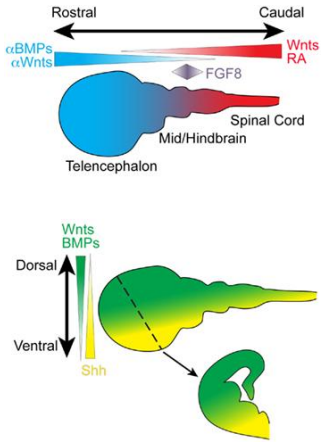

Figure 8. Patterning the vertebrate embryonic central nervous system by secreted signaling factors.

BMP, bone morphogenetic protein; FGF, fibroblast growth factor; RA, retinoic acid; Shh, Sonic hedgehog

Taken from (Petros, Tyson et al. 2011).

3. Retinoic acid receptors in pluripotency and differentiation

Intense research on NRs in the last two decades has uncovered diverse roles of these receptors in developmental biology and metabolism of stem cells (Sonoda, Pei et al. 2008; Simandi, Cuaranta-Monroy et al. 2013). Increasing evidence shows that certain

NRs function in regulating stemness or differentiation of embryonic stem cells (Jeong and Mangelsdorf 2009). A detailed recent review describes several factors belonging to the nuclear receptor class of transcription factors that have been implicated in the promotion or suppression of pluripotency (Wagner and Cooney 2013). Dax1, Lrh1, Sf1 and Esrrβ are linked directly to the maintenance of pluripotency in mouse ESCs and recent genome-wide studies now allow a deeper insight into their regulatory role (Simandi, Cuaranta-Monroy et al. 2013). A systematic profiling of nuclear receptors (Xie, Jeong et al. 2009) and our gene expression characterization of mouse ESCs (re-analysis of microarray data published by (Simandi, Balint et al. 2010) revealed in addition the high expression of Tr4, Ngf1β, Lxrβ, Rarα and Rarγ in undifferentiated ESCs (Figure 9). The importance of these nuclear receptors in stem cell biology remains largely unknown.

More is known about the differentiation promoting role of NRs. For instance, during neural differentiation, thyroid hormone receptors (TRs), the retinoic acid receptors, the retinoid X receptors, the peroxisome proliferator-activated receptors (PPARs), the liver X receptors, the estrogen receptors (ERs), the mineralocorticoid receptor (MR), the glucocorticoid receptor (GR), as well as orphan nuclear receptors have been identified as important regulators (Gonzales and Ng 2013; Stergiopoulos and Politis 2013).

Recently, genes targeted by RAR in early embryonic stem cell to neural differentiation has been identified in a genome-wide chromatin immunoprecipitation study (Mahony, Mazzoni et al. 2011). By profiling the binding of active RAR isoforms in both the absence and presence of retinoid ligands, it was found that only a small subset of sites are constitutively bound. Importantly, the binding of pre-RA bound RAR sites showed high correlation with core ESC regulators. Determinants of post-RA binding site has not been identified so far.

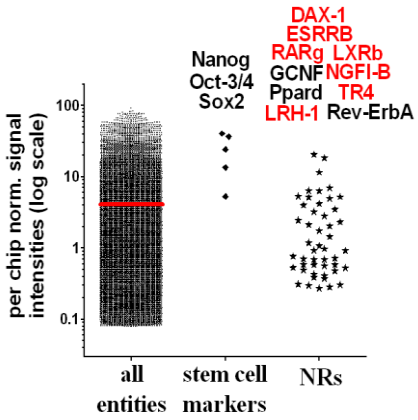

Figure 9. Expression of nuclear receptors in ESCs. Per chip normalized signal intensity data of nuclear receptors in undifferentiated ESCs. Values of all entities and some known embryonic stem cell markers (eg. Nanog, Oct3/4, Sox2) are also shown. Red line indicates the median value of all entities. One dot represents one gene. Gene symbols on the top of the figure indicate the dominantly expressed genes from each group and are considered to be highly expressed in embryonic stem cells. T*aken from* (Simandi, Cuaranta-Monroy et al. 2013).

Little is known about the expression and dynamic behavior of RA co-regulators in ES cells and early differentiation. Present models propose that unliganded RAR form a heterodimer with RXR and binds to DNA. In the absence of ligand the heterodimer is often complexed with co-repressor proteins (such as SMRT, NCoR, etc.) (Nagy, Kao et al. 1997) and inhibits gene expression. Indeed, RA receptors are present not only where RA signaling is active, but also in anterior regions, in which RA is fully absent (Sharma and Kim 1995). Unliganded RAR:RXR thought to have a repressor function. Such active repression of RAR signaling has important regulatory role, for instance it is required for the proper head development (Koide, Downes et al. 2001). The accepted model of RAR:RXR-mediated repression, as we will show later, only partially true for undifferentiated embryonic stem cells.

Importance of P300 in retinoic acid induced differentiation has already been demonstrated. F9 embryonal carcinoma cells expressing a P300-specific ribozyme are

resistant to retinoic acid-induced differentiation (Kawasaki, Eckner et al. 1998). Strikingly, RA-induced protein ubiquitination and degradation of P300 was observed in these cells, however, its significance has not been addressed (Iwao, Kawasaki et al. 1999). An intriguing question is why distinct cells respond differently to the same stimuli. Strikingly, while in embryonic carcinoma (EC) or HL60 cells RA induces cell cycle arrests and differentiation (Flynn, Miller et al. 1983; Mummery, van Rooijen et al. 1987), in hepatocytes RA promotes cell proliferation (Ledda-Columbano, Pibiri et al. 2004). Differences in the chromatin landscape are potentially responsible to regulate the context-dependent biological effects of RA (Delacroix, Moutier et al. 2009). Cell-type specific expression of co-regulators may also resolve this contradiction and provide an additional context-dependent level of RA-signaling control.

Gudas recently showed an example for the complexity of the mechanisms that control RA-mediated transcription in fibroblasts versus stem cells. It was found that while $Rar\beta_2$ is induced in both cell types by RA, Hoxa1 and Cyp26a1 are induced only in F9 stem cells, but not in fibroblasts. Co-activators and RNA Pol II recruitment followed the same pattern. Strikingly, recruitment of Suz12 (a polycomb protein), H3K27me3 (repressive epigenetic modification), H3K9 and K14 acetylation (activation) and methylated CpG islands were just partly able to explain this phenomenon (Kashyap and Gudas 2010). Thus, simple gene expression studies are not sufficient to clarify the context-dependent role of co-regulators in cell fate commitment (Gudas 2012).

A recent study demonstrated that different HDACs bind to the promoter and enhancer regions of RA regulated genes (Urvalek and Gudas 2014). These data also suggest that not only HDAC3 is critical in regulating transcriptional repression of RA-regulated genes, but HDAC1 and HDAC2 also play major role in RA-induced transcription.

V. ARGININE METHYLATION IN EMBRYOGENESIS

Asymmetric arginine methylation is associated with cellular differentiation (Wu, Bruce et al. 2009; Lee, Ma et al. 2012). Early studies revealed high level of aDMA in the nervous system (Nakajima, Matsuoka et al. 1971), but the expression profile of Type I PRMTs and their functional contribution to neurogenesis remained largely uncharacterized (Pawlak, Scherer et al. 2000; Cimato, Tang et al. 2002; Miyata, Mori et al. 2008; Tsai, Pan et al. 2011).

PRMT1 is a major type of protein arginine methyltransferase and the most studied one (Bedford and Clarke 2009). PRMT1 was implicated in RNA processing, and some nucleic acid-binding proteins such as hnRNPA1, EWS and Sam68 are among the earliest reported substrates of PRMT1 (Cote, Boisvert et al. 2003; Araya, Hiraga et al. 2005; Yu 2011). Substrates involved in DNA repair and signal transduction have also been identified. PRMT1 null mice die at an early stage, indicating its essential role in embryonic development (Pawlak, Scherer et al. 2000). PRMT1-dependent methylation of Arg3 on H4 tail peptides facilitates P300-mediated histone H4 acetylation *in vitro* (Wang, Huang et al. 2001; An, Kim et al. 2004; Huang, Litt et al. 2005). These studies collectively suggest that PRMT1 is likely to interact with P300 to regulate transcription.

Mechanistically, PRMT1 dimerization/oligomerization or heterodimerization may be required for PRMT1 to achieve its co-activator function (Lee, Ianculescu et al. 2007). A comparison of mammalian PRMTs revealed that PRMT8 is PRMT1's closest paralogue within this enzyme family, with an identical exon structure and a brain specific expression pattern, and the two paralogs can form a heterodimer (Lee, Sayegh et al. 2005) (Figure 10). Catalytic activity and substrate specificity of PRMT8 is also very similar to PRMT1 (Kim, Kako et al. 2008).

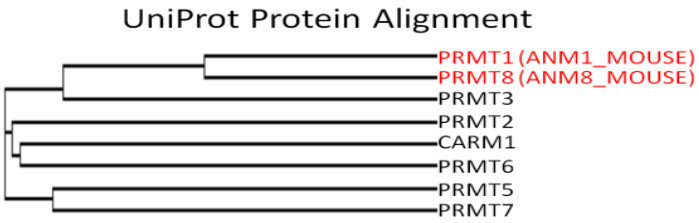

Figure 10. UniProt protein alignment of mouse PRMT1-8 (human alignment results in similar picture).

A remarkable difference is that while PRMT1 is expressed almost in every tissue, PRMT8 expression seems to be highly restricted to the brain, based on animal experiments (Pawlak, Scherer et al. 2000; Taneda, Miyata et al. 2007; Kousaka, Mori et al. 2009). Several studies implicated the importance of arginine methylation in neurogenesis (Cimato, Ettinger et al. 1997; Pawlak, Scherer et al. 2000; Miyata, Mori et al. 2008). Thus, a potential question is, how PRMT1 and PRMT8 contribute to neuronal differentiation and whether they show any functional redundancy.

A mouse study has been published about the immunoreactivity of endogenous PRMT8. It showed a broad distribution of PRMT8 in the mouse CNS neurons with markedly intense signals in the cerebellum, hippocampal formation, and cortex, but the protein was not detected in the cerebellar granular layer and in the astrocytes. In some subset of the neurons, the immunoreactivity was observed in the dendrites and axon bundles (Kousaka, Mori et al. 2009). A single study has investigated the distribution of H4R3me2a histone mark in mouse cortex (Chittka 2010). This latter study showed loss of H4R3me2a staining in the proliferative neuroepithelium and positive staining of post-mitotic neurons. Thus, it is very likely, that an H4R3me2a modification, catalyzed by PRMT1 and PRMT8, specifies the onset of neurogenesis and the commitment of the neural stem cells to differentiate.

Recently, PRMT1 and PRMT8 have been shown to have a non-redundant function in the neural development of zebrafish (Lin, Tsai et al. 2013). The intriguing question is the

molecular and functional consequences of a potential shift from PRMT1/PRMT1 to PRMT1/PRMT8 complex in the context of neural differentiation. Whether PRMT8 has any role in gene expression regulation similar to PRMT1 is not known.

A recent study investigated the role of PRMT1 in gliomagenesis comparing protein level of PRMT1 in 2 normal brain tissues versus 17 primary glioma samples (Wang, Tan et al. 2012). These results are not very conclusive, especially due to the low number of healthy controls. Furthermore, the authors investigated the loss of PRMT1 function in the proliferation of glioma cell lines (T98G, U87MG, and A172). As it was earlier described, loss of PRMT1 resulted in a decreased cell proliferation. The same group also tried to identify targets of PRMT1 in glioma by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Wang, Tan et al. 2012).

PRMT8 has not yet been directly linked to any disease. High-throughput sequencing revealed the importance of PRMT8 related SNPs in telomere length (Kim, Parks et al.), rs12299470, located in intron 1 of PRMT8, was found to be associated with long telomeres. The role of PRMT8 in telomere stability and function remains to be fully elucidated, but it might be linked to tumor progression.

VI. UNDERSTANDING TRANSCRIPTIONAL REGULATION: METHODS

1. Gene silencing

Since the first application of RNA interference (RNAi) in mammalian cells, the expression of short hairpin RNAs (shRNAs) for targeted gene silencing has become a benchmark technology. Using plasmid and viral vectoring systems, the transcription of shRNA precursors that are effectively processed by the RNAi pathway can lead to potent gene knockdown (Lambeth and Smith 2012). The introduction of siRNAs into cultured cells provides a fast but transient gene expression knockdown and has allowed siRNAs to quickly become a ubiquitous tool in molecular biology. In contrast, shRNA offers an opportunity to silence gene expression stably and efficiently. Long term, stable gene silencing can be achieved by shRNA lentiviral constructs which can easily transduce cell lines that are difficult to transfect, such as primary cells, stem cells and non-dividing cells and readily integrate the shRNA into the genome of these cells for stable gene silencing (Rubinson, Dillon et al. 2003). Another advantage of lentiviral transduction that the method avoids the side effects produced by harsh, disruptive, and inefficient transfection protocols using chemically synthesized siRNA. Vector integration into the host genome is followed by the shRNA transcription in the nucleus by polymerase II or polymerase III. The RNA product mimics pri-microRNA and is processed by Drosha. The resulting pre-shRNA is exported from the nucleus by Exportin 5 and then processed by Dicer. The specialized ribonuclease Dicer initiates RNA interference by cleaving double-stranded RNA substrates into small fragments about 25 nucleotides in length (Macrae, Zhou et al. 2006). Dicer catalyzes the first step in the RNA interference pathway and initiates formation of the RNA-induced silencing complex (RISC). The sense strand is targeted resulting in its degradation, whereas the antisense strand directs RISC to a mRNA that has a complementary sequence. In the case of perfect complementarity, RISC

cleaves the mRNA. In the case of imperfect complementarity, RISC represses translation of the mRNA. In both of these cases, the shRNA leads to target gene silencing (Figure 11).

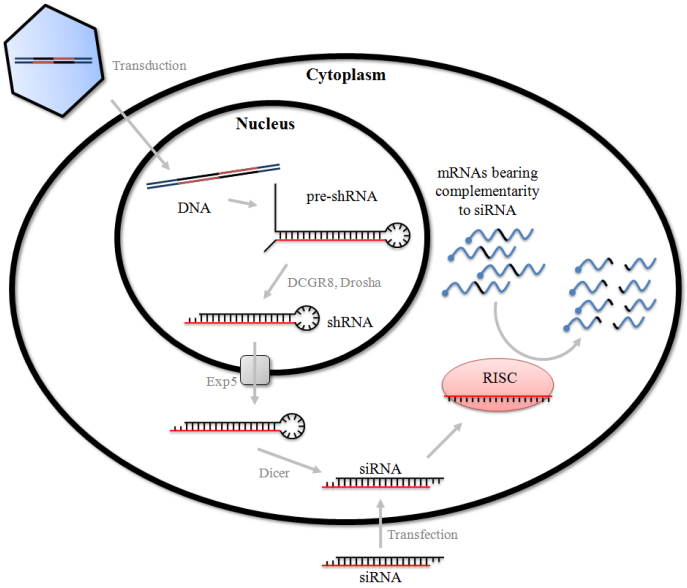

Figure 11. Mechanism of RNAi induced gene silencing. Expressed shRNAs are processed by Drosha and its double-stranded RNA-binding partner DGCR8 in the nucleus and then exported by Exportin-5 to the cytoplasm where they associate with Dicer, resulting in the removal of the loop sequence. siRNAs are introduced into cell as short duplexes and recognized by the Dicer. After association with RISC and removal of one of the RNA strands, they target mRNAs possessing a complementary sequence, resulting in their degradation. *Taken from* [http://link.springer.com/chapter/10.1007%2F978-90-481-3415-1_5/fulltext.html].

2. Genome-wide transcriptional analysis (Microarray and RNA-seq)

Since the invention of DNA microarrays in the 1990s, it has been the technology of choice for large-scale studies of gene expression. Advances in microarray technology enabled massively parallel mining of biological data, with biological chips providing hybridization-based expression monitoring, polymorphism detection and genotyping on a genomic scale (Schena, Heller et al. 1998). The ability of these arrays to simultaneously interrogate tens of thousands of transcripts has led to important advances in tackling a

wide range of biological problems, including the identification of genes in developmental processes (Zhao, Fung-Leung et al. 2014). Currently, microarrays remain the most popular approach for transcript profiling and can be readily afforded by many laboratories. Nonetheless, array technology has several limitations. For example, background hybridization limits the accuracy of expression measurements, particularly for transcripts present in low abundance. Furthermore, probes differ considerably in their hybridization properties, and arrays are limited to interrogating only those genes for which probes are designed.

RNA-seq is a recently developed approach to transcriptome profiling that uses deep-sequencing technologies (Wang, Gerstein et al. 2009) and it may well become a replacement to microarrays for whole-genome transcriptome profiling.

A comparison of data sets derived from RNA-seq and Affymetrix platforms using the same set of samples showed a high correlation between gene expression profiles generated by the two platforms (Zhao, Fung-Leung et al. 2014). However, it also demonstrated that RNA-seq was superior in detecting low abundance transcripts, differentiating biologically critical isoforms, and allowing the identification of genetic variants. RNA-seq also demonstrated a broader dynamic range than microarray, which allowed for the detection of more differentially expressed genes with higher fold-change. However, RNA-seq poses novel algorithmic and logistical challenges for data analysis and storage.

3. Chromatin Immunprecipitation followed by sequencing (ChIP-seq)

The technique called chromatin immunoprecipitation (ChIP) is a type of immunoprecipitation experimental technique used to investigate the interaction between proteins and DNA in the cell. It aims to determine whether specific proteins (eg.

transcription factors, co-regulators) are associated with specific genomic regions. ChIP also aims to determine the specific location in the genome that various histone modifications are associated with, indicating the target of the histone modifying enzymes. Briefly, the method is as follows: protein and associated chromatin in a cell lysate are temporarily cross-linked by formaldehyde, the DNA-protein complexes (chromatin-protein) are then sheared by sonication. DNA fragments associated with the protein of interest are selectively immunoprecipitated by a protein specific antibody. Finally, DNA fragments are purified and used for PCR or sequenced (Figure 12).

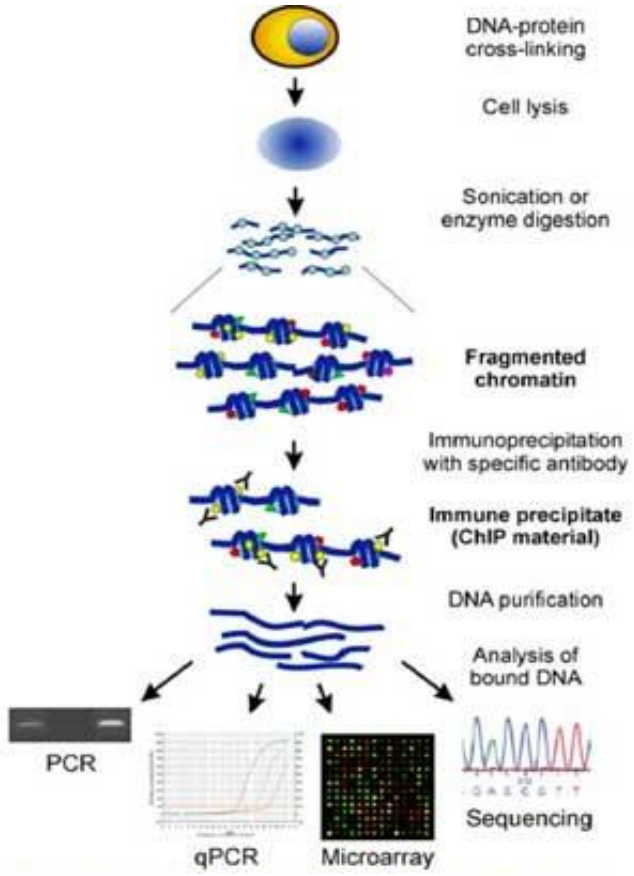

Figure 12. Overview of chromatin immunoprecipitation (ChIP) *Taken from* (Collas 2010).

Raw data obtained from ChIP followed by sequencing (ChIP-seq) experiments requires downstream bioinformatic analysis, which includes the alignment of the sequenced reads. The primary processed data can be visualized with different software (eg. Integrative

Genomics Viewer, IGV, Broad Institute). IGV displays data in horizontal rows called tracks. Typically, each track represents one sample or experiment. For instance, panel shown on Figure 20. shows 4 tracks: P300 and RXR ChIP-seq data of untreated (veh) and treated (RA) samples. By default, IGV displays data showing one genomic region, but offer the possibility to compare and visualize more genomic regions in the same time, using the same scale (eg. Cyp26a1, Dleu7, Rbp1, etc. on Figure 20). This type of visualization allows the direct comparison of distinct genomic regions and thus most of the figures presented in this dissertation represent more ChIP-enriched regions (enrichment of a transcription factor or a co-regulator). Importantly, in case of each ChIP the same scale was used for the comparison of treated and untreated samples. Thus, difference between the height of eg. P300 peak is comparable between treated and untreated samples. A detailed description for the software is available here: http://www.broadinstitute.org/igv/book/export/html/6.

VII. MATERIALS AND METHODS

1. Embryonic stem cell culture

D3 and E14 embryonic stem cells (ESCs) were kind gift of Tomo Saric and Istvan Szatmari. ESCs were grown on a layer of mitotically inactivated mouse primary embryonic fibroblasts (PMEF) or SNL feeder cells to promote growth and prevent differentiation. ESCs were cultured on 0.1% gelatin-coated plates in feeder-free condition for at least three passages prior to RT-qPCR or Western blot analysis. Cell cultures were incubated in a humidified atmosphere with 5% CO_2 at 37˚C. The ESC medium was prepared by supplementing DMEM Glutamax media with 15% FBS (Hyclone), 1000U of leukemia inhibitory factor (LIF), penicillin/streptomycin, non-essential amino acids and 2-mercaptoethanol.

2. Neural differentiation of embryonic stem cell cultures

The protocol has been adopted from a publication (Bibel, Richter et al. 2007). Briefly, ESCs were cultured on feeder cells for at least two passages after thawing and for three passages prior to differentiation without feeder cells in ESC medium. Embryoid body (EB) formation was induced by plating 4 $*10^6$ cells onto bacteriological Greiner Petri dishes in 15 ml ES differentiation media (10% FBS). Medium was changed after 2 days of aggregation by transferring the entire EB suspension into a 50 ml Falcon tube and letting EBs settle by leaving them for about 3–5 min at room temperature. Supernatant was removed and EBs were resuspended gently with 15 ml ES differentiation medium again by pipetting up and down twice. This procedure was repeated at day 4 and day 6, but medium was complemented with retinoic acid 1:2000 from 10 mM stock solution (final concentration 5 μM). EBs were disaggregated with 0.05% trypsine at day 8, cell suspension was filtered through a 40 mm nylon cell strainer and cells were plated onto

poly-L-ornithin (PORN)-laminin pre-coated culture dishes in N2 medium (DMEM, F-12, L-glutamine, insulin, transferrine, selenite, putrescine, progesterone, BSA) at about 2 *10^5 cells per cm^2density. N2 medium was changed 2 h, 1 day and 2 days after plating. Cells in long-term cultures were grown in complete medium, containing B27 supplement.

3. shRNA-based stable gene silencing assays

shRNA lentiviral plasmids for targeting the mouse PRMT1 and PRMT8 (MISSION shRNA, TRCN0000018490-493 and TRCN 0000097479-483, respectively) were purchased from Sigma. Lentiviruses were produced by transient co-transfection of shRNA lentiviral plasmids and helper plasmids (pMD2.G, pRSV-Rev, pMDLg/pRRE)into 293T. The medium was replaced after 12 h following transfection to fresh ESC media; the conditioned medium was collected after another 24 h, cleared by low-speed centrifugation, and filtered through 0.4-mm-pore-size cellulose acetate filters. Serial dilutions of freshly harvested conditioned medium were used to infect 10^5 ES cells in a six-well plate. Cells were then maintained under puromycin selection. Non-target shRNA was used as a control (SHC016, Sigma).

4. Plasmids and transient transfection

The plasmid expressing full-length wild-type human PRMT1 was described earlier (Balint, Szanto et al. 2005). NHf-290-Hoxb1-luciferase and 2xDR2-luciferase plasmids were a kind gift of Toshihiko Ogura and Ronald M. Evans (Ogura and Evans 1995). Luciferase reporter were generated by insertion of PCR amplified enhancer region of Hoxb1, Cyp26a1, Pmp22, Spsb1 or PRMT8 (PCR amplified from genomic DNA or BAC clone RP23-280P20, respectively). Full length human PRMT8 was PCR amplified from GFP-PRMT8 (Lee, Sayegh et al. 2005) and inserted into EcoRI/SalI site of pCMV-Tag2

(Stratagene). All constructs were verified by DNA sequencing. Embryonic stem cells were passaged three times without feeder cells and plated onto 0.1% gelatin coated plates in ES media. Transfection was carried out using FuGENE® transfection reagent (Promega), according to the manufacturer's instructions. Luciferase activity was determined by Luciferase Assay System (Promega) and normalized to β-galactosidase activity.

5. Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from the cell cultures and tissue samples with TRIZOL reagent (Invitrogen). All experiments were carried out on biological replicates. cDNA synthesis was performed with High Capacity cDNA Reverse Trancription kit (Applied Biosystems) according to the manufacturer's recommendation. Quantitative PCR was performed using real-time PCR (ABI PRISM 7900, Applied Biosystems). Gene expression was quantified by the comparative C_T method and normalized to Gapdh expression. Gapdh expression levels did not vary between cell types or treatments. Values are expressed as mean ± SD of the mean. GraphPad Prism version 5.02 was used for data interpretation.

6. Microarray analysis

Microarray experiments were performed by the UD-GENOMED Medical Genomic Technologies Ltd. Briefly, total RNA was purified by using the RNeasy kit (Qiagen) or by TRIZOL reagents according to the manufacturer's recommendations. Sense-strand cDNA was generated from 250 ng total RNA using Ambion® WT Expression Kit. Fragmentation and labeling was performed using the Affymetrix GeneChip WT terminal Labeling Kit. All kits were used according to the manufacturer's recommendation. GeneChip Mouse Gene 1.0 ST Arrays were used to obtain whole-genome gene-level

expression data. Data analysis was performed using GeneSpring GX 12.6 software (Agilent, Santa Clara, CA). Affymetrix data files were imported to the GeneSpring by RMA16 algorithm. Normalization to the median of control samples was performed. Differential expression analysis was carried out to determine the number of changing entities during EB formation by using unpaired T-test, Asymptotic p-value computation and Benjamini-Hochberg multiple testing correction. Test correction was not used in case of day 16 neuronal cells. Hierarchical cluster analysis was performed using Euclidean distance metric (dendrograms are not shown on the figures). All microarray data from this study have been submitted to the Gene Expression Omnibus database (Series accession numbers: GSE37060 and GSE47214). Data were further analyzed with the IPA software (Ingenuity® Systems, www.ingenuity.com).

7. RNA-seq

Illumina RNA-sequencing was performed using standard procedures at the Centre National de Genotypage (CNG) Paris, France. RNA-seq library was prepared from two biological replicates by using TruSeq RNA Sample Preparation Kit (Illumina) according to the manufacturer's protocol. Briefly, 2.5µg total RNA was used for the library preparation. In the first step poly-A tailed RNA molecules (mRNA) were purified with poly-T oligo-attached magnetic beads. Following the purification mRNA was fragmented in the presence of divalent cations at 85ºC, then first strand cDNA was generated using random primers and SuperScript II reverse transcriptase (Invitrogen, Life Technologies). This was followed by the second strand cDNA synthesis, then double stranded cDNA fragments went through an end repair process, the addition of a single ‘A’ base and then barcode-indexed adapter ligation. Adapter-ligated products were enriched with adapter-specific PCR to create cDNA library. Agarose gel electrophoresis was performed on E-

Gel EX 2% agarose gel (Invitrogen, Life Technologies) and the library was purified from the gel using QIAquick Gel Extraction Kit (Qiagen). Fragment size distribution and concentration were determined on Agilent BioAnalyzer using DNA1000 chip (Agilent Technologies).

Analysis of RNA-seq data has been carried out at the Center for Clinical Genomics and Personalized Medicine, University of Debrecen by Attila Horvath. The TopHat-Cufflinks-CummeRbund toolkit trio (Trapnell, Hendrickson et al. 2013) was used for mapping spliced reads, making transcript assemblies, and getting, sorting and visualizing gene expression data. Downstream data analysis were performed in R. Expression Heatmap was created using R package pheatmap.

Sequencing data has been submitted to SRA under accession number: SRP042072 / PRJNA248061.

8. Western blot analysis

Whole cell extract containing 20µg protein was separated by SDS- gel electrophoresis in 10 or 12.5% polyacrylamide gel and then transferred to Immobilon-P Transfer Membrane (Millipore Crp., Billerica, Massachusetts). Membranes were probed with anti-PRMT1 (07-404; Millipore), anti-PRMT8 (ab73686; Abcam), anti-ASYM24 (07-414; Millipore), anti-SYM11 (07-413; Millipore), anti-OCT3/4 (sc5279; Santa Cruz), anti-ACTIN (A2066, Sigma), anti-GAPDH (sc32233; Santa Cruz) antibodies, according to the manufacturer's recommendations. Nuclear and cytoplasmic proteins were isolated by a protocol adapted from Panomics, Inc. for their Nuclear Extrcation Kit.

9. Immunfluorescence detection of stem cell and differentiation markers

Undifferentiated or differentiated embryonic stem cells were fixed in 4% paraformaldehyde and incubated for 1 h in blocking buffer (PBS, 10% FBS, and 0.1% TritonX-100). Primary antibodies were diluted in blocking buffer and applied overnight at 4°C. After three washes in PBS, FITC-conjugated secondary antibodies were diluted at 1:200 in blocking buffer and applied for 1h at room temperature. Cells were washed three times in PBS and visualized on an AxioVision inverted fluorescence microscope. Primary antibodies were obtained from the following sources: anti-OCT3/4 (sc-5279; Santa Cruz), anti-SSEA (MC-480; DSHB), anti-TUJ1 (MMS-435P; Covance), anti-PRMT1 (07-404; Millipore), anti-PRMT8 (HPA039747; Sigma), anti-H4R3me2a (Active Motif, Cat. no 39705), anti-SYN1 (Millipore, AB1543P and BD Biosciences Cat. no 611393).

10. Chromatin immunoprecipitation - Sample preparation

ChIP and ChIP-qPCR experiments were carried out according to a previously described protocol (Barish, Yu et al. 2012), with minor modifications. Embryonic stem cells were cultured in the presence or absence of RA for various time points and then cross-linked in two steps: (1) disuccinimidyl glutarate (DSG) (ProteoChem) for 30 min then (2) 1% methanol-free ultrapure formaldehyde (Thermo Scientific, #28908) for 10 min at room temperature. Glycine was added for 5 min in 125 mM final concentration. After fixation chromatin was sonicated with Diagenode Bioraptor to generate 200-1000 bp fragments. Chromatin was immunoprecipitated with antibodies against pre-immune IgG (Millipore,12-370), RXR (Santa Cruz, sc-774), RAR (Santa Cruz, sc-773), P300 (Santa Cruz, sc-585), HDAC3 (Santa Cruz, sc-11417), Oct3/4 (Santa Cruz, sc-8628), H3K27ac (Abcam, ab4729) and H4R3me2a (Active Motif, Cat. no 39705). Beads were washed 6 times with cell lysis buffer and DNA was eluted and RNase treated, protease treated and

purified (Qiagen, MinElute). Qubit fluorometer (Invitrogen) was used to determine the final DNA concentration prior to library preparation. ChIP-qPCR data was normalized to input gDNA (INPUT) samples.

11. Chromatin immunoprecipitation - Library preparation

Library preparations were carried out at the Center for Clinical Genomics and Personalized Medicine, University of Debrecen. ChIP-seq libraries were prepared by Ovation Ultralow Library Systems (Nugen) or TruSeq ChIP sample preparation kit according to manufacturer's instructions. Briefly, 1-10 ng immunoprecipitated DNA per sample was submitted to end repair. Adaptors were ligated to the end-repaired DNA fragments and the libraries were amplified with adaptor-specific primers in 16 PCR cycles. Libraries were gel-purified with E-gel systems (Life Technologies) to remove primers. Libraries were then quantified by Qubit fluorometer and their quality was assessed with Agilent 1000 DNA Chip.

12. ChIP-seq data analysis

Primary analysis of ChIP-seq data was done at the Center for Clinical Genomics and Personalized Medicine, University of Debrecen by Erik Czipa, Gergely Nagy and Endre Barta using the ChIP-seq analyze command line pipeline (Barta 2011). Briefly, Burrows-Wheeler Alignment Tool (BWA) was used to align the reads to mm9 genome assembly (Li and Durbin 2009). Peaks were predicted by the findPeaks.pl (HOMER), and filtered based on the HOMER score. Peaks predicted in D3 ES input control were subtracted as well (Liu, Scannell et al. 2011) (see Table below). Peakset overlaps were defined by intersectBed (BEDTools) (Quinlan and Hall 2010). Differential binding analysis was carried out by DiffBind (Ross-Innes, Stark et al. 2012). The fullLibrarySize parameter

was used to separate the peaks with significantly changing 'binding affinity'. The normalization was made by the total read numbers of samples (calculated from the source BAM files). Genome coverage files for visualization were made by makeUCSCfile.pl. Integrative Genomics Viewer (IGV2.3, Broad Institute) was used for data browsing (Thorvaldsdottir, Robinson et al. 2012). Numbers of overlapping peaks were visualized by VennMaster (Kestler, Muller et al. 2008). HOMER was used for making meta-histograms, and finding motif enrichments.

RNA Pol, Oct3/4, Sox2, Klf4 and Nanog ChIP-seq were used from (Chen, Xu et al. 2008). Sequencing data has been submitted to SRA under accession number: SRP042072 / PRJNA248061.

Antibody	Cell line	Knockdown	Treatment	Predicted	Filtered	Score
P300	D3	shSCR	vehicle	7062	5820	10-
P300	D3	shPRMT1	vehicle	8675	7276	10-
P300	D3	shSCR	RA	5646	1897	10-
P300	E14	shSCR	vehicle	18809	8258	15-
P300	E14	shPRMT1	vehicle	8235	5473	15-

13. Calcium imaging, loose-patch and whole-cell patch-clamp recording

All measurements were carried out at the Department of Phsyology, University of Debrecen.

ESCs were differentiated to neurons for 16 days, and loaded with the calcium indicator dye Oregon Green 488 BAPTA-1, AM in 1.5 micromolar concentration, disolved in artificial cerebrospinal fluid (aCSF; composition in mM: NaCl, 125; KCl, 2.5; $NaHCO_3$, 26; glucose, 10; NaH_2PO_4, 1.25; $CaCl_2$, 2; $MgCl_2$, 1; myo-inositol, 3; ascorbic acid, 0.5; sodium pyruvate, 2). Calcium imaging measurements has been carried out as described

by Koszeghy et al (Koszeghy, Pal et al. 2009). Briefly, cells were viewed using a Zeiss Axioskop microscope with a 40× water dipping objective. The microscope was equipped with a fluorescent imaging system (Till Photonics GmbH, Gräfelfing, Germany) containing a xenon bulb-based Polychrome V light source, and a CCD camera. In the experiments monitoring Ca^{2+} concentration fluctuations, the excitation wavelength was set to 488 nm by the monochromator of the Polychrome V light source. Throughout the calcium imaging, 4×4 binning of the 1.4-megapixel camera was employed (resulting in frames with 344×260 pixel resolution), the frame rate was set to 10 Hz.

For extracellular loose-patch recording of the neuronal action potential firing, micropipettes filled with aCSF were used (resistance ≈5 MΩ). After achieving a seal resistance of ≥40 MΩ, the electrical activity of the cells was recorded in voltage-clamp mode using an Axopatch 200A amplifier (Molecular Devices, Union City, CA, USA). Data acquisition was performed at 10-kHz sampling rate.

For whole-cell patch clamp recordings patch pipettes with 5 MΩ pipette resistance were pulled with a Narishige vertical puller, and filled with a solution containing (in mM): K-gluconate, 120; NaCl, 5; 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES), 10; EGTA, 2; $CaCl_2$, 0.1; Mg-ATP, 5; Na_3-GTP, 0.3; Na_2- phosphocreatinine, 10; biocytin, 8; pH 7.3. Whole-cell patch-clamp recordings were performed using an Axopatch 200A amplifier, with 10 KHz sampling rate.

14. Tissue samples

83 samples from glioblastoma and 54 samples of normal brain tissue were collected during neurosurgical operations in the Department of Neurosurgery, University of Debrecen. Each sample was collected from a different patient. Normal samples were collected either during functional neurosurgery for epilepsy or non-tumor herniated brain

tissue during tumor surgery. Sections for histological analysis were cut from the same samples used for mRNA analysis. The samples were frozen promptly after removal on the surface of liquid nitrogen and were stored at –80°C until processing. All procedures were approved by the National Ethical Committee, and every patient signed an informed consent form.

15. Enrichment of histone modification

Engraved microscopic slides were agarose-coated by dipping slides into hot (80°C) 1% SeaKem LE agarose (Lonza Inc., Allendale, New Jersey, USA) dissolved in distilled water and drying on air. 2×10^5 cells in 100 µl of 0.75% LMP (low melting point) agarose (Sigma-Aldrich, Saint Louis, Missouri, USA) were layered onto the agarose-coated slides and were incubated for 4 min. at 37ºC to let the cells sediment. Slides were placed on ice for 2 min. to solidify the agarose and were put in ice-cold 1% Triton X-100 / 1×PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) / 5mM EDTA for 10 min. Cells were washed with 1×PBS / 5mM EDTA and blocked with 5% Blotto Non-Fat Dry Milk (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) dissolved in 0,2% Tween / 1×PBS on ice for 30 min. Cells were washed with 1×PBS / 5mM EDTA and labeled with rabbit anti-H4R3me2 primary antibody (Cat.no 39705, Active Motif, Carlsbad, California, USA, 1mg/ml stock) diluted 800× in 1% BSA / 1×PBS / 5mM EDTA at 4ºC overnight using a total volume of 150 µl labeling solution on each slides. Cells were washed with 1×PBS / 5mM EDTA and labeled with Alexa 647-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen / Life Technologies, Carlsbad, California, USA, 2mg/ml stock) diluted 800× in 1% BSA / 1×PBS / 5mM EDTA on ice for 2 hours. Cells were washed with 1×PBS / 5mM EDTA and nuclei were stained with 25 µg/ml propidium-iodide. Mean fluorescence intensities were measured using an iCys

laser scanning cytometer (CompuCyte Corp, Cambridge, MA, USA). Argon-ion laser (488nm) was used for the excitation of propidium-iodine and HeNe laser (633nm) was used for the excitation of Alexa 647 dye. For identifying single nuclei, contouring was based on propidium-iodine detected in the long red channel (>650nm) and samples were analyzed according to cell cycle phases. Fluorescence of Alexa 647 was detected in the long red channel (>650nm) as well. Data evaluation and hardware control was performed by the iCys 3.4 software for Windows XP.

16. Statistical analysis

All experiments were conducted as biological triplicates. Values are expressed as mean ± SD of the mean. GraphPad Prism version 5.02 was used for data interpretation. We performed unpaired t-tests unless otherwise indicated and results were considered to be significant with $p<0.05$. Details of the statistical analysis is discussed along with the description of the certain method.

VIII. HYPOTHESIS AND RESEARCH QUESTIONS

Hypotheses

- Components of retinoic acid pathways are expressed in undifferentiated embryonic stem cells and addition of retinoic acid to these cells regulates large sets of genes and initiate dynamic epigenetic reorganization that contribute to cellular differentiation
- Retinoic acid target genes are already bound by retinoic acid receptors and generally regulated by dynamic recruitment of co-repressors and co-activators
- PRMT1 affect P300-dependent transcriptional events by arginine methylating P300 or members of the co-regulatory complex
- PRMT8 is cell-type specific arginine methyltransferase that is involved in neurogenesis
- PRMT1 and PRMT8 contribute to retinoic acid induced neural differentiation.

Research questions

- Which components of the retinoic acid pathway are expressed at mRNA level in mouse embryonic stem cells?
- How many genes are regulated by the addition of retinoic acid?
- Is P300 a general co-activator of retinoic acid induced transcriptional program?
- Is there a crosstalk between arginine methyltransferase PRMT1 and P300 in retinoic acid induced differentiation?
- What are the functional and transcriptomic consequences of loss of PRMT1?
- Is PRMT8 involved in neural differentiation?

IX. AIMS

Our study focused on the transcriptomic and epigenetic events of retinoic acid induced differentiation of mouse embryonic stem cells.

The general aim was to understand the following concepts in transcriptional regulation:

1. gene selective transcriptional regulation
2. fine-tuning of signal responsiveness
3. cell-type specific interpretation of the same signal
4. functional consequences of signal misinterpretation

As more specific goals, we aimed to:

1. Determine components of retinoic acid signaling that are expressed in undifferentiated embryonic stem cells
2. Identify retinoic acid induced genes and their epigenetic regulation
3. Identify putative cross-talk signaling between P300 and PRMT1
4. Characterize PRMT1-dependent transcriptional events in cellular differentiation
5. Establish a multistage differentiation model of mouse embryonic stem cells to neurons to explore the mechanistic and functional role of novel co-regulator PRMT1 and PRMT8 in neurogenesis.

X. RESULTS

Expression pattern of elements of retinoic acid signaling in undifferentiated ESCs

As a first step of our work we identified which elements of the retinoid signaling pathway (depicted on Figure 5) are present in undifferentiated ESCs. Our global gene expression analyses showed that only Crabp1, Rarγ and Rxrα are expressed at higher levels in ESCs. RBPs, ALDHs and Cyp26s are present at very low mRNA levels (Figure 13). These results suggest that although the receptors of the retinoic acid are present in undifferentiated ESCs, enzymes required for endogenous production of RA are missing, thus it is unlikely that these cells synthetitize retinoic acid, making it a suitable model system to investigate retinoic acid induced transcriptional and epigenetic events.

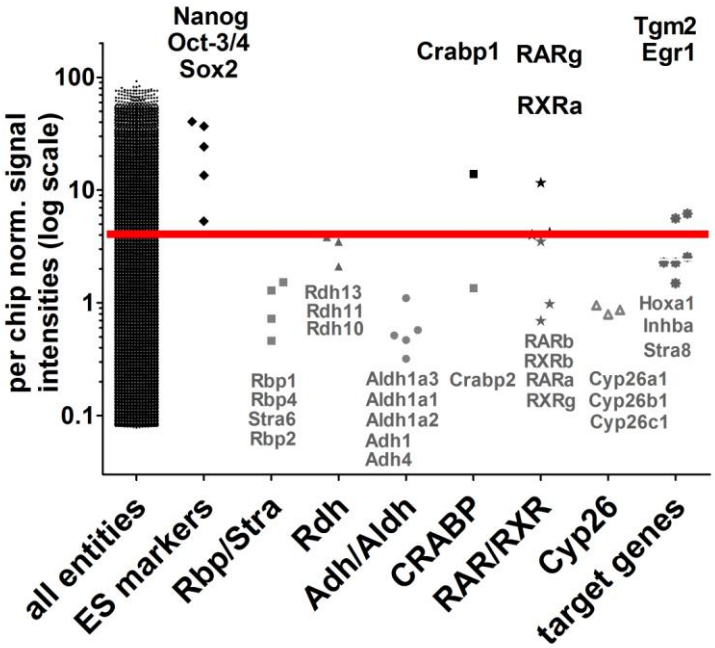

Figure 13. Expression level of components of the retinoic acid signaling. Per chip normalized microarray signal intensity data of genes involved in retinoid signaling are shown. Values of all entities and some known ES markers are also shown. Red line indicates the median value of all probes. One dot represents one gene. Genes showing expression above the median were considered as present in ESCs. Gene symbols on the top of the figure indicate the dominantly expressed genes from each group, while the genes with lower expression are colored in gray.

Identification of retinoic acid induced genes

Having established that ESCs express the receptors required for retinoic acid response, we next characterized the consequences of RA treatment. 24 hrs of RA resulted in obvious phenotypic changes, such as enlarged flattened cellular morphology, indicating early differentiation of the cell culture (Figure 14).

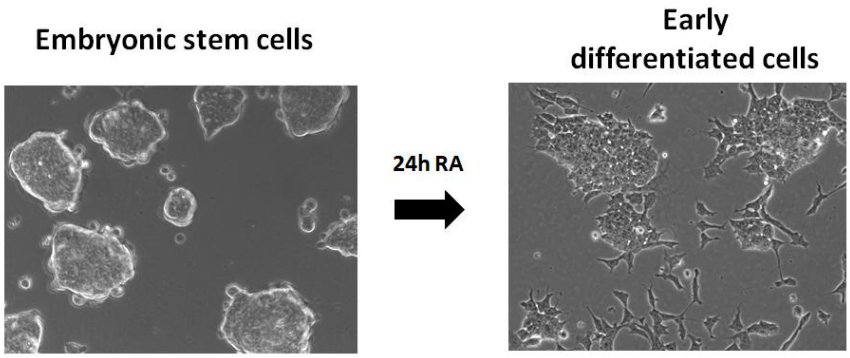

Figure 14. Morphology of RA-treated embryonic stem cell culture. Undifferentiated embryonic stem cells cultured in feeder-free condition (*left*) and culture treated with 1 μM RA for 24 hrs (*right*).

To determine the global transcriptomic effect of RA treatment in wild-type ESCs we carried out RNA-seq analysis. Harvesting the cells at 1 h, 3 h and 24 h following ligand treatment allowed us to categorize the regulated genes as early induced, late induced and late repressed genes. In accordance with other studies, only low number of genes (35 genes) were identified as early RA regulated genes (regulated at 1 h or 3 h). Several previously identified direct RA target genes are represented on the list, eg. Cyp26a1, Dhrs3, Hoxa1, Hoxb1, Rarβ, Stra6 or Stra8 (Figure 15) (Martinez-Ceballos, Chambon et al. 2005; Kashyap and Gudas 2010; Mendoza-Parra, Walia et al. 2011).

Genes regulated by retinoic acid are known to play essential roles in neuronal differentiation. Hoxa1 activity is essential for the neuronal differentiation of ES cells in the presence of RA (Martinez-Ceballos and Gudas 2008), Hoxb1 and downstream signaling is required for proper hindbrain segmentation (Bell, Wingate et al. 1999), while

the RA catabolizing enzyme Cyp26a1 is dispensable for correct A-P patterning and production of migratory cranial neural crest cells in the developing mammalian brain (Uehara, Yashiro et al. 2007). As part of our analysis, we also found so far uncharacterized putative targets, such as Pmp22 or Dleu7. Pmp22 (Peripheral myelin protein 22) is known to primarily expressed in the compact myelin of the peripheral nervous system (Li, Parker et al. 2012) and thus potentially contribute to the proper neurogenesis in vivo. Almost no information is available about the function of Dleu7 (deleted in lymphocytic leukemia, 7) (Zhu, Li et al. 2012).

As Figure 15 shows, expression changes of all selected genes could be validated by RT-qPCR on independent sample sets.

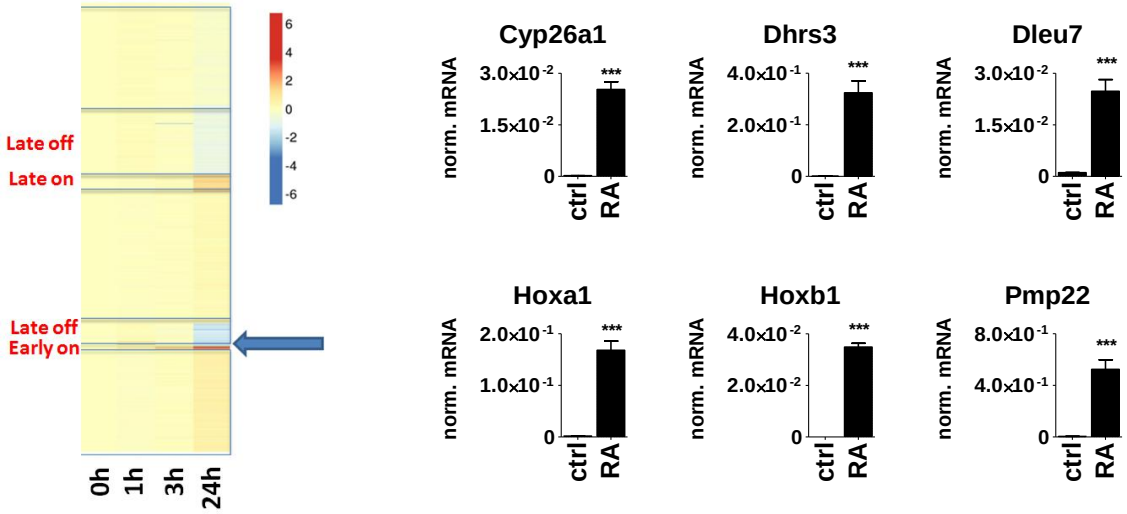

Figure 15. Retinoic acid induced gene expression in embryonic stem cells. *Left:* Heatmap visualization of clustered RNA-seq data. Blue arrow indicate the cluster of the identified 35 early responding, RA-regulated genes. *Right:* RT-qPCR validation of retinoic acid-induced gene expression. ESCs were treated with RA in 1 µM concentration or vehicle (DMSO) for 12 h. *** P ≤0.001

Retinoic acid-dependent epigenetic changes

Epigenetic events induced by retinoic acid remain poorly characterized in stem cells with the exception of few recent studies (Urvalek, Laursen et al. 2014; Urvalek and Gudas

2014). According to the classical model identified in distinct cell types, unliganded RAR:RXR heterodimers are bound to the retinoic acid response elements and repress gene activation via the recruitment of co-repressor complexes containing HDAC3. Upon ligand activation co-repressors are replaced by co-activators, such as P300. In order to revisit this classical model of retinoid induced epigenetic changes in embryonic stem cell differentiation, we used the well characterized RA response elements of the Cyp26a1 and Hoxa1 genes as representative examples (Figure 16).

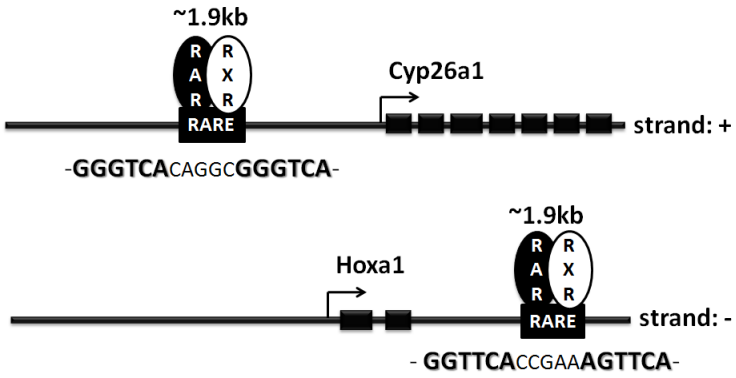

Figure 16. Schematic representation of previously identified retinoic acid response elements (RARE) of Cyp26a1 and Hoxa1.

Previous studies in different cell types suggest a rather static model that already unliganded RAR:RXR are bound to the DNA and keep the chromatin repressed. As a first step, we determined the RA-dependent dynamics of RAR and RXR using chromatin immunoprecipitation followed by qPCR. We could detect RAR and RXR binding to the RARE site of Cyp26a1 already at 0 h which was slightly further induced by retinoic acid (Figure 17). Unexpectedly, we found no enrichment of RAR and RXR on the Hoxa1 enhancer prior to retinoic acid treatment, however even short-term (15 min) retinoic acid treatment initiated the binding of the receptors to the response elements. Such dynamic redistribution of the RAR:RXR receptors were not described previously.

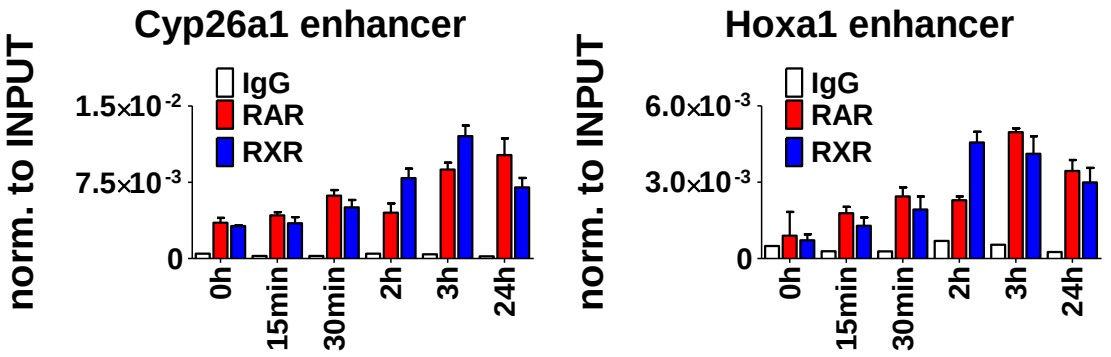

Figure 17. Ligand induced recruitment of RAR:RXR. Embryonic stem **c**ells were treated with 1 μM RA for the indicated time points after treatment initiation. RAR and RXR binding is shown as determined by chromatin immunprecipitation followed by RT-qPCR (ChIP-qPCR). Measurement were done in biological triplicates, results of a representative example are shown.

Next, we determined the dynamics of the co-repressor HDAC3 and co-activator P300 binding to the same sites in the same model system. Analysis of Hoxa1 enhancer confirmed the classical model, showing ligand induced exchange between HDAC3 and P300 (Figure 18). Strikingly, we found no signs of binding of P300 and HDAC3 at any time point on the Cyp26a1 enhancer (see later on Figure 20). Since Cyp26a1 and Hoxa1 were similarly upregulated upon RA treatment (Figure 15), this observation suggests an enhancer-specific selective epigenetic regulation of retinoid target genes.

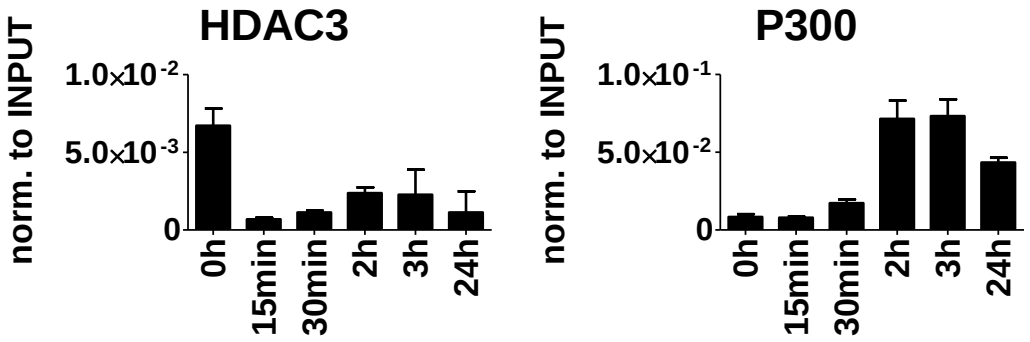

Figure 18. Ligand induced co-regulator cycle. HDAC3 and P300 binding are shown on the retinoic acid response element of Hoxa1 as determined by chromatin immunprecipitation followed by RT-qPCR. Embryonic stem **c**ells were treated with 1 μM RA for the indicated time points.

This conclusion led us to hypothesize that retinoic acid induced epigenetic events are not identical on each enhancer and there are enhancers activated independent of the presence of P300. To extend our analysis and identify more examples where retinoic acid induces target gene expression without the recruitment of P300, we determined P300 cistrome genome-wide by ChIP-seq analysis.

We could identify 3420 consensus P300 binding sites present in both D3 and E14 untreated ESCs. Bioinformatic analysis of the DNA sequences occupied by P300 revealed that P300 is mainly recruited to binding sites of stem cell specific transcription factors (eg. Oct3/4, Sox2, Klf4, Esrrb and Nanog). Upon RA treatment there was a ~3 fold reduction relative to the number of P300 binding sites in control cells and the occupancy was also substantially decreased, but new P300 sites also could be identified (Figure 19).

	D3	E14
Oct3/4	36.85%	34.36%
Sox2	28.57%	30.73%
Klf4	40.05%	29.61%
Esrrb	22.16%	21.65%
Nanog	10.55%	6.28%

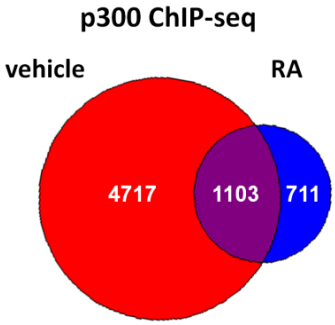

Figure 19. P300 cistrome in mouse embryonic stem cells. E14 and D3 ESCs were used to identify genome-wide P300 binding by ChIP-seq. *Left:* Motif analysis of P300-occupied genomic regions in untreated ESCs. Presence of predicted transcription factor binding sites in P300-occupied regions are shown in percentage. *Right:* Area-proportional Venn-diagram of RA induced P300 redistribution in D3 ESCs. Number of identified binding sites in vehicle and RA treated (1 μM, 24 h) samples are shown.

The results obtained in genome-wide analysis confirmed the selective recruitment of P300 to Hoxa1 retinoic acid response element. Including the RA-induced genes previously identified in the RNA-seq analysis into our analysis, we could identify more examples showing P300 binding upon RA treatment similar to Hoxa1. Examples of

retinoid-induced genes without P300 recruitment could be also detected. We classified these genes as P300-independent (eg. Cyp26a1, Dleu7, Stra8) or P300-dependent (eg. Hoxa1, Hoxb1, Pmp22) genes. Data obtained from RXR ChIP-seq were used to confirm the presence of RXR, obligate heterodimer of RAR, at the retinoic acid response elements (Figure 20).

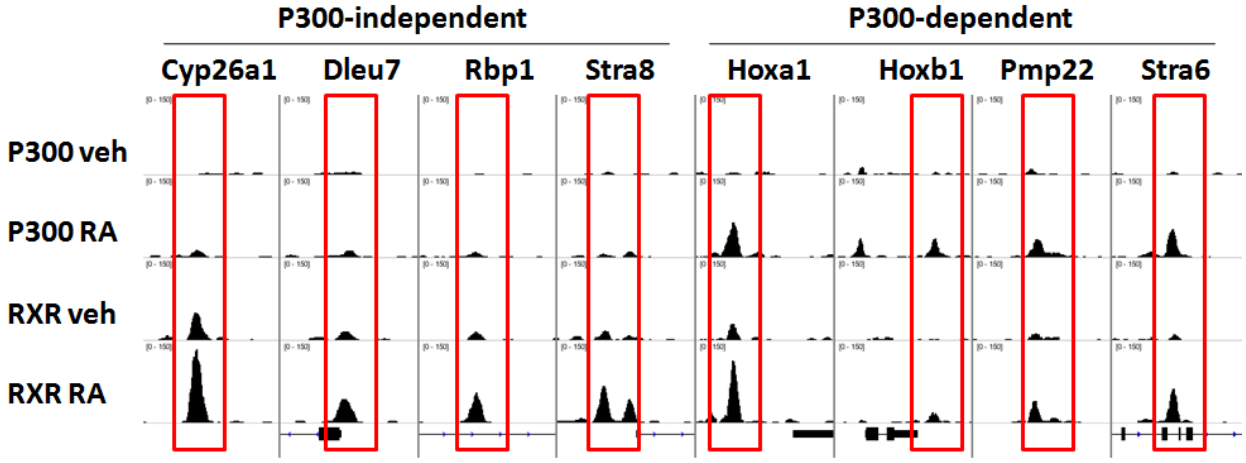

Figure 20. Selective P300 recruitment of P300 to enhancers of retinoic acid target genes. Undifferentiated (veh) and RA-treated (1 μM, 24 h) ESCs were used to determine the RXR and P300 cistrome. Genome browser view of RXR and P300 ChIP-seq data. Known and predicted retinoic acid response elements of the indicated retinoic acid target genes are shown (see *page 45* for further description).

Results of motif analysis shown on Figure 19 suggested that P300 is primarily recruited by the stem cell specific transcription factors rather than by the RAR:RXR, however there is no experimental evidence supporting this idea. Nevertheless, recent studies described the existence of super enhancers and regulatory "hot spots" that are occupied by large number of cell-type specific transcription factors and also recruit in addition P300 and other co-regulators (Hnisz, Abraham et al. 2013; Siersbaek, Rabiee et al. 2014). Analysis and visualization of published Oct3/4, Nanog and Sox2 ChIP-seq data obtained in undifferentiated ESCs (Chen, Xu et al. 2008) revealed that P300-occupied enhancers indeed show the binding of at least one of the stem cell-specific transcription factors

(Figure 21). Binding of ESC-specific transcription factor Oct3/4 could be validated by ChIP-qPCR on selected regions (Figure 21). Strikingly, Oct3/4 binding did not change in the investigated time-frame, suggesting the potential importance of Oct3/4 in the determination of differentiation beside its well-established role in maintenance of the pluripotent stage.

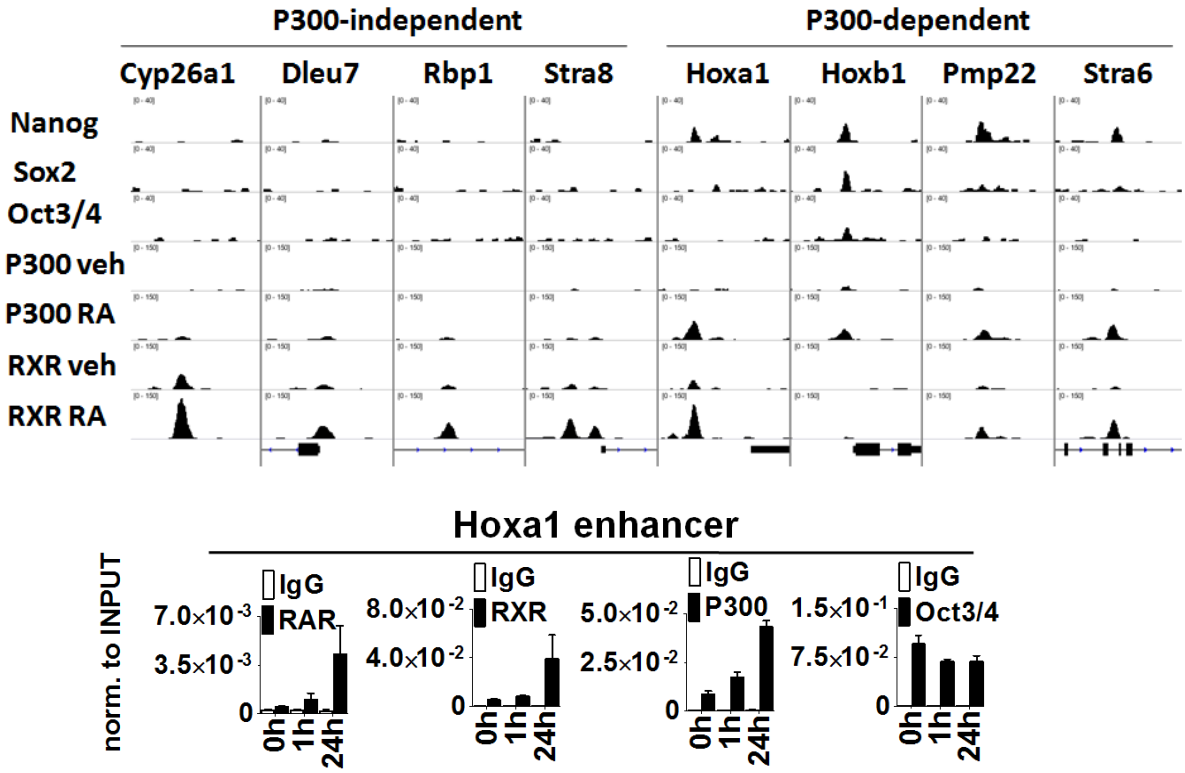

Figure 21. Selective P300 and ESC-specific transcription factor recruitment to retinoic acid target genes. Top: Nanog, Sox2 and Oct3/4 ChIP-seq data obtained and re-analyzed from (Chen, Xu et al. 2008) were visualized in genome browser. Bottom: a representative ChIP-qPCR analysis of Oct3/4, RAR, RXR and P300 binding on the RARE of Hoxa1.

Taken together, we concluded from these observations that P300 is selectively bound and used by retinoic acid response elements. This observation raise the possibility that additional co-regulators might have similar enhancer selectivity as well.

Identification of PRMT1 as a selective co-repressor of "regulatory hotspots"

Crosstalk between protein arginine methyltransferases (PRMTs) and P300 was demonstrated by others (Chevillard-Briet, Trouche et al. 2002; Ceschin, Walia et al. 2011). It has been also shown previously that methylation of Arg3 on Histone 4 tail peptides by PRMT1 facilitates P300-mediated histone acetylation *in vitro* (Wang, Huang et al. 2001; An, Kim et al. 2004; Huang, Litt et al. 2005). Based on this our hypothesis was that by targeting PRMT1-mediated arginine methylation we can modulate the retinoic acid response of P300-dependent genes.

As the first step, we assessed the expression profile of PRMT-family members in undifferentiated embryonic stem cells. Gene expression data obtained from RNA-seq in undifferentiated ESCs revealed the high expression level of PRMT1 and to a lesser extent PRMT5, suggesting their dominant role in ESCs arginine methylation (Figure 22).

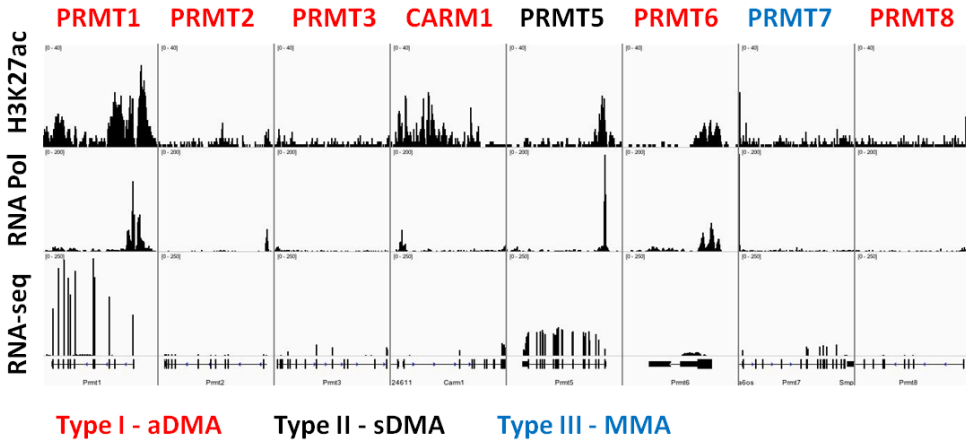

Figure 22. Comparison of RNA-seq signal intensity, and histone acetylation level and RNA Pol binding of PRMTs, determined by ChIP-seq analysis. Genome browser view of Type I (PRMT1, 2, 3, 6, 8 and CARM1), Type II (PRMT5) and Type III (PRMT7) protein arginine methyltransferases are shown.

Although PRMT1 knockout ESCs (labeled on figures as -/-) were established previously (Pawlak, Scherer et al. 2000), a proper genetic control was not available for comparative studies. Thus, to understand the contribution of PRMT1-mediated arginine methylation to

P300-mediated transcriptional and epigenetic program of ESCs in a well-controlled system, we established stable PRMT1 knockdown ESCs using lentiviral-based approach.

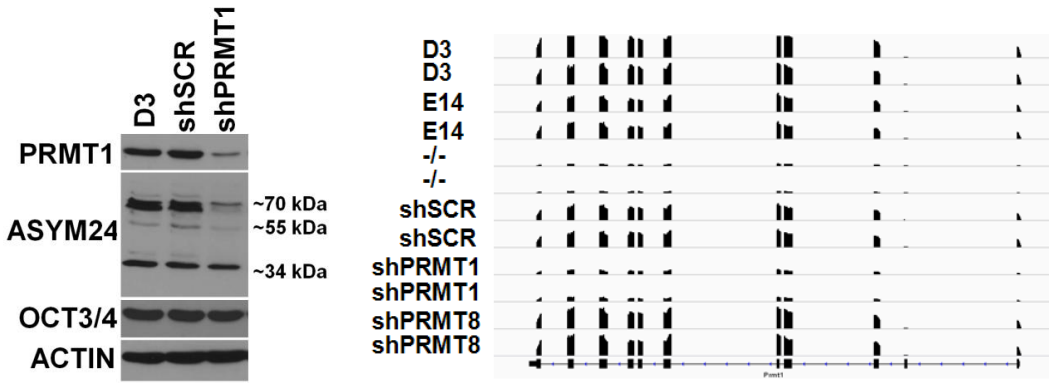

Figure 23. Establishment of stable PRMT1 knockdown ESCs. *Left:* Immunoblots of protein samples from the indicated D3 ESC lines; probed for expression of PRMT1, ASYM24 and OCT3/4. ACTIN was used as a loading control. *Right:* Genome browser view of RNA-seq data comparing the PRMT1 coding locus in the indicated ESCs in the presence or absence of knockdowns. -/- represents transgenic knockout of PRMT1. Biological duplicates are shown.

We could observe efficient gene silencing at both the mRNA and protein level (Figure 23). As a functional consequence of loss of the dominant arginine methyltransferase, we we next investigated the arginine methylation level of total cellular proteins using anti-ASYM24 antibody that specifically recognizes asymmetrically arginine-methylated proteins (Boisvert, Cote et al. 2003). With the esception of a protein with 34 kDa size, we found substantially decresed signal in PRMT1 knockdown cells. Subcellular fractionation revealed that however PRMT1 was mainly localized in the cytoplasm, nucleus also contained proteins arginine methylated by PRMT1. ASYM24 signal intensity decreased in total in both cytoplasmic and nuclear protein fractions as the result of PRMT1 silencing (Figure 24). The total amount of PRMT1-mediated Histone 4 aRginine 3 methylation (H4R3me2a) was also decreased, further demonstrating the functional consequences of the loss of PRMT1 activity (Figure 24).

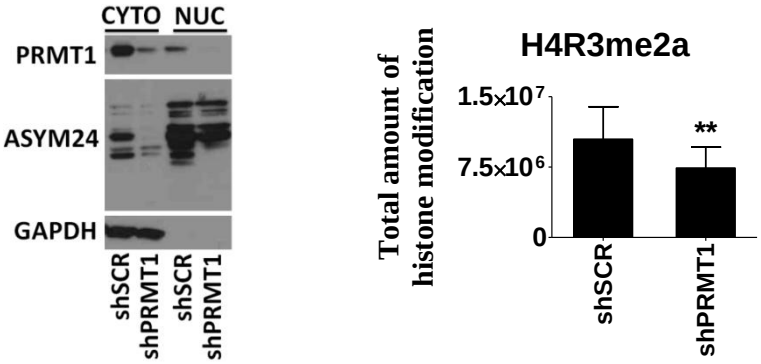

Figure 24. Hypomethylation of cellular proteins in PRMT1 knockdown ESCs. Left: Western blot analysis of subcellular fractionations. Cytoplasmic (CYTO) and nuclear fractions (NUC) of undifferentiated shSCR and shPRMT1 D3 ESCs were isolated and probed for PRMT1 and ASYM24. GAPDH is a loading control for cytoplasm. Right: Total intranuclear levels of H4R3 asymmetric dimethylation as compared by indirect immunofluorescence between undifferentiated shSCR and shPRMT1 ESCs. See *page 55* for the description of the method. **P ≤0.01

In order to characterize whether the early response of RA is affected in PRMT1-depleted cells, we performed genome-wide comparison of gene expression in RA treated embryoid bodies. While most of the established retinoid targets, such as Cyp26a1, Dhrs3, Dleu7, Rbp1, Stra8 were induced to the same degree in PRMT1-depleted and control cells, Hoxa1, Hoxb1, Stra6, Pmp22 and Spsb1 showed significantly higher induction in PRMT1-depleted, hypomethylated cells ($FC \geqslant 1.5$, $P \leqslant 0.05$) (Figure 25).

The selective effect of PRMT1 on gene expression and the results obtained in previous analysis of P300 binding showed remarkable overlap between the genesets. These results indicate a functional crosstalk between PRMT1 and P300 in the regulation of many retinoic acid induced genes, including the Hox genes.

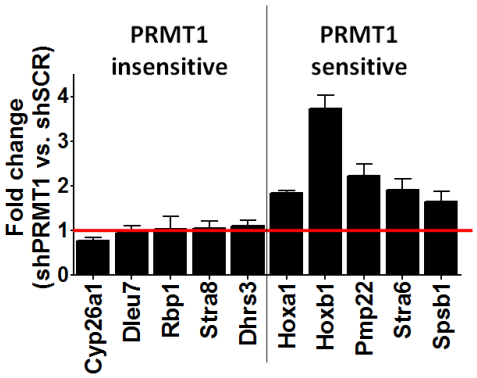

Figure 25. Gene expression changes in control and PRMT1-depleted cells upon RA-treatment. Cells were spontaneously differentiated for four days and then treated with RA or vehicle for 12 h. Relative expressions were calculated from per chip normalized microarray data (vehicle vs. RA treated, n=3 per condition) for each gene. Calculated relative expression values of PRMT1-depleted cells then were normalized by values calculated for the control samples. Red line shows when the induction is equal in both PRMT1-depleted and control cell types (eg. Cyp26a1, Dhrs3).

To further investigate the observed selectivity at the promoter level, we transfected ESCs with the Hoxb1-promoter containing the endogenous DR2 RA-response element, linked to a luciferase reporter gene (Ogura and Evans 1995). Transient overexpression of PRMT1 resulted in a decrease in basal transcription level of Hoxb1-luciferase construct. To a lesser extent Pmp22 and Spsb1 enhancers were also suppressed in the presence of PRMT1. The ligand-induced retinoid response of Hoxb1 and Spsb1 enhancers were also decreased (Figure 26).

In order to see whether P300 and PRMT1 are present in one complex in ESCs we carried out co-immunprecipitation experiments. Despite to our several attempts, we could not detect the presence of PRMT1 and P300 in one complex. FCS or FRET analysis should be carried out to further investigate the potential co-binding of the two proteins.

However, we found that several asymmetrically arginine methylated proteins could be co-immunprecipitated along with P300. Although the detected signals were weak, this data was reproducible (Figure 27).

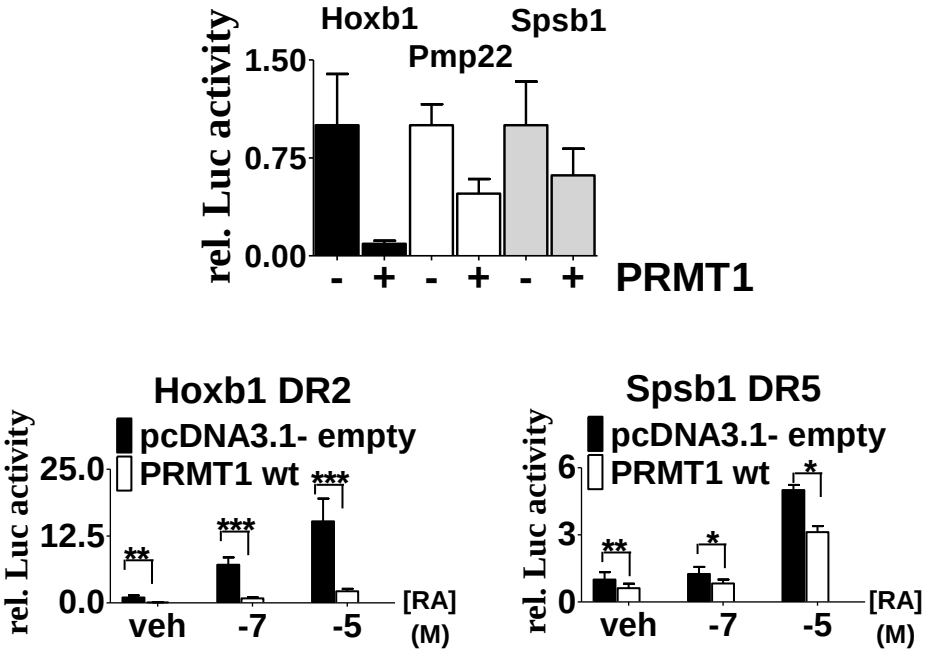

Figure 26. PRMT1-dependent transcriptional repression. pcDNA.3.1-PRMT1 (PRMT1) or empty pcDNA3.1 (empty) plasmids were co-transfected with NHf290-Hoxb1-Luciferase plasmid (Ogura and Evans 1995) or Pmp22 and Spsb1 enhancers cloned into Luciferase encoding vector. Luciferase signal intensity was determined and normalized to βgal signal (rel.luciferase activity). Treatments: -7 = 10^{-7} M RA, -5 = 10^{-5} M RA. *P ≤0.05, **P ≤0.01 *** P ≤0.001.

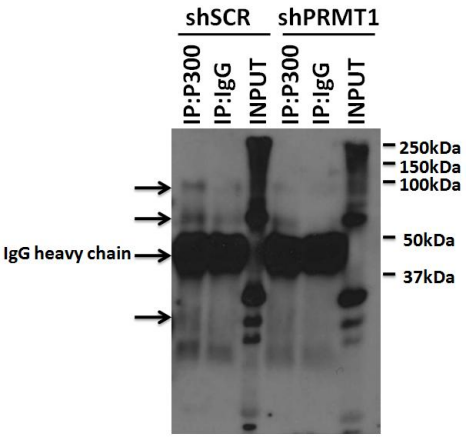

Figure 27. Asym24 immunoblot of P300 interacting complex in ESCs. Nuclear extract of control and PRMT1-depleted cells were used to co-immunprecipitate proteins with anti-P300 or IgG isotype control.

Importantly, the asymmetric arginine methylation of these proteins were PRMT1-dependent, confirming the existence of proteins that are in one complex with P300 and

asymmetrically arginine methylated by PRMT1. Identification of these proteins will require further analysis, eg. mass spectrometry.

ChIP grade antibody was not available to confirm the co-binding of P300 and PRMT1. To get further mechanistic evidence that P300 occupied regions are targeted by PRMT1, we determined the level of PRMT1-dependent H4R3me2a signal in the proximity of PRMT1-sensitive and insensitive genes. As shown in Figure 28, Hoxa1 and Spsb1 enhancers showed clear PRMT1-dependent enrichment of H4R3me2a at these genomic regions.

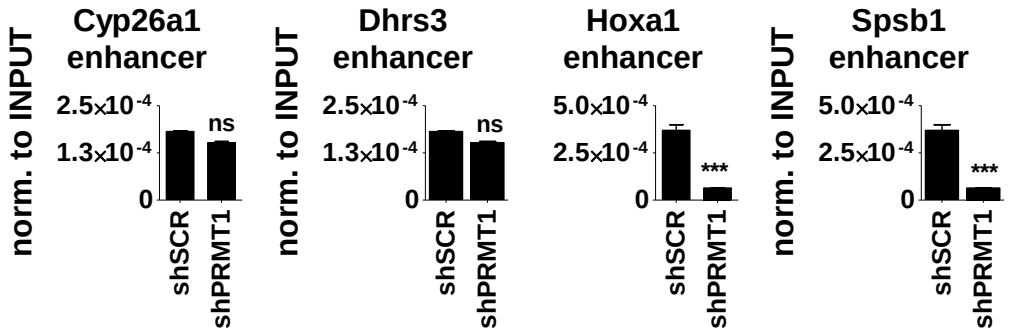

Figure 28. Enhancer selective enrichment of H4R3me2a signal. ChIP-qPCR signals on the indicated individual enhancers as detected in untreated shSCR and shPRMT1 ESCs. ns - non significant, *** P ≤0.001.

These data collectively suggest that RA reguleted enhancers can be grouped into two categories: PRMT1-sensitive and insensitive ones, with distinct transcription factor complexes. The PRMT1-sensitive ones are characterized by PRMT1-dependent H4R3me2a, ESC-specific transcription factors and P300 binding. Importantly, loss of PRMT1 results in increased retinoic acid responsiveness affecting developmental-related genes, such as Hoxa1 or Hoxb1.

RA has coordinating effects on multiple lineages during early embryoid body formation

As the next step in our study we sought to identify how PRMT1 contributes to spontaneous and retinoic acid induced early ESCs differentiation. Differentiation of ESCs is often induced by cell aggregation resulting in the formation of embryoid bodies (EBs). This spontaneous differentiation of wild-type ESCs induces significant changes in the expression level of approximately 1000 genes (Figure 29).

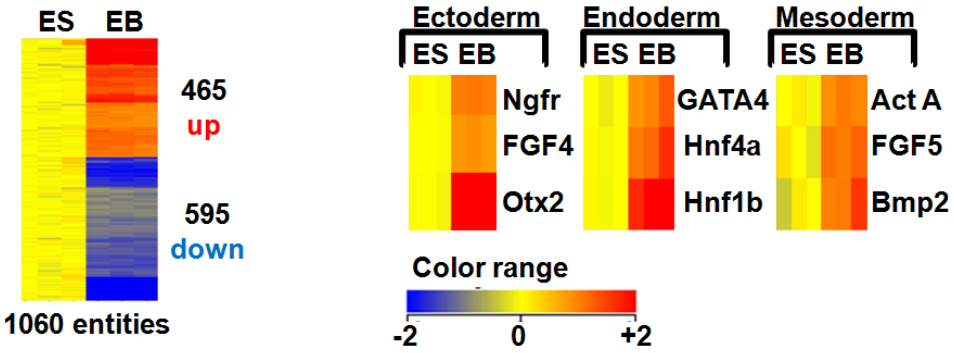

Figure 29. Transcriptional changes in early ESC differentiation. *Left:* Heat map analysis of gene expression changes during the ES–EB transition. Expression of 1060 probes has changed significantly (FC $\geqslant$ 2, $P \leqslant 0.05$). *Right:* Heat map analysis of the expression of early ectodermal (Ngfr, Fgf4, Otx2), endodermal (Gata4, Hnf4a, Hnf1b) and mesodermal (Activin C, Fgf5, Bmp2) differentiation markers during ES–EB transition. Color intensities reflect the ratios of signal intensities.

These ~1000 genes include upregulation of germline specific markers such as Ngfr, Fgf4 and Otx2 for ectoderm, Activin C, Fgf5 and Bmp2 for mesoderm and Gata4, Hnf4a and Hnf1b for endoderm differentiation. Importantly, the presence of these prototypic markers suggests that our differentiation protocol allows the appearance of precursors of all three germ layers.

Compared to spontaneous differentiation, cells differentiated in the presence of RA upregulate markers of neuroectoderm (Tuj1), pancreatic β cells (Pdx1) and hepatocytes (Hnf1b). These results are in line with previously published results showing that RA can

be used *in vitro* to promote lineage commitment into these directions. On the other hand, Otx2 (required for proper forebrain development) is repressed by RA. Therefore, EB differentiation in the presence of RA biases the spontaneous differentiation of ESCs into certain directions, whilst other pathways appear to be repressed by the same signal.

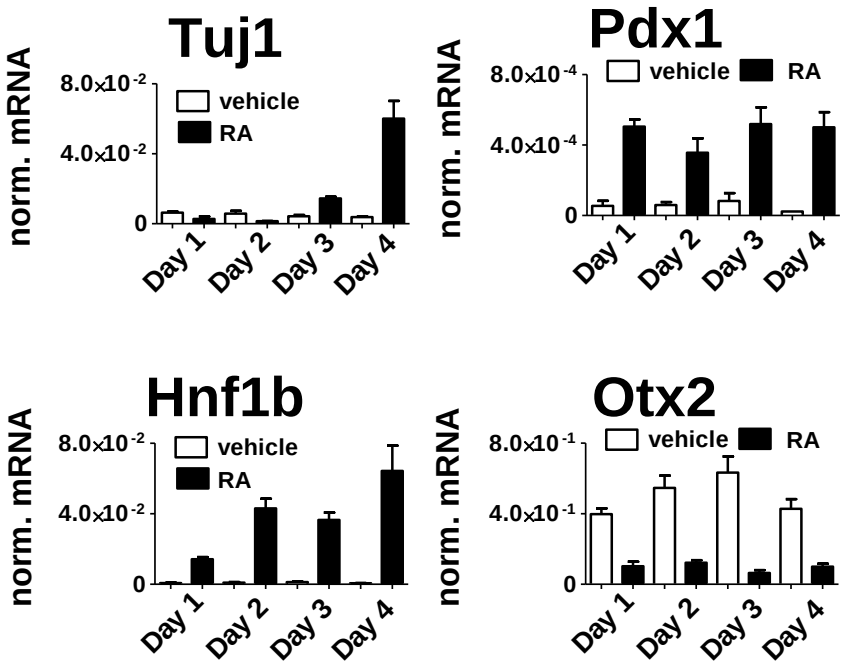

Figure 30. Differentiation promoting effect of RA during early EB formation. Expressional changes of neural (Tuj1), hepatocyte (Hnf1b), pancreatic (Pdx1) and Otx2 markers shown as detected by RT-qPCR. RA was added in 5 μM final concentration or DMSO as a control to ESCs and samples were collected at day 1, 2, 3 and 4 following the first treatment.

We next evaluated the differentiation potential of the PRMT1-depleted cells. Genome-wide comparison and RT-qPCR validation of spontaneously differentiated control and hypomethylated cells showed that classical lineage markers of endoderm, mesoderm and ectoderm were similarly induced upon spontaneous differentiation, indicating no essential function of PRMT1 in spontaneous, non-retinoic acid driven differentiation (Figure 31).

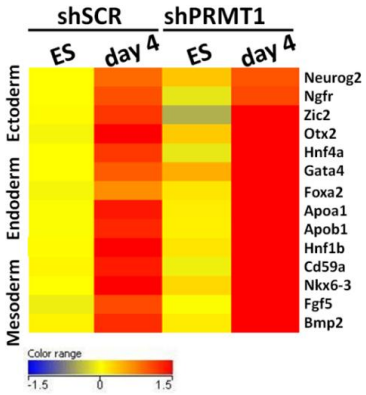

Figure 31. Expression of lineage markers in spontaneously differentiating PRMT1-depleted ESCs. Heatmap display of the expression of lineage-specific markers. Microarray analysis of D3 shSCR and shPRMT1 ESCs and day 4 spontaneously differentiated cells are shown. Expression level is normalized to shSCR day 0 (ES).

To further evaluate the differentiation potential of PRMT1-depleted cells we injected PRMT1-/- ESCs into immunodeficient (SCID) mice. Histological evaluation of the teratomas revealed obvious differentiated structures from all three germinal layers, excluding the possibility that residual PRMT1, present in knockdown cells, is sufficient for differentiation (Figure 32).

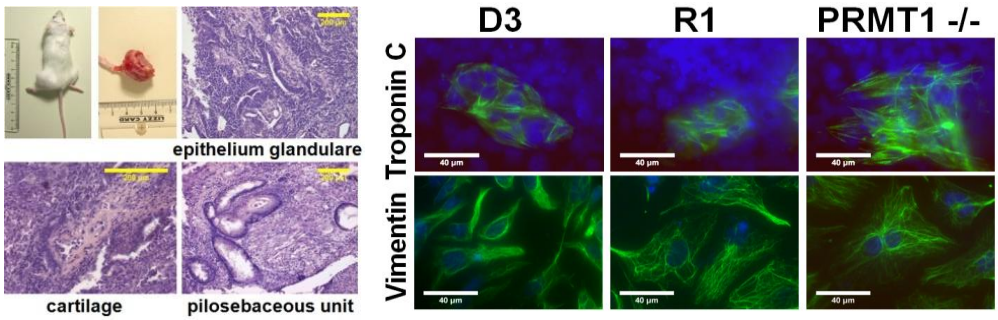

Figure 32. Characterization of differentiation potential of PRMT1-depleted ESC. Hematoxylin and eosin staining of teratoma derived from PRMT1-/- ESCs. Cells were injected into lower leg of SCID mice at a concentration of $5x10^6$ cells/ml..Troponin C (cardiomyocyte marker) and Vimentin (mesenchymal cell) immunocytochemical staining of D3, R1 and PRMT1-/- derived ESCs. Cells were spontaneously differentiated for 4 days as embryoid bodies, then disaggregated and plated for the staining. Cells were co-stained by DAPI (blue). Scale bar = 40 µm (right).

PRMT1-/- cells were also able to differentiate to Vimentin+ mesenchymal cells and TroponinC+ beating cardiomyocytes, suggesting that cells could undergo differentiation to various cell types in the absence of PRMT1.

Asymmetric arginine methylation is present in distinct stages of retinoic acid induced neural differentiation

PRMT1 null mice die at an early developmental stage, indicating its essential role in embryonic development. Several studies demonstrated the high expression of PRMT1 in the developing nervous system as well (Pawlak, Scherer et al. 2000; Cimato, Tang et al. 2002; Miyata, Mori et al. 2008; Tsai, Pan et al. 2011). Having established that PRMT1 affected retinoic acid responsiveness indicated in neurogenesis and also affected gene expression of Hoxa1 and Hoxb1, known factors for proper neural differentiation, we next focused on the role of PRMT1 in neural differentiation specifically.

To explore the involvment of arginine methylation in neuronal differentiation, we set up an embryonic stem cell-based model system. This can be dissected into 4 stages: (I) undifferentiated embryonic stem cells, (II) aggregates of spontaneously differentiating cells (III) cells committed to neuroectoderm as a result of retinoic acid treatment and (IV) fully differentiated neuronal cells (Bibel, Richter et al. 2007) (Figure 33). Our results discussed above did not indicate essential role of PRMT1 in stage 1 and 2. Analysis of stage 3 and 4 allowed us to investigate the role of PRMT1 in the early and late stages of neural differentiation.

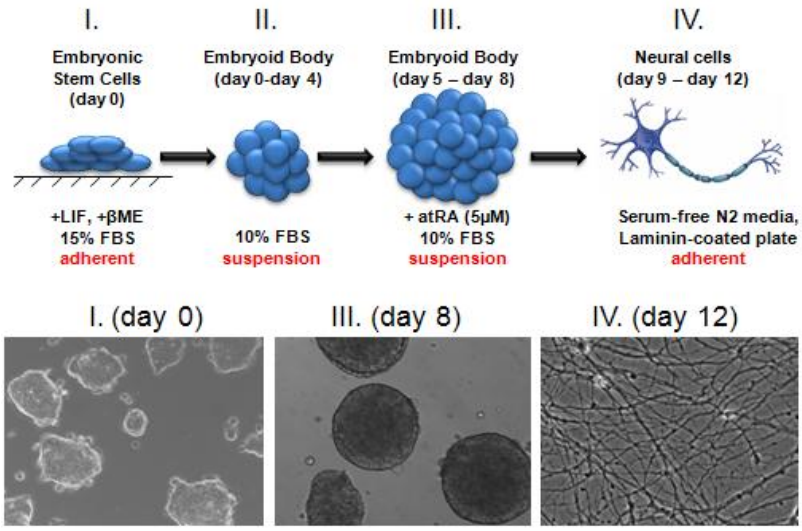

Figure 33. Flow diagram of the multistage differentiation procedure(I) undifferentiated stem cell culture, (II) embryoid body formation, (III) all-trans retinoic acid (RA) treatment and (IV) neuronal culture on poly-L-ornithin/laminin-coated plates. Indicated stages are shown with bright field microscopy.

Importantly, patch clamp analysis of these terminally differentiated neurons suggested that these cells have electrophysiological properties similar to those brain-derived neurons (Barth, Sutterlin et al. 2014) (Figure 34).

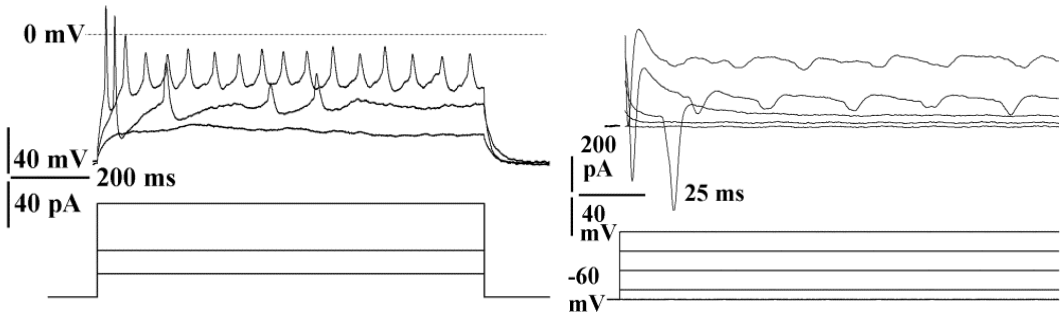

Figure 34. Typical single-cell voltage clamp and current clamp measurements of terminally differentiated (day 16) neurons. *Left panel*: Voltage-gated inactivating and non-inactivating inward currents (from -30mV) are marked on the figure. *Right panel:* ESC-derived neurons were used in depolarising current injection steps. AP-trains with over-shoot are shown.

The model system allows a systematic step-by-step analysis of early cell fate commitment, as well as late neural cell type specification (Niwa, Miyazaki et al. 2000;

Bibel, Richter et al. 2007). The progression of cellular differentiation is also reflected at the level of active chromatin, as it is detected by H3K27ac ChIP-seq analysis. Genomic regions of pluripotency genes, such as Oct3/4, Nanog, Zfp42 become deacetylated, while neural markers (Lhx1, Tuj1, Vamp2, Ntrk3) show enriched H3K27ac upon differentiation (Figure 35).

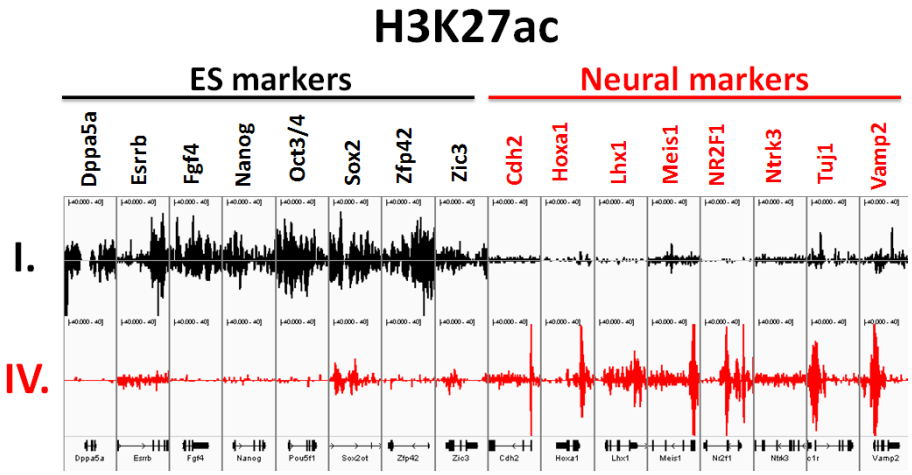

Figure 35. Visualization of active histone mark H3K27ac in genome browser. ESC markers and neural markers are shown in undifferentiated ESC stage (I.) and in terminally differentiated neurons (IV.).

Using this multistage differentiation system of neural differentiation, we have found that proteins with asymmetric and symmetric dimethylated arginine residues (representing Type I and Type II PRMTs, respectively) are present in ESCs. Asymmetric arginine methylation level of these proteins was changing dynamically during retinoic acid induced neural differentiation, suggesting either an increased level of target proteins or increased Type I enzyme-activity. A 68 kDa protein (likely Sam68, a previously identified PRMT1 target) (Cote, Boisvert et al. 2003) showed dramatically increased level of arginine methylation as detected by ASM24 during differentiation. Importantly, symmetric arginine methylation was not altered significantly (Figure 36).

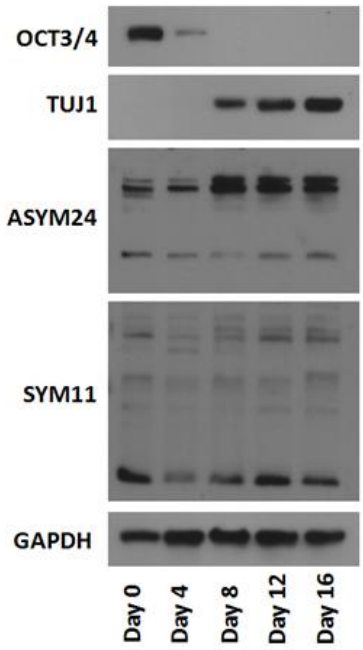

Figure 36. Immunoblot analysis of different stages of neural differentiation. Samples were collected at day 0, 4, 8, 12 and 16 of neural differentiation. Anti-ASYM24 antibody recognizes proteins that contain arginines that are asymmetrically dimethylated (aDMA). Anti-SYM11 antibody recognizes proteins that contain arginines that are symmetrically dimethylated (sDMA). GAPDH serves as a loading control.

As the next step, we assessed the expression profile of 8 members of the PRMT-family. RT-qPCR validation confirmed that PRMT1 is highly expressed in ESCs as we could detect it earlier by RNA-seq and its expression remained constant during neural differentiation showing a slight increase upon RA-treatment. Other Type I PRMTs, putatively responsible for aDMA were less abundant in ESCs, but PRMT2, PRMT6 and PRMT8 showed significantly increased gene expression in differentiated neurons. In line with the unaltered sDMA level detected by SYM11 (Figure 36), expression of Type II PRMT5 was not changed (Figure 37).

As it was shown in Figure 36, retinoic acid treatment resulted in changes in asymmetric arginine methylation. Comparison of the expressional profile of PRMTs upon short-term

RA-induction of day 4 EBs revealed the early upregulation of PRMT8 (Figure 38).

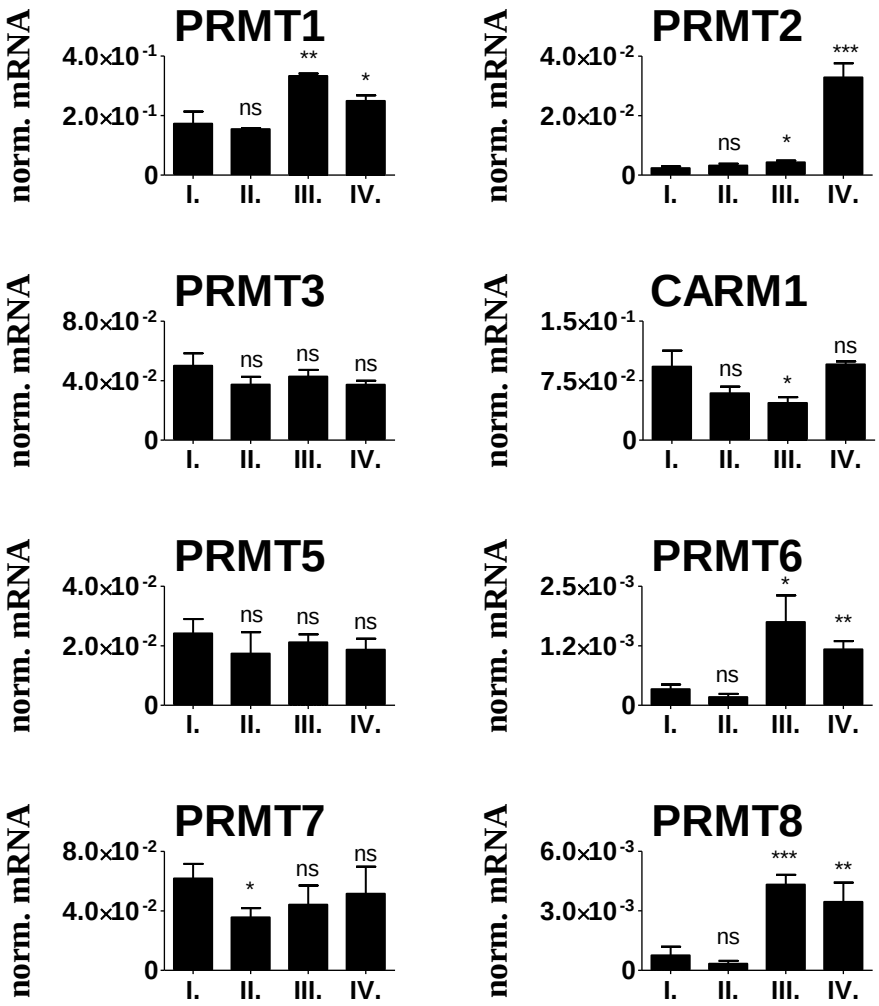

Figure 37. Expression profile of PRMTs in neural differentiation. RNA samples were collected at day 0 (I), day 4 (II), day 8 (III) and day 12 (IV). Gene expression data are expressed as a ratio of the indicated genes' transcript relative to Gapdh. ns - non significant, *P ≤0.05, **P ≤0.01 *** P ≤0.001

PRMT8 is retinoic acid induced and act as a co-activator

To prove that PRMT8 is regulated in a RA-dependent manner, ESCs were treated with RAR and RXR specific ligands. PRMT8 expression could be induced by RAR specific ligand AM580, but the RXR specific LG268 had no effect (Figure 38).

A recently published RAR and RXR ChIP-seq in F9 cells (Mendoza-Parra, Walia et al. 2011) allowed us to identify a putative RARE in the promoter region of PRMT8. This region (-1400 to -1450 bp relative to TSS) contains a direct repeat with no spacer

(AGGTCAAGGTCA, DR0), that can bind RAR:RXR. Transfecting an enhancer trap vector that contained this ~300bp genomic region of the PRMT8 promoter, we could validate that the element is responding to RA treatment in ESCs functionally. These results confirmed PRMT8 as a direct RA-regulated gene (Figure 38).

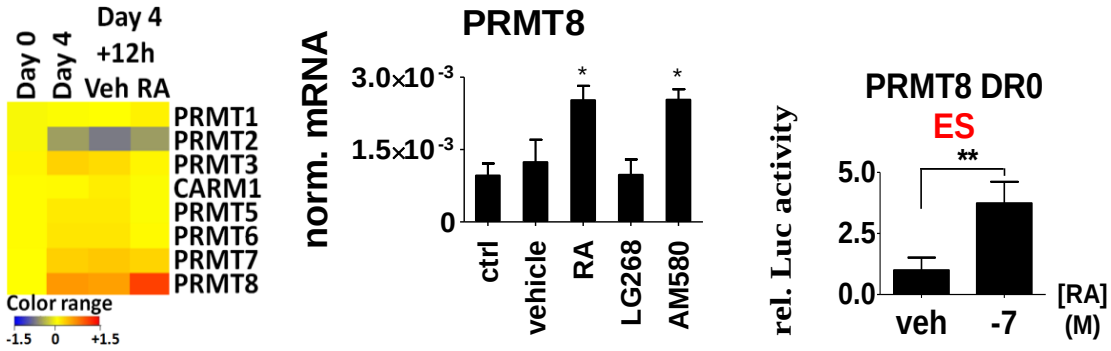

Figure 38. PRMT8 is regulated by retinoic acid. *Left:* Heatmap analysis of gene expression microarray data of PRMT family members. Values are normalized to undifferentiated ESCs (day 0). *Middle:* PRMT8 level as detected by RT-qPCR in ESCs treated with the indicated ligands. *Right:* TK-Luc-PRMT8 was constructed by cloning ~300bp promoter region of PRMT8, containing the DR0 element, into a TK-Luc-empty plasmid. ESCs were transiently transfected and treated with 10^{-7} M RA (labelled as -7) for 24 h.
*P ≤0.05, **P ≤0.01.

PRMT1 and PRMT8 exhibit high sequence similarities (Lee, Sayegh et al. 2005); thus, we were interested in whether loss of PRMT8 may also affect retinoid response similat to as we have seen in case of PRMT1. To establish the co-regulatory function of PRMT8 in RAR:RXR signaling, enhancer trap vector of Hoxb1 RARE was used in a luciferase reporter assay. As shown in Figure 39, ESCs transfected with the reporter alone showed RA-dependent induction, which was further stimulated by co-transfection of PRMT8 expressing vector. In contrast, loss of PRMT8 resulted in a decreased signal intensity. Moreover, the decrease observed in PRMT8-depleted cells could be restored by the overexpression of PRMT8 (Figure 39).

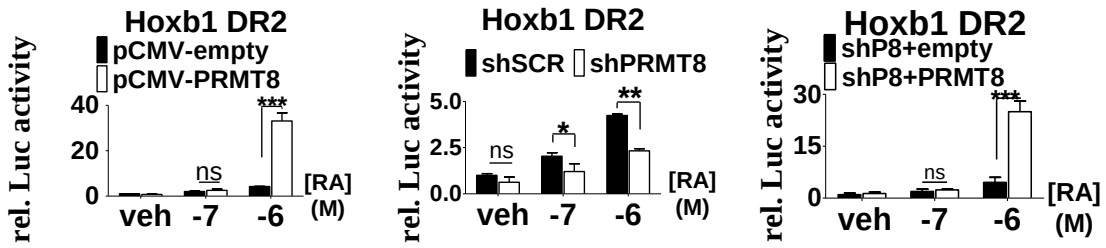

Figure 39. PRMT8 is a co-activator of retinoic acid signaling. TK-Luc Hoxb1 enhancer trap (Hoxb1 DR2) was constructed by cloning a ~300bp regions of Hoxb1 RARE in the luciferase vector. *Left:* ESCs were transfected with Hoxb1 DR2 alone, or in combination with pCMV-Tag2-empty or pCMV-Tag2-PRMT8 expression plasmid. *Middle:* Hoxb1 DR2 was transfected into shSCR or shPRMT8 ESCs. *Right:* shPRMT8 ESCs were transfected with Hoxb1 DR2 alone or in combination with pCMV-Tag2-empty or pCMV-Tag2-PRMT8 expression plasmid. Cells were treated with RA for 24h using the indicated ligand concentrations. ns - non significant, *P ≤0.05, **P ≤0.01 *** P ≤0.001.

PRMT1 and PRMT8 regulates subtype specification of differentiating neural cells

Next, we studied if loss of either PRMT1 or PRMT8 effects the retinoic acid induced neuronal differentiation and neurogenesis-specific gene expression changes. The stability of the knockdowns has been confirmed in differentiated neurons. Importantly, loss of either PRMT1 or PRMT8 resulted in hypomethylation of neurons as detected by anti-ASYM24 antibody (Figure 40).

At day 12 of differentiation neural cells differentiated from the knockdown cells showed similar morphology to wild-type differentiated cells and similarly high expression of typical neural markers, such as Lhx1, Pax6 or Tuj1 to wild-type cells, suggesting no dramatic decrease in differentiation ability resulted by the loss of PRMT1 or PRMT8 (Figure 41).

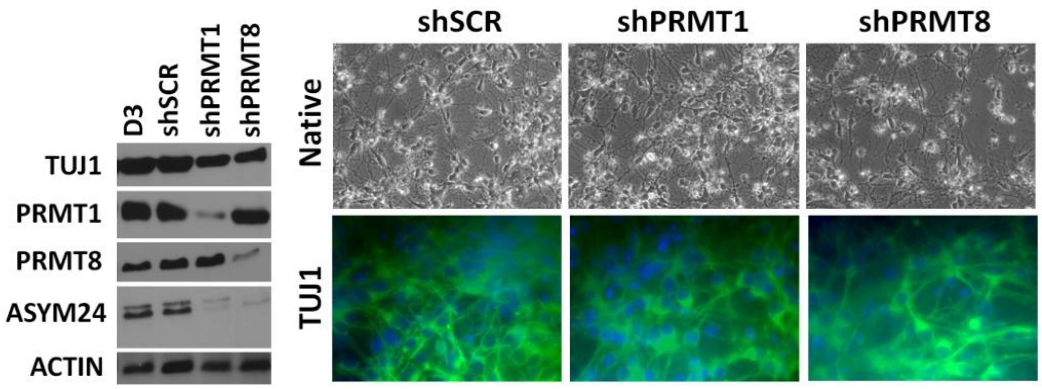

Figure 40. Neural differentiation of PRMT1 or PRMT8 depleted cells. Immunoblot analysis of day 16 differentiated neurons derived from the indicated cell types probed for the indicated proteins. TUJ1-staining of day 12 neurons. DAPI co-staining was used to visualize cell nuclei.

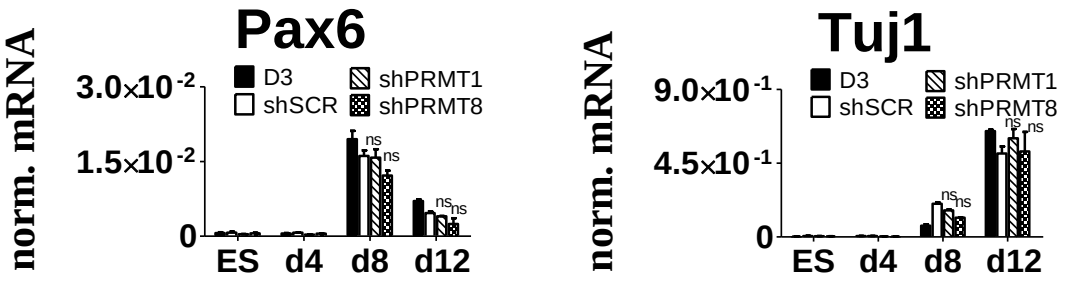

Figure 41. Neural differentiation of PRMT1 or PRMT8 depleted cells. Expression level of Pax6 and Tuj1 as measured by RT-qPCR at day 0 (ES), day 4 (d4), day 8 (d8) and day 12 (d12). ns- non significant.

In contrast, genome-wide analysis of day 16 samples revealed dysregulation of several genes in PRMT1 or PRMT8-depleted neurons. Loss of either PRMT1 or PRMT8 resulted mainly in the downregulation of genes. Interestingly, grouping of the differentially expressed genes identified a large fraction of the genes (473 out of 947) showing similar dysregulation in both knockdown cell types (Figure 42).

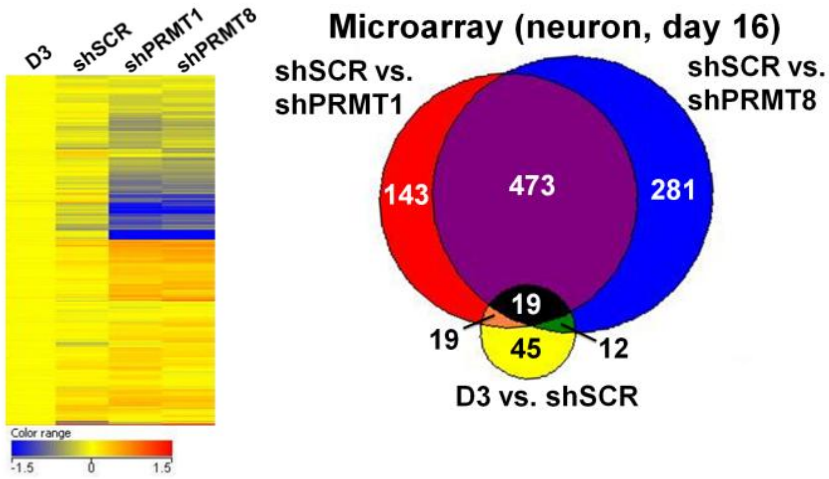

Figure 42. Gene expressional analysis of neurons differentiated from PRMT1 or PRMT8-depleted cells. *Left:* Heatmap display of microarray gene expression data obtained from D3, shSCR, shPRMT1 and shPRMT8-derived neurons at day 16, normalized to D3 neurons. Blue color indicates downregulated, red shows upregulated genes compared to D3-derived neurons. Dendrogram is not shown. *Right:* Area-proportional Venn-diagram compares PRMT1-dependent and PRMT8-dependent gene expression changes in differentiated neurons. Cells differentiated for 16 days were used for the microarray experiments. Significantly changing genes (FC≥1.5, p<0.05) were identified by comparing shSCR vs. shPRMT1 or shSCR vs. shPRMT8-derived neurons.

We validated expression changes of Phox2b (Paired-Like Homeobox 2b), an important transcription factor of neural specification (Brunet and Pattyn 2002) and Gfra2 (GDNF Family Receptor Alpha), a regulator of neurite outgrowth (Yoong and Too 2007) by RT-qPCR (Figure 43).

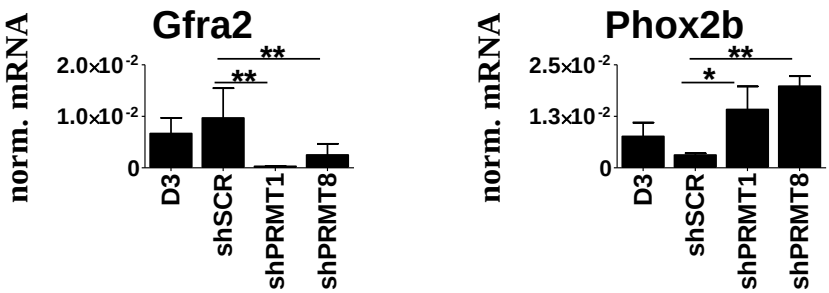

Figure 43. RT-qPCR validation of expression level of Phox2b and Gfra2 in the indicated cells. *P ≤0.05, **P ≤0.01

Ingenuity Pathway Analysis of the 473 genes implicated PRMT1 and PRMT8 in neuronal synaptic formation, glutamate receptor signaling and axonal guidence (data not shown). Thus, we next studied functional properties of PRMT1 or PRMT8-depleted neurons by calcium imaging. This method allowed us to compare >200 neural cells per condition. Importantly, the frequency of calcium peaks and the frequency of action potentials recorded in the same neuron show a significant correlation (Helmchen, Imoto et al. 1996; Stosiek, Garaschuk et al. 2003; Koszeghy, Vincze et al. 2012). Based on this, approximately 50% of the cells showed neural activity in the control and PRMT8 knockdown cells, while in the PRMT1-depleted cell culture the percentage of active cells was close to 100% (Figure 44). As a remarkable difference, we found that the frequency of fast calcium signals per active cells was dramatically dropped in PRMT8-knockdowns, suggesting an important role of PRMT8 in the establishement of neuronal excitability. Another interesting observation was the frequent occurance of synchronous activity among the cells of the PRMT1 knockdown cultures (see synchronous events labelled with asterisks in Figure 44). These data indicated that PRMT1 and PRMT8 have non-overlapping functions in neural functions.

Loss of PRMT8 results in PRMT1-independent transcriptional changes

Gene expression analysis also revealed that several genes, such as Cxcr4, Dhfr or Efemp1 previously linked to glial differentiation and gliomagenesis (Idbaih, Carvalho Silva et al. 2008; Huang, Vogel et al. 2011; Ehtesham and Thompson 2013) were expressed differentially only in PRMT8-knockdown cells, further demonstrating the existence of non-overlapping functions between the two gene products (Figure 45).

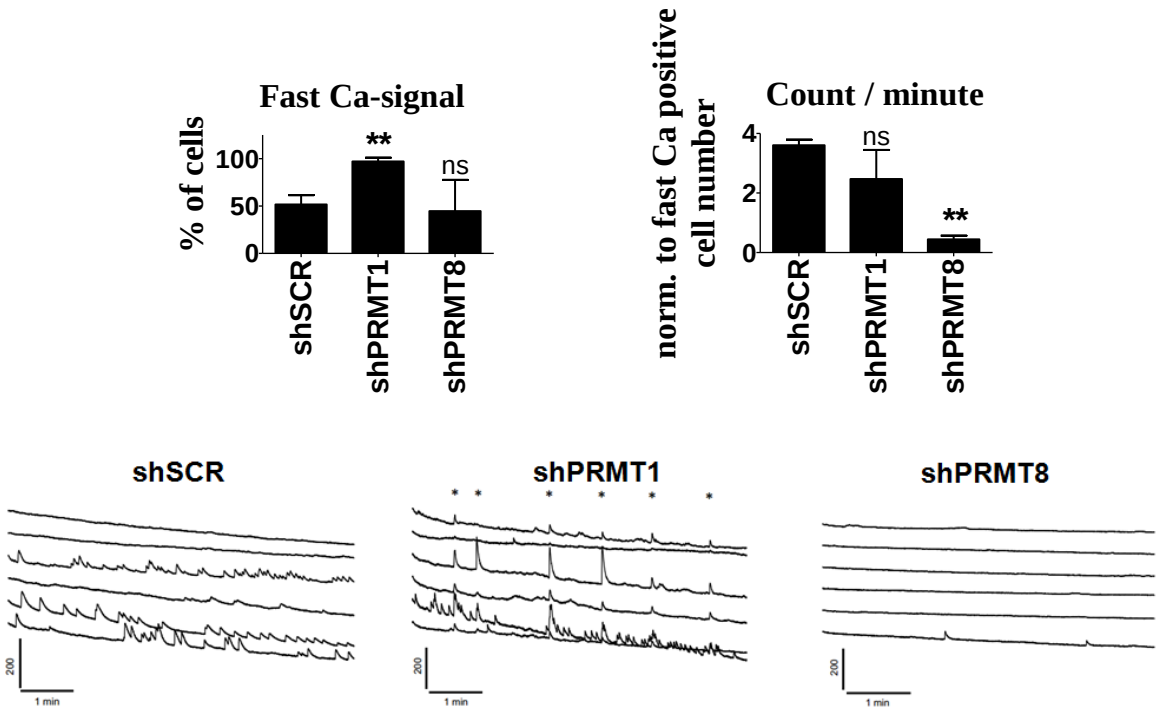

Figure 44. Electrophysiological properties of PRMT-depleted cells. Recordings and analysis has been carried out in the indicated ESC-derived neural cultures. Percentage of cells with recorded fast calcium signal. A representative experiment is shown (>200 measured neurons/condition) (upper left).Counts of calcium signals per minute normalized to the number of cells with positive calcium signal. A representative experiment is shown (>200 measured neurons/condition) (upper right). Synchronous activity among the cells of the PRMT1 knockdown cultures. Synchronous events labelled with asterisks. Calcium imaging records of 6 randomly chosen ROIs (regions of interest) per condition are shown (bottom). ns-non significant; **P ≤0.01.

To demonstrate that PRMT8 indeed has a PRMT1-independent effect, we established double knockdown cells by knocking down PRMT8 in PRMT1 KO ESCs (shSCR was used as a control) (named as PRMT1-/- shPRMT8 and PRMT1-/- shSCR, respectively). Using these PRMT1-PRMT8 double knockdown cells, we found that Cxcr4, Efemp1 and Dhfr were dysregulated in a PRMT8-dependent manner (Figure 46). Double knockdown cells had a more pronounced phenotype regarding the expression level of neural markers (eg. Tuj1, Mapt) compared to single PRMT1-/- cells (Figure 46).

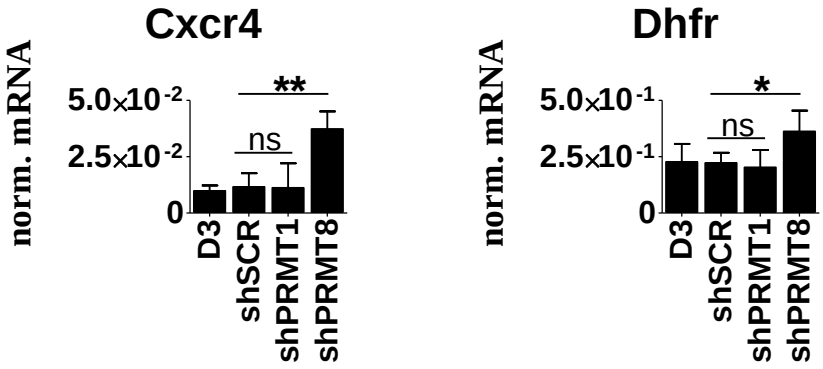

Figure 45. PRMT8-dependent gene expression program. RT-qPCR validation of expression level of Cxcr4 and Dhfr in the indicated cells. ns-non significant, *P ≤0.05, **P ≤0.01.

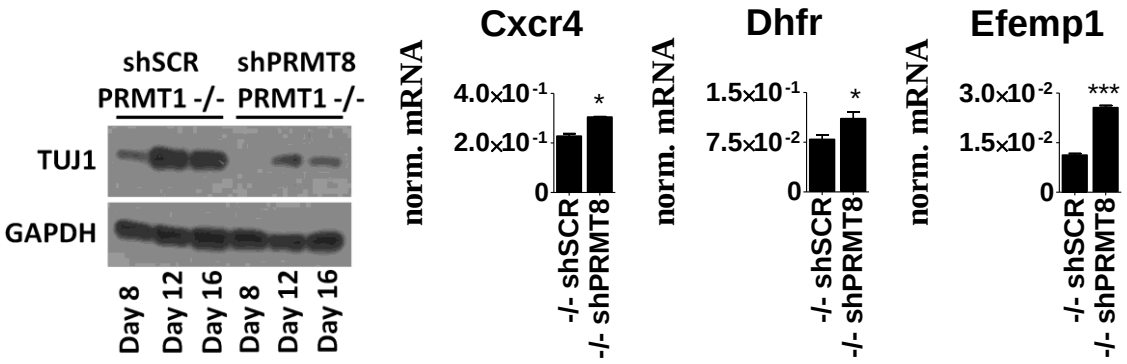

Figure 46. Characterization of PRMT1/PRMT8 double knockdown cells. *Left:*Immunoblot analysis of TUJ1 in different stages of PRMT1 -/- shSCR vs. PRMT1 -/- shPRMT8 neural differentiation. Samples were collected at day 8, 12 and 16 during differentiation. GAPDH serves as a loading control. *Right:* Gene expression level of indicated genes in PRMT1 -/- shSCR vs. PRMT1 -/- shPRMT8 ESC-derived neurons as measured by RT-qPCR at day 14 of differentiation. *P ≤0.05, ***P ≤0.001.

Potential role of PRMT8 in nervous system function in health and disease

PRMT1 has been previously indicated in gliomagenesis (Wang, Tan et al. 2012). Moreover, we found PRMT8-dependent altered expression of Cxcr4 and Efemp1, which have been previously described in brain malignancies, including astrocytoma. Prompted by this finding and a result of a recent study that linked Single Nucleotide Polymorphism

(SNP) variation in the PRMT8 promoter to familial gliomagenesis (Liu, Melin et al. 2012), we hypothesized that PRMT8 might contribute to astrocytic differentiation pathway and disease progression. To determine whether loss of PRMT8 relates to the progression of astrocytoma we collected and used a large number of human primary glioma samples (grade IV astrocytoma). Our results confirmed that PRMT8-dependent Cxcr4 and Efemp1 indeed show dysregulation in glioblastoma multiforme (GBM). Next, we determined expression of PRMT8 in these samples by RT-qPCR. Very strikingly, PRMT8 showed a substantially lower expression level in GBM samples, while PRMT1 did not show a difference between the groups. These data indicate that loss of PRMT8 and genes regulated by it are putative markers in GBM and might participate in its development (Figure 47).

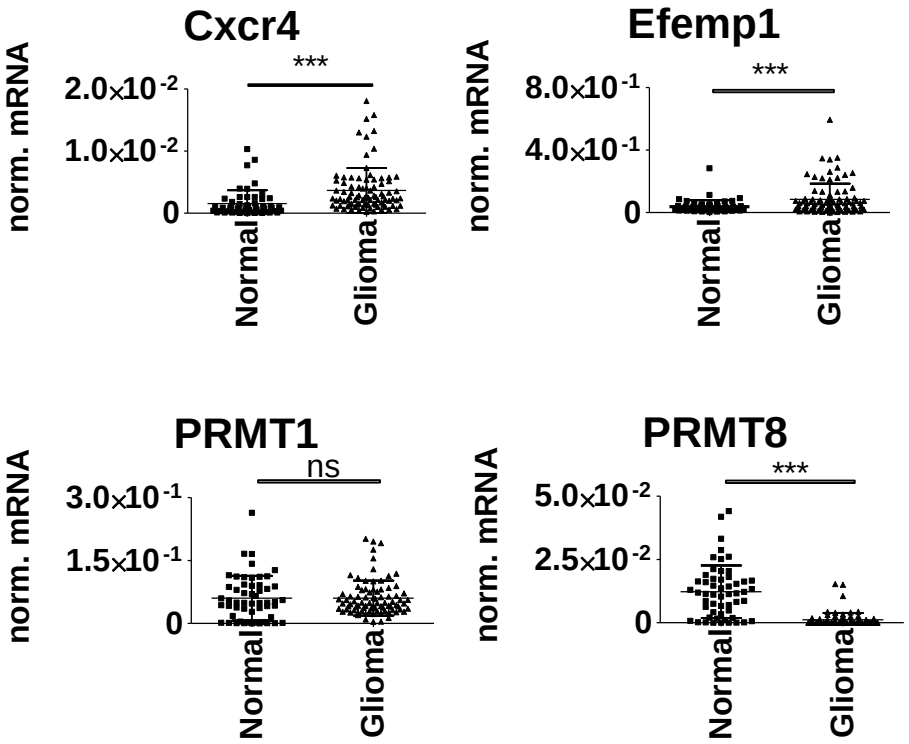

Figure 47. Loss of PRMT8 is a marker of glioblastoma multiforme. Gene expression level of Cxcr4, Efemp1, PRMT1 and PRMT8 as measured by RT-qPCR. Human normal (n=54) and glioblastoma multiforme (GBM) (n=83) samples were compared. Each dot represents an individual sample. ns - non significant, *** P ≤0.001.

XI. DISCUSSION

It has become well established that retinoids and retinoid signaling has an essential role and impact on ESC differentiation and lineage commitment. Retinoids are now widely used as inducers of certain differentiation pathways, however early transcriptomic and epigenetic events of this signaling pathway have remained largely uncharacterized. Our global gene expression analyses provide a more detailed map of this pathway and the contribution of retinoid signaling to pluripotency and lineage specific differentiation. It appears that retinoic acid receptors are present in the ESCs while the enzymes involved in processing of retinoic acid are largely absent. Genome-wide transcriptomic analysis helped us to characterize the early target genes that are induced upon short-term retinoic acid treatment. The low number of genes that is regulated early on following induction is somewhat surprising. However, several important transcription factor are included in the list. A likely scenario is, that retinoic acid turns on master regulators (such as the Hox-cluster) as the first of wave of response and these factors will be responsible later on for the dramatic changes that can be easily observed even phenotypically in the cell cultures.

Characterization of receptor and co-regulator binding upon retinoic acid treatment led us to question the validity of the recent accepted model of retinoic acid induced transcription. Unexpectidely, we found that RAR:RXR binding is inducible, and does not represent a static model. We also observed that P300, which was so far accepted as a general co-activator of RAR:RXR signaling pathway, show enhancer selectivity. Selectivity is likely provided by the cell-type specific transcription factors bound to the close proximity of these regions in ESCs. This example of cell-type specific transcription factor driven enhancer selective co-regulator binding suggest a novel mechanism of how a cell-type specific transcriptional response might be achieved.

We also identified PRMT1 as a novel regulator of P300-dependent retinoic acid responsiveness. We propose that PRMT1 acts as a selective repressor of a large set of retinoic acid induced genes (eg. Hoxa1, Hoxb1, Pmp22 and Spsb1). The promoter regions of these PRMT1-sensitive genes are regulatory "hot spots" as they are occupied by Oct3/4, Sox2 or Nanog and show P300 recruitment upon RA-treatment. This way PRMT1 arms the cells with a negative-feedback mechanism and selectively limit the effect of retinoic acid on its target genes.

Subsequently, the RA signal directly induces the expression of PRMT8,an other member of the PRMT-family. In this late stage, PRMT8 forms a complex with PRMT1, but PRMT8 might also exist as a homodimer (Lee, Sayegh et al. 2005). PRMT8 in such complex acts as a co-activator that potentiates retinoid response. Thus, PRMT1 and its closest paralogue PRMT8, integrate the retinoic acid signal in a temporal manner acting as a rheostat. Loss of PRMT1 or PRMT8 results in mostly similar changes in neural specification, but PRMT1 and PRMT8 have independent regulatory potential as well.

One of the novel finding is the identification of PRMT1 as a selective co-repressor of the retinoic acid signaling. PRMT1 has been previously identified as a ubiquitously expressed secondary co-activator for many nuclear receptors. It has been also shown to bind the activation domain (AD2) of primary co-activators and enhance transcription (Koh, Chen et al. 2001). In contrast to this, our results now show a repressive function of PRMT1. Previous studies already indicated a co-repressor role for PRMT1 in different cellular context (Kleinschmidt, Streubel et al. 2008; Lafleur, Richard et al. 2014). Such opposing roles in gene expression regulation are not unique to PRMT1, similar context-dependent co-regulatory functions have been reported in case of other co-activators as well (Rogatsky, Luecke et al. 2002).

Gene and enhancer selectivity is also a novel and striking feature of PRMT1, and although some aspects of the mechanism has been addressed by us, still many details remains unclear. Our results provide evidence to the overlapping function and localization of P300 and PRMT1. Due to the lack of reliable ChIP-grade anti-PRMT1 antibody, we could not get reproducible data so far which would give us direct evidence for the presence of PRMT1 at the P300 occupied sites. However, we could detect a remarkable decrease in the level of H4R3me2a mark at these sites in PRMT1-depleted cells, suggesting the presence of enzimatically active PRMT1 at these sites. Importantly, P300 genomic occupancy is not affected by loss of PRMT1. Based on these observations, a potential mechanism for the enhancer selectivity of PRMT1 is that P300 is arginine methylated and this affects its co-activator function. This possibility would also explain why only P300-recruiting genomic regions are PRMT1-sensitive. Such arginine methylation dependent regulation of CBP has been demonstrated by others (Chevillard-Briet, Trouche et al. 2002; Ceschin, Walia et al. 2011). Prompted by these possible connections we used ASYM24 antibody to detect putative arginine methylated proteins among the ones that are immunoprecipitated along with P300. However arginine methylation of P300 could not be observed, we found that PRMT1-dependent arginine methylation of certain P300-interacting proteins were detectable, suggesting an indirect crosstalk between P300 and PRMT1. Further mass spectrometry experiments will be necessary to determine the identity of these proteins.

We combined genetic approaches with genome-wide gene expression technologies to unravel the contribution of PRMT1 and PRMT8 to *in vitro* neuronal differentiation. A previous comparison of mammalian PRMTs revealed that PRMT1 and PRMT8 share the highest degree of identity within this enzyme family (Lee, Sayegh et al. 2005). Not only the amino acid sequence of the two proteins, but also the intron-exon boundaries are well

conserved, suggesting that PRMT8 evolved by the duplication of PRMT1. Importantly, a recent study demonstrated that despite the similarities, PRMT1 and PRMT8 have non-redundant functions in the neural development of zebrafish (Lin, Tsai et al. 2013), suggesting that PRMT8 has acquired novel functions since its duplication. Our genome-wide screen and double knockdown experiments also provide evidence of a PRMT1-independent program of PRMT8.

Loss of PRMT1 and PRMT8 function in differentiating neurons significantly altered the transcriptome. Gene expression data obtained can be used in further studies to mechanistically describe the PRMT-dependent transcriptional network in neurogenesis. Importantly, the detected gene expression differences could be linked to various functional defects of the neurons, as demonstrated by the differencies in electrophysiological properties. A recent study identified methylation of brain sodium channel Nav1.2 in response to seizures (Baek, Rubinstein et al. 2014). Our preliminary comparison of electrophysiological properties of the knockdown cells by calcium imaging led us to the conclusion that PRMT1 and PRMT8 are likely responsible for the proper function of certain ion channels. Studies aiming to identify PRMT1 and PRMT8 methylated proteins in the brain will be required to get further insights into the function of these proteins in the developing and adult brain.

Dysregulation of RA-signaling has been also implicated in progression of different subtypes of cancers (Ali, Campos et al. 2012). In a recent study, up-regulation of PRMT1 has been reported in glioma tissues and glioma cell lines (Wang, Tan et al. 2012). Our genome-wide analysis indicated the involvement of PRMT8 instead. Our results show that loss of PRMT8 results in a decrease in the level of Gfra2 and increase in the level of Cxcr4, Dhfr and Efemp1 in a single and double knockdown cells as well. These are established markers of astrocyte-derived glioma (Idbaih, Carvalho Silva et al. 2008; Hu,

Thirtamara-Rajamani et al. 2009; do Carmo, Patricio et al. 2010). Furthermore, a recent genome-wide linkage study of glioma families linked a PRMT8-related SNP to gliomagenesis (Liu, Melin et al. 2012). Detailed functional analysis of this SNP has not been carried out, thus the downstream effect of the SNP could not be linked to the expression of PRMT8 yet. In order to revisit this issue we used a large patient cohort and found that the RNA levels of PRMT8 were almost completely down-regulated in glioma tissues. A trivial explanation may be that PRMT8 is a highly specific neural marker and it is simply not expressed in astrocyte-derived tumors (Kousaka, Mori et al. 2009). Alternatively, loss of PRMT8 positively affects astrocyte differentiation, resulting in a shift in cell fate commitment. Although further work is required, these results already provide strong support to the notion that altered expression of PRMT8 might be a genetic risk factor in gliomagenesis and PRMT8 might be a novel putative therapeutic target. One of our future goal is to establish PRMT8 transgenic animal model to understand the gene function in more details. Regardless of the mechanism, our results implicate PRMT8 as a biomarker of glioma tissues.

XII. SUMMARY

In summary, the results of my work presented here provide insight into the early transcriptomic and epigenetic events of retinoic acid mediated stem cell differentiation. Moreover, we suggest a novel and so far unprecedented mechanism of how two evolutionarily linked proteins with similar enzymatic activity can have distinct effects on cellular differentiation through the integration of retinoid signaling acting as parts of a rheostat. These results provide a new conceptual framework for the interpretation of retinoid signaling in neuronal differentiation and potentially in other tissues as well. These proteins, PRMT1 and PRMT8, can also be targeted pharmacologically to modulate neuronal differentiation *in vitro* or *in vivo* and might also be a relevant target in a major unresolved clinical issues such as gliomas.

XIII. NEW FINDINGS

Identification of retinoic acid mediated transcriptional events in stem cell differentiation.

A comprehensive picture of ligand-induced dynamics of co-activator P300.

PRMT1 is a selective repressor of early retinoid response in ESCs.

PRMT8 is a direct retinoid target gene.

PRMT8 is a co-activator of retinoid response in differentiating neurons.

PRMT1 and PRMT8 act together and regulate neuron cell type specification.

PRMT1 and PRMT8 affects functions of terminally differentiated neurons.

Human glioblastoma tissues lack PRMT8 expression.

XIV. ÖSSZEFOGLALÁS

Az RAR:RXR magreceptorok szerepét vizsgáltuk egér embrionális őssejt differenciációja során. Azonosítottuk a retinsav útvonal komponenseinek expresszióját a differenciálatlan őssejtekben, továbbá genom szintű analízissel meghatároztuk a sejtekben kialakuló génexpressziós változásokat a korai időpontokra fókuszálva. Az így azonosított gének esetén vizsgáltuk individuális gének és genom szintjén is az epigenetikai változásokat.

Azonosítottuk a PRMT1 és PRMT8 fehérjéket, mint a retinsav útvonal ko-regulátorait. A PRMT1 szerepet játszik a P300-mediált retinsav útvonalban. A P300-al egy komplexben kimutathatóak arginin metilált fehérjék.

A PRMT8 egy idegsejt-specifikus expressziót mutató fehérje, ami retinsavra indukálódik a differenciáció során. A PRMT1 és PRMT8 egymással heterodimert alkotva számos gén expresszióját szabályozza, de a PRMT8 esetén megfigyelhető egy PRMT1-független szabályozó hatás is. A PRMT8 által szabályozott gének szerepet játszanak a neuron-asztrocita differenciációs útvonal meghatározásában. A PRMT8 expresszió jelentős csökkenése figyelhető meg humán glioma minták esetén.

XIV. LIST OF KEY WORDS

nuclear receptor, transcription factor, co-regulator, PRMT1, PRMT8, arginine methylation, retinoic acid, glioblastoma multiforme

XV. KULCSSZAVAK

magreceptor, transzkripciós faktor, ko-regulátor, PRMT1, PRMT8, arginin metiláció, retinsav, glioblastoma multiforme

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XVII. REFERENCES

Ait-Si-Ali, S., S. Ramirez, et al. (1998). "Histone acetyltransferase activity of CBP is controlled by cycle-dependent kinases and oncoprotein E1A." Nature **396**(6707): 184-6.

Ali, R., B. Campos, et al. (2012). "Quantification of retinoid concentrations in human serum and brain tumor tissues." Anal Chim Acta **725**: 57-66.

Allfrey, V. G., R. Faulkner, et al. (1964). "Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis." Proc Natl Acad Sci U S A **51**: 786-94.

An, W., J. Kim, et al. (2004). "Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53." Cell **117**(6): 735-48.

Araya, N., H. Hiraga, et al. (2005). "Transcriptional down-regulation through nuclear exclusion of EWS methylated by PRMT1." Biochem Biophys Res Commun **329**(2): 653-60.

Babu, M. M., N. M. Luscombe, et al. (2004). "Structure and evolution of transcriptional regulatory networks." Curr Opin Struct Biol **14**(3): 283-91.

Baek, J. H., M. Rubinstein, et al. (2014). "Reciprocal Changes in Phosphorylation and Methylation of Mammalian Brain Sodium Channels in Response to Seizures." J Biol Chem **289**(22): 15363-15373.

Bahari-Javan, S., F. Sananbenesi, et al. (2014). "Histone-acetylation: a link between Alzheimer's disease and post-traumatic stress disorder?" Front Neurosci **8**: 160.

Baldwin, R. M., A. Morettin, et al. (2014). "Role of PRMTs in cancer: Could minor isoforms be leaving a mark?" World J Biol Chem **5**(2): 115-29.

Balint, B. L., A. Szanto, et al. (2005). "Arginine methylation provides epigenetic transcription memory for retinoid-induced differentiation in myeloid cells." Mol Cell Biol **25**(13): 5648-63.

Bannister, A. J. and T. Kouzarides (1996). "The CBP co-activator is a histone acetyltransferase." Nature **384**(6610): 641-3.

Bannister, A. J. and T. Kouzarides (2011). "Regulation of chromatin by histone modifications." Cell Res **21**(3): 381-95.

Barberi, T., P. Klivenyi, et al. (2003). "Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice." Nat Biotechnol **21**(10): 1200-7.

Barish, G. D., R. T. Yu, et al. (2012). "The Bcl6-SMRT/NCoR cistrome represses inflammation to attenuate atherosclerosis." Cell Metab **15**(4): 554-62.

Barrero, M. J. and S. Malik (2006). "Two functional modes of a nuclear receptor-recruited arginine methyltransferase in transcriptional activation." Mol Cell **24**(2): 233-43.

Barta, E. (2011). Command line analysis of ChIP-seq results.

Barth, L., R. Sutterlin, et al. (2014). "Functional differentiation of stem cell-derived neurons from different murine backgrounds." Front Cell Neurosci **8**: 49.

Bedford, M. T. and S. G. Clarke (2009). "Protein arginine methylation in mammals: who, what, and why." Mol Cell **33**(1): 1-13.

Begemann, G. and A. Meyer (2001). "Hindbrain patterning revisited: timing and effects of retinoic acid signalling." Bioessays **23**(11): 981-6.

Bell, E., R. J. Wingate, et al. (1999). "Homeotic transformation of rhombomere identity after localized Hoxb1 misexpression." Science **284**(5423): 2168-71.

Berghagen, H., E. Ragnhildstveit, et al. (2002). "Corepressor SMRT functions as a coactivator for thyroid hormone receptor T3Ralpha from a negative hormone response element." J Biol Chem **277**(51): 49517-22.
Bernstein, B. E., T. S. Mikkelsen, et al. (2006). "A bivalent chromatin structure marks key developmental genes in embryonic stem cells." Cell **125**(2): 315-26.
Bibel, M., J. Richter, et al. (2007). "Generation of a defined and uniform population of CNS progenitors and neurons from mouse embryonic stem cells." Nat Protoc **2**(5): 1034-43.
Blomhoff, R. and H. K. Blomhoff (2006). "Overview of retinoid metabolism and function." J Neurobiol **66**(7): 606-30.
Boisvert, F. M., C. A. Chenard, et al. (2005). "Protein interfaces in signaling regulated by arginine methylation." Sci STKE **2005**(271): re2.
Boisvert, F. M., J. Cote, et al. (2003). "A proteomic analysis of arginine-methylated protein complexes." Mol Cell Proteomics **2**(12): 1319-30.
Brunet, J. F. and A. Pattyn (2002). "Phox2 genes - from patterning to connectivity." Curr Opin Genet Dev **12**(4): 435-40.
Casci, T. (2008). "Developmental biology: Retinoic acid passes the morphogen test." Nature Reviews Genetics (9): 7-7.
Cedar, H. and Y. Bergman (2009). "Linking DNA methylation and histone modification: patterns and paradigms." Nat Rev Genet **10**(5): 295-304.
Ceschin, D. G., M. Walia, et al. (2011). "Methylation specifies distinct estrogen-induced binding site repertoires of CBP to chromatin." Genes Dev **25**(11): 1132-46.
Chambon, P. (2005). "The nuclear receptor superfamily: a personal retrospect on the first two decades." Mol Endocrinol **19**(6): 1418-28.
Chen, J. D. and R. M. Evans (1995). "A transcriptional co-repressor that interacts with nuclear hormone receptors." Nature **377**(6548): 454-7.
Chen, X., H. Xu, et al. (2008). "Integration of external signaling pathways with the core transcriptional network in embryonic stem cells." Cell **133**(6): 1106-17.
Chevillard-Briet, M., D. Trouche, et al. (2002). "Control of CBP co-activating activity by arginine methylation." EMBO J **21**(20): 5457-66.
Chittka, A. (2010). "Dynamic distribution of histone H4 arginine 3 methylation marks in the developing murine cortex." PLoS One **5**(11): e13807.
Christophersen, N. S. and K. Helin (2010). "Epigenetic control of embryonic stem cell fate." J Exp Med **207**(11): 2287-95.
Chuang, D. M., Y. Leng, et al. (2009). "Multiple roles of HDAC inhibition in neurodegenerative conditions." Trends Neurosci **32**(11): 591-601.
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.
Collas, P. (2010). "The current state of chromatin immunoprecipitation." Mol Biotechnol **45**(1): 87-100.
Consortium, I. H. G. S. (2004). "Finishing the euchromatic sequence of the human genome." Nature **431**(7011): 931-45.
Cote, J., F. M. Boisvert, et al. (2003). "Sam68 RNA binding protein is an in vivo substrate for protein arginine N-methyltransferase 1." Mol Biol Cell **14**(1): 274-87.

Cuaranta-Monroy, I., Z. Simandi, et al. (2014). "Highly efficient differentiation of embryonic stem cells into adipocytes by ascorbic acid." Stem Cell Res **13**(1): 88-97.
Cullum, M. E. and M. H. Zile (1985). "Metabolism of all-trans-retinoic acid and all-trans-retinyl acetate. Demonstration of common physiological metabolites in rat small intestinal mucosa and circulation." J Biol Chem **260**(19): 10590-6.
D'Ambrosio, D. N., R. D. Clugston, et al. (2011). "Vitamin A Metabolism: An Update." **3**(1): 63-103.
Del Rizzo, P. A. and R. C. Trievel (2014). "Molecular basis for substrate recognition by lysine methyltransferases and demethylases." Biochim Biophys Acta.
Delacroix, L., E. Moutier, et al. (2009). "Cell-specific interaction of retinoic acid receptors with target genes in mouse embryonic fibroblasts and embryonic stem cells." Mol Cell Biol **30**(1): 231-44.
do Carmo, A., I. Patricio, et al. (2010). "CXCL12/CXCR4 promotes motility and proliferation of glioma cells." Cancer Biol Ther **9**(1): 56-65.
Drager, U. C. (2006). "Retinoic acid signaling in the functioning brain." Sci STKE **2006**(324): pe10.
Duester, G. (2008). "Retinoic acid synthesis and signaling during early organogenesis." Cell **134**(6): 921-31.
Duester, G. (2013). "Retinoid signaling in control of progenitor cell differentiation during mouse development." Semin Cell Dev Biol **24**(10-12): 694-700.
Ehtesham, M. and R. C. Thompson (2013). "CXCR4-Expressing Glial Precursor Cells Demonstrate Enhanced Migratory Tropism for Glioma." J Cancer Ther **3**(6): 1086-1091.
Evans, M. J. and M. H. Kaufman (1981). "Establishment in culture of pluripotential cells from mouse embryos." Nature **292**(5819): 154-6.
Fan, X., D. Sun, et al. (2014). "Stem-cell challenges in the treatment of Alzheimer's disease: a long way from bench to bedside." Med Res Rev **34**(5): 957-78.
Feng, Q., P. Yi, et al. (2006). "Signaling within a coactivator complex: methylation of SRC-3/AIB1 is a molecular switch for complex disassembly." Mol Cell Biol **26**(21): 7846-57.
Flynn, P. J., W. J. Miller, et al. (1983). "Retinoic acid treatment of acute promyelocytic leukemia: in vitro and in vivo observations." Blood **62**(6): 1211-7.
Gaspard, N., T. Bouschet, et al. (2008). "An intrinsic mechanism of corticogenesis from embryonic stem cells." Nature **455**(7211): 351-7.
Gayatri, S. and M. T. Bedford (2014). "Readers of histone methylarginine marks." Biochim Biophys Acta **1839**(8): 702-10.
Gayatri, S. and M. T. Bedford (2014). "Readers of histone methylarginine marks." Biochim Biophys Acta.
Germain, N., E. Banda, et al. (2010). "Embryonic stem cell neurogenesis and neural specification." J Cell Biochem **111**(3): 535-42.
Glass, C. K. (1994). "Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers." Endocr Rev **15**(3): 391-407.
Glass, C. K. and M. G. Rosenfeld (2000). "The coregulator exchange in transcriptional functions of nuclear receptors." Genes Dev **14**(2): 121-41.
Glover, J. C., J. S. Renaud, et al. (2006). "Retinoic acid and hindbrain patterning." J Neurobiol **66**(7): 705-25.
Gonzales, K. A. and H. H. Ng (2013). "Driving pluripotency and reprogramming: nuclear receptors at the helm." Semin Cell Dev Biol **24**(10-12): 670-8.

Graf, T. (2011). "Historical origins of transdifferentiation and reprogramming." Cell Stem Cell **9**(6): 504-16.
Graf, T. and T. Enver (2009). "Forcing cells to change lineages." Nature **462**(7273): 587-94.
Gudas, L. J. (2012). "Emerging roles for retinoids in regeneration and differentiation in normal and disease states." Biochim Biophys Acta **1821**(1): 213-21.
Haberland, M., R. L. Montgomery, et al. (2009). "The many roles of histone deacetylases in development and physiology: implications for disease and therapy." Nat Rev Genet **10**(1): 32-42.
Halachmi, S., E. Marden, et al. (1994). "Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription." Science **264**(5164): 1455-8.
Helmchen, F., K. Imoto, et al. (1996). "Ca2+ buffering and action potential-evoked Ca2+ signaling in dendrites of pyramidal neurons." Biophys J **70**(2): 1069-81.
Hnisz, D., B. J. Abraham, et al. (2013). "Super-enhancers in the control of cell identity and disease." Cell **155**(4): 934-47.
Ho, J. W., Y. L. Jung, et al. (2014). "Comparative analysis of metazoan chromatin organization." Nature **512**(7515): 449-52.
Holmqvist, P. H. and M. Mannervik (2012). "Genomic occupancy of the transcriptional co-activators p300 and CBP." Transcription **4**(1): 18-23.
Horlein, A. J., A. M. Naar, et al. (1995). "Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor." Nature **377**(6548): 397-404.
Horst, R. L., T. A. Reinhardt, et al. (1995). "Identification of 9-cis,13-cis-retinoic acid as a major circulating retinoid in plasma." Biochemistry **34**(4): 1203-9.
Hu, B., K. K. Thirtamara-Rajamani, et al. (2009). "Fibulin-3 is uniquely upregulated in malignant gliomas and promotes tumor cell motility and invasion." Mol Cancer Res **7**(11): 1756-70.
Huang, J., G. Vogel, et al. (2011). "Type II arginine methyltransferase PRMT5 regulates gene expression of inhibitors of differentiation/DNA binding Id2 and Id4 during glial cell differentiation." J Biol Chem **286**(52): 44424-32.
Huang, S., M. Litt, et al. (2005). "Methylation of histone H4 by arginine methyltransferase PRMT1 is essential in vivo for many subsequent histone modifications." Genes Dev **19**(16): 1885-93.
Idbaih, A., R. Carvalho Silva, et al. (2008). "Genomic changes in progression of low-grade gliomas." J Neurooncol **90**(2): 133-40.
Iwao, K., H. Kawasaki, et al. (1999). "Ubiquitination of the transcriptional coactivator p300 during retinic acid induced differentiation." Nucleic Acids Symp Ser(42): 207-8.
Jamaladdin, S., R. D. Kelly, et al. (2014). "Histone deacetylase (HDAC) 1 and 2 are essential for accurate cell division and the pluripotency of embryonic stem cells." Proc Natl Acad Sci U S A **111**(27): 9840-5.
Jeong, Y. and D. J. Mangelsdorf (2009). "Nuclear receptor regulation of stemness and stem cell differentiation." Exp Mol Med **41**(8): 525-37.
Kashyap, V. and L. J. Gudas (2010). "Epigenetic regulatory mechanisms distinguish retinoic acid-mediated transcriptional responses in stem cells and fibroblasts." J Biol Chem **285**(19): 14534-48.
Kato, S., K. Inoue, et al. (2010). "Emergence of the osteo-epigenome in bone biology." IBMS BoneKEy **7**(9): 314-324.
Kawaguchi, R., J. Yu, et al. (2007). "A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A." Science **315**(5813): 820-5.

Kawasaki, H., R. Eckner, et al. (1998). "Distinct roles of the co-activators p300 and CBP in retinoic-acid-induced F9-cell differentiation." Nature **393**(6682): 284-9.
Kestler, H. A., A. Muller, et al. (2008). "VennMaster: area-proportional Euler diagrams for functional GO analysis of microarrays." BMC Bioinformatics **9**: 67.
Khorasanizadeh, S. and F. Rastinejad (2001). "Nuclear-receptor interactions on DNA-response elements." Trends Biochem Sci **26**(6): 384-90.
Kim, J. D., K. Kako, et al. (2008). "EWS is a substrate of type I protein arginine methyltransferase, PRMT8." Int J Mol Med **22**(3): 309-15.
Kim, S., C. G. Parks, et al. "Association between genetic variants in DNA and histone methylation and telomere length." PLoS One **7**(7): e40504.
Kim, Y. J., C. B. Greer, et al. (2013). "HDAC inhibitors induce transcriptional repression of high copy number genes in breast cancer through elongation blockade." Oncogene **32**(23): 2828-35.
Kleinschmidt, M. A., G. Streubel, et al. (2008). "The protein arginine methyltransferases CARM1 and PRMT1 cooperate in gene regulation." Nucleic Acids Res **36**(10): 3202-13.
Koh, S. S., D. Chen, et al. (2001). "Synergistic enhancement of nuclear receptor function by p160 coactivators and two coactivators with protein methyltransferase activities." J Biol Chem **276**(2): 1089-98.
Koide, T., M. Downes, et al. (2001). "Active repression of RAR signaling is required for head formation." Genes Dev **15**(16): 2111-21.
Koleske, A. J. and R. A. Young (1994). "An RNA polymerase II holoenzyme responsive to activators." Nature **368**(6470): 466-9.
Koszeghy, A., B. Pal, et al. (2009). "Purkinje-like cells of the rat cochlear nucleus: a combined functional and morphological study." Brain Res **1297**: 57-69.
Koszeghy, A., J. Vincze, et al. (2012). "Activation of muscarinic receptors increases the activity of the granule neurones of the rat dorsal cochlear nucleus--a calcium imaging study." Pflugers Arch **463**(6): 829-44.
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.
Kouzarides, T. (2007). "Chromatin modifications and their function." Cell **128**(4): 693-705.
Kumar, R. and E. B. Thompson (2003). "Transactivation functions of the N-terminal domains of nuclear hormone receptors: protein folding and coactivator interactions." Mol Endocrinol **17**(1): 1-10.
Ladewig, J., P. Koch, et al. (2013). "Leveling Waddington: the emergence of direct programming and the loss of cell fate hierarchies." Nat Rev Mol Cell Biol **14**(4): 225-36.
Lafleur, V. N., S. Richard, et al. (2014). "Transcriptional repression of hypoxia-inducible factor-1 (HIF-1) by the protein arginine methyltransferase PRMT1." Mol Biol Cell **25**(6): 925-35.
Lambeth, L. S. and C. A. Smith (2012). "Short hairpin RNA-mediated gene silencing." Methods Mol Biol **942**: 205-32.
Ledda-Columbano, G. M., M. Pibiri, et al. (2004). "Induction of hepatocyte proliferation by retinoic acid." Carcinogenesis **25**(11): 2061-6.
Lee, D. Y., I. Ianculescu, et al. (2007). "Surface-scanning mutational analysis of protein arginine methyltransferase 1: roles of specific amino acids in methyltransferase substrate specificity, oligomerization, and coactivator function." Mol Endocrinol **21**(6): 1381-93.

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.
Lee, Y. H., H. Ma, et al. (2012). "Protein arginine methyltransferase 6 regulates embryonic stem cell identity." Stem Cells Dev **21**(14): 2613-22.
Li, H. and R. Durbin (2009). "Fast and accurate short read alignment with Burrows-Wheeler transform." Bioinformatics **25**(14): 1754-60.
Li, J., B. Parker, et al. (2012). "The PMP22 gene and its related diseases." Mol Neurobiol **47**(2): 673-98.
Lin, C., A. S. Garrett, et al. (2011). "Dynamic transcriptional events in embryonic stem cells mediated by the super elongation complex (SEC)." Genes Dev **25**(14): 1486-98.
Lin, Y. L., Y. J. Tsai, et al. (2013). "The Critical Role of Protein Arginine Methyltransferase prmt8 in Zebrafish Embryonic and Neural Development Is Non-Redundant with Its Paralogue prmt1." PLoS One **8**(3): e55221.
Litt, M., Y. Qiu, et al. (2009). "Histone arginine methylations: their roles in chromatin dynamics and transcriptional regulation." Biosci Rep **29**(2): 131-41.
Liu, Y., B. S. Melin, et al. (2012). "Insight in glioma susceptibility through an analysis of 6p22.3, 12p13.33-12.1, 17q22-23.2 and 18q23 SNP genotypes in familial and non-familial glioma." Hum Genet **131**(9): 1507-17.
Liu, Z., D. Merkurjev, et al. (2014). "Enhancer Activation Requires trans-Recruitment of a Mega Transcription Factor Complex." Cell **159**(2): 358-73.
Liu, Z., D. R. Scannell, et al. (2011). "Control of embryonic stem cell lineage commitment by core promoter factor, TAF3." Cell **146**(5): 720-31.
Lonard, D. M. and W. O'Malley B (2007). "Nuclear receptor coregulators: judges, juries, and executioners of cellular regulation." Mol Cell **27**(5): 691-700.
Macrae, I. J., K. Zhou, et al. (2006). "Structural basis for double-stranded RNA processing by Dicer." Science **311**(5758): 195-8.
Maden, M. (2007). "Retinoic acid in the development, regeneration and maintenance of the nervous system." Nat Rev Neurosci **8**(10): 755-65.
Mahony, S., E. O. Mazzoni, et al. (2011). "Ligand-dependent dynamics of retinoic acid receptor binding during early neurogenesis." Genome Biol **12**(1): R2.
Mark, M., N. B. Ghyselinck, et al. (2009). "Function of retinoic acid receptors during embryonic development." Nucl Recept Signal **7**: e002.
Martens, J. A. and F. Winston (2003). "Recent advances in understanding chromatin remodeling by Swi/Snf complexes." Curr Opin Genet Dev **13**(2): 136-42.
Martinez-Ceballos, E., P. Chambon, et al. (2005). "Differences in gene expression between wild type and Hoxa1 knockout embryonic stem cells after retinoic acid treatment or leukemia inhibitory factor (LIF) removal." J Biol Chem **280**(16): 16484-98.
Martinez-Ceballos, E. and L. J. Gudas (2008). "Hoxa1 is required for the retinoic acid-induced differentiation of embryonic stem cells into neurons." J Neurosci Res **86**(13): 2809-19.
McKenna, N. J., R. B. Lanz, et al. (1999). "Nuclear receptor coregulators: cellular and molecular biology." Endocr Rev **20**(3): 321-44.
McKenna, N. J. and B. W. O'Malley (2002). "Combinatorial control of gene expression by nuclear receptors and coregulators." Cell **108**(4): 465-74.
Mendoza-Parra, M. A., M. Walia, et al. (2011). "Dissecting the retinoid-induced differentiation of F9 embryonal stem cells by integrative genomics." Mol Syst Biol **7**: 538.

Mersfelder, E. L. and M. R. Parthun (2006). "The tale beyond the tail: histone core domain modifications and the regulation of chromatin structure." Nucleic Acids Res **34**(9): 2653-62.
Meyer, M. E., H. Gronemeyer, et al. (1989). "Steroid hormone receptors compete for factors that mediate their enhancer function." Cell **57**(3): 433-42.
Mic, F. A., A. Molotkov, et al. (2003). "Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis." Proc Natl Acad Sci U S A **100**(12): 7135-40.
Millard, C. J., P. J. Watson, et al. (2013). "An evolving understanding of nuclear receptor coregulator proteins." J Mol Endocrinol **51**(3): T23-36.
Miyata, S., Y. Mori, et al. (2008). "PRMT1 and Btg2 regulates neurite outgrowth of Neuro2a cells." Neurosci Lett **445**(2): 162-5.
Molina-Serrano, D., V. Schiza, et al. (2013). "Cross-talk among epigenetic modifications: lessons from histone arginine methylation." Biochem Soc Trans **41**(3): 751-9.
Molotkov, A., N. B. Ghyselinck, et al. (2004). "Opposing actions of cellular retinol-binding protein and alcohol dehydrogenase control the balance between retinol storage and degradation." Biochem J **383**(Pt 2): 295-302.
Moutier, E., T. Ye, et al. (2012). "Retinoic acid receptors recognize the mouse genome through binding elements with diverse spacing and topology." J Biol Chem **287**(31): 26328-41.
MuhChyi, C., B. Juliandi, et al. (2013). "Epigenetic regulation of neural stem cell fate during corticogenesis." Int J Dev Neurosci **31**(6): 424-33.
Mummery, C. L., M. A. van Rooijen, et al. (1987). "Cell cycle analysis during retinoic acid induced differentiation of a human embryonal carcinoma-derived cell line." Cell Differ **20**(2-3): 153-60.
Nagy, L., H. Y. Kao, et al. (1997). "Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase." Cell **89**(3): 373-80.
Nagy, L., H. Y. Kao, et al. (1999). "Mechanism of corepressor binding and release from nuclear hormone receptors." Genes Dev **13**(24): 3209-16.
Nagy, L. and J. W. Schwabe (2004). "Mechanism of the nuclear receptor molecular switch." Trends Biochem Sci **29**(6): 317-24.
Nakajima, T., Y. Matsuoka, et al. (1971). "Isolation and identification of N-G-monomethyl, N-G, N-G-dimethyl- and N-G,N' G-dimethylarginine from the hydrolysate of proteins of bovine brain." Biochim Biophys Acta **230**(2): 212-22.
Niederreither, K. and P. Dolle (2008). "Retinoic acid in development: towards an integrated view." Nat Rev Genet **9**(7): 541-53.
Niederreither, K., V. Subbarayan, et al. (1999). "Embryonic retinoic acid synthesis is essential for early mouse post-implantation development." Nat Genet **21**(4): 444-8.
Niederreither, K., J. Vermot, et al. (2000). "Retinoic acid synthesis and hindbrain patterning in the mouse embryo." Development **127**(1): 75-85.
Niwa, H., J. Miyazaki, et al. (2000). "Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells." Nat Genet **24**(4): 372-6.
Novitch, B. G., H. Wichterle, et al. (2003). "A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification." Neuron **40**(1): 81-95.
Noy, N. (1992). "The ionization behavior of retinoic acid in aqueous environments and bound to serum albumin." Biochim Biophys Acta **1106**(1): 151-8.

Ogryzko, V. V., R. L. Schiltz, et al. (1996). "The transcriptional coactivators p300 and CBP are histone acetyltransferases." Cell **87**(5): 953-9.
Ogura, T. and R. M. Evans (1995). "Evidence for two distinct retinoic acid response pathways for HOXB1 gene regulation." Proc Natl Acad Sci U S A **92**(2): 392-6.
Onate, S. A., S. Y. Tsai, et al. (1995). "Sequence and characterization of a coactivator for the steroid hormone receptor superfamily." Science **270**(5240): 1354-7.
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.
Peng, L. and E. Seto (2011). "Deacetylation of nonhistone proteins by HDACs and the implications in cancer." Handb Exp Pharmacol **206**: 39-56.
Perissi, V. and M. G. Rosenfeld (2005). "Controlling nuclear receptors: the circular logic of cofactor cycles." Nat Rev Mol Cell Biol **6**(7): 542-54.
Petros, T. J., J. A. Tyson, et al. (2011). "Pluripotent stem cells for the study of CNS development." Front Mol Neurosci **4**: 30.
Qiu, Y., Y. Zhao, et al. (2006). "HDAC1 acetylation is linked to progressive modulation of steroid receptor-induced gene transcription." Mol Cell **22**(5): 669-79.
Quinlan, A. R. and I. M. Hall (2010). "BEDTools: a flexible suite of utilities for comparing genomic features." Bioinformatics **26**(6): 841-2.
Rhinn, M. and P. Dolle (2012). "Retinoic acid signalling during development." Development **139**(5): 843-58.
Rizzo, G., B. Renga, et al. (2005). "The methyl transferase PRMT1 functions as co-activator of farnesoid X receptor (FXR)/9-cis retinoid X receptor and regulates transcription of FXR responsive genes." Mol Pharmacol **68**(2): 551-8.
Rochette-Egly, C. (2005). "Dynamic combinatorial networks in nuclear receptor-mediated transcription." J Biol Chem **280**(38): 32565-8.
Rogatsky, I., H. F. Luecke, et al. (2002). "Alternate surfaces of transcriptional coregulator GRIP1 function in different glucocorticoid receptor activation and repression contexts." Proc Natl Acad Sci U S A **99**(26): 16701-6.
Ross-Innes, C. S., R. Stark, et al. (2012). "Differential oestrogen receptor binding is associated with clinical outcome in breast cancer." Nature **481**(7381): 389-93.
Ross, A. C. and R. Zolfaghari (2011). "Cytochrome P450s in the regulation of cellular retinoic acid metabolism." Annu Rev Nutr **31**: 65-87.
Rubinson, D. A., C. P. Dillon, et al. (2003). "A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference." Nat Genet **33**(3): 401-6.
Schena, M., R. A. Heller, et al. (1998). "Microarrays: biotechnology's discovery platform for functional genomics." Trends Biotechnol **16**(7): 301-6.
Sharma, R. P. and Y. W. Kim (1995). "Localization of retinoic acid receptors in anterior-human embryo." Exp Mol Pathol **62**(3): 180-9.
Shi, Z. and J. Jiao (2012). "Direct lineage conversion: induced neuronal cells and induced neural stem cells." Protein Cell **3**(11): 826-33.
Shimozono, S., T. Iimura, et al. (2013). "Visualization of an endogenous retinoic acid gradient across embryonic development." Nature **496**(7445): 363-6.
Siersbaek, R., A. Rabiee, et al. (2014). "Transcription factor cooperativity in early adipogenic hotspots and super-enhancers." Cell Rep **7**(5): 1443-55.
Simandi, Z., B. L. Balint, et al. (2010). "Activation of retinoic acid receptor signaling coordinates lineage commitment of spontaneously differentiating mouse embryonic stem cells in embryoid bodies." FEBS Lett **584**(14): 3123-30.

Simandi, Z., I. Cuaranta-Monroy, et al. (2013). "Nuclear receptors as regulators of stem cell and cancer stem cell metabolism." Semin Cell Dev Biol **24**(10-12): 716-23.
Sockanathan, S. and T. M. Jessell (1998). "Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons." Cell **94**(4): 503-14.
Sommer, A. (2008). "Vitamin a deficiency and clinical disease: an historical overview." J Nutr **138**(10): 1835-9.
Song, Y., J. Ahn, et al. (2013). "Identification of novel tissue-specific genes by analysis of microarray databases: a human and mouse model." PLoS One **8**(5): e64483.
Sonoda, J., L. Pei, et al. (2008). "Nuclear receptors: decoding metabolic disease." FEBS Lett **582**(1): 2-9.
Stergiopoulos, A. and P. K. Politis (2013). "The role of nuclear receptors in controlling the fine balance between proliferation and differentiation of neural stem cells." Arch Biochem Biophys **534**(1-2): 27-37.
Stosiek, C., O. Garaschuk, et al. (2003). "In vivo two-photon calcium imaging of neuronal networks." Proc Natl Acad Sci U S A **100**(12): 7319-24.
Takahashi, K. and S. Yamanaka (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." Cell **126**(4): 663-76.
Takeda, A., O. P. Nyssen, et al. (2013). "Vitamin A and carotenoids and the risk of Parkinson's disease: a systematic review and meta-analysis." Neuroepidemiology **42**(1): 25-38.
Taneda, T., S. Miyata, et al. (2007). "Specific regional distribution of protein arginine methyltransferase 8 (PRMT8) in the mouse brain." Brain Res **1155**: 1-9.
Tapscott, S. J., R. L. Davis, et al. (1988). "MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts." Science **242**(4877): 405-11.
ten Berge, D., W. Koole, et al. (2008). "Wnt signaling mediates self-organization and axis formation in embryoid bodies." Cell Stem Cell **3**(5): 508-18.
Thorvaldsdottir, H., J. T. Robinson, et al. (2012). "Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration." Brief Bioinform **14**(2): 178-92.
Trapnell, C., D. G. Hendrickson, et al. (2013). "Differential analysis of gene regulation at transcript resolution with RNA-seq." Nat Biotechnol **31**(1): 46-53.
Tsai, M. J. and B. W. O'Malley (1994). "Molecular mechanisms of action of steroid/thyroid receptor superfamily members." Annu Rev Biochem **63**: 451-86.
Tsai, Y. J., H. Pan, et al. (2011). "The predominant protein arginine methyltransferase PRMT1 is critical for zebrafish convergence and extension during gastrulation." FEBS J **278**(6): 905-17.
Uehara, M., K. Yashiro, et al. (2007). "CYP26A1 and CYP26C1 cooperatively regulate anterior-posterior patterning of the developing brain and the production of migratory cranial neural crest cells in the mouse." Dev Biol **302**(2): 399-411.
Umesono, K., V. Giguere, et al. (1988). "Retinoic acid and thyroid hormone induce gene expression through a common responsive element." Nature **336**(6196): 262-5.
Urvalek, A., K. B. Laursen, et al. (2014). "The roles of retinoic acid and retinoic acid receptors in inducing epigenetic changes." Subcell Biochem **70**: 129-49.
Urvalek, A. M. and L. J. Gudas (2014). "Retinoic acid and histone deacetylases regulate epigenetic changes in embryonic stem cells." J Biol Chem **289**(28): 19519-30.
van Oevelen, C., E. M. Kallin, et al. (2013). "Transcription factor-induced enhancer modulations during cell fate conversions." Curr Opin Genet Dev **23**(5): 562-7.
Vaquerizas, J. M., S. K. Kummerfeld, et al. (2009). "A census of human transcription factors: function, expression and evolution." Nat Rev Genet **10**(4): 252-63.

Visel, A., M. J. Blow, et al. (2009). "ChIP-seq accurately predicts tissue-specific activity of enhancers." Nature **457**(7231): 854-8.
Wagner, E. J. and P. B. Carpenter (2012). "Understanding the language of Lys36 methylation at histone H3." Nat Rev Mol Cell Biol **13**(2): 115-26.
Wagner, R. T. and A. J. Cooney (2013). "Minireview: the diverse roles of nuclear receptors in the regulation of embryonic stem cell pluripotency." Mol Endocrinol **27**(6): 864-78.
Wang, H., Z. Q. Huang, et al. (2001). "Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor." Science **293**(5531): 853-7.
Wang, S., X. Tan, et al. (2012). "The role of protein arginine-methyltransferase 1 in gliomagenesis." BMB Rep **45**(8): 470-5.
Wang, S., X. C. Tan, et al. (2012). "Screening of substrates of protein arginine methyltransferase 1 in glioma." Chin Med Sci J **27**(1): 1-6.
Wang, Z., M. Gerstein, et al. (2009). "RNA-Seq: a revolutionary tool for transcriptomics." Nat Rev Genet **10**(1): 57-63.
Wang, Z., C. Zang, et al. (2009). "Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes." Cell **138**(5): 1019-31.
Watanabe, K., D. Kamiya, et al. (2005). "Directed differentiation of telencephalic precursors from embryonic stem cells." Nat Neurosci **8**(3): 288-96.
White, J. A., B. Beckett-Jones, et al. (1997). "cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450." J Biol Chem **272**(30): 18538-41.
Whyte, W. A., D. A. Orlando, et al. (2013). "Master transcription factors and mediator establish super-enhancers at key cell identity genes." Cell **153**(2): 307-19.
Wu, Q., A. W. Bruce, et al. (2009). "CARM1 is required in embryonic stem cells to maintain pluripotency and resist differentiation." Stem Cells **27**(11): 2637-45.
Xie, C. Q., Y. Jeong, et al. (2009). "Expression profiling of nuclear receptors in human and mouse embryonic stem cells." Mol Endocrinol **23**(5): 724-33.
Xu, J. and Q. Li (2003). "Review of the in vivo functions of the p160 steroid receptor coactivator family." Mol Endocrinol **17**(9): 1681-92.
Xu, W., H. Chen, et al. (2001). "A transcriptional switch mediated by cofactor methylation." Science **294**(5551): 2507-11.
Yao, T. P., S. P. Oh, et al. (1998). "Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300." Cell **93**(3): 361-72.
Yokoyama, A., K. Igarashi, et al. (2014). "Identification of myelin transcription factor 1 (MyT1) as a subunit of the neural cell type-specific lysine-specific demethylase 1 (LSD1) complex." J Biol Chem **289**(26): 18152-62.
Yoong, L. F. and H. P. Too (2007). "Glial cell line-derived neurotrophic factor and neurturin inhibit neurite outgrowth and activate RhoA through GFR alpha 2b, an alternatively spliced isoform of GFR alpha 2." J Neurosci **27**(21): 5603-14.
Yu, M. C. (2011). "The Role of Protein Arginine Methylation in mRNP Dynamics." Mol Biol Int **2011**: 163827.
Zhang, X., Y. V. Bernatavichute, et al. (2009). "Genome-wide analysis of mono-, di- and trimethylation of histone H3 lysine 4 in Arabidopsis thaliana." Genome Biol **10**(6): R62.
Zhao, S., W. P. Fung-Leung, et al. (2014). "Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells." PLoS One **9**(1): e78644.

Zhu, X., Z. Li, et al. (2012). "Characterization and expressional analysis of Dleu7 during Xenopus tropicalis embryogenesis." Gene **509**(1): 77-84.

XIX. LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

Candidate: Zoltán Simándi
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Doctoral School: Doctoral School of Molecular Cell and Immune Biology
MTMT ID: 10040517

List of publications related to the dissertation

1. **Simándi, Z.**, Czipa, E., Horváth, A., Kőszeghy, Á., Bordás, C., Póliska, S., Juhász, I., Imre, L., Szabó, G., Dezső, B., Barta, E., Sauer, S., Károlyi, K., Kovács, I., Hutóczky, G., Bognár, L., Klekner, Á., Szűcs, P., Bálint, B.L., Nagy, L.: PRMT1 and PRMT8 regulate retinoic acid dependent neuronal differentiation with implication to neuropathology.
   Stem Cells. "accepted by publisher" (2014)
   IF:7.133 (2013)

2. **Simándi, Z.**, Cuaranta-Monroy, I., Nagy, L.: Nuclear receptors as regulators of stem cell and cancer stem cell metabolism.
   Semin. Cell Dev. Biol. 24 (10-12), 716-723, 2013.
   DOI: http://dx.doi.org/10.1016/j.semcdb.2013.10.002
   IF:5.971

3. **Simándi, Z.**, Bálint, B.L., Póliska, S., Rühl, R., Nagy, L.: Activation of retinoic acid receptor signaling coordinates lineage commitment of spontaneously differentiating mouse embryonic stem cells in embryoid bodies.
   FEBS Lett. 584 (14), 3123-3130, 2010.
   DOI: http://dx.doi.org/10.1016/j.febslet.2010.05.052
   IF:3.601

XX. LIST OF OTHER PUBLICATIONS

UNIVERSITY OF DEBRECEN
UNIVERSITY AND NATIONAL LIBRARY
PUBLICATIONS

List of other publications

4. Cuaranta-Monroy, I., **Simándi, Z.**, Kolostyák, Z., Doan-Xuan, Q., Póliska, S., Horváth, A., Nagy, G., Bacsó, Z., Nagy, L.: Highly efficient differentiation of embryonic stem cells into adipocytes by ascorbic acid.
Stem Cell Res. 13 (1), 88-97, 2014.
DOI: http://dx.doi.org/10.1016/j.scr.2014.04.015
IF:3.912 (2013)

5. Czimmerer, Z., Hulvely, J., **Simándi, Z.**, Varallyay, É., Havelda, Z., Szabó, E., Varga, A., Dezső, B., Balogh, M., Horváth, A., Domokos, B., Török, Z., Nagy, L., Bálint, B.L.: A versatile method to design stem-loop primer-based quantitative PCR assays for detecting small regulatory RNA molecules.
PLoS One. 8 (1), 10 p., 2013.
IF:3.534

6. **Simándi, Z.**, Nagy, L.: Retinoid signaling is a context-dependent regulator of embryonic stem cells. In: Embryonic Stem Cells - Differentiation and Pluripotent Alternatives. Ed.: Michael S. Kallos, InTech - Open Access Publisher, Rijeka, 55-78, 2011.

Total IF of journals (all publications): 24,151
Total IF of journals (publications related to the dissertation): 16,705

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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