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Combinatorial knockout of RARα, RARβ, and RARγ completely abrogates transcriptional responses to retinoic acid in murine embryonic stem cells

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All-*trans*–retinoic acid (RA), a potent inducer of cellular differentiation, functions as a ligand for retinoic acid receptors (RARα, β, and γ). RARs are activated by ligand binding, which induces transcription of direct genomic targets. However, whether embryonic stem cells respond to RA through routes that do not involve RARs is unknown. Here, we used CRISPR technology to introduce biallelic frameshift mutations in RARα, RARβ, and RARγ, thereby abrogating all RAR functions in murine embryonic stem cells. We then evaluated RA-responsiveness of the RAR-null cells using RNA-Seq transcriptome analysis. We found that the RAR-null cells display no changes in transcripts in response to RA, demonstrating that the RARs are essential for the regulation of all transcripts in murine embryonic stem cells in response to RA. Our key finding, that in embryonic stem cells the transcriptional effects of RA all depend on RARs, addresses a long-standing topic of discussion in the field of retinoic acid signaling.

Cells respond to environmental cues in a number of ways (*e.g.* proliferation, apoptosis, and differentiation). All-*trans*–retinoic acid (RA)[2] is a metabolite of vitamin A (retinol) and functions as a potent inducer of stem cell differentiation (1, 2). We and others have shown that RA can induce differentiation of both human and murine ES cells into various specified lineages (3–10). The extensive cell culture applications of RA are reflections of the plethora of developmental processes in which RA is involved (11–16). *In vitro* RA signaling has been extensively evaluated in highly diverse cell culture systems, from multipotent ES, F9, and P19 cells to differentiated fibroblasts, embryoid bodies, and complex organoids. In F9 cells the loss of RARγ diminishes the RA-responsiveness of *RARβ_2*, *Cyp26a1*, *Hoxa1*, *Hoxa5*, *CoupTF1* (*Nr2f1*), and *CoupTF2* (*Nr2f2*) (17, 18). Similarly, the induction of *Stra6*, *Meis1*, *Cyp26a1*, and *Hoxa1* mRNA in ES cells depends on RARγ (19–21). Transcriptome analysis of ES cells confirmed that RARγ is required to increase transcript levels of most RA-responsive genes, including Hoxa1 and Cyp26a1 (19). However, RA can induce transcripts of various targets (*e.g. Cdx1*, *Stra8*, *Lefty1*, *Pitx2*, and *Dleu7*) even in the absence of RARγ (19), and consequently for a subset of RA target genes, RARα (and/or RARβ) may be able to functionally substitute for RARγ. We have previously used microarray profiling to demonstrate that RA induces the same set of genes in RARα-null F9 cells as in WT cells (22), suggesting that in F9 cells RARγ/RARβ can fully substitute for RARα function. In RAR$\beta_2{}^{-/-}$ F9 cells *Hoxa1*, *Hoxb5*, *RARα*, and *Sfrp2* are induced, albeit to a lesser extent, whereas *Cdx1* and *Aurkc* are fully induced (23). Overall, induction of specific RA-responsive target genes is affected by genetic abrogation of individual RARs, whereas other aspects of RA signaling persist. To explain this, several models have been presented, but most commonly, residual target gene induction is attributed to functional and possibly context-dependent redundancy among the RARs (24–27).

RARα, β, and γ are all activated by the endogenous ligand RA, and the development of synthetic ligands with preferences for specific RARs provided valuable tools for probing target specificity of the RARs (28). However, as with the genomic targets, specific RARs have a preference for particular ligands rather than a stringent specificity.

The mechanism of RA induction is firmly established for direct (primary) targets of RARs (1, 29). In brief, RAR/RXR heterodimers associate with genomic retinoic acid response elements (RAREs), and the binding of the RA ligand induces a conformational shift which displaces corepressors and favors coactivator recruitment (19, 21, 30–32). In addition, primary targets of RA may induce transcription of secondary targets, *e.g.* RA-induced genes that lack RAREs. We have identified a group of genes (including *CoupTF1* and *CoupTF2*) that exhibits delayed RA-responsiveness. Characteristic of these genes, the RA-induced dissociation of polycomb-group proteins (PcG) from DNA was significantly delayed relative to that of direct genomic targets of RA, such as *Hoxa1* and *Hoxa5* (17).

Transcriptional activation by RA is well understood, but more elusive mechanisms of RA-mediated repression govern

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This article contains Figs. S1 and S2.

The genome-wide RNA-Seq files can be accessed can be accessed through the NCBI under Gene Expression Omnibus (GEO) accession number GSE112412.

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[2] The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; LIFR, leukemia inhibitory factor receptor; TKO, triple knockout; RXR, retinoid X receptor; DKO, double knockout; iPS, induced pluripotent stem; RPKM, reads per kilobase per million.

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the regulation of several pluripotency genes. We originally identified *Rex1* (*Zfp42*) as repressed in RA-treated cells (33), and determined that the RA-responsiveness is controlled by an Oct4 site in the *Rex1* promoter (34). Additional genes that are negatively regulated by RA include *Otx2, Bcl11a*, and pluripotency markers *Nanog, Oct4,* and *Klf4* (19, 22, 34–36). Notably, the RARs do not associate with the regulatory elements of these stem cell markers (35), pointing to an indirect regulation by RA. We found that RA disrupts the leukemia inhibitory factor receptor (LIFR) signaling, altering cellular phosphorylation (8), but the exact mechanisms of RA-dependent transcriptional gene repression have yet to be determined.

RA also induces protein phosphorylation through the activation of cellular kinases (37). Possibly functioning through cytoplasmic RARs (38), this activates a nongenomic signaling cascade independent of the direct transcriptional regulation by RA (39). In HepG2 cells, the translocation of Rbp4 (*Srbp*) bound retinol to Rbp1 (*Crbp1*) was demonstrated to activate the Jak/Stat phosphorylation cascade through the Stra6 membrane receptor (40). It remains to be determined whether this intersects with the LIFR pathway, but if so, this pathway could potentially account for the altered cellular phosphorylation in response to vitamin A. Finally, RA can regulate transcription through the orphan receptors CoupTF2, PPARβ/δ, and RORβ (41–43). In summary, RA may elicit transcriptional responses 1) through RARs α, β, and γ; 2) through nuclear receptors other than the RARs; and 3) through a nongenomic phosphorylation cascade, possibly involving RARs located in the cytoplasm (38). These three routes of RA signaling were identified in various biochemical assays and cell types, yet it remains to be determined how many of these routes are functional in ES cells.

We wanted to determine the potential of embryonic stem cells to respond to RA through routes not involving RARs. We therefore generated an RAR triple knockout (TKO) ES cell line that lacks RARs α, β, and γ. We then mapped RA-induced changes in the transcriptomes of WT and RAR TKO ES cells and found that upon biallelic abrogation of all three RAR genes, the ES cells fail to respond transcriptionally to RA, *i.e.* the transcriptome profile does not change in response to RA. We conclude that embryonic stem cells that lack functional RARs α, β, and γ fail to initiate the transcriptional changes commonly associated with RA-responsiveness in stem cells. Consequently, in murine ES cells the RARs provide the only route for RA to regulate transcript levels.

Results

Generation of an ES cell line that harbors biallelic abrogation of RARα, RARβ, and RARγ

As outlined above, RA may elicit transcriptional responses through one or several mechanisms. We wanted to determine the roles of RARα, β, and γ in cellular RA-responsiveness, and thus evaluate potential non–RAR-mediated signaling routes of RA. We therefore sequentially abrogated RARγ, RARβ, and RARα using CRISPR technology (Fig. 1*A*). Following each step of genome edits, single cells were expanded and genotyped by PCR, and allelic modifications were mapped to ascertain biallelic introduction of frameshift mutations (Fig. 1*B*). The six resulting alleles in the established RAR$\gamma^{-/-}$, RAR$\beta^{-/-}$, RAR$\alpha^{-/-}$ TKO ES cell line are depicted (Fig. 1*C*). Importantly, the genome edits impact all known isoforms of each of the three receptors, and the resulting cell line is thus null for all RAR isoforms and isotypes.

Direct RA targets are differentially affected by the loss of RARs

We wanted to evaluate the induction of direct targets of RA. We therefore determined the transcript levels of *Cdx1*, *Cyp26a1*, *RARβ2*, and *Hoxa1* in RAR$\gamma^{-/-}$ (KO; 33643); RAR$\beta^{-/-}$;RAR$\gamma^{-/-}$ (double knockout (DKO); 34523); and RAR$\alpha^{-/-}$;RAR$\beta^{-/-}$;RAR$\gamma^{-/-}$ (triple knockout (TKO); 40479) cell lines following 24 and 48 h of RA treatment (Fig. 1*D*). We found that *Cyp26a1* and *Hoxa1*, both RARγ targets (19, 21), were induced only in WT cells, whereas *RARβ2* and *Cdx1* were induced in both RAR$\gamma^{-/-}$ and DKO cells, albeit to a lesser extent.

Cellular proliferation of the RAR TKO cell line is not affected by RA

The ability to induce cellular growth arrest is one of the hallmark features of RA (44, 45). We evaluated the ability of the RAR TKO cell line for growth arrest, and found that whereas the WT cells (and RARγ KO cells, data not shown) growth arrested, the RAR TKO cells showed unimpaired proliferation in the presence of RA (Fig. 2*A*).

The genome-wide transcriptional profile of the RAR TKO cell line is not affected by RA

We next wanted to identify any transcriptional changes elicited by RA treatment of the TKO cells (*i.e.* RAR-independent signaling of RA). We therefore performed genome-wide transcriptional profiling of untreated and RA-treated TKO cells (in triplicate independent experimental setups). For reference, untreated and RA-treated WT ES cells were analyzed in parallel. We used unsupervised clustering analysis to evaluate the relatedness of the samples (Fig. 2*B*). The WT groups clustered together in a manner corresponding to the treatment. In contrast, the TKO groups clustered together pair-wise according to the biological replicates, indicating that the RA-treated TKO cells did not constitute a group different from the untreated TKO cells.

Inspection of the read-abundance at the CRISPR-targeted sites of RARα and RARγ confirmed the presence of frameshift mutations in RARα and RARγ (Fig. S2) corresponding to the deletions identified on a genomic template. The read-abundance also demonstrates biallelic expression of both RARα and RARγ in the ES cell line.

RA-responsive genes are not modulated by RA in the RAR TKO cell line

We next evaluated individual transcripts for differential expression between untreated and RA-treated cells. When plotting the -fold change (RA-responsiveness) against mean expression level it is evident that numerous genes in the WT cells are differentially expressed in response to RA (statistically significant changes are marked in *red* in Fig. 2*C*). In contrast,

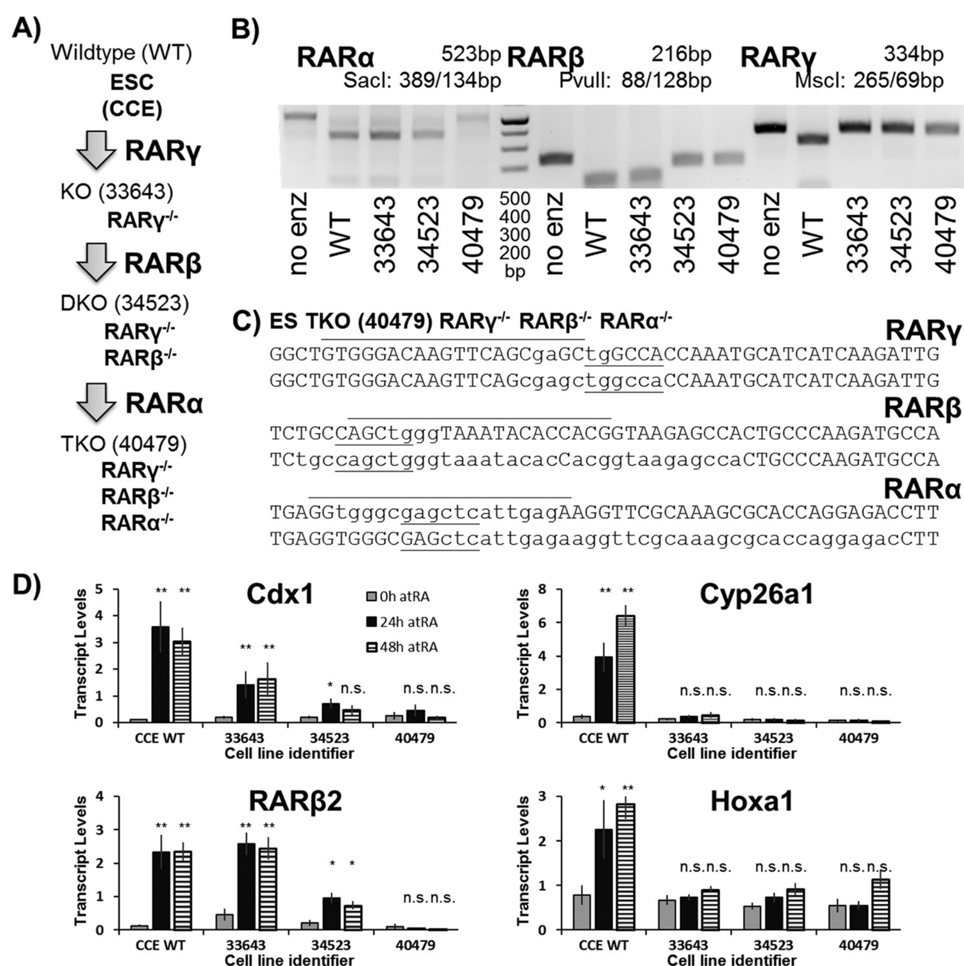


Figure 1. Generation of RAR single, double, and triple knockout cell lines. *A*, strategy and experimental outline. We generated an RAR TKO ES cell line by sequentially abrogating *RARγ* (33643), *RARβ* (34523), and *RARα* (40479) using transiently introduced CRISPR vectors. *B*, genotyping of RAR KO, DKO, and TKO. In brief, we used PCR to amplify the CRISPR target and assayed for the absence of specific restriction sites (MscI, PcuII, and SacI) present in the WT sequence. *C*, allelic mapping of introduced CRISPR edits. Two alleles for each gene are depicted and deleted bases are specified by lowercase letters. Note that each genome edit introduces a frameshift mutation in the targeted allele. The CRISPR guide-RNA target sites are indicated above (note that the RARβ guide-RNA targets the complementary strand). Restriction sites employed for genome-edit detections are *underlined*. Allelic mapping of the additional RAR TKO clone (40453) is described in Fig. S1. *D*, transcript levels of *Cdx1, RARβ2, Cyp26a1,* and *Hoxa1* RA-responsive genes in CCE WT cells, RARγ knockout (33643), RARγ;RARβ double knockout (34523), and RARγ;RARβ;RARα triple knockout (40479) cells treated with vehicle or RA for 24 or 48 h. *, $p \leq 0.05$; **, $p \leq 0.01$.

RA treatment of TKO cells induced no statistically significant changes in any of the gene transcripts (Fig. 2*C*). For further visualization we plotted the 20 most up- or down-regulated transcripts in RA-treated WT cells relative to these transcript levels in TKO cells. We note that the levels of these 40 transcripts change in response to RA only in the WT and not in the TKO cells (Fig. 2*D*).

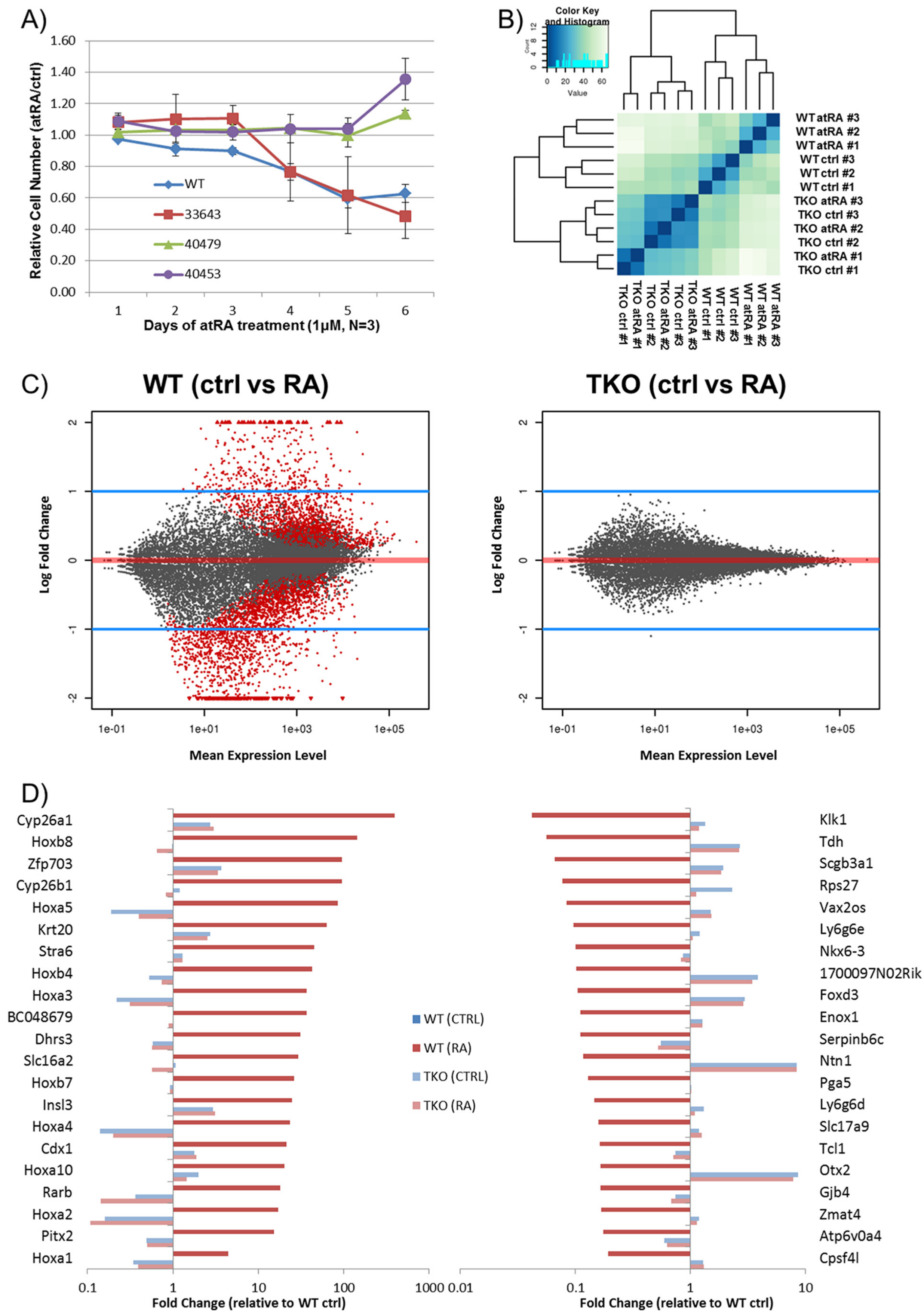
Induction of RA-inducible differentiation markers is abrogated in the RAR TKO cell line

RA induces expression of several differentiation markers. Specifically, RARγ is required for the induction of *Cyp26a1* and *Hoxa1*, but not for the induction of *Cdx1* and *Stra8* transcripts (19). Induction of *CoupTF1* and *Meis1* by RA is enhanced by polycomb depletion, which in contrast does not affect *Cyp26a1* and *Hoxa1* transcript levels (17). Consequently, *Cyp26a1/Hoxa1*, *Cdx1/Stra8*, and *CoupTF1/Meis1* represent three mechanistically different types of RA induction. We determined the levels of these transcripts in WT and TKO cells in response to RA. All six transcripts were increased in WT cells (6-fold or more) but exhibited no increase by RA in the TKO cells (Fig. 3*A*). Furthermore, these six transcript levels in TKO cells were comparable to those of untreated WT cells.

Repression of stem cell markers is perturbed in the RAR TKO cell line

In addition to inducing differentiation, RA decreases stem cell marker transcript levels. We therefore determined the transcript levels of key stem cell markers *Nanog, Oct4, Zfp42, Sox2, Klf4,* and *Sall4* (35, 46–48) in untreated and RA-treated WT and TKO cells. We found that all six transcripts were decreased in WT cells (57 ± 5%, 61 ± 3%, 28 ± 3%, 66 ± 3%, 57 ± 5%, and 73 ± 5%, respectively), but showed no RA-dependent decreases in the TKO cells (Fig. 3*B*). The transcript levels of *Nanog* and *Oct4* in TKO cells were decreased (86 ± 15% and 80 ± 8%, respectively) compared with those of untreated WT cells, whereas *Sall4* and *Klf4* transcript levels were increased in TKO relative to untreated WT cells (123 ± 2% and 125 ± 14%, respectively). The *Zfp42* and *Sox2* transcript levels in untreated TKO cells were comparable to those in WT cells (102 ± 11%

Transcriptional response to RA is abrogated in RAR TKO cells



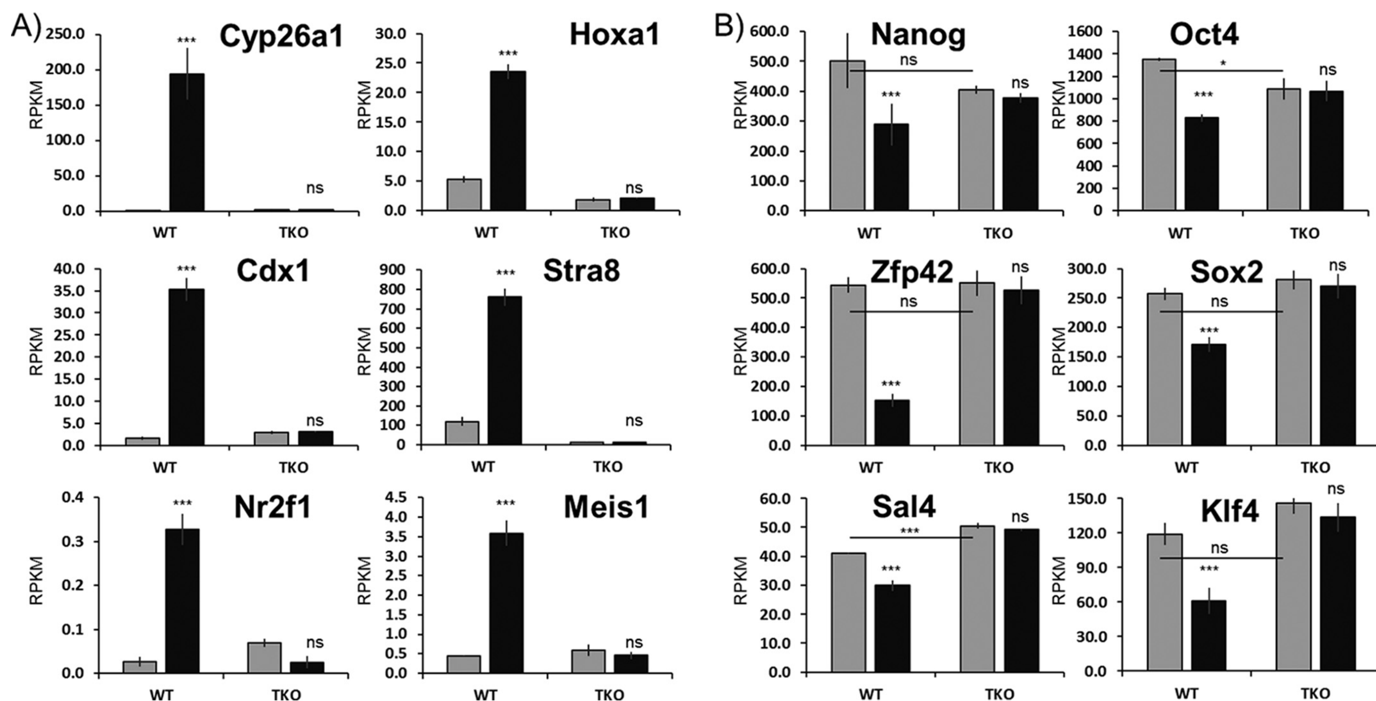


Figure 3. Transcript levels of RA-responsive genes in WT and RAR TKO ES cells. *A*, transcript levels of RA-responsive differentiation markers (*Cyp26a1, Hoxa1, Cdx1, Stra8, CoupTF1*, and *Meis1*). *B*, transcript levels of RA-responsive stem cell markers (*Nanog, Oct4, Zfp42, Sox2, Sall4*, and *Klf4*) in untreated and RA-treated RAR triple knockout cells. RA-treatment was with 1.0 μM RA for 24 h. The transcriptome data set is derived from triplicate independent experiments, each starting with freshly thawed cells (*, *p* value ≤ 0.05 relative to untreated cells, or as indicated; ***, *p* value ≤ 0.001).

and 110 ± 9%, respectively) (Fig. 3*B*). Consequently, the RARs are not required to maintain high expression levels of stem cell markers, but are essential for RA to negatively regulate transcript levels of these key pluripotency markers.

Expression of ligand-independent targets of RARs is deregulated in the RAR TKO cell line

F9 cell lines deficient for specific RARs exhibit impaired expression of certain RA-responsive genes, but they also display altered transcript levels of genes which do not respond to RA (22). Indeed, we have identified a number of imprinted genes, including *Mest, Stmn2, Tex13, Mobp, Gtl2,* and *H19*, which show deregulated expression in one or more RAR-deficient F9 cell lines (22, 49). Therefore we determined the transcript levels (and RA-responsiveness) of *Mest, Stmn2, Tex13, Mobp, Gtl2,* and *H19* in control and RA-treated WT and TKO ES cells and found that *Stmn2* and *Mest* transcripts were elevated in TKO cells (282 ± 50% and 290 ± 31%, respectively), whereas *Tex13* and *Mobp* transcripts were decreased (to 2 ± 1% and 14 ± 4%, respectively) in TKO cells (Fig. 4*A*). *Gtl2* and *H19*, which are markers for ES and induced pluripotent stem (iPS) cells, respectively (46), both exhibited elevated transcript levels in the TKO cells relative to WT cells (Fig. 4*A*). It is interesting to note that the same imprinted genes that were identified in RAR-deficient F9 are misregulated in the RAR TKO ES cells.

Expression of *RARβ* (and to a lesser extent *RARα* and *RARγ*) increases in response to RA (24, 25, 50). We evaluated transcript levels in WT and RAR TKO ES cells, and found that transcript levels of *RARα, RARβ*, and *RARγ* were increased in WT cells, but unchanged in the TKO cells in response to RA (Fig. 4*B*). In contrast, the transcript levels of *RXRα, RXRβ*, and *RXRγ* did not respond to RA in WT cells, and were not significantly changed in the RAR TKO ES cells. Orphan receptors CoupTF2, PPARβ/δ, and RORβ have been reported to bind RA (41–43). We therefore assessed the transcript levels of these receptors in WT ES cells. We found low levels of expression of CoupTF2 and RORβ (0.05 ± 0.02 and 0.57 ± 0.05 RPKM, respectively). In contrast, the levels of *PPARβ/δ* transcripts were comparable to those of *RARα* (13.5 ± 0.9 and 15.0 ± 1.9 RPKM, respectively), yet the absence of RA-induced transcription suggests that in ES cells the PPARβ/δ does not function as a receptor for RA.

Discussion

Our transcriptional profiling of RAR TKO ES cells revealed impaired responses to RA on two counts: 1) failure to induce

Figure 2. Growth and transcriptome analysis in WT and RAR TKO ES cells. *A*, cell numbers in RA-treated conditions relative to untreated conditions. Note that WT cells and RARγ knockout cells (33643) growth arrest in response to RA, whereas no growth arrest is observed for the RAR triple knockout cell lines (40479 and 40453). *B*, unsupervised clustering analysis groups WT cells into untreated and RA-treated populations, whereas the TKO cells group into clusters of the specific biological repeats. *C*, genome-wide plot of the effects of RA in WT (*left*) and RAR TKO (triple knockout ES cells, *right*). Note that the TKO untreated *versus* RA-treated cells depict a mirrored distribution around a log -fold change of 1 (10^0). Statistically significant changes are marked in *red* ($p < 0.01$). The few changes observed in transcript levels between vehicle and RA-treated TKO cells were not statistically significant, and were generally associated with low read coverage. Consequently, these nonsignificant differences most likely reflect variations in sequencing coverage. *D*, transcriptome reads for the top 20 most RA-responsive genes in WT ES cells (up- or down-regulated, *left* and *right*, respectively) plotted relative to untreated cells (set as 1.0). Note the absence of RA-responsiveness in the RAR triple knockout cells.

Transcriptional response to RA is abrogated in RAR TKO cells

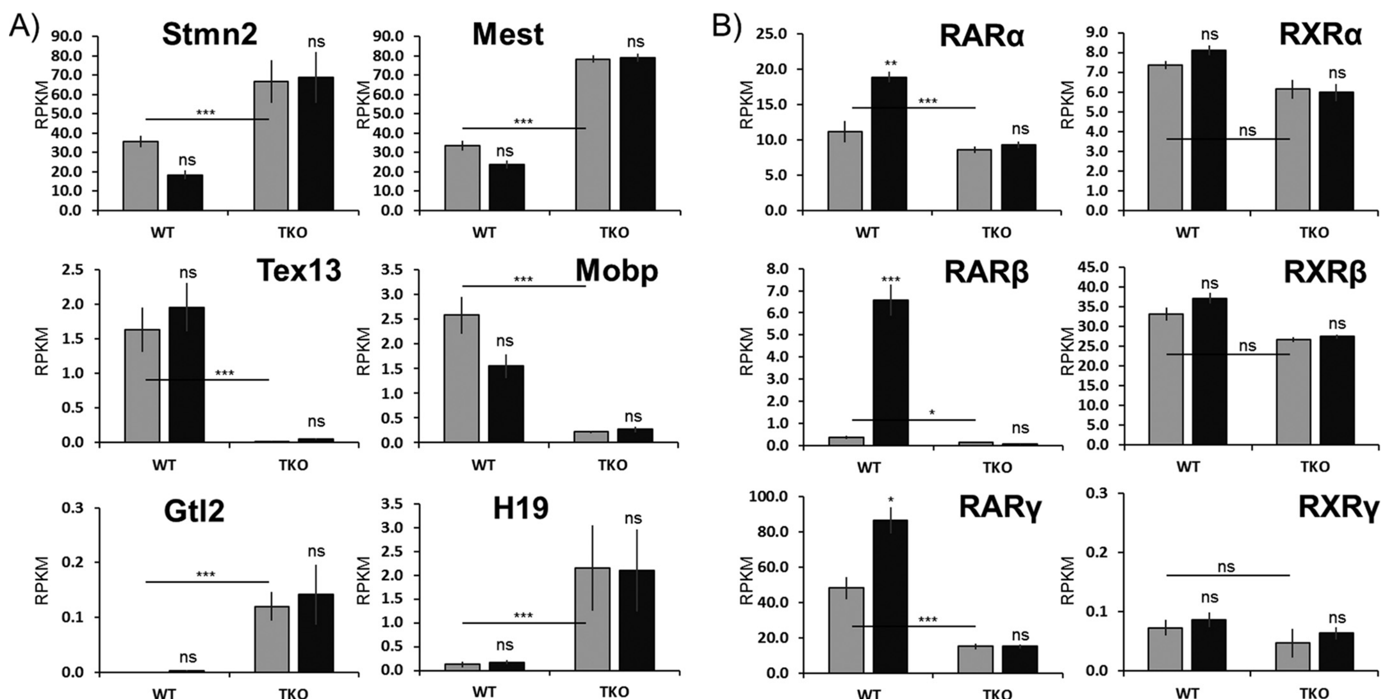


Figure 4. Transcript levels of RARs, RXRs, and imprinted genes in WT and RAR TKO ES cells. *A*, transcript levels of RAR-dependent imprinted genes (*Stmn2, Mest, Tex13, Mobp, Gtl2,* and *H19*). *B*, transcript levels of RAR and RXR nuclear receptors (*RARα, RARβ, RARγ, RXRα, RXRβ,* and *RXRγ*) in untreated and RA-treated RAR triple knockout cells. RA treatment was with 1.0 μM RA for 24 h. The transcriptome data set is derived from triplicate independent experiments, each starting with freshly thawed cells (*, p value $\leq$ 0.05 relative to untreated cells, or as indicated; **, p value $\leq$ 0.01; ***, p value $\leq$ 0.001).

differentiation markers and 2) persistent expression of pluripotency markers. This was physiologically evident by the ability of the TKO cells to bypass RA-induced growth arrest.

Unlike the WT ES cells, which growth arrest in the presence of RA, the TKO ES cells continue to proliferate. The inability of F9 cells to growth arrest was found to be dependent on RARβ_2, whereas no deficiencies were detected in RARα or RARγ knockout cell lines (24, 51). The induction of *p27* (*Cdkn1b*) is a key feature in the RA-mediated growth arrest (52), and consistently, *p27* induction was severely impaired in RARβ_2 knockout F9 cells (51). In WT ES cells we detected no induction of *p27* upon 24 h of RA treatment (data not shown), suggesting that *p27* may be a late or a secondary target of RA. RARβ_2 is itself induced by RA, and consequently, RARα/RARγ–specific targets may be induced more rapidly than RARβ-specific targets. The inability of the TKO cells to growth arrest is consistent with a dependence on RARβ (51).

In cells deficient for one receptor (RARα, β, or γ), the mRNA levels of specific targets, including *Cdx1, Stra8,* and *Aurkc* are increased by RA (19, 23). Even in ES cells deficient for both RARγ and RARβ we observed residual induction of Cdx1 and RARβ (Fig. 1*D*), suggesting that in the absence of functional RARγ and RARβ protein, the RAREs of Cdx1 and RARβ2 are targeted by RARα. In the RAR TKO ES cells all three RARs are absent, and RA treatment for 24 h failed to induce statistically any significant increases in the levels of these (Fig. 1*D*) or any other transcripts (Fig. 2*C*). Consequently, in murine ES cells RAR function is required for the induction of all RA-responsive target genes, *i.e.* genes that are differentially regulated upon 24 h of RA treatment.

In contrast to the RA-induced increases in transcript levels, the RA-induced decreases in stem cell markers *Nanog, Oct4, Zfp42, Sox2,* and *Sall4* persist even in the absence of any one receptor (RAR α, β, or γ) (17, 19, 23, 35). However, RA treatment failed to induce any significant decreases in these transcript levels in the RAR TKO ES cells. Consequently, the decreases in transcript levels of stem cell markers are dependent on the presence of functional RARs, even if no RAR-binding sites are present in the promoter proximal regions of these stem cell markers (35). These results point to the RARs mediating a ligand-dependent function unrelated to the canonical genomic function (*i.e.* transcriptional activation through an RARE). In this context, the binding of RA may enable RARs to block transcriptional activators such as Oct4 and AP-1 (34, 53, 54). Alternatively, direct targets of RAR could function as repressors that decrease the levels of stem cell markers.

It is interesting to note that the estrogen nuclear receptors modulate the activities of several key transcription factors (*i.e.* AP-1, NF-κB, and SP1) and can regulate cellular phosphorylation in a ligand-dependent manner (55). The modulation of kinase activities parallels the regulation of PI3K, ERK1/2, and AKT1 kinases by RARs (37, 56). Consequently, RA could potentially induce phosphorylation in the absence of RARs, but the lack of a transcriptional response suggests that in the TKO ES cell the cellular phosphorylation is not responsive to RA and/or does not result in any transcript changes. This is indeed consistent with a dependence of RA-induced phosphorylation on RARγ (37).

The process of somatic reprogramming has facilitated a comparison of pluripotency markers in cells from different origins. Specifically, when somatic cells are induced to pluripotency, the resulting iPS cells are highly similar to ES cells, and key pluripotency markers (*e.g. Nanog, Sox2,* and *Oct4*) are

Table 1
Gene-specific primers for cDNA quantification (qRT-PCR)
All primer pairs were designed to span intronic elements.

RT-qPCR	Sense and antisense primers (5′–3′)	Product (bp)	
		cDNA	gDNA
mCdx1(+)E mCdx1(−)F	GACGCCCTACGAATGGATG CTCTGTGAGCCCCAGGTTAG	196	15,352
mHoxa1(+)E mHoxa1(−)D	TAACTCCTTATCCCCTCTCCAC ACCCACGTAGCCGTACTCTCCA	151	628
mCyp26a1(+)A mCyp26a1(−)B	GAAACATTGCAGATGGTGCTTCAG CGGCTGAAGGCCTGCATAATCAC	272	728
mHprt(+)C mHprt(−)D	GTTGAAGATATAATTGACACTGG CAAGGGCATATCCAACAAC	184	4021

expressed at similar levels. However, a few imprinted genes, including *Glt2* and *H19*, show differential expression (46). Although unable to respond to RA, the TKO ES cells maintain several stem cell characteristics: 1) rapid proliferation; 2) high expression of key stem cell markers, including *Nanog, Oct4, Zfp42, Sox2*, and *Sall4*; and 3) expression of imprinted genes commonly expressed in ES (*Gtl2*) and iPS (*H19*) cells. RA antagonizes several aspects of stem cell signaling, thereby decreasing expression of stem cell markers (8, 53, 54), but it is currently not clear whether this occurs through RAR-dependent or -independent pathways. We show here that RARs are required for RA to decrease the mRNA levels of pluripotency markers. We demonstrate that in the absence of functional RARs the ES cells display no changes in transcript levels in response to RA. We therefore conclude that the RARs provide the only route for RA to regulate transcript levels in murine ES cells.

Experimental procedures

Cell culture and retinoic acid treatment of ES cells

The ES WT cell lines were described previously (21). All-*trans* retinoic acid (Sigma-Aldrich) was added to the cells 24 h after plating (1 μM final concentration) and ethanol (EtOH, 0.1%) served as a vehicle control. For time course evaluations ES cells were plated on gelatin-coated tissue culture dishes and treated with RA (1.0 μM) for various times up to 72 h prior to harvesting.

RNA isolation and reverse transcription

Total RNA was isolated from ES cells using TRIzol reagent (Invitrogen) and RNA was quantitated by optical density at 260 nm. The RNA (1 μg) was reverse transcribed (Quanta Biosciences, Beverly, MD), and then diluted 1:10 with H_2O. The cDNA obtained was diluted 10-fold and 3 μl of this cDNA was utilized for PCR reactions.

Generation of cDNA, semi-quantitative and real-time PCR

Real-time PCR was performed using SYBR Green Supermix (Quanta Biosciences) in a 15 μl reaction containing reaction mix (1×), 0.2 μM of each primer, and 3 μl of cDNA template. The reactions were run on a MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad). Amplification in the linear range was demonstrated by a serial dilution of cDNA from RA-treated WT cells included in each reaction (1:1, 1:5, 1:10, 1:50, 1:100, 1:500). Reactions with H_2O and template without reverse transcriptase, respectively, served as negative controls for primer-dimer and for amplification of residual genomic DNA (gDNA). All real-time PCR primers were designed to span intronic regions. Primer sequences are listed in Table 1. Each expression analysis was performed at least three times (*e.g.* $n \geq 3$ in independently propagated cells, experiment repeated three times). Within each PCR analysis, samples were run in triplicate. PCR products were verified by DNA sequencing.

Construction of plasmids for genome editing (pX330)

Double-stranded oligomers with the appropriate overhang were ordered and cloned into BbsI opened pX330 vector (Addgene, catalog no. 42230) as previously described (57, 58). We identified positive clones using standard bacterial screening and confirmed construct integrity by DNA sequencing. The pX330 constructs contain the following gRNA sequences: GTGGGACAAGTTCAGCGAGC (RARγ) CGTGGTGTATTTACCCAGC (RARβ), and GGTGGGCGAGCTCATTGAGA (RARα), each of which targets the genomic DNA proximal to endogenous MscI, PvuII, and SacI sites, respectively.

Generation of single, double, and triple RAR knockout cell lines

We transiently introduced the constructs into ES cells using Lipofectamine 2000 (Invitrogen), after which we trypsinized and seeded the transfected cells at low density (2×10^3 cells per 150-mm dish) to obtain clonal lines. The resulting colonies were transferred to 24-well plates and upon further clonal expansion, genomic DNA was harvested for genotyping.

Genome editing and screening

We screened the colonies by PCR followed by endonuclease restriction digest. The PCR bands with modified restriction sites were sequenced to map the exact mutations introduced. We subjected cell lines with mutations in both alleles to further analysis and/or genome edits. In brief, sequential inactivation of RARγ, RARβ, and RARα was performed in CCE WT ES cells, thus generating RAR$\gamma^{-/-}$ (KO); RAR$\gamma^{-/-}$;RAR$\beta^{-/-}$ (DKO); and RAR$\gamma^{-/-}$;RAR$\beta^{-/-}$; RAR$\alpha^{-/-}$ (TKO). Details are in Table 2.

Mapping of allelic edits

We purified the PCR products resistant to restriction digest using a PCR purification kit (Qiagen, Germantown, MD), and

Table 2
Primer sequences and targeted regions
Genotyping primers for detection of CRISPR edit of RARs α, β, and γ. The sequences of the genotyping primers are specified together with the CRISPR-targeted region. The restriction endonuclease used to detect genome edit and the amplicon sizes pre- and post-restriction digest are indicated in the last two columns. Note that the exact sizes of the resistant amplicons vary depending on the specific genomic edit.

Genotyping	Sense and antisense primers (5′–3′)	Restriction site	Targeted region (gRNA, restriction site)	Product (bp)	
				Resistant	Digested
mRARγE7(+) mRARγE8(−)	CCGACAGCTATGAACTGAGT GCAATGCTGAGCCCTGTAAAACCA	MscI	CTGTGGGACAAGTTCAGCGAGC**TGGCCA**CC	334	265 70
mRARβE7(+) mRARβI7(−)	AGAGAGCTATGAGATGACAGCG GCTGACAGGCCACTGAGCATA	PvuI	CTGC**CAGCTG**GGTAAATACACCACGGTAAG	216	128 88
mRARαI5(+) mRARαE7(−)	AAGCAGTCACTCTCTAACCAACCCA ATGGTGAGGGTGGTGAAGCCG	SacI	CCTGAGGTGGGC**GAGCTC**ATTGAGAAGGTT	523	378 145
SP6(+) T7(+)	ATTTAGGTGACACTATAGAATAC TAATACGACTCACTATAGGGCGA				

the eluted DNA was ligated into the pGEM-T Easy Vector (Promega, Madison, WI). The ligated products were recovered by bacterial transformation. We picked single colonies and isolated plasmid DNAs. Insert-containing plasmids were identified by restriction digestion and sequenced using T7(+) and SP6(+) primers.

Genome-wide transcriptome sequencing and analysis

CCE WT and RAR triple knockout ES cells (clone 40479) were treated with vehicle (EtOH) or RA (1.0 μM, 24 h). We performed the treatment three times, and RNA from a total of 12 samples (triplicates of four conditions) was prepared using RNeasy kits (Qiagen). The RNA-Seq was performed by the Weill Cornell Medical College Genomics Core (50-bp single-end reads) on an Illumina HiSeq 2000. In brief, the reads ($>2.5 \times 10^7$ per sample) were aligned to the mouse genome (mm10 with a unique mapping of >90% of the reads), and data analysis was performed with the Tuxedo tools software (59).

Data analysis and statistics

Data from at least three independent experiments were analyzed using one-way analysis of variance (ANOVA) in the expression analyses. The mean ± S.E. was determined for each of the data sets (at least three biological, independent repeats, each in triplicate, plotted as error bars in the graphs), and ANOVA values of $p < 0.05$ among compared samples were assigned statistical significance.

The cDNA libraries were generated using the Illumina TruSeq RNA Sample Preparation kit and sequenced with single-end 51 bp on HiSeq 4000 sequencer. The sequencing reads were cleaned by trimming adapter sequences and low-quality bases, and were aligned to the mouse reference genome (GRCm38) using STAR (60), and Cufflinks (61, 62) was used to measure transcript abundances in fragments per kilobase of exon model per million mapped reads (FPKM). The aligned RNA-Seq data were visualized using the Integrative Genomics Viewer from the Broad Institute (63, 64).

Accession numbers

NCBI Gene identifiers for the murine retinoic acid receptors are *RARα* (ID: 19401), *RARβ* (ID: 218772), and *RARγ* (ID: 5916).

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References

1. Gudas, L. J., and Wagner, J. A. (2011) Retinoids regulate stem cell differentiation. *J. Cell. Physiol.* **226,** 322–330 CrossRef Medline
2. Soprano, D. R., Teets, B. W., and Soprano, K. J. (2007) Role of retinoic acid in the differentiation of embryonal carcinoma and embryonic stem cells. *Vitam. Horm.* **75,** 69–95 CrossRef Medline
3. Silva, C., Wood, J. R., Salvador, L., Zhang, Z., Kostetskii, I., Williams, C. J., and Strauss, J. F., 3rd (2009) Expression profile of male germ cell-associated genes in mouse embryonic stem cell cultures treated with all-trans retinoic acid and testosterone. *Mol. Reprod. Dev.* **76,** 11–21 CrossRef Medline
4. Martinez-Ceballos, E., and Gudas, L. J. (2008) Hoxa1 is required for the retinoic acid-induced differentiation of embryonic stem cells into neurons. *J. Neurosci. Res.* **86,** 2809–2819 CrossRef Medline
5. Leung, A. Y., and Verfaillie, C. M. (2005) All-*trans* retinoic acid (ATRA) enhances maintenance of primitive human hematopoietic progenitors and skews them towards myeloid differentiation in a stroma-noncontact culture system. *Exp. Hematol.* **33,** 422–427 CrossRef Medline
6. Phillips, B. W., Vernochet, C., and Dani, C. (2003) Differentiation of embryonic stem cells for pharmacological studies on adipose cells. *Pharmacol. Res.* **47,** 263–268 CrossRef Medline
7. Wan, D. C., Shi, Y. Y., Nacamuli, R. P., Quarto, N., Lyons, K. M., and Longaker, M. T. (2006) Osteogenic differentiation of mouse adipose-derived adult stromal cells requires retinoic acid and bone morphogenetic protein receptor type IB signaling. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 12335–12340 CrossRef Medline
8. Tighe, A. P., and Gudas, L. J. (2004) Retinoic acid inhibits leukemia inhibitory factor signaling pathways in mouse embryonic stem cells. *J. Cell. Physiol.* **198,** 223–229 CrossRef Medline
9. Ricard, M. J., and Gudas, L. J. (2013) Cytochrome p450 cyp26a1 alters spinal motor neuron subtype identity in differentiating embryonic stem cells. *J. Biol. Chem.* **288,** 28801–28813 CrossRef Medline
10. Pérez, R. J., Benoit, Y. D., and Gudas, L. J. (2013) Deletion of retinoic acid receptor β (RARβ) impairs pancreatic endocrine differentiation. *Exp. Cell Res.* **319,** 2196–2204 CrossRef Medline

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11. Matt, N., Dupé, V., Garnier, J. M., Dennefeld, C., Chambon, P., Mark, M., and Ghyselinck, N. B. (2005) Retinoic acid-dependent eye morphogenesis is orchestrated by neural crest cells. *Development* **132,** 4789–4800 CrossRef Medline
12. Lee, G. S., Kochhar, D. M., and Collins, M. D. (2004) Retinoid-induced limb malformations. *Curr. Pharm. Des.* **10,** 2657–2699 CrossRef Medline
13. Mark, M., Ghyselinck, N. B., and Chambon, P. (2004) Retinoic acid signalling in the development of branchial arches. *Curr. Opin. Genet. Dev.* **14,** 591–598 CrossRef Medline
14. Drumea, K., Yang, Z. F., and Rosmarin, A. (2008) Retinoic acid signaling in myelopoiesis. *Curr. Opin. Hematol.* **15,** 37–41 CrossRef Medline
15. Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsaksophak, K., Wilson, M. J., Rossant, J., Hamada, H., and Koopman, P. (2006) Retinoid signaling determines germ cell fate in mice. *Science* **312,** 596–600 CrossRef Medline
16. Snyder, J. M., Jenkins-Moore, M., Jackson, S. K., Goss, K. L., Dai, H. H., Bangsund, P. J., Giguere, V., and McGowan, S. E. (2005) Alveolarization in retinoic acid receptor-β-deficient mice. *Pediatr. Res.* **57,** 384–391 CrossRef Medline
17. Laursen, K. B., Mongan, N. P., Zhuang, Y., Ng, M. M., Benoit, Y. D., and Gudas, L. J. (2013) Polycomb recruitment attenuates retinoic acid–induced transcription of the bivalent *NR2F1* gene. *Nucleic Acids Res.* **41,** 6430–6443 CrossRef Medline
18. Gillespie, R. F., and Gudas, L. J. (2007) Retinoid regulated association of transcriptional co-regulators and the polycomb group protein SUZ12 with the retinoic acid response elements of *Hoxa1*, *RAR*β_2, and *Cyp26A1* in F9 embryonal carcinoma cells. *J. Mol. Biol.* **372,** 298–316 CrossRef Medline
19. Kashyap, V., Laursen, K. B., Brenet, F., Viale, A. J., Scandura, J. M., and Gudas, L. J. (2013) RARγ is essential for retinoic acid induced chromatin remodeling and transcriptional activation in embryonic stem cells. *J. Cell Sci.* **126,** 999–1008 CrossRef Medline
20. Laursen, K. B., Kashyap, V., Scandura, J., and Gudas, L. J. (2015) An alternative retinoic acid-responsive *Stra6* promoter regulated in response to retinol deficiency. *J. Biol. Chem.* **290,** 4356–4366 CrossRef Medline
21. Kashyap, V., Gudas, L. J., Brenet, F., Funk, P., Viale, A., and Scandura, J. M. (2011) Epigenomic reorganization of the clustered Hox genes in embryonic stem cells induced by retinoic acid. *J. Biol. Chem.* **286,** 3250–3260 CrossRef Medline
22. Laursen, K. B., Wong, P. M., and Gudas, L. J. (2012) Epigenetic regulation by RARα maintains ligand-independent transcriptional activity. *Nucleic Acids Res.* **40,** 102–115 CrossRef Medline
23. Faria, T. N., Mendelsohn, C., Chambon, P., and Gudas, L. J. (1999) The targeted disruption of both alleles of RARβ_2 in F9 cells results in the loss of retinoic acid-associated growth arrest. *J. Biol. Chem.* **274,** 26783–26788 CrossRef Medline
24. Boylan, J. F., Lufkin, T., Achkar, C. C., Taneja, R., Chambon, P., and Gudas, L. J. (1995) Targeted disruption of retinoic acid receptor α (RAR α) and RAR γ results in receptor-specific alterations in retinoic acid-mediated differentiation and retinoic acid metabolism. *Mol. Cell. Biol.* **15,** 843–851 CrossRef Medline
25. Taneja, R., Bouillet, P., Boylan, J. F., Gaub, M. P., Roy, B., Gudas, L. J., and Chambon, P. (1995) Reexpression of retinoic acid receptor (RAR) gamma or overexpression of RAR alpha or RAR beta in RAR gamma-null F9 cells reveals a partial functional redundancy between the three RAR types. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7854–7858 CrossRef Medline
26. Marinelli, A., Bossi, D., Pelicci, P. G., and Minucci, S. (2009) Redundant function of retinoic acid receptor isoforms in leukemogenesis unravels a prominent function of genome topology and architecture in the selection of mutagenic events in cancer. *Leukemia* **23,** 417–419 CrossRef Medline
27. Chiba, H., Clifford, J., Metzger, D., and Chambon, P. (1997) Specific and redundant functions of retinoid X receptor/retinoic acid receptor heterodimers in differentiation, proliferation, and apoptosis of F9 embryonal carcinoma cells. *J. Cell Biol.* **139,** 735–747 CrossRef Medline
28. Delescluse, C., Cavey, M. T., Martin, B., Bernard, B. A., Reichert, U., Maignan, J., Darmon, M., and Shroot, B. (1991) Selective high affinity retinoic acid receptor alpha or beta-gamma ligands. *Mol. Pharmacol.* **40,** 556–562 Medline
29. Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10,** 940–954 CrossRef Medline
30. Urvalek, A. M., and Gudas, L. J. (2014) Retinoic acid and histone deacetylases regulate epigenetic changes in embryonic stem cells. *J. Biol. Chem.* **289,** 19519–19530 CrossRef Medline
31. Dilworth, F. J., and Chambon, P. (2001) Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. *Oncogene* **20,** 3047–3054 CrossRef Medline
32. Urvalek, A., Laursen, K. B., and Gudas, L. J. (2014) The roles of retinoic acid and retinoic acid receptors in inducing epigenetic changes. *Subcell. Biochem.* **70,** 129–149 CrossRef Medline
33. Hosler, B. A., LaRosa, G. J., Grippo, J. F., and Gudas, L. J. (1989) Expression of *REX-1*, a gene containing zinc finger motifs, is rapidly reduced by retinoic acid in F9 teratocarcinoma cells. *Mol. Cell. Biol.* **9,** 5623–5629 CrossRef Medline
34. Ben-Shushan, E., Thompson, J. R., Gudas, L. J., and Bergman, Y. (1998) *Rex-1*, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. *Mol. Cell. Biol.* **18,** 1866–1878 CrossRef Medline
35. Mendoza-Parra, M. A., Walia, M., Sankar, M., and Gronemeyer, H. (2011) Dissecting the retinoid-induced differentiation of F9 embryonal stem cells by integrative genomics. *Mol. Syst. Biol.* **7,** 538 CrossRef Medline
36. Ben-Shushan, E., Sharir, H., Pikarsky, E., and Bergman, Y. (1995) A dynamic balance between ARP-1/COUP-TFII, EAR-3/COUP-TFI, and retinoic acid receptor:retinoid X receptor heterodimers regulates Oct-3/4 expression in embryonal carcinoma cells. *Mol. Cell. Biol.* **15,** 1034–1048 CrossRef Medline
37. Bastien, J., Plassat, J. L., Payrastre, B., and Rochette-Egly, C. (2006) The phosphoinositide 3-kinase/Akt pathway is essential for the retinoic acid-induced differentiation of F9 cells. *Oncogene* **25,** 2040–2047 CrossRef Medline
38. Han, Y. H., Zhou, H., Kim, J. H., Yan, T. D., Lee, K. H., Wu, H., Lin, F., Lu, N., Liu, J., Zeng, J. Z., and Zhang, X. K. (2009) A unique cytoplasmic localization of retinoic acid receptor-γ and its regulations. *J. Biol. Chem.* **284,** 18503–18514 CrossRef Medline
39. Rochette-Egly, C., and Germain, P. (2009) Dynamic and combinatorial control of gene expression by nuclear retinoic acid receptors (RARs). *Nucl. Recept. Signal* **7,** e005 CrossRef Medline
40. Berry, D. C., O'Byrne, S. M., Vreeland, A. C., Blaner, W. S., and Noy, N. (2012) Cross-talk between signalling and vitamin A transport by the retinol-binding protein receptor STRA6. *Mol. Cell. Biol.* **32,** 3164–3175 CrossRef Medline
41. Kruse, S. W., Suino-Powell, K., Zhou, X. E., Kretschman, J. E., Reynolds, R., Vonrhein, C., Xu, Y., Wang, L., Tsai, S. Y., Tsai, M. J., and Xu, H. E. (2008) Identification of COUP-TFII orphan nuclear receptor as a retinoic acid-activated receptor. *PLoS Biol.* **6,** e227 CrossRef Medline
42. Stehlin-Gaon, C., Willmann, D., Zeyer, D., Sanglier, S., Van Dorsselaer, A., Renaud, J. P., Moras, D., and Schüle, R. (2003) All-*trans* retinoic acid is a ligand for the orphan nuclear receptor RORβ. *Nat. Struct. Biol.* **10,** 820–825 CrossRef Medline
43. Schug, T. T., Berry, D. C., Shaw, N. S., Travis, S. N., and Noy, N. (2007) Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. *Cell* **129,** 723–733 CrossRef Medline
44. Wightman, J., Roberson, M. S., Lamkin, T. J., Varvayanis, S., and Yen, A. (2002) Retinoic acid-induced growth arrest and differentiation: Retinoic acid up-regulates CD32 (FcγRII) expression, the ectopic expression of which retards the cell cycle. *Mol. Cancer Ther.* **1,** 493–506 Medline
45. Campochiaro, P. A., Hackett, S. F., and Conway, B. P. (1991) Retinoic acid promotes density-dependent growth arrest in human retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **32,** 65–72 Medline
46. Stadtfeld, M., Apostolou, E., Akutsu, H., Fukuda, A., Follett, P., Natesan, S., Kono, T., Shioda, T., and Hochedlinger, K. (2010) Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. *Nature* **465,** 175–181 CrossRef Medline
47. Sharov, A. A., Masui, S., Sharova, L. V., Piao, Y., Aiba, K., Matoba, R., Xin, L., Niwa, H., and Ko, M. S. (2008) Identification of *Pou5f1*, *Sox2*, and *Nanog* downstream target genes with statistical confidence by applying a

novel algorithm to time course microarray and genome-wide chromatin immunoprecipitation data. *BMC Genomics* **9,** 269 CrossRef Medline
48. Shi, W., Wang, H., Pan, G., Geng, Y., Guo, Y., and Pei, D. (2006) Regulation of the pluripotency marker *Rex-1* by Nanog and Sox2. *J. Biol. Chem.* **281,** 23319–23325 CrossRef Medline
49. Su, D., and Gudas, L. J. (2008) Gene expression profiling elucidates a specific role for RARγ in the retinoic acid-induced differentiation of F9 teratocarcinoma stem cells. *Biochem. Pharmacol.* **75,** 1129–1160 CrossRef Medline
50. Boylan, J. F., Lohnes, D., Taneja, R., Chambon, P., and Gudas, L. J. (1993) Loss of retinoic acid receptor gamma function in F9 cells by gene disruption results in aberrant Hoxa-1 expression and differentiation upon retinoic acid treatment. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 9601–9605 CrossRef Medline
51. Li, R., Faria, T. N., Boehm, M., Nabel, E. G., and Gudas, L. J. (2004) Retinoic acid causes cell growth arrest and an increase in p27 in F9 wild type but not in F9 retinoic acid receptor β_2 knockout cells. *Exp. Cell Res.* **294,** 290–300 CrossRef Medline
52. Matsuo, T., and Thiele, C. J. (1998) p27Kip1: A key mediator of retinoic acid induced growth arrest in the SMS-KCNR human neuroblastoma cell line. *Oncogene* **16,** 3337–3343 CrossRef Medline
53. Nicholson, R. C., Mader, S., Nagpal, S., Leid, M., Rochette-Egly, C., and Chambon, P. (1990) Negative regulation of the rat stromelysin gene promoter by retinoic acid is mediated by an AP1 binding site. *EMBO J.* **9,** 4443–4454 Medline
54. Yang-Yen, H. F., Zhang, X. K., Graupner, G., Tzukerman, M., Sakamoto, B., Karin, M., and Pfahl, M. (1991) Antagonism between retinoic acid receptors and AP-1: Implications for tumor promotion and inflammation. *New Biol.* **3,** 1206–1219 Medline
55. Marino, M., Galluzzo, P., and Ascenzi, P. (2006) Estrogen signaling multiple pathways to impact gene transcription. *Curr. Genomics* **7,** 497–508 CrossRef Medline
56. Masiá, S., Alvarez, S., de Lera, A. R., and Barettino, D. (2007) Rapid, nongenomic actions of retinoic acid on phosphatidylinositol-3-kinase signaling pathway mediated by the retinoic acid receptor. *Mol. Endocrinol.* **21,** 2391–2402 CrossRef Medline
57. Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., and Zhang, F. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* **339,** 819–823 CrossRef Medline
58. Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8,** 2281–2308 CrossRef Medline
59. Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., Pimentel, H., Salzberg, S. L., Rinn, J. L., and Pachter, L. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7,** 562–578 CrossRef Medline
60. Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T. R. (2013) STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29,** 15–21 CrossRef Medline
61. Roberts, A., Trapnell, C., Donaghey, J., Rinn, J. L., and Pachter, L. (2011) Improving RNA-Seq expression estimates by correcting for fragment bias. *Genome Biol.* **12,** R22 CrossRef Medline
62. Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M. J., Salzberg, S. L., Wold, B. J., and Pachter, L. (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **28,** 511–515 CrossRef Medline
63. Thorvaldsdóttir, H., Robinson, J. T., and Mesirov, J. P. (2013) Integrative Genomics Viewer (IGV): High-performance genomics data visualization and exploration. *Brief. Bioinform.* **14,** 178–192 CrossRef Medline
64. Robinson, J. T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G., and Mesirov, J. P. (2011) Integrative genomics viewer. *Nat. Biotechnol.* **29,** 24–26 CrossRef Medline