RARγ is essential for retinoic acid induced chromatin remodeling and transcriptional activation in embryonic stem cells

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Summary

We have utilized retinoic acid receptor γ (gamma) knockout (RARγ$^{-/-}$) embryonic stem (ES) cells as a model system to analyze RARγ mediated transcriptional regulation of stem cell differentiation. Most of the transcripts regulated by all-trans retinoic acid (RA) in ES cells are dependent upon functional RARγ signaling. Notably, many of these RA–RARγ target genes are implicated in retinoid uptake and metabolism. For instance, Lrat (lecithin:retinol acyltransferase), Stra6 (stimulated by retinoic acid 6), Crabp2 (cellular retinoic acid binding protein 2), and Cyp26a1 (cytochrome p450 26a1) transcripts are induced in wild type (WT), but not in RARγ$^{-/-}$ cells. Transcripts for the transcription factors Pbx1 (pre-B cell leukemia homeobox-1), Wt1 (Wilm's tumor gene-1), and Meis1 (myeloid ecotropic viral integration site-1) increase upon RA treatment of WT, but not RARγ$^{-/-}$ cells. In contrast, Stra8, Dleu7, Leftb, Pitx2, and Cdx1 mRNAs are induced by RA even in the absence of RARγ. Mapping of the epigenetic signature of Meis1 revealed that RA induces a rapid increase in the H3K9/K14ac epigenetic mark at the proximal promoter and at two sites downstream of the transcription start site in WT, but not in RARγ$^{-/-}$ cells. Thus, RA-associated increases in H3K9/K14ac epigenetic marks require RARγ and are associated with increased Meis1 transcript levels, whereas H3K4me3 is present at the Meis1 proximal promoter even in the absence of RARγ. In contrast, at the Lrat proximal promoter primarily the H3K4me3 mark, and not the H3K9/K14ac mark, increases in response to RA, independently of the presence of RARγ. Our data show major epigenetic changes associated with addition of the RARγ agonist RA in ES cells.

Key words: Meis1, Differentiation, Transcription, Nuclear receptor, Retinoic acid receptor, Epigenetics

Introduction

Retinoic acid receptors (RARs) belong to the family of nuclear receptors that regulates transcription. There are three RAR isotypes (RARα, RARβ, RARγ) that heterodimerize with RXRs (RXRα, RXRβ, RXRγ) and bind the cis-acting retinoic acid response elements (RAREs) to execute the biological functions of RA during embryonic development and postnatally (Clagett-Dame and Knutson, 2011; Means and Gudas, 1995; Samarut and Rochette-Egly, 2012). While single RAR mutant mice are viable and show relatively mild phenotypes, compound mutants of RARs display an array of congenital abnormalities and die shortly after birth (Mark et al., 2009).

RARγ null mice exhibit growth deficiency (Lohnes et al., 1993). Recently, RARγ was shown to be highly expressed in the growth plate, and ablation of RARγ is associated with reduced chondrocyte proliferation and decreased expression and deposition of proteoglycans (Williams et al., 2009). These findings provide some mechanistic understanding of the growth retardation phenotype observed in the RARγ null mice. RARγ regulates hindbrain and axial patterning, and its loss results in several malformations of the axial skeleton, including anteriorization of the cervical and thoracic vertebrae (Lohnes et al., 1993; Wendling et al., 2001). RARγ is required for the formation of normal alveoli and alveoli elastic fibers in the lung (McGowan et al., 2000). Genetic ablation of RARγ results in male sterility and is associated with squamous metaplasia of seminal vesicles and the prostate glands and keratinization of glandular epithelia (Lohnes et al., 1993).

Retinoids also regulate hematopoietic development, which is dependent on distinct functions mediated by RARα and RARγ (Purton, 2007). While RARα induces granulocytic differentiation, RARγ plays a critical role in maintaining the balance between the self-renewal state of HSCs and their differentiation (Purton, 2007; Purton et al., 2006). Many of the molecular targets and pathways downstream of RARγ that mediate its effects on hematopoiesis remain to be determined.

RARγ mediates the anti-proliferative and apoptotic effects of retinoids in certain tissues and cancer cells, such as melanoma and neuroblastoma cells (Meister et al., 1998; Spanjaard et al., 1997). RARγ is the principal receptor that functions in RA mediated growth arrest in keratinocytes (Goyette et al., 2000). In a model of epidermal tumorigenesis, ablation of RARγ enhanced

the tumor incidence of Ras transformed keratinocytes and was associated with resistance to retinoid mediated growth arrest and apoptosis (Chen et al., 2004).

Studies conducted in our laboratory have shown that the lack of both alleles of RARγ in F9 teratocarcinoma stem cells is associated both with impaired differentiation and greatly reduced expression of genes involved in cell differentiation, such as Hoxa1, laminin B1, and collagen IV (α1) (Boylan et al., 1993; Boylan et al., 1995). A microarray analysis of F9 RARγ null teratocarcinoma stem cells revealed novel RARγ regulated genes, reinforcing its important role in retinoid signaling and differentiation (Su and Gudas, 2008a; Su and Gudas, 2008b).

We have now utilized murine RARγ knockout ($RAR\gamma^{-/-}$) embryonic stem (ES) cells as a model system to study RARγ mediated transcriptional regulation in development and cell differentiation. ES cells are derived from the inner cell mass of blastocysts and have the unique ability to self-renew under defined conditions (Smith, 2001). ES cells have a stable genome and possess the capacity to differentiate into the three germ layers, thus making them an excellent cell culture system to study RA mediated differentiation *in vitro* (Gudas and Wagner, 2011; Soprano et al., 2007). We previously reported that RARγ null ES cells do not differentiate into parietal endoderm, an epithelial cell type, in response to RA (Kashyap et al., 2011).

To delineate further the functions of RARγ in ES cells, we performed microarray analysis of wild type (WT) and $RAR\gamma^{-/-}$ ES cells and identified differentially regulated genes. We characterized the transcriptional and epigenetic regulation of the RARγ target gene Meis1 using chromatin immunoprecipitation (ChIP) and ChIP-chip technologies. Furthermore, functional depletion of RARγ in WT ES cells, combined with restored RARγ expression in $RAR\gamma^{-/-}$ cells, confirmed the requirement for RARγ in the RA induced transcription of Meis1.

Results

Identification of differentially expressed genes in WT and RARγ knockout ES cells by microarray analysis

To identify RARγ regulated genes in ES cells, we performed microarray analyses of ES RARγ knockout ($RAR\gamma^{-/-}$) compared to wild type (WT) ES cells under different culture conditions. We cultured the WT and $RAR\gamma^{-/-}$ cells with vehicle control or 1 μM RA for 8 or 24 h. The 8 h and 24 h time points allowed us to examine the kinetics of RA induced transcript changes. The 8 h time point also provides the advantage of capturing early transcript changes that are less likely to be secondary changes related to cell differentiation.

A total of 152 transcripts were differentially regulated by threefold or more in untreated WT versus $RAR\gamma^{-/-}$ ES cells (Fig. 1). Of these 152 differentially expressed transcripts, 80 transcripts showed reduced levels (Fig. 1; supplementary material Table S2A), and 78 transcripts showed elevated levels in the $RAR\gamma^{-/-}$ cells compared to the WT cells in vehicle treated (untreated) conditions (Fig. 1; supplementary material Table S2B). These data suggest a role for RARγ in regulating gene expression in the absence of the ligand, as we demonstrated in teratocarcinoma stem cells for RARα (Laursen et al., 2012). Furthermore, a total of 56 and 72 transcripts were differentially regulated by threefold or more in WT versus $RAR\gamma^{-/-}$ ES cells upon 8 and 24 h of RA treatment, respectively (Fig. 1; supplementary material Table S3A,B, Table S4A,B).

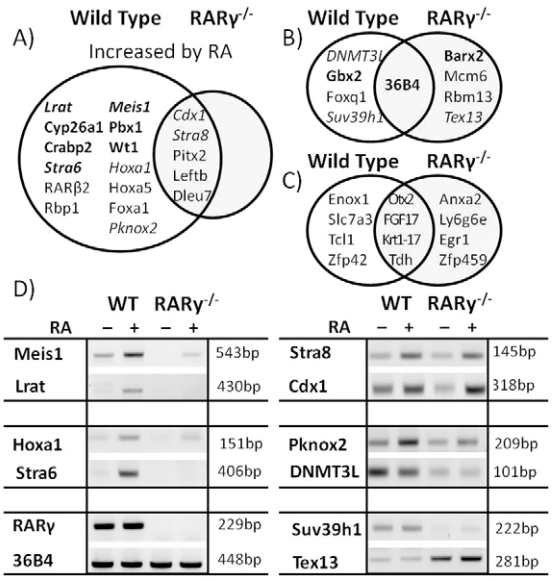

Fig. 1. Microarray analysis of transcript levels in WT and $RAR\gamma^{-/-}$ ES cells. (**A**) Genes that show elevated transcript levels upon RA treatment for 24 h. The white circle shows genes induced by RA in WT ES cells; the gray circle shows genes induced by RA in RARγ null ES cells. The $RAR\gamma^{-/-}$ ES cells were generated as described previously (Kashyap et al., 2011). The microarray data were generated from a single-cell cloned ES cell line. (**B**) Genes that are differentially expressed between untreated (vehicle only) WT and $RAR\gamma^{-/-}$ cells at 24 h. The white circles show genes expressed at a higher level in untreated WT than $RAR\gamma^{-/-}$ cells. The gray circles show genes expressed at a higher level in untreated $RAR\gamma^{-/-}$ than in WT. (**C**) Genes that show decreased transcript levels upon RA treatment for 24 h. The white circle shows genes repressed by RA in WT cells. The gray circle shows genes repressed by RA in $RAR\gamma^{-/-}$ cells. Genes validated by real time RT-PCR are shown in bold. Note that the Venn diagrams depict only a subset of the genes identified in each group (for complete lists, refer to the supplementary material Tables S2–S7). (**D**) Validation of transcript levels in WT and $RAR\gamma^{-/-}$ ES cells by end-point PCR. Meis1, Lrat, Hoxa1, Stra6, and Pknox2 display reduced RA dependent induction in $RAR\gamma^{-/-}$ cells relative to WT, whereas Stra8 and Cdx1 are induced in the absence of RARγ. DNMT3L, Suv39h1, and Tex13 are differentially expressed between WT and $RAR\gamma^{-/-}$ ES cells in an RA independent manner. Experiments in D were performed multiple times with identical results; representative samples are shown.

Transcripts are differentially regulated by RA in WT and RARγ knockout cells

To identify the genes that were regulated by RA treatment, we analyzed the fold changes in WT and $RAR\gamma^{-/-}$ ES cells independently upon 8 and 24 h of RA treatment in comparison to the untreated controls for each cell line. A total of 29 and 91 transcripts were increased 2.5-fold in WT ES cells upon 8 and 24 h of RA treatment, respectively, compared to untreated WT (Fig. 1A; supplementary material Tables S5, S6). The 2.5-fold cut-off was selected based on advice from the Weill Cornell Bioinformatics Core staff. Transcript levels of many known RA target genes increased in WT ES cells in response to RA treatment, such as genes of the Hoxa and Hoxb clusters and Cdx1, $RAR\beta_2$, and Gata6 (Fig. 1A,D; supplementary material Table S5, Table S6A). Transcript levels of 40 genes decreased upon 24 h of RA treatment in WT cells (Fig. 1C; supplementary material Table S6B, Table S7B). In accord with previous studies, transcript levels of Zfp42 (Rex1), a stem cell marker, were

reduced by 2.7-fold upon 24 h of RA treatment of WT cells (Fig. 1C; supplementary material Table S6B). Only a few genes (Otx2, FGF17, Krt1-17, and Tdh) showed reduced expression levels in both WT and RARγ$^{-/-}$ cells treated with RA for 24 h (Fig. 1C; supplementary material Table S6B, Table S7B).

In contrast, only 23 transcripts were increased by 2.5-fold or more in RARγ$^{-/-}$ ES cells upon treatment with RA for 24 h compared to untreated RARγ$^{-/-}$ (supplementary material Table S7A). The 8 h RA treatment did not induce significant (>2.5-fold) changes in the levels of any transcripts in the RARγ$^{-/-}$ ES cells compared to untreated RARγ$^{-/-}$ ES cells. A large number of transcripts that were differentially regulated by 2.5-fold or more in WT cells upon 24 h of RA treatment did not show statistically significant fold changes of 2.5-fold or more in the RARγ$^{-/-}$ ES cells (Fig. 1A; supplementary material Table S4A). Thus, RARγ is implicated in regulating this group of genes and our results also suggest that other RARs, i.e. RARα and RARβ, incompletely compensate for the loss of RARγ. However, Stra8, Dleu7, Leftb, Pitx2, and Cdx1 were induced by RA by more than 3.8-fold even in the absence of RARγ.

Gene ontology revealed that the vast majority of genes which exhibit reduced expression in RARγ$^{-/-}$ cells are homeobox genes involved in morphogenesis, axis formation, and tissue patterning (Meis1, Pknox2, Pbx1, Foxq1, Gbx2, and Hox genes). RARγ plays a key role in axis specification by RA (Bayha et al., 2009). The induction of Hoxa1, Hoxa2, Hoxb1, and Hoxb2, which are involved in rhombomere/hindbrain formation (Gavalas et al., 2003), is lost in RARγ$^{-/-}$ ES cells. Reduced levels of Anxa5, Anxa2, F2r, F2rl1, and Gap43 suggest impaired wound healing in RARγ$^{-/-}$ mice. Also, the transcript levels of several P450 cytochromes (Cyp1b1, Cyp26a1, and Cyp7b1) are reduced in RARγ$^{-/-}$ cells, pointing to abnormal metabolism. Finally, reduced expression of Col4a1, Col4a2, Ccnd2, Lama1, Pik3r1, PDGFRα, and Zyxin in RARγ$^{-/-}$ versus WT suggests that focal adhesion may be impaired in RARγ$^{-/-}$ ES cells, which could lead to increased cellular mobility and/or invasiveness.

RARγ regulates RA mediated changes in the transcript levels of genes involved in retinoid metabolism, including *Stra6*, *Cyp26a1*, *Lrat* and *Crabp2*, in ES cells

Because we previously observed alterations in the expression of several genes involved in retinol metabolism during ES differentiation (Langton and Gudas, 2008), we assessed the mRNA levels of several genes that function in the retinoid metabolism pathway by real time RT-PCR to determine if they were regulated by RARγ. RA greatly increased transcript levels of Stra6, Lrat, Crabp2, and Cyp26a1 in WT cells but not in the RARγ$^{-/-}$ cells, implicating RARγ in the regulation of retinoid metabolism (Fig. 2). Stra6 is a membrane bound receptor that binds to the serum retinol binding protein (RBP4), a carrier of retinol in the blood, and Stra6 both facilitates uptake of retinol and cell signaling via STAT5 (Berry et al., 2011; Kawaguchi et al., 2007). Mutations in Stra6 cause a wide range of defects that include anophthalmia, pulmonary agenesis, diaphragmatic hernia, pancreatic malformations, mental retardation, and congenital heart defects (Golzio et al., 2007; Pasutto et al., 2007). Lrat is an enzyme that converts intracellular retinol to retinyl esters; such esters are a storage form of retinoids in the cell (Amengual et al., 2012; Batten et al., 2004; Guo and Gudas, 1998; Liu and Gudas, 2005; O'Byrne et al., 2005; Zolfaghari and Ross, 2000). Crabp2 is involved in transporting RA from the cytoplasm into the nucleus (Noy, 2000), and Cyp26a1 metabolizes RA into polar metabolites in ES cells (Langton and Gudas, 2008; White et al., 1997). Real time PCR validation showed that the kinetics of the RA mediated increases in transcript levels of Stra6, Crabp2, and Cyp26a1 are similar (Fig. 2B–D). All three transcripts increase as early as 8 h after RA addition and show increased expression at 48 h of RA treatment in WT ES cells (Fig. 2A–E). In contrast, Stra6, Crabp2, and Cyp26a1 transcripts remained much lower in the RARγ$^{-/-}$ cells (Fig. 2A–E). Lrat mRNA levels in WT ES cells increased as early as 8 h after RA addition and reached a maximum at 24 h of RA treatment, after which Lrat mRNA levels declined (Fig. 2A, black square). Lrat transcript levels did not increase in the RARγ$^{-/-}$ cells treated with RA (Fig. 2A, open triangle). The mRNA levels of the reference gene 36B4 (Rplp0) were measured as an internal control and were unchanged with RA treatment (Fig. 2E).

The Lrat gene is epigenetically altered by RA treatment

Transcriptional induction by RA is frequently associated with increased levels of transcriptional permissive marks, such as H3K9/14ac and H3K4me3, whereas the levels of transcriptional repressive marks, such as the polycomb (PcG) deposited H3K27me3, are decreased (Gillespie and Gudas, 2007b; Kashyap and Gudas, 2010; Wu et al., 2009). RA treatment for 24 h causes a ~10-fold increase in the Lrat transcript level in WT ES cells (Fig. 2A), yet the levels of H3K9/14ac marks did not significantly increase in response to RA (Fig. 2F). The levels of the epigenetic mark H3K4me3 were similar in WT and RARγ$^{-/-}$ ES cells (Fig. 2F). We also found that the Lrat gene is associated with a repressive H3K27me3 mark in untreated WT and RARγ$^{-/-}$ ES cells. While RA treatment leads to a reduction in the H3K27me3 mark in the WT ES cells, H3K27me3 levels remain high in the RARγ$^{-/-}$ ES cells and in fact, the levels of H3K27me3 at 24 h of RA treatment are higher than those in untreated RARγ$^{-/-}$ cells (Fig. 2A). Thus, our data indicate a role for RA and RARγ in the transcriptional activation of Lrat by antagonizing PcG mediated repression. We also show that RARγ is not required for the placement of H3K4me3, an epigenetic mark generally associated with transcriptionally active chromatin (Sims and Reinberg, 2006), and that the presence of this mark is not sufficient for active transcription of Lrat in RA treated RARγ$^{-/-}$ cells.

RARγ regulates transcript levels of transcription factors

We further validated the transcript levels of several transcription factors that play key roles in different aspects of differentiation during development and are differentially expressed in the WT and RARγ$^{-/-}$ cells (Fig. 3A–E). Meis1 and Pbx1 are homeodomain transcription factors that function as cofactors of Hox proteins and regulate distinct aspects of differentiation (Chang et al., 1996; Featherstone, 2003; Shanmugam et al., 1999; Shen et al., 1996; Shen et al., 1997; Soprano et al., 2007). Dysregulated expression of these cofactors in combination with Hox proteins is observed in different types of leukemia (Eklund, 2007; Wang et al., 2006). Pbx1 is required during embryogenesis for skeletal patterning, development of adrenal glands and pancreas, urogenital differentiation, nephrogenesis, and maintenance of hematopoiesis in the liver of the developing fetus (Moens and Selleri, 2006). Meis1 disruption in mice is embryonic lethal and Meis1 deficient embryos have defects in the eye, angiogenesis, and hematopoiesis (Azcoitia et al., 2005; Hisa

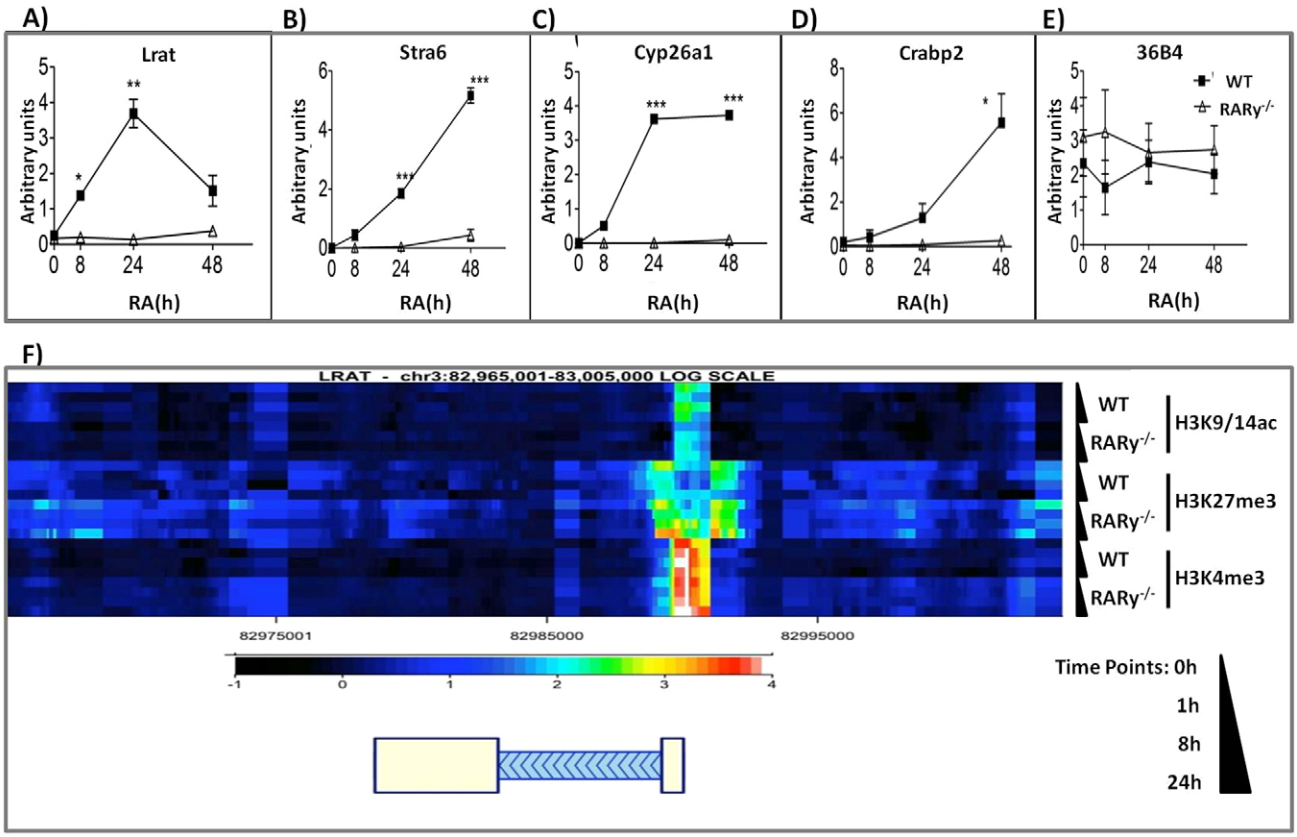

Fig. 2. RA regulated induction of retinoid metabolism genes. (**A–E**) Transcript levels of Lrat, Stra6, Cyp26a1, Crabp2, and 36B4 (a control) in WT and RARγ$^{-/-}$ ES cells. Experiments were performed three times using independent RNA samples (■ WT; △ RARγ$^{-/-}$); the error bars represent the standard error of the mean (s.e.m.). (**F**) ChIP-chip heatmap of the Lrat genomic region showing H3K9/14ac, H3K27me3, and H3K4me3 histone marks in WT and RARγ$^{-/-}$ cells upon increasing times of exposure to RA (0, 1, 8, and 24 h, black triangle). The colors represents $\log_2$-transformed ChIP enrichment in ChIP-chip data sets (replicate means). Columns show genomic loci and rows show IP condition. Statistical significance: *$P<0.05$, **$P<0.01$, ***$P<0.005$. The color scale of the $\log_2$ enrichment is indicated below the ChIP-chip data panel. The Lrat genomic location (blue tones) is indicated schematically at the bottom.

et al., 2004). Expression of Meis1 is elevated in acute myeloid leukemias, and Meis1 has oncogenic potential in leukemias that harbor fusion proteins with the translocation of MLL (mixed lineage leukemia) family members (Kawagoe et al., 1999; Wong et al., 2007). Pbx1 and Meis1 mRNA levels increase upon 24 h of RA treatment in WT cells, and transcript levels continue to increase up to 48 h of RA treatment (Fig. 3A,B). In contrast, RA did not increase Pbx1 and Meis1 transcript levels in the RARγ$^{-/-}$ cells (Fig. 3A,B).

Wt1 (Wilm's tumor gene) encodes a transcription factor that has an essential role in the normal development of the urogenital system, and is mutated in a subset of patients with Wilm's tumors, a type of kidney tumor (Kreidberg et al., 1993; Pelletier et al., 1991a; Pelletier et al., 1991b). RA increased Wt1 mRNA levels at 48 h in WT cells, but no RA dependent increase in the Wt1 mRNA levels occurred in the RARγ$^{-/-}$ cells (Fig. 3C).

Gbx2 (gastrulation brain homeobox 2) is required for normal development of mid/hindbrain region and morphogenesis of the inner ear (Lin et al., 2005; Wassarman et al., 1997). In WT cells RA treatment caused less than a twofold increase in the Gbx2 mRNA levels. Gbx2 mRNA levels were consistently lower in RARγ$^{-/-}$ cells compared to WT cells (Fig. 3D).

Barx1 is a homeodomain transcription factor that is expressed in the stomach mesenchyme and molar dental cells of mesenchymal origin (Makarenkova and Meech, 2012). Barx1 mRNA levels were 70-fold lower in untreated WT cells than in the untreated RARγ$^{-/-}$ cells (Fig. 3E). At 48 h of RA treatment Barx1 mRNA levels decreased by ~25% in the RARγ$^{-/-}$ cells, whereas Barx1 transcript levels did not change in WT cells (Fig. 3E). Thus, RA mediates changes in the transcript levels of multiple genes involved in differentiation and development that require RARγ. Why these specific genes are highly regulated by RA in differentiating WT ES cells is not yet clear.

RA induces RARγ dependent epigenomic re-organization of the Meis1 gene in WT cells

Since aberrant regulation of Meis1 is implicated in leukemia (Kawagoe et al., 1999; Wong et al., 2007) and we are interested in differentiation therapy for cancer, we next examined the dynamics of the RA induced increase in Meis1 transcript levels in further detail. We hypothesized that RA signaling would induce epigenetic changes at the Meis1 gene and that loss of RARγ would prevent such chromatin changes at this gene. In WT cells RA induced a rapid increase in H3K9/14ac levels at the proximal promoter and at specific regions (DS1 and DS2) located

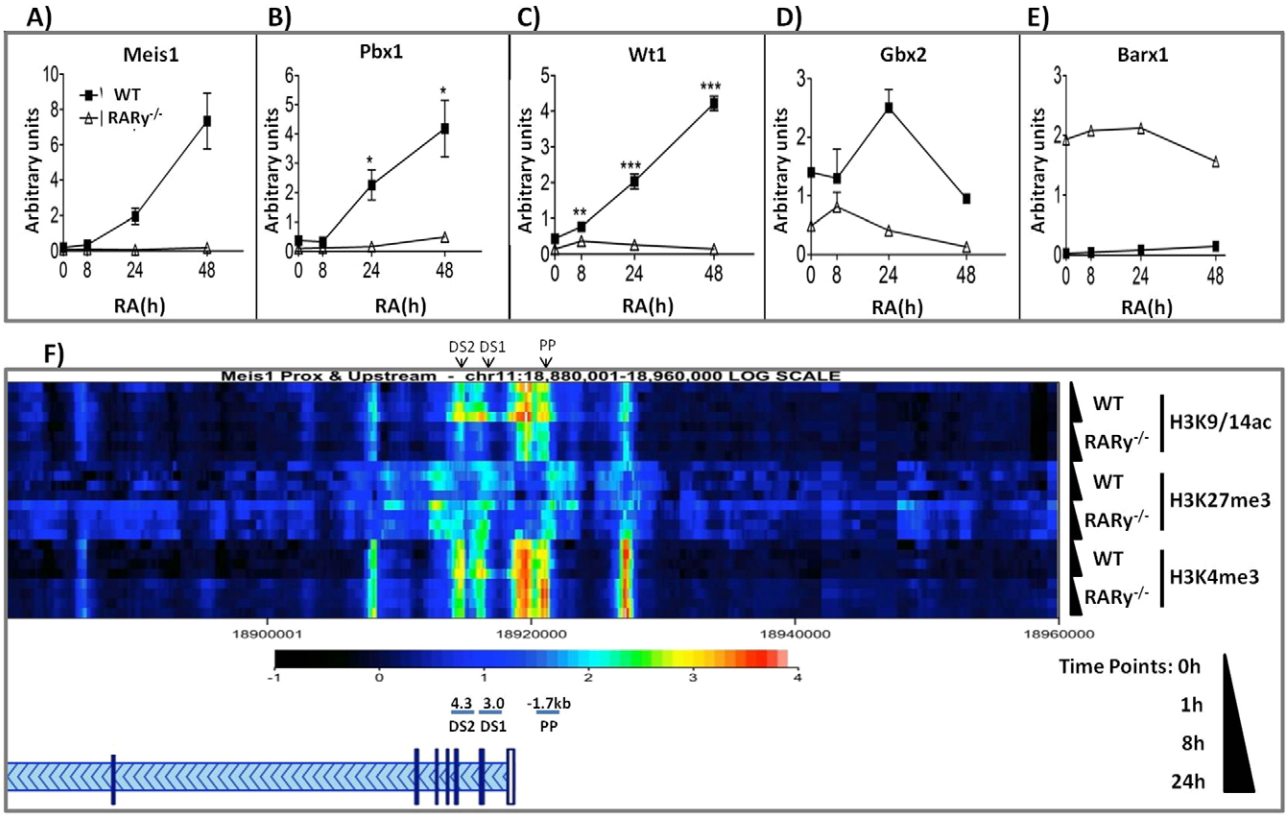

Fig. 3. RA regulated induction of transcription factors. (**A–E**) Transcript levels in WT and RARγ$^{-/-}$ ES cells of Meis1, Pbx1, Wt1, Gbx2, and Barx1. Experiments were performed three times using independent RNA samples (filled squares, WT; open triangles, RARγ$^{-/-}$); error bars indicate s.e.m. (**F**) ChIP-chip heat-map of the Meis1 proximal promoter region showing H3K9/14ac, H3K27me3, and H3K4me3 histone marks in WT and RARγ$^{-/-}$ cells upon increasing times of exposure to RA (0, 1, 8, and 24 h, black triangle). The colors represents $\log_2$-transformed ChIP enrichment in ChIP-chip data sets (replicate means). DS1 and DS2 indicate downstream sites 1 and 2; PP indicates the proximal promoter region. Columns show genomic loci and rows show IP condition. Statistical significance: $P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$. The color scale of the $\log_2$ enrichment is indicated below the ChIP-chip data panel. The Meis1 exon (broad) and intron (narrow) locations (blue tones) are indicated schematically at the bottom.

downstream of the transcription start site (TSS) in the Meis1 gene (Fig. 3F, arrows; Fig. 4A). H3K9/14ac epigenetic marks are generally associated with transcriptionally active genes (Jenuwein and Allis, 2001). In contrast, the H3K9/14ac levels at the promoter and downstream intragenic regions of the Meis1 gene were much lower in the RARγ cells compared to the WT cells (Fig. 3F; Fig. 4A), correlating with Meis1 transcript levels in WT versus RARγ$^{-/-}$ cells (Fig. 3A). The H3K4me3 mark is elevated in a region surrounding the promoter of the Meis1 gene in both untreated and RA treated WT and RARγ$^{-/-}$ cells (Fig. 3F; Fig. 4C).

Meis1 resides in a bivalent chromatin domain, e.g. it is associated with both with the repressive H3K27me3 mark deposited by PcG proteins (Boyer et al., 2006) and with the permissive H3K4me3 mark described above. By 24 h of RA treatment there is a reduction in the H3K27me3 mark (Fig. 3F), and in the levels of Suz12 protein (Fig. 4B) at the promoter and in the gene body of Meis1 in WT cells. However, in RARγ$^{-/-}$ cells both the H3K27me3 mark and Suz12 levels remain high at 24 h of RA treatment (Fig. 3F; Fig. 4B). Thus, RA signaling via RARγ antagonizes the PcG mediated repression of the Meis1 gene, and the lack of RARγ prevents the removal of the H3K27me3 mark from the Meis1 gene (Fig. 3F).

Taken together, these data suggest that at the Meis1 gene RARγ (or its downstream targets) is required for RA induced changes in the epigenetic configuration comprising the H3K9/14ac marks. However, RARγ is not needed for the deposition of the H3K4me3 mark at the Meis1 promoter and the H3K4me3 mark does not correlate with transcription of Meis1 in RA treated RARγ$^{-/-}$ cells (Fig. 3F).

The Meis1 promoter proximal region appears devoid of functional retinoic acid response elements (RAREs)

The effects of RA are mediated through RAREs, and consequently the epigenomic structure exhibits dramatic changes in response to RA, generating hot-spots in the ChIP-chip maps. We evaluated the Meis1 proximal promoter region (defined as TSS −/+ 40 kb), and identified three sites exhibiting dramatic epigenetic changes in response to RA (Fig. 3F, arrows). Given the critical role of RARγ in the regulation of the Meis1 gene, we further characterized the levels of retinoic acid receptor γ (RARγ) and retinoid receptor α (RXRα) at these three sites (Fig. 5A,B). RARγ levels were slightly elevated in WT versus RARγ$^{-/-}$ cells at all evaluated regions, but showed no specific enrichment at any one particular region. The levels of RXRα at all of these regions were similar to those of IgG (Fig. 5A,B versus Fig. 5F). We detected binding of RXRα at the Cyp26a1 promoter, our positive control (supplementary material Fig. S1), consistent with our previous studies indicating that Cyp26a1 is a direct RA target gene (Kashyap et al., 2011). Consequently, the Meis1 proximal promoter region appears to be devoid of functional RAREs.

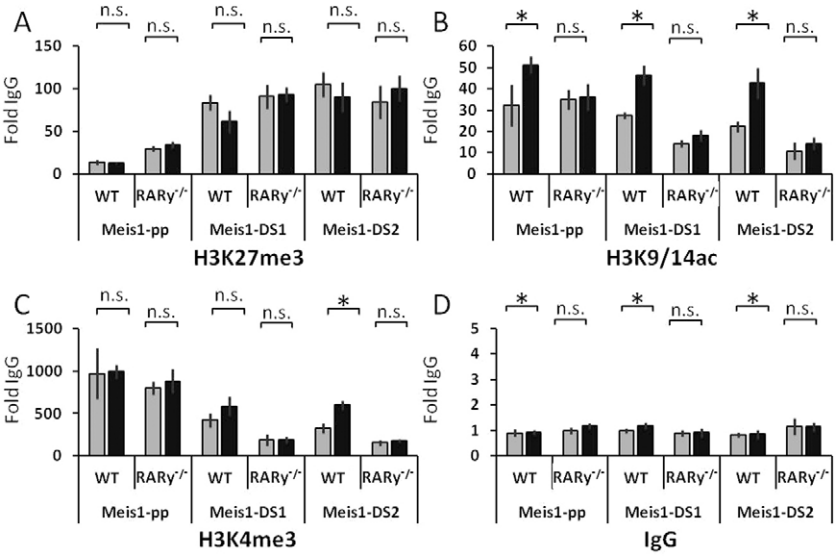

Fig. 4. H3K9/14ac, Suz12, and H3K4me3 associate with specific Meis1 elements. ChIP assays of Meis1 P_{refseq} (pp) and Meis1 downstream elements (DS1 and DS2) in WT and RARγ$^{-/-}$ ES cells evaluating (**A**) H3K9/14ac; (**B**) Suz12; and (**C**) H3K4me3 association with each of the three Meis1 elements. (**D**) IgG was used as a negative control. Each experiment was repeated at least three times, starting with freshly plated cells, and evaluated by quantitative PCR. The data are plotted as fold enrichment relative to IgG background. Note that the y-axes are different in each panel. Values are means ± s.e.m. of three independent experiments. Statistical significance: *$P<0.05$; n.s., not significant. Untreated, gray bars; treated with RA for 24 h, black bars.

Pol II, p300, and histone acetylation are decreased in RARγ knockout cells compared to WT cells

In the WT cells we detected RA induced enrichment of Pol II, most pronounced at the promoter proximal region of Meis1. In contrast to the WT cells, we detected low levels of Pol II in both untreated and RA treated RARγ$^{-/-}$ cells, suggesting that RA fails to increase the levels of Pol II in the RARγ$^{-/-}$ cells (Fig. 5C).

We also assessed the association of the H3K27ac mark and the recruitment of the co-activator p300 (KAT3B) in WT and RARγ$^{-/-}$ ES cells by ChIP. The H3K27ac mark is associated with active enhancers and promoters and this epigenetic mark antagonizes the repressive H3K27me3 mark associated with PcG silencing (Creyghton et al., 2010; Pasini et al., 2010; Vernimmen et al., 2011). In addition, the transcriptional co-activator p300 possesses histone acetyltransferase activity and is recruited to

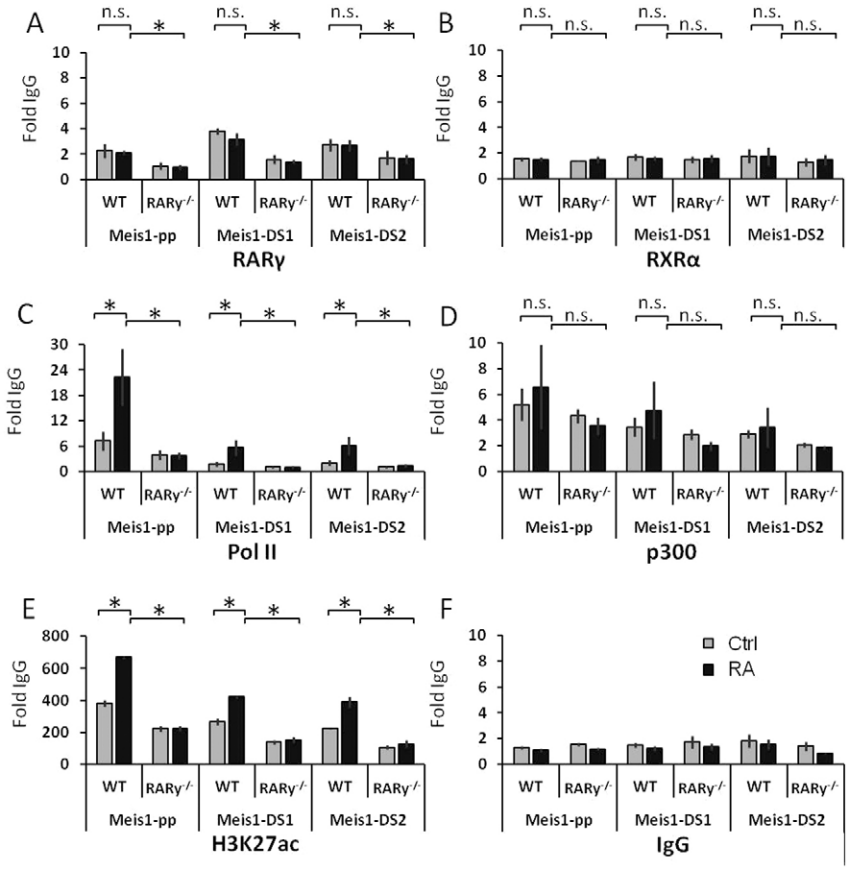

Fig. 5. RARγ, RXRα, H3K27ac, p300, and PolII-CTD association with specific Meis1 elements. ChIP assays of Meis1 P_{refseq} (pp) and Meis1 downstream elements (DS1 and DS2) in WT and RARγ$^{-/-}$ ES cells to evaluate the association of (**A**) RARγ, (**B**) RXRα, (**C**) H3K27ac, (**D**) p300, and (**E**) PolII-CTD with each of the three Meis1 elements. (**F**) IgG was used as a negative control. Each experiment was repeated at least three times, starting with freshly plated cells, and evaluated by quantitative PCR performed in triplicate. The data are plotted as fold enrichment over IgG background. Values are means ± s.e.m. of three independent experiments. Statistical significance: *$P<0.05$; n.s., not significant. Untreated, gray bars; treated with RA for 24 h, black bars.

regulatory enhancer elements in the genome (Heintzman et al., 2009; Visel et al., 2009; Wang et al., 2005). Levels of the H3K27ac mark at the proximal promoter and at the DS1 and DS2 downstream sites of the Meis1 gene increased upon RA treatment of WT, but not RARγ$^{-/-}$ cells (Fig. 5D). We observed low levels of enrichment of p300 at the proximal promoter and the two downstream regions in untreated WT cells. Levels of p300 at the Meis1 gene did not increase significantly with RA treatment, but the levels of both H3K27ac and p300 association were consistently higher in WT compared to RARγ$^{-/-}$ cells (Fig. 5D,E).

RARγ$_2$ is necessary and sufficient for RA induced transcription of Meis1

Because no significant binding of RARγ (or RXRα) was detected in the Meis1 DS1 and DS2 regions identified by the ChIP-chip analysis we decided to further validate the requirement for RARγ. We employed shRNA to deplete RARγ functionally and specifically, thereby providing an independent validation of the RARγ dependent induction of Meis1. The RARγ shRNA knockdown reduced RARγ transcript levels to ~25% of the levels in WT cells, which decreased Meis1 transcript levels to 21% and 45% of the shLuc control with 24 and 48 h of RA treatment, respectively (Fig. 6A). Additionally, we show that knockdown of RARγ by shRNA does not reduce RARα and RARβ transcript levels (supplementary material Fig. S2). These data confirm that RA induces transcription of Meis1 through the actions of RARγ in ES cells.

We next determined if the RA responsiveness of Meis1 can be restored by reintroducing RARγ$_2$, which is the predominantly expressed RARγ isoform. We generated two independent cell lines which ectopically express RARγ$_2$ in the RARγ null background. We then assessed Meis1 mRNA levels in each of the two RARγ$_2$ restoration cell lines (Fig. 6B). We observed Meis1 transcripts in both RARγ$_2$ restoration cell lines relative to the RARγ$^{-/-}$ ES cell line (WT: 19- to 22-fold, clone 1: 10- to 12-fold, and clone 2: 19- to 33-fold, each relative to Meis1 transcript levels in RARγ$^{-/-}$ cells, $P<0.005$ for either treatment condition). Expression of the ectopic RARγ$_2$ cassette was confirmed by assessing RARγ$_2$ transcript levels in the restoration cell lines. The restored RA responsiveness of Meis1 upon ectopic RARγ$_2$ expression demonstrates that RARγ$_2$ is both necessary and sufficient for the RA-associated increase in Meis1 transcripts.

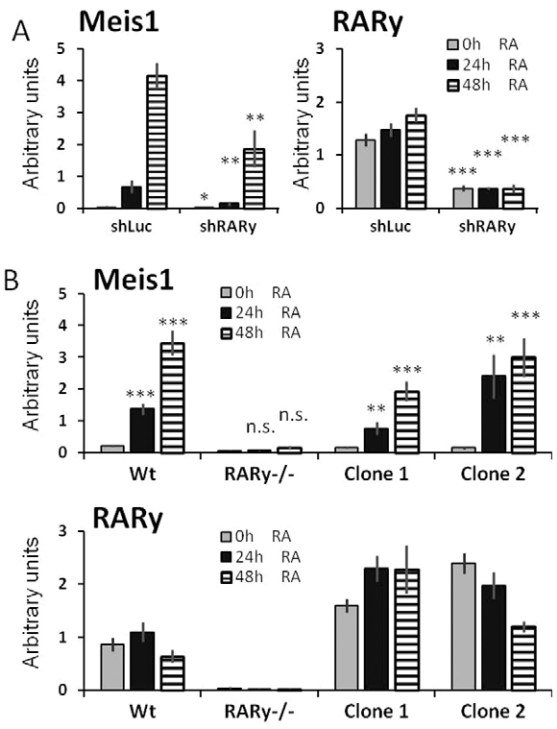

Fig. 6. RARγ knockdown decreases Meis1 transcript levels, and ectopic RARγ$_2$ in RARγ$^{-/-}$ cells restores Meis1 transcript levels. (**A**) In WT ES cells, RARγ knockdown by stable shRNA decreased endogenous Meis1 transcript levels to 21% and 45% upon 24 and 48 h of RA treatment, respectively, as assessed by real time RT-PCR. RARγ transcript levels were reduced to 25% by expression of a RARγ-specific shRNA (shRARγ) relative to a control shRNA targeting luciferase (shLuc). Statistical significance: *$P<0.05$, **$P<0.01$, ***$P<0.005$ relative to WT cells under similar conditions. (**B**) In RARγ$^{-/-}$ ES cells ectopic expression of RARγ$_2$ restored RA responsive levels of Meis1 transcripts. Results are shown from two independent clones (1 and 2). Statistical significance is indicated by *$P<0.05$, **$P<0.01$, ***$P<0.005$ relative to untreated cells.

Discussion

We utilized microarray analysis to identify RARγ regulated genes in WT and RARγ$^{-/-}$ ES cells cultured in the presence versus the absence of RA. The microarray data analysis revealed the important role of RARγ in gene regulation in both the presence of the RA agonist (Fig. 1A; supplementary material Table S4A,B), and in the absence of the ligand, as we observed large numbers of genes that were differentially regulated even in untreated RARγ$^{-/-}$ versus WT ES cells (Fig. 1B; supplementary material Table S2A,B). Additionally, we show that transcript levels of many genes decrease upon 24 h of RA treatment in WT ES cells, thus implicating RA in gene repression (Fig. 1C; supplementary material Table S6B).

The interaction of RARγ with transcription factors and polycomb repressive complexes

The antagonistic, functional cross-talk between RARs and polycomb group (PcG) protein regulated transcription in stem cells has raised many questions concerning the mechanistic details of the recruitment and displacement of the PcG proteins from retinoid regulated genes. It is worth noting that the PcG protein EZH2 has also been shown to associate directly with the repressor of estrogen receptor activity (REA), a co-repressor of ER, and EZH2 repressed estrogen-induced transcription from a reporter (Hwang et al., 2008). These findings call for the identification of co-regulators of RAR mediated transcription that may bind and target PcG proteins to RA target genes in stem cells. We showed that the polycomb protein Suz12 interacts with RARγ in the absence of RA in ES cells and that this interaction is abrogated upon RA addition; however this interaction may not be by direct binding and could be bridged through association with other transcriptional regulators, such as co-repressors (Amat and Gudas, 2011). Importantly, by using the RARγ$^{-/-}$ ES cells we have shown that the lack of RARγ does not interfere with the basal association of the PcG mediated H3K27me3 mark at the Hoxa and Hoxb clusters and other RA targets, such as Cyp26a1 (Kashyap et al., 2011) and Meis1 (Fig. 3F).

Given the differential chromatin signatures and transcript profiles across different cell lineages (Kashyap and Gudas, 2010), it is likely that RARs exhibit cross-talk with other transcription factors in a cell-type dependent manner. In fact, the cell-specific

functions of RARs may be executed in conjunction with transcription factors that play key roles in the biological functions of their respective lineages. The interplay of RARs with other transcription factors, such as Foxa1 (Hua et al., 2009), calls for interrogation and identification of additional transcription factors that may regulate the functions of RARs in ES cells. Some of the transcription factors that we have identified as being transcriptionally activated by RARγ (Figs 1, 3) in ES cells in response to RA are candidates for playing such roles.

RARγ is required for RA-associated epigenetic changes at the Lrat gene

Our data show that RA increases Lrat transcript levels in WT, but not in RARγ$^{-/-}$ cells (Fig. 2A). We show that RARγ is required for the removal of the H3K27me3 mark from the Lrat gene, and that failure to deplete the repressive H3K27me3 mark specifically at the Lrat proximal promoter region is associated with the lack of transcriptional activation by RA in the RARγ$^{-/-}$ cells (Fig. 2F). RARγ is not required for the placement of the H3K4me3 mark and the presence of this mark is not sufficient for Lrat transcriptional activation in RARγ$^{-/-}$ cells (Fig. 2F). We did not detect a DR2 or DR5 RARE within 2 kb 5′ or 3′ of the Lrat start site of transcription, suggesting that Lrat may possess an RARE at some distance from the coding region or that Lrat is a secondary RAR target gene. The absence of H3K9/14ac marks in the Lrat proximal promoter region suggests that RA induction of Lrat is regulated mainly by dissociation of the H3K27me3 repressive mark, a feature which is observed in WT, but not in RARγ$^{-/-}$ cells (Fig. 2F).

Meis1 transcriptional activation by RA involves loss of PcG mediated repression and RARγ mediated epigenetic activation

RA signaling in WT ES cells leads to increased association of the transcriptional activation marks H3K9/14ac at the Meis1 gene, concomitant with increased levels of Pol II (Figs 3–5). These RA dependent epigenetic changes are attenuated or absent in the RARγ$^{-/-}$ cells, in accord with the significantly lower Meis1 transcript levels in RA treated RARγ$^{-/-}$ cells (Figs 3–5). Importantly, we did not find any correlation between placement of the H3K4me3 mark and transcriptional activation of Meis1 by RA. Like the H3K27me3 mark, the H3K4me3 mark is recruited independently of RARγ, thus generating a bivalent domain (Fig. 3F). In this environment in WT cells the activation of RARγ by RA induces local depletion of the H3K27me3 mark, thus shifting the balance between repressive and permissive H3K4me3 histone marks. In addition, the RA induced recruitment of co-activators favors histone acetylation, further potentiating transcriptional induction. We confirmed the requirement for RARγ in the induction of Meis1 through shRARγ depletion of RARγ (Fig. 6A), but we did not detect binding of RARγ or RXRα in the DS1 and DS2 regions of the Meis1 proximal promoter region (Fig. 3F; Fig. 5). This indicates that Meis1 may be an indirect, secondary target of RARγ in ES cells. Alternatively, RAR/RXR binding could occur at an enhancer region distant (+40 kb) from the Meis1 proximal promoter region. The presence of a conserved Pbx1 binding site in the Meis1 proximal promoter region (Magnani et al., 2011) suggests that RARγ may induce Meis1 through or possibly in cooperation with Pbx1.

Conclusions

Our analysis shows that many genes exhibit reduced expression upon RA treatment of WT ES cells; in fact, while 91 genes showed upregulation by RA, 40 genes, including Otx2 and Zfp42, exhibited downregulation by RA at 24 h. This points to non-consensus RA signaling in addition to ligand-induced transcription. Several genes, including DNMT3L, Suv39h1, and Tex13, were differentially expressed between WT and RARγ$^{-/-}$ ES cells independent of RA treatment. Consequently, RARγ may have ligand-independent functions similar to those recently reported for RARα (Laursen et al., 2012). Our research data also lead to novel conclusions about epigenetic modifications of RA-responsive genes in ES cells. First, RARγ is not required for placement of the H3K4me3 epigenetic mark in ES cells and the presence of this mark is not sufficient for transcriptional activation of the RA responsive genes Lrat and Meis1. Additionally, the lack of RARγ increases the association of the H3K27me3 mark with the proximal promoter of Meis1, but not with the downstream elements DS1 and DS2. In conclusion, these data provide new insights into the types of RA induced epigenetic changes in embryonic stem cells.

Materials and Methods

Derivation and culture of the ES cell lines

The cell lines were derived and cultured as described previously (Kashyap et al., 2011). All-trans retinoic acid (RA; Cat. no. 2625, Sigma Chemical Co., MO). RA (1 μM) was added to the cells 24 h after cell-plating and ethanol (0.1%) was used as a vehicle control.

Generation of RARγ knockdown cell lines

Generation of viral particles and transduction of ES cells was previously described (Benoit et al., 2009). In brief, knockdown vectors pLKO shRARγ (hair-pin sequence 5′-CCCAGAGGAAGCCTCTATTTA-3′) or pLKO shLuc (control), together with packaging vectors pCMVΔ8.9 and pVSV-G (Cat. no. 631530, Clontech, CA), were transfected into HEK293T cells using Lipofectamine 2000 (Cat. no. 11668, Invitrogen, CA). After overnight recovery the medium was replaced with fresh medium and the cells were allowed to produce virus for an additional 48 h before the supernatant was harvested, filtered through 0.45 μm filters, and supplemented with polybrene. WT ES cells were transduced with viral supernatant in a 1:1 ratio with 2× growth medium. About 16 h later, the medium was replaced with medium supplemented with puromycin (Cat. no. P7255, Sigma Chemical Co., MO) at a final concentration of 0.5 μg/ml for 10 days of propagation in the selection medium. After this, puromycin was not included in the medium.

Generation of stable clones

The pRosa26-SV40 mRARγ$_2$ expression vector was stably introduced into RARγ$^{-/-}$ ES cells. In brief, the pRosa26 expression vector, which contains a Hygromycin expression cassette was transfected into RARγ$^{-/-}$ ES cells using LTX Plus reagent (Cat. no. 15338, Invitrogen, CA) according to manufacturer's instructions. Selection of stable clones was performed using hygromycin (Cat. no. 10687, Invitrogen, CA) at a final concentration of 100 μg/ml for 10 days. Colonies were picked and screened by PCR using the mRARγE7(+)/mRARγE8(−) primer pair. Successful gDNA purification was evident by a 334 bp PCR product, whereas integration of the transgene was evident by an additional PCR product of 241 bp. Transgene expression in positive clones was verified by the presence of a 162 bp PCR product using the rβ-globin5′C/rβ-globin3′B primer pair, which spans the β-globin intron of the pRosa26-SV40 vector. In addition, the generation of RARγ protein was validated by western blotting (data not shown).

RNA isolation and reverse transcription

Total RNA was extracted using Trizol reagent (Cat. no. 15596, Invitrogen, CA). The RNA was quantitated by optical density at 260 nm. The RNA (1 μg) was reverse transcribed to cDNA using the Quanta reverse transcription mix (Cat. no. 95048, Quanta Biosciences, MD). The cDNA obtained was diluted tenfold and 2 μl of diluted cDNA was utilized for quantitative PCR reactions.

Real time PCR and primers

Real time PCR was carried out in a total volume of 20 μl using the Sybr Green mix (Cat. no. 84091, Quanta Biosciences, MD) according to Kashyap et al. (Kashyap et al., 2011). The primers were designed using the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgPc) and all real-time PCR primers were designed

around the introns. The primer sequences can be found in supplementary material Table S1.

Chromatin immunoprecipitation

Cells were treated with RA for 24 h, cross-linked (1% formaldehyde, 10 min), quenched (200 mM glycine, 5 min), washed with ice cold phosphate-buffered saline (PBS), and harvested by scraping. ChIP was performed according to Gillespie and Gudas (Gillespie and Gudas, 2007a; Gillespie and Gudas, 2007b). At least three biological replicate ChIP experiments were performed.

Antibodies and chemicals

Anti-H3K27ac (07-360), anti-H3K4me3 (07-473) and anti-H3K9/14ac (06–599) antibodies were purchased from Millipore (Billerica, MA). Anti-RXRα (D-20, sc-553), anti-p300 (N-15, sc-584), and anti-IgG (sc-2030) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Ser-5 carboxyl-terminal domain (CTD) of RNA polymerase II (pCTDser5) was purchased from Covance Research Products (Richmond, CA). Anti-RARγ (ab12012) and Anti-H3K27me3 (ab6002) were purchased from Abcam Inc. (Cambridge, MA).

Microarray expression profiling and analysis

Cells were treated with RA for various times (0, 8 and 24 h) prior to harvesting. Total RNA was isolated using Trizol. RNA quality was assessed using the RNA 6000 NanoAssay and a Bioanalyzer 2100 (Agilent). Samples with a 28S/18S ribosomal peak ratio of 1.8–2.0 were considered suitable for labeling. 200 ng of total RNA from each sample was labeled using the Illumina Total Prep RNA Amplification kit (Ambion), according to the manufacturer's instructions. Labeled and fragmented cRNAs (3 µg) were then hybridized to the mouse-ref8 array (Illumina), which incorporates 22,000 transcripts of known mouse genes. The raw data obtained from the Illumina microarray platform were imported into Genespring 11 (Agilent) and were normalized using the quantile normalization procedure. Following the normalization, the data were filtered for expression values. The data across replicates were averaged and subsequently, the list of genes that showed statistically significant fold changes ($P<0.05$) was obtained. The generation of the ChIP-chip data has been previously described (Kashyap et al., 2011). We obtained bioinformatics advice on data analyses from Dr Piali Mukherjee at the Epigenomics Core at Weill Cornell Medical College. Gene expression profiles were deposited at GEO with the accession code GSE43221 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43221).

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Author contributions

V.K., K.B.L., F.B., A.J.V., and J.M.S. performed experiments. V.K., K.B.L., J.M.S., and L.J.G. wrote the manuscript. V.K., K.B.L., A.J.V., J.M.S., and L.J.G. analyzed and interpreted the data.

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