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) 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 RARβ2 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 Dieu7) 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 RARγ function. In RARβ2−/− F9 cells Hoxa1, Hoxb5, RARα, and Sfrp2 are induced, albeit to a lesser extent, whereas Cdx1 and Aurrk 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 polycyto-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...
the regulation of several pluripotency genes. We originally identified \textit{Rex1} (Zfp42) as repressed in RA-treated cells (33), and determined that the RA-responsiveness is controlled by an Oct4 site in the \textit{Rex1} promoter (34). Additional genes that are negatively regulated by RA include \textit{Otx2}, \textit{Bcl11a}, and pluripotency markers \textit{Nanog}, \textit{Oct4}, and \textit{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.

RAR 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 Sra5 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, PPAR, and ROR (41–43). In summary, RA may elicit transcriptional responses 1) through RAR\(\alpha\), \(\beta\), and \(\gamma\); 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 RAR\(\alpha\), \(\beta\), and \(\gamma\). 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 RAR\(\alpha\), \(\beta\), and \(\gamma\) 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 RAR\(\alpha\), RAR\(\beta\), and RAR\(\gamma\)**

As outlined above, RA may elicit transcriptional responses through one or several mechanisms. We wanted to determine the roles of RAR\(\alpha\), \(\beta\), and \(\gamma\) in cellular RA-responsiveness, and thus evaluate potential non--RAR-mediated signaling routes of RA. We therefore sequentially abrogated RAR\(\gamma\), RAR\(\beta\), and RAR\(\alpha\) using CRISPR technology (Fig. 1A). 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. 1B). The six resulting alleles in the established RAR\(\gamma^{−/−}\), RAR\(\beta^{−/−}\), RAR\(\alpha^{−/−}\) TKO ES cell line are depicted (Fig. 1C). 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, RAR\(\beta\), 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. 1D). We found that Cyp26a1 and Hoxa1, both RAR\(\gamma\) targets (19, 21), were induced only in WT cells, whereas RAR\(\beta\) 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 RAR\(\gamma\) KO cells, data not shown) growth arrested, the RAR TKO cells showed unimpaired proliferation in the presence of RA (Fig. 2A).

**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. 2B). The WT groups clustered together in a manner corresponding to the treatment. In contrast, the TKO groups clustered together pairwise 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 RAR\(\alpha\) and RAR\(\gamma\) confirmed the presence of frameshift mutations in RAR\(\alpha\) and RAR\(\gamma\) (Fig. S2) corresponding to the deletions identified on a genomic template. The read-abundance also demonstrates biallelic expression of both RAR\(\alpha\) and RAR\(\gamma\) 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. 2C). In contrast,
RA treatment of TKO cells induced no statistically significant changes in any of the gene transcripts (Fig. 2C). 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. 2D).

**RA treatment is abrogated in RAR TKO cells**

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. 3A). 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. 3B). 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 ± 4%, 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

A) Relative Cell Number (atRA/ctrl) vs Days of atRA treatment (1μM, N=3)

B) Heatmap of gene expression comparison between WT and TKO cells with and without RA treatment.

C) Scatter plot showing Log Fold Change vs Mean Expression Level for WT (ctrl vs RA) cells.

D) Bar chart showing Fold Change (relative to WT ctrl) for various genes under different treatment conditions.
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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 RARy 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 (40479 and 40453). In contrast, the levels of transcripts were decreased (to 2 ± 1% and 14 ± 4%, respectively) in TKO cells (Fig. 4A). Gtll2 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. 4A). 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 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, Smmn2, 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, Smmn2, Tex13, Mobp, Gtl2, and H19 in control and RA-treated WT and TKO ES cells and found that Smmn2 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. 4A). Gtll2 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. 4A). 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. 4B). 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
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 (RAα, RARβ, RARγ, RXRa, 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 ≤ 0.05 relative to untreated cells, or as indicated; **, p value ≤ 0.01; ***, p value ≤ 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 RARβ2, 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 RARβ2 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. RARβ2 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. 1D), suggesting that in the absence of functional RARγ and RARβ protein, the RAREs of Cdx1 and RARβ 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. 1D) or any other transcripts (Fig. 2C). 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
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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) |
|---------|-------------------------------------|--------------|
| mCdx1(+)/E | GACGCCCTACGAAAGATG | cDNA 196 gDNA 15,352 |
| mCdx1(−)/F | CTCTGTTGAGGTCAGAGTAG | |
| mHoxa1(+)/E | TAACTCCTTACCCCTCTCACA | |
| mHoxa1(−)/D | ACCACGTTGACCTCTCACA | |
| mCyp26A1(+)/A | GAAAACATTGAGAAGGTTGAG | |
| mCyp26A1(−)/B | CGCGTGAAGCCCTGCTAAACAC | |
| mHprt(+)/C | GTTGAAGATATAATTGACACTG | |
| mHprt(−)/D | CGGAAGCCTATTCAAGCAC | |

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 (Glt2) 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 H2O. 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 H2O 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 = 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) CGTGTTGTATT-TACCCAGC (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 tetracyclined and seeded the transfected cells at low density (2 × 10^5 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 RARγ−/− (KO); RARγ−/−;RARβ−/− (DKO); and RARγ−/−;RARβ−/−; RARα−/− (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
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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) |
|--------------|------------------------------------|------------------|------------------------------------------|--------------|
| mRARγE7(+)   | CCGCAGACCTAAGACTGAGT              | MscI             | CTGGCGACAAAGTCAGCGCAAC₅GCGCA           | 334          |
| mRARγE8(−)   | GCCATGGCGCGCCCTGTAACCCA           |                  | CTGGCGACAAAGTCAGCGCAAC₅GCGCAG         | 265          |
| mRARβE7(+)   | AGAGGCTGTTACAGATGACCG            | PvuII            | CTGGCGACAAAGTCAGCGCAAC₅GCGCAG         | 34           |
| mRARβ(−)     | GCGTAGCGGCTAGACCATATA             |                  | CTGGCGACAAAGTCAGCGCAAC₅GCGCAG         | 128          |
| mRARαs(+5)   | AACAGCTACCTCTCTACCAAC      | SacI             | CTGGCGACAAAGTCAGCGCAAC₅GCGCAG         | 88           |
| mRARα(−)     | AGGTAGCGGTAGTGAGACCG             |                  | CTGGCGACAAAGTCAGCGCAAC₅GCGCAG         | 70           |
| SP6(+)       | ATTTAGGCGACACTATAGATAC           |                  | CTGGCGACAAAGTCAGCGCAAC₅GCGCAG         | 145          |
| T7(+)        | TAATACGACTCACTATAGG             |                  | CTGGCGACAAAGTCAGCGCAAC₅GCGCAG         | 378          |

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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 × 10⁷ 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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