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## RESEARCH ARTICLE

# Genes involved in cell adhesion and signaling: a new repertoire of retinoic acid receptor target genes in mouse embryonic fibroblasts

Ziad Al Tanoury\*, Aleksandr Piskunov\*, Dina Andriamoratsiresy, Samia Gaouar, Régis Lutzings, Tao Ye, Bernard Jost, Céline Keime and Cécile Rochette-Egly<sup>‡</sup>

## ABSTRACT

Nuclear retinoic acid (RA) receptors (RAR $\alpha$ ,  $\beta$  and  $\gamma$ ) are ligand-dependent transcription factors that regulate the expression of a battery of genes involved in cell differentiation and proliferation. They are also phosphoproteins and we previously showed the importance of their phosphorylation in their transcriptional activity. In the study reported here, we conducted a genome-wide analysis of the genes that are regulated by RARs in mouse embryonic fibroblasts (MEFs) by comparing wild-type MEFs to MEFs lacking the three RARs. We found that in the absence of RA, RARs control the expression of several gene transcripts associated with cell adhesion. Consequently the knockout MEFs are unable to adhere and to spread on substrates and they display a disrupted network of actin filaments, compared with the WT cells. In contrast, in the presence of the ligand, RARs control the expression of other genes involved in signaling and in RA metabolism. Taking advantage of rescue cell lines expressing the RAR $\alpha$  or RAR $\gamma$  subtypes (either wild-type or mutated at the N-terminal phosphorylation sites) in the null background, we found that the expression of RA-target genes can be controlled either by a specific single RAR or by a combination of RAR isoforms, depending on the gene. We also selected genes that require the phosphorylation of the receptors for their regulation by RA. Our results increase the repertoire of genes that are regulated by RARs and highlight the complexity and diversity of the transcriptional programs regulated by RARs, depending on the gene.

**KEY WORDS:** Retinoic acid, Nuclear receptors, Transcription, Adhesion

## INTRODUCTION

Retinoic acid (RA), the active metabolite of Vitamin A, regulates multiple biological processes and plays key roles in embryonic development, cell growth and differentiation, and in tissue homeostasis through modifications in the expression of target genes. It exerts its effects through two classes of nuclear receptors, the retinoic acid receptors (RARs;  $\alpha$ ,  $\beta$  and  $\gamma$ ) and the retinoid X receptors (RXRs;  $\alpha$ ,  $\beta$  and  $\gamma$ ), which function as ligand-dependent heterodimeric RAR/RXR transcription regulators (Germain et al., 2006a; Germain et al., 2006b; Germain et al., 2006c). For each subtype, there are at least two known isoforms, which are

generated by differential promoter usage and alternative splicing, and differ in their N-terminal regions (Chambon, 1996).

Similar to most nuclear receptors, RARs have a well-defined domain organization and structure, consisting of a variable N-terminal domain (NTD), and two well-structured and highly conserved domains, a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) (Bastien and Rochette-Egly, 2004). RARs that are heterodimerized with RXRs control the expression of target genes through binding specific and polymorphic RA response elements (RAREs) located in the promoters (Moutier et al., 2012). According to the canonical model of gene regulation by RARs, in the absence of ligand, DNA-bound RARs repress transcription through the recruitment of corepressor complexes, which maintain chromatin in a repressed state. In contrast, upon ligand binding, RARs undergo conformational changes in their LBD, which induces the dissociation of co-repressors and the recruitment of other coregulator complexes, which alter the chromatin structure surrounding the promoter of target genes and pave the way for the recruitment of the transcription machinery (for reviews see Al Tanoury et al., 2013; Rochette-Egly and Germain, 2009).

During the last decade, this scenario became more complicated with the discovery that RARs are phosphoproteins (Al Tanoury et al., 2013; Rochette-Egly, 2003). Indeed, we identified two main phosphorylation sites, one in the LBD (S369 in RAR $\alpha$  and S371 in RAR $\gamma$ 1) (Gaillard et al., 2006; Rochette-Egly et al., 1995) and the other one in the NTD (S77 in RAR $\alpha$  and S79 in RAR $\gamma$ 1) (Bastien et al., 2000; Rochette-Egly et al., 1997). These two sites are rapidly phosphorylated in response to RA (Bruck et al., 2009) subsequent to extranuclear and non-genomic effects of RA that lead to the activation of the p38MAPK–MSK1 pathway (Piskunov and Rochette-Egly, 2012). RA-activated MSK1 phosphorylates RARs at the serine residue located in the LBD and phosphorylation of this residue allows the recruitment of the cyclin–H–cdk7 subcomplex of TFIIH, which then can phosphorylate the second site located in the NTD (Chebaro et al., 2013; Samarut et al., 2011). This final phosphorylation step is crucial for the binding of the receptor to DR5 RAREs and the transcription of canonical RA target genes (Bruck et al., 2009).

Recently, genome-wide chromatin immunoprecipitation coupled with deep sequencing (ChIP-seq) and high throughput sequencing (RNA-seq) technologies expanded the repertoire of the RAR-target genes and RAR binding sites in breast cancer cell lines (Hua et al., 2009; Ross-Innes et al., 2010) and during the RA-induced differentiation of F9 cells (Mendoza-Parra et al., 2011; Su and Gudas, 2008) and ES cells into neurons (Mahony et al., 2011; Moutier et al., 2012). The RA response has been correlated to the DNA binding of RARs and to the transcriptional regulation of cell specific target genes (Delacroix et al., 2010).

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Here we conducted a genome-wide analysis of the genes that are regulated by RARs, in mouse embryonic fibroblasts (MEFs), by comparing wild-type (WT) MEFs with MEFs in which the three RARs had been knocked out. We observed that, in the absence of RA, the lack of RARs is associated with reduced levels of several gene transcripts associated with cell adhesion and with the disruption of the morphology and adhesion properties of the cells. The lack of RARs also prevented the expression of all the genes that are regulated by RA and that are associated with cell signaling in MEFs. Finally, taking advantage of rescue cell lines expressing the RAR $\alpha$  or RAR $\gamma$  subtypes (either WT or mutated at the N-terminal phosphorylation sites) in the null background, we selected genes that require phosphorylation of the receptors for their regulation by RA.

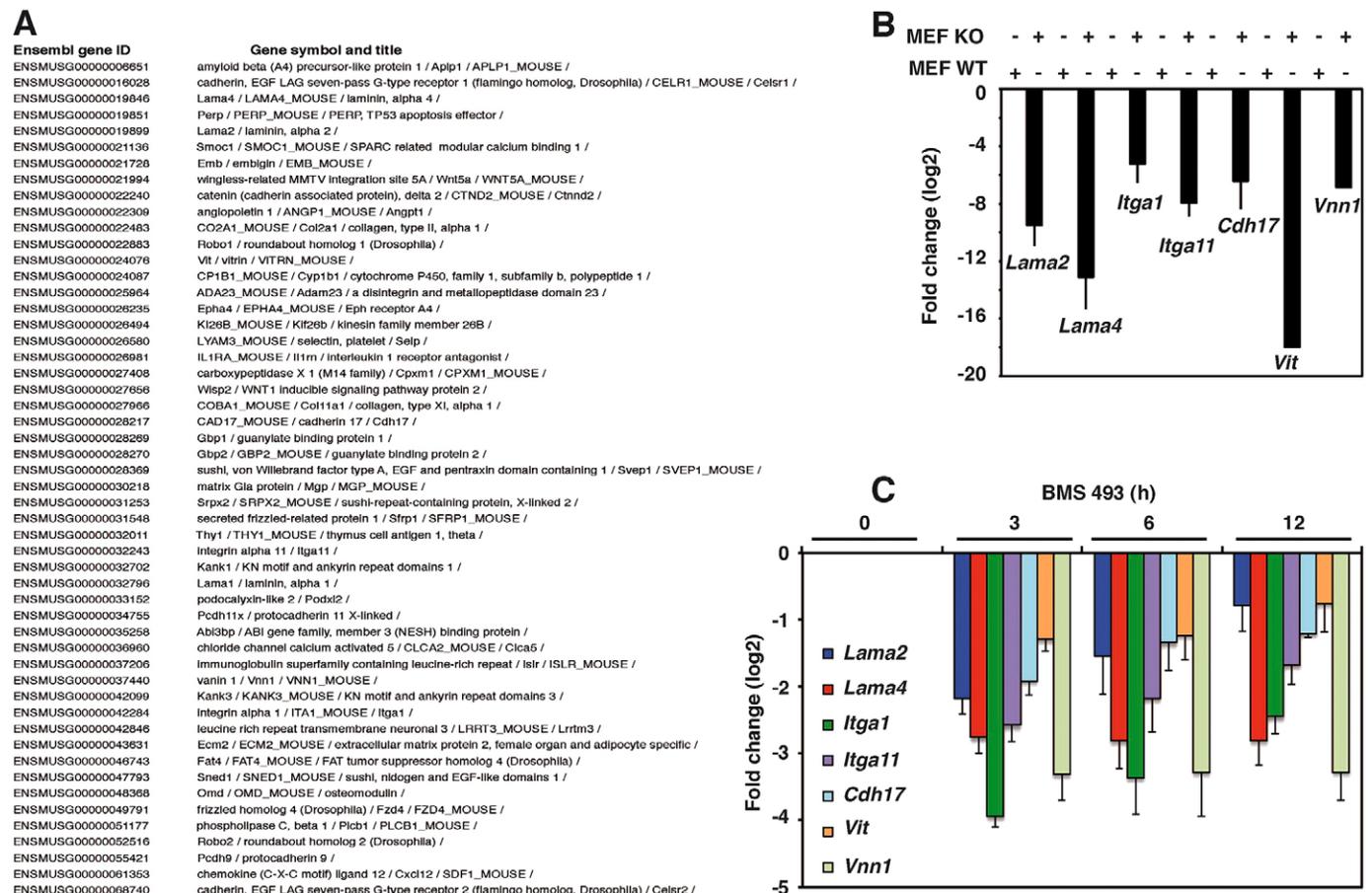
## RESULTS

### In MEFs, RARs contribute to the expression of genes involved in cell adhesion

The repertoire of genes that are regulated by RARs in the absence of RA was profiled by comparing WT MEFs and *Rara*<sup>-/-</sup> *Rarb*<sup>-/-</sup> *Rarg*<sup>-/-</sup> MEFs [referred to as RAR( $\alpha,\beta,\gamma$ )-/- MEFs hereafter] in high throughput sequencing (RNA-seq) experiments (supplementary material Table S1). This analysis revealed a

number of genes that are downregulated in the knockout cells. Remarkably, these genes predominantly encode proteins involved in cell adhesion, such as integrins (alpha 1 and alpha 11), cadherin 17, protocadherin 9, laminins (alpha 1, alpha 2 and alpha 4), collagens (type II alpha 1, type 11 alpha 1), vitrin, vanin-1, robo 1 and robo 2 (Fig. 1A). That these genes are downregulated in RAR knockout MEFs was corroborated in RT-qPCR experiments (Fig. 1B).

Such results suggest that in the absence of ligand, RARs would sustain the transcription of genes associated to adhesion. Therefore, we analyzed whether these genes had RA response elements in their promoters. The analysis of the regions located  $\pm 10$  kb from the gene limits revealed the presence of several direct repeats (DRs) and inverted repeats (IRs) (Moutier et al., 2012) with spacing of between 0 and 8 base pairs (data not shown). We also treated the WT MEFs with the panRAR antagonist BMS493. In fact BMS493 is as an inverse agonist that displaces the positioning of helix 12 so that the interaction with corepressors is strongly enhanced (Bourguet et al., 2010; le Maire et al., 2010). Treatment of WT MEFs with this compound strongly repressed the expression of the genes involved in adhesion (Fig. 1C). Altogether, these data indicate that RARs can mediate a substantial level of transcriptional activation in the absence of RA.



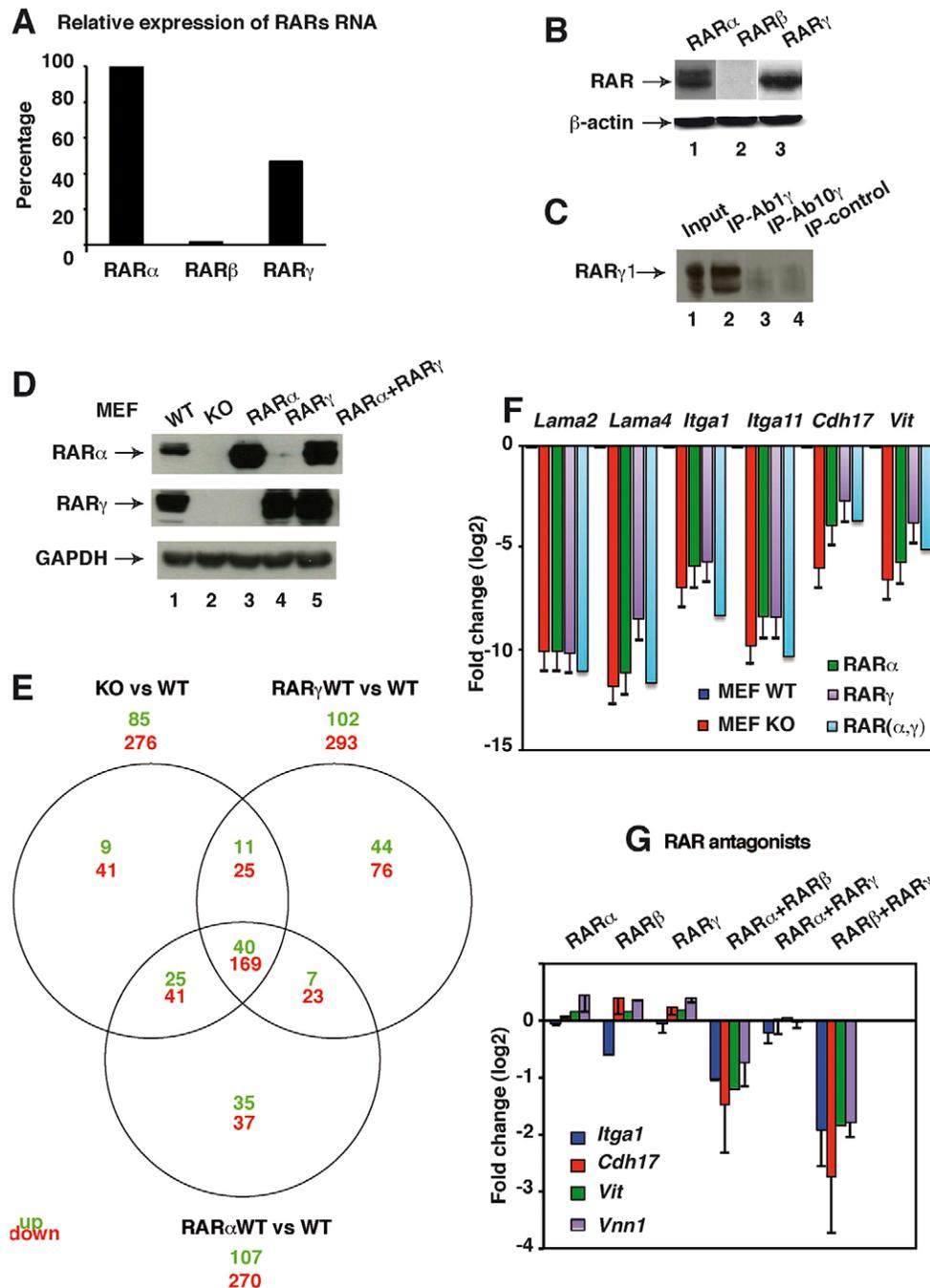
**Fig. 1. In the absence of RA, RARs control the expression of genes involved in cell adhesion.** (A) Cell adhesion is the major biological process, which is downregulated in RAR( $\alpha,\beta,\gamma$ )-/- MEFs (MEF KO) compared with WT cells. The genes involved are listed. (B) RT-qPCR experiments confirming the downregulation of genes involved in cell adhesion in RAR( $\alpha,\beta,\gamma$ )-/- MEFs compared with WT MEFs. Values are the means  $\pm$  s.d. of triplicates from two or three separate experiments. (C) RT-qPCR experiments showing that treatment of WT MEFs with the pan RAR antagonist BMS 493 (1  $\mu$ M) inhibits the expression of the genes involved in cell adhesion. Values are the means  $\pm$  s.d. of triplicates from three separate experiments.

Then the question was whether the expression of these genes is regulated by a specific RAR subtype,  $\alpha$ ,  $\beta$  or  $\gamma$ , in the absence of RA. MEFs express constitutively the RAR $\alpha$  and RAR $\gamma$  subtypes at both the mRNA and proteins levels, whereas RAR $\beta$  is hardly detectable (Fig. 2A,B). Immunoprecipitation experiments performed with monoclonal antibodies recognizing specifically the two main RAR $\gamma$  isoforms (RAR $\gamma$ 1 and RAR $\gamma$ 2) indicated that RAR $\gamma$ 1 is expressed in MEFs and not RAR $\gamma$ 2 (Fig. 2C). Such results suggest that the RAR $\alpha$  and/or RAR $\gamma$ 1 subtypes might control the expression of the genes involved in cell adhesion.

In order to determine which RAR, alpha or gamma1, contributes to the expression of these genes, we took advantage of MEF rescue cell lines stably expressing RAR $\alpha$  or RAR $\gamma$ 1 in the triple RAR null background. The rescue line expressing RAR $\alpha$  has already been

characterized [(Bruck et al., 2009) and Fig. 2D, lane 3] and we established stable rescue lines expressing RAR $\gamma$ 1. Three clones expressing the receptor at levels similar to the endogenous receptor were obtained and one was selected (Fig. 2D, lane 4).

The RAR $\alpha$  and RAR $\gamma$  rescue cell lines were then compared with the WT and triple RAR knockout cells in RNA-seq experiments (supplementary material Table S1). The upregulated or downregulated genes of the knockout versus wild-type cells were crossed with the RAR $\alpha$  rescue cells (versus the WT cells) and with the RAR $\gamma$  rescue cells (versus the WT cells). The Venn diagram in Fig. 2E shows that among the genes that were downregulated in the triple RAR knockout cells, 15% were restored in the two rescue cell lines, 15% in the RAR $\gamma$  rescue cell line (and not in the RAR $\alpha$  line) and 10% in the RAR $\alpha$  line (and not in the RAR $\gamma$  line). From



**Fig. 2. Comparison of WT MEFs, RAR( $\alpha,\beta,\gamma$ )-/- MEFs and MEFs expressing RAR $\alpha$  and RAR $\gamma$ , in the absence of RA.** (A) Comparison of RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$  RNA levels in WT MEFs by RT-qPCR analysis. (B) Immunoblotting analysis of RAR $\alpha$ , RAR $\gamma$  and RAR $\beta$  levels in WT MEFs. (C) MEFs express the RAR $\gamma$ 1 isoform and not the RAR $\gamma$ 2 isoform as assessed by immunoprecipitation (IP) with antibodies specifically recognizing the isoforms, Ab1 $\gamma$  (A1) and Ab10 $\gamma$  (A2), respectively. (D) Expression of RAR $\alpha$  and RAR $\gamma$ 1, alone or in combination, compared with WT levels in the triple RAR knockout background. (E) Venn diagram comparing the transcripts of the knockout (KO), RAR $\alpha$  and RAR $\gamma$  rescue lines versus the WT cells. (F) RT-qPCR experiments showing that the expression of the genes involved in cell adhesion is not restored in the rescue lines expressing RAR $\alpha$ , RAR $\gamma$  or both RAR $\alpha$  and RAR $\gamma$ . Values are the means  $\pm$  s.d. of triplicates from three separate experiments. (G) RT-qPCR experiments showing that treatment of WT MEFs with RAR $\alpha$  or RAR $\gamma$  antagonists combined with a RAR $\beta$  antagonist inhibits the expression of the genes involved in cell adhesion. Values are the means  $\pm$  s.d. of triplicates from two separate experiments.

these results, one can suggest that the expression of these restored genes is regulated by a specific single receptor, RAR $\alpha$  or RAR $\gamma$ .

In contrast, 60% of the downregulated genes (including most of the adhesion genes) were not restored upon re-expression of RAR $\alpha$  or RAR $\gamma$ . We first hypothesized that the expression of these genes would require both RAR $\alpha$  and RAR $\gamma$  and/or a pioneer factor, itself under the control of RAR $\alpha$  or RAR $\gamma$  (Delacroix et al., 2010; Hua et al., 2009; Mahony et al., 2011). Therefore we established an additional stable rescue line expressing both RAR $\alpha$  and RAR $\gamma$  (Fig. 2D, lane 5). Unfortunately, the adhesion genes that were downregulated in the triple knockout cells, were not restored in either double rescue line (Fig. 2F), suggesting a role for the third RAR, RAR $\beta$ , despite its low and hardly detectable level of expression. In order to corroborate such a hypothesis, WT MEFs were treated with antagonists for each RAR subtype, either alone or in combination. When added alone, none of the antagonists significantly affected the expression of the genes involved in adhesion, as exemplified by *Itga1*, *Cdh17*, *Vit* and *Vnn1* (Fig. 2G), and neither did the combination of the RAR $\alpha$  and RAR $\gamma$  antagonists (Fig. 2G). However, the combination of RAR $\beta$  with either RAR $\gamma$  or RAR $\alpha$  significantly decreased their expression (Fig. 2G). Collectively, these results highlight the complexity of the mechanisms regulating genes expression in the absence of ligand, some genes requiring RAR $\alpha$  or RAR $\gamma$  and others a cooperation between RAR $\alpha$  or RAR $\gamma$  with RAR $\beta$ .

Finally, genes that were not affected in the triple RAR knockout cells were induced or repressed upon expression of RAR $\alpha$  or RAR $\gamma$  in the triple RAR knockout background (Fig. 2E), highlighting an additional class of genes, which can be regulated by a single RAR upon inactivation of the others.

#### RARs contribute to the morphology, adhesion and migration of MEFs in the absence of RA

Collectively, the above results suggest that RARs would control the adhesion properties of MEFs. To corroborate this hypothesis, we monitored the ability of WT and RAR( $\alpha,\beta,\gamma$ )-/- MEFs to attach to different extracellular matrix (ECM) proteins (collagen I, collagen IV, laminin, fibrinogen and fibronectin) with *in vitro* adhesion assays. Both cell types attached as efficiently to laminin, fibronectin and fibrinogen (Fig. 3A). However, the triple knockout cells did not attach well to collagen I and collagen IV (Fig. 3A).

Then WT and RAR( $\alpha,\beta,\gamma$ )-/- MEFs were also compared for cell shape and spreading in fluorescence experiments performed after incubation of the cells with fluorescent green 488 phalloidin. Although the WT cells spread rapidly, RAR( $\alpha,\beta,\gamma$ )-/- MEFs remained compact and round without substantial spreading (Fig. 4A). Moreover, in the triple knockout cells the network of actin filaments was disrupted, compared with the WT cells (Fig. 4A). Focal adhesions, labeled with vinculin antibodies, were also disrupted (Fig. 4B). Note that co-labeling with our purified RAR $\alpha$  or RAR $\gamma$  antibodies corroborated the absence of the receptors in the knockout cells (Fig. 4A). That RAR( $\alpha,\beta,\gamma$ )-/- MEF cells are more compact round cells was corroborated by phase-contrast time-lapse video microscopy after splitting each cell line on microplates coated with different ECM proteins (Fig. 3B; Fig. 5; supplementary material Movie 1).

It is interesting to note that the triple knockout cells also seemed more motile than the WT counterparts (supplementary material Movie 1). Therefore, we addressed whether their migration properties were affected or not in a Radius<sup>TM</sup> Cell Migration Assay with different ECM substrates (collagen I, laminin, fibronectin) or on plastic. Remarkably, the triple RAR null MEFs migrated less

efficiently than the WT MEFs when seeded on laminin or plastic (Fig. 5; supplementary material Movie 2).

As most of the genes involved in cell adhesion that were downregulated in the knockout MEFs, were not restored upon expression of RAR $\alpha$ , RAR $\gamma$  or both RAR $\alpha$  and RAR $\gamma$ , one could expect that the rescue lines do not recover the adhesion, morphology and migration of the WT cells. Accordingly, the rescue lines did not recover the ability to attach to collagen I and collagen IV (Fig. 3A) and to migrate on laminin and plastic (Fig. 3C). They did not fully recover the morphology of the WT cells either (Fig. 3B). However, it is interesting to note that the RAR $\gamma$  and RAR( $\alpha,\gamma$ ) rescue lines recovered a more dense network of actin fibers than the RAR $\alpha$  rescue line (Fig. 4B and data not shown). Such observations were unexpected but in fact could reflect the fact that RAR $\gamma$  restores more genes than RAR $\alpha$  (Fig. 2E).

Collectively such results corroborate the RNA-seq results and highlight a new role for RARs in the control of cell adhesion, migration and morphology through the regulation of the expression of adhesion proteins.

#### The addition of RA does not affect the adhesion of MEFs but enhances the expression of genes involved in signaling

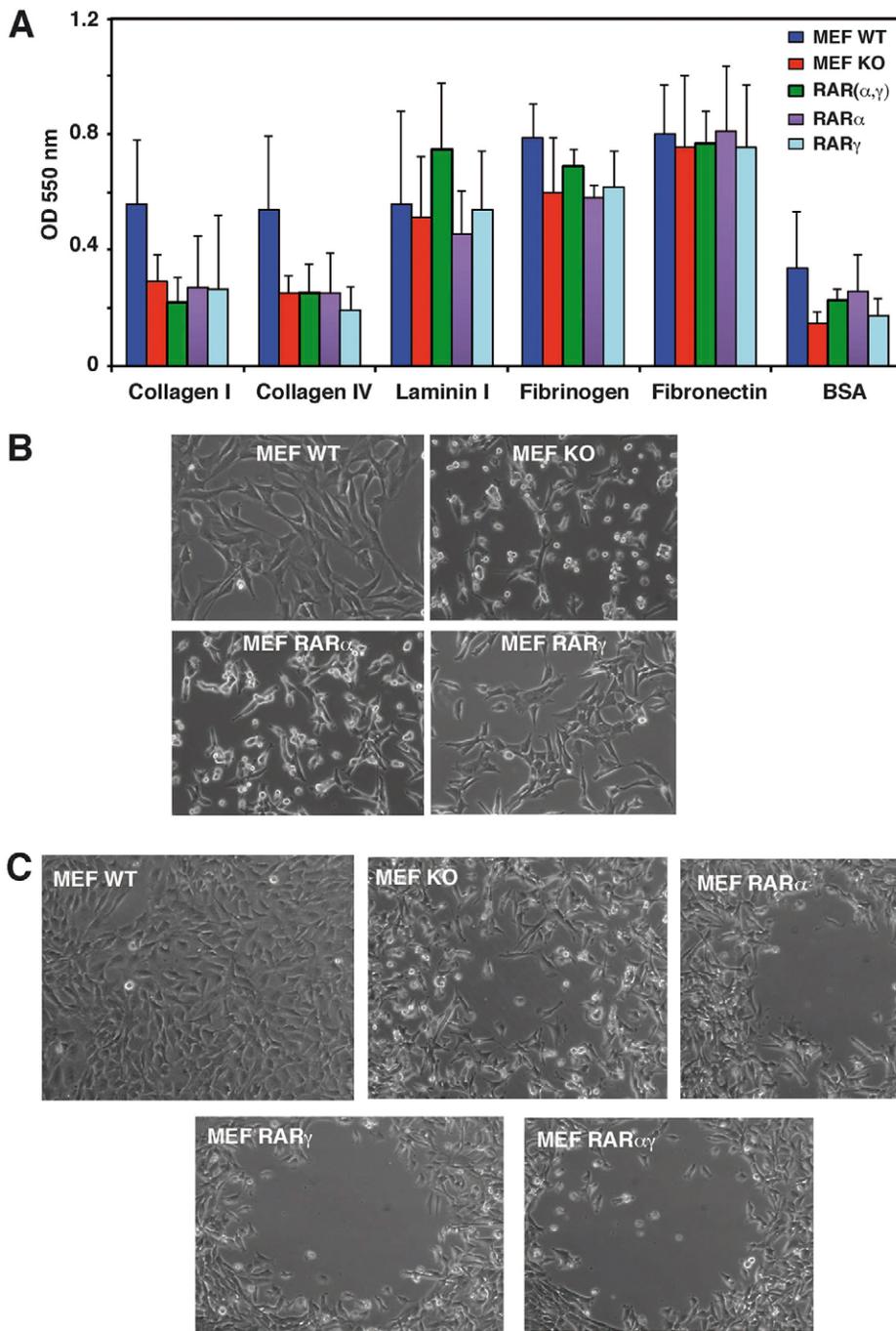
Then the repertoire of genes that are regulated by RARs in response to RA was profiled by comparing WT MEFs and RAR( $\alpha,\beta,\gamma$ )-/- MEFs, RA-treated versus untreated, in RNA-seq experiments. In order to select the primary target genes, the two MEF lines were treated with RA for 2 hours only.

A list of up- and downregulated genes was generated with WT MEFs (supplementary material Table S2). Remarkably, this list did not include genes involved in cell adhesion and migration. Accordingly, adhesion and spreading of MEFs were the same in the absence and presence of RA (supplementary material Movie 3). In fact, the list of upregulated genes included the *Rarb2* gene and the canonical RA-target genes involved in RA metabolism such as *Cyp26b1* (cytochrome P450, family 26, subfamily b, polypeptide 1) and *Dhrs3* [dehydrogenase/reductase (SDR family) member 3]. It also included several genes encoding proteins involved in signaling such as *Pfkfb3* (6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3), *Tnfrsf1b* (tumor necrosis factor receptor super family, member 1b), *Cxcr7* [chemokine (C-X-C motif) receptor 7], *Adora2b* (adenosine A2b receptor), *Rab20* (RAB20 member RAS oncogene family), *Rhpn2* (rhophilin, Rho GTPase binding protein 2) and *Stab1* (stabilin 1). Note that the list did not include the canonical homeobox genes involved in development and activated in other cell lines (Kashyap and Gudas, 2010; Mahony et al., 2011; Moutier et al., 2012; Su and Gudas, 2008) (Z.A.T., S.G., A.P., T.Y., Sylvia Urban, B.J., C.K., Irwin Davidson, Andree Dierich, C.R.E., unpublished data), in line with the fact that fibroblasts are already differentiated cells.

Remarkably all the genes that were RA-regulated in the WT cells did not have any significant differential expression in the triple-knockout cells (supplementary material Table S2). Moreover analysis of their promoters revealed the presence of several DR motifs with spacing between 0 and 8 base pairs (data not shown), indicating that they are targets for RARs (Moutier et al., 2012).

Then in order to investigate which genes are specifically regulated by RAR $\alpha$  or RAR $\gamma$ , WT MEFs were compared to the different rescue lines, i.e. expressing RAR $\alpha$  or RAR $\gamma$  in the triple-null background, in RNA-seq experiments (supplementary material Table S2; Fig. 6). The results were corroborated in RT-qPCR experiments (Fig. 7 and Table 1).

The Venn diagram (Fig. 6) shows that among the genes that were upregulated in WT MEFs, 25% (exemplified by *Cyp26b1*,



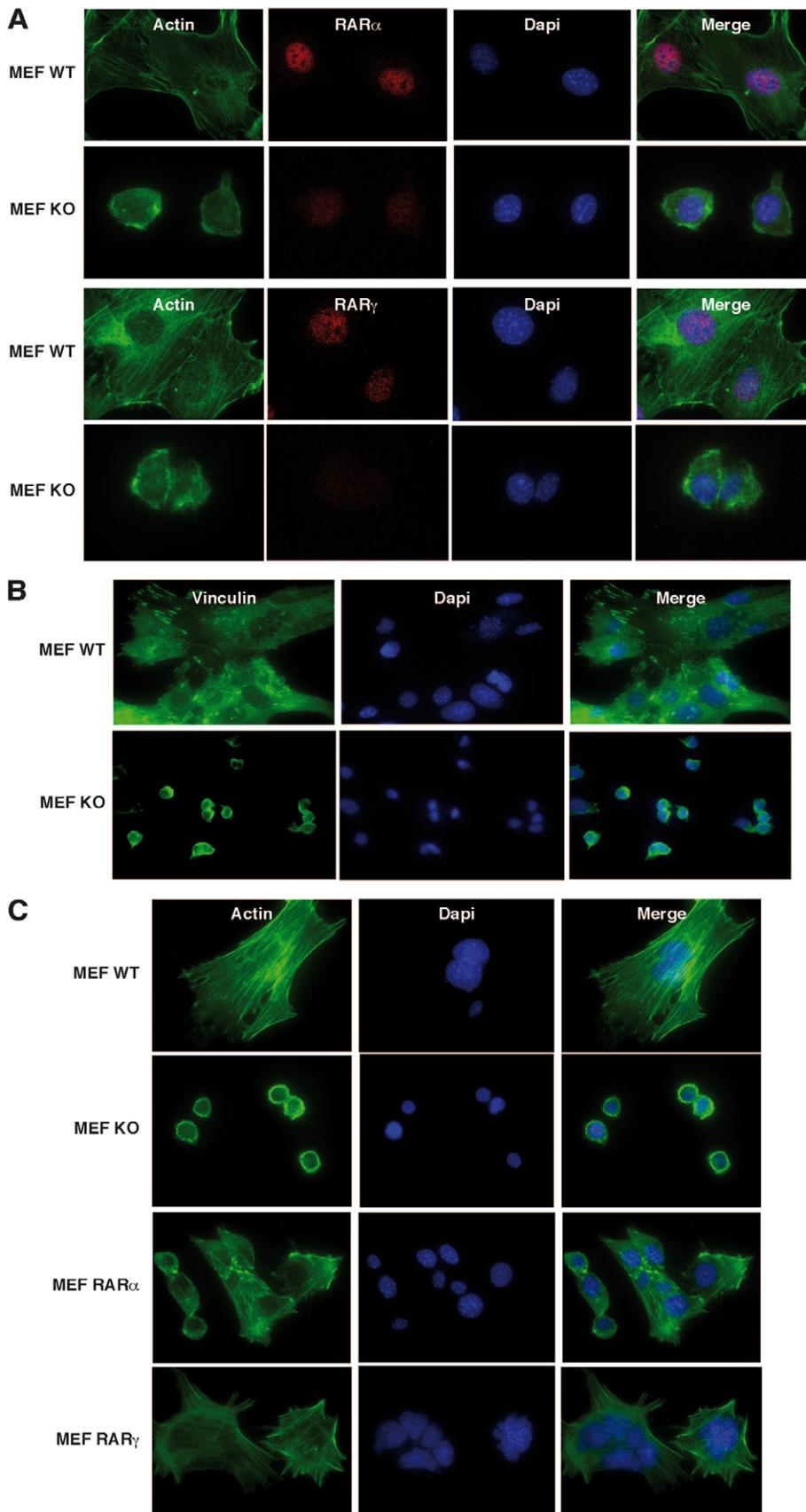
**Fig. 3. RARs control MEF adhesion, morphology and migration.**

(A) Adhesion of WT MEFs, RAR( $\alpha,\beta,\gamma$ )-/- MEFs and of the rescue lines expressing RAR $\alpha$ , RAR $\gamma$  or both RAR $\alpha$  and RAR $\gamma$  on different substrates. Values are the means  $\pm$  s.d. of duplicates from three separate experiments. (B) MEFs WT, RAR( $\alpha,\beta,\gamma$ )-/- MEFs and MEFs expressing RAR $\alpha$  or RAR $\gamma$  in the null background, were compared for their morphology by phase-contrast microscopy, 6 hours after seeding on plastic micro plates. (C) Invasion of WT MEFs, RAR( $\alpha,\beta,\gamma$ )-/- MEFs and of the rescue lines expressing RAR $\alpha$ , RAR $\gamma$  or both RAR $\alpha$  and RAR $\gamma$  in a Radius<sup>TM</sup> Cell Migration Assay on plastic (12 hours after removal of the hydrogel spot).

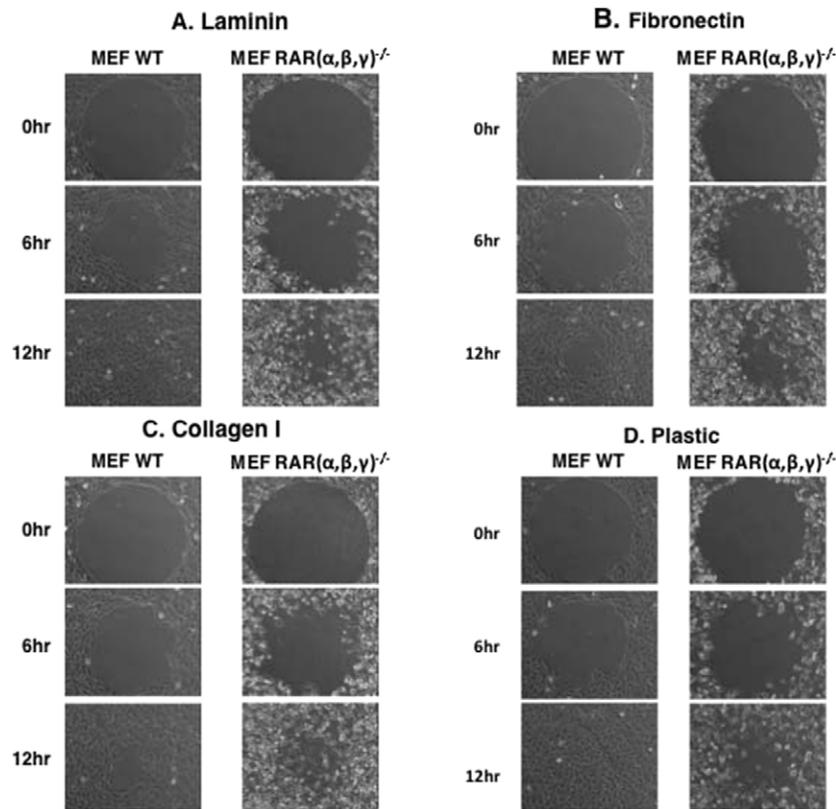
*Dhrs3*, *Cxcr7* and *Tnfrsf1b*) were restored in the two rescue lines (Fig. 7A). Thus the expression of these genes could be mediated by either RAR $\alpha$  or RAR $\gamma$ , each RAR being able to substitute for the other. Consequently, the expression of these genes was not further regulated in the double RAR ( $\alpha,\gamma$ ) rescue line (supplementary material Fig. S1A). In contrast, 5% (exemplified by *Rab20* and *Rhpn2*) were restored only in the RAR $\gamma$  rescue cell line (Fig. 7B) and 15% (exemplified by *Stab1*) in the RAR $\alpha$  rescue line (Fig. 7C). Thus the RA regulation of these genes appears to involve a single RAR (RAR $\alpha$  or RAR $\gamma$ ). This conclusion was corroborated in the double RAR( $\alpha,\gamma$ ) rescue line and by using specific RAR $\alpha$  or RAR $\gamma$  agonists (supplementary material Fig. S1A,B).

Remarkably, 55% of the upregulated genes were not restored (Fig. 6). One can suggest that both RAR $\alpha$  and RAR $\gamma$  in combination would control their expression in response to RA (Su and Gudas, 2008; Taneja et al., 1996) but a role for RAR $\beta$ 2 cannot be excluded.

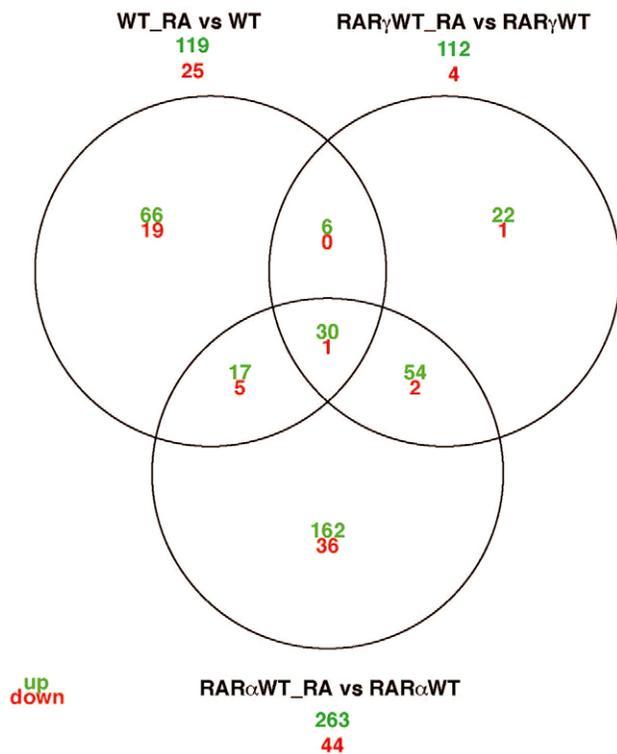
Finally, several other genes, which were not RA-regulated in WT MEFs, were activated in the RAR $\alpha$  rescue line (*Dlx3*; distal-less homeobox 3), in the RAR $\gamma$  rescue line (*Ngfr*; nerve growth factor receptor; TNFR super family, member 16) or in the two rescue lines [*Cyp26a1* (cytochrome P450, family 26, subfamily a, polypeptide 1); *Wnt7b* (wingless-related MMTV integration site 7B); and *Nrip1* (nuclear receptor interacting protein 1)] (Fig. 7D). Most interestingly, the expression of these genes decreased to the



**Fig. 4. RARs control MEF morphology and the actin filament network.** (A) Fluorescence experiments performed with green 488 phalloidin showing the disruption of the actin network in RAR( $\alpha,\beta,\gamma$ )-/- MEFs. Co-labeling with purified polyclonal antibodies raised against RAR $\alpha$  or RAR $\gamma$  confirmed the absence of these RARs in the KO cells. (B) Immunofluorescence experiments performed with vinculin antibodies showing that the microfilaments network is disturbed in MEFs knocked out for the three RARs. (C) WT MEFs, RAR( $\alpha,\beta,\gamma$ )-/- MEFs, and MEFs expressing RAR $\alpha$  or RAR $\gamma$  in the null background, were compared in fluorescence experiments performed with green 488 phalloidin.



**Fig. 5. RARs control the migration of MEFs.** WT MEFs and  $RAR(\alpha,\beta,\gamma)^{-/-}$  MEFs were compared in a Radius™ Cell Migration Assay with different substrates: collagen I, laminin, fibronectin and plastic.



**Fig. 6. Venn diagram comparing the genes that are regulated by RA in WT MEFs and in the rescue MEFs expressing RAR $\alpha$  WT or RAR $\gamma$  WT in the null background.**

WT levels in the double rescue cell line (supplementary material Fig. S1A), indicating that in the WT cells, the two receptors antagonize each other for the induction of these genes, which have DR motifs in their promoters (data not shown) (Taneja et al., 1996).

#### RAR phosphorylation and the RA regulation of their target genes

RAR $\alpha$  and RAR $\gamma$  have a conserved phosphorylation site (S77 and S79, respectively) in their NTD (Fig. 8A). Upon treatment of MEFs with RA, RAR $\alpha$  becomes rapidly phosphorylated at this site (Bruck et al., 2009). RAR $\gamma$  also becomes rapidly phosphorylated at the same conserved site. Indeed, the amount of RAR $\gamma$  phosphorylated at S79 markedly increased within minutes after RA addition, as assessed by immunoprecipitation with antibodies recognizing specifically RAR $\gamma$  phosphorylated at this residue (Fig. 8B).

In order to determine whether phosphorylation plays a role in the activation of the RAR $\alpha$  and/or RAR $\gamma$  target genes, we generated additional stable rescue lines in the triple RAR knockout cells, which expressed RAR $\alpha$  or RAR $\gamma$  mutated at the N-terminal phosphorylation site. The rescue line expressing RAR $\alpha$  with S77 replaced with an alanine (RAR $\alpha$  S77A) has already been characterized (Bruck et al., 2009) (Fig. 8C, lane 4, D). We also established stable rescue lines expressing RAR $\gamma$  S79A. Three clones expressing the mutated receptor at levels similar to the endogenous receptor were obtained and one was selected and analyzed (Fig. 8C, lane 6, B).

Then the RAR $\alpha$  S77A and RAR $\gamma$  S79A cell lines, RA-treated versus untreated were compared with the WT RAR $\alpha$  and RAR $\gamma$  rescue lines, respectively, and with the WT cells in RNA-seq

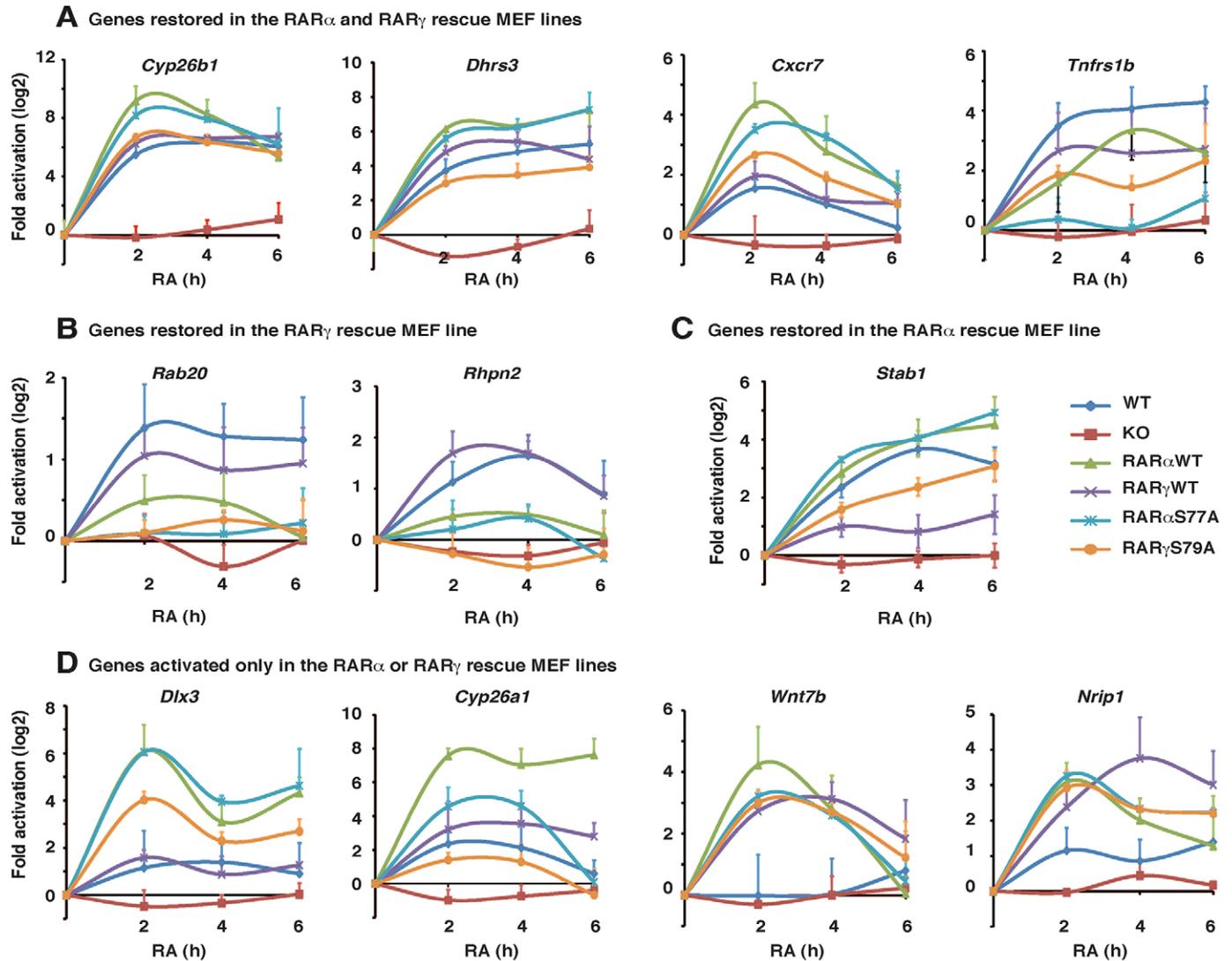


Fig. 7. RT-qPCR analysis of the genes regulated by RA in the different MEF lines that were selected in the RNA-seq experiments. Values are the means  $\pm$  s.d. of triplicates from three to five separate experiments.

(supplementary material Table S2; Fig. 8E,F) and RT-qPCR experiments (Fig. 7). The results show that among the upregulated genes that were restored by WT RAR $\alpha$  or by WT RAR $\gamma$ , only 13% and 20% were not restored by RAR $\alpha$  S77A or RAR $\gamma$  S79A, respectively, and thus depend on the phosphorylation of the receptors for their activation by RA (Fig. 8E,F). As an example, the *Rab20* and *Rhpn2* genes are controlled by the phosphorylation of RAR $\gamma$  (Fig. 7B) and the *Tnfrs1b* gene by that of RAR $\alpha$  (Fig. 7A). Reciprocally, other genes were restored only in the cell lines expressing the phospho-mutants, indicating that phosphorylation can also have inhibitory effects. This is exemplified by the *Stab1* gene, which was restored by RAR $\gamma$  S79A but not by the WT receptor (Fig. 6C). Note that this gene was also restored by RAR $\alpha$  but independently of its phosphorylation state (Fig. 7C). Thus RAR $\alpha$  and RAR $\gamma$  can regulate the expression of a same gene with distinct phosphorylation requirements.

Similarly, among the genes that were activated only in the WT RAR $\alpha$  and WT RAR $\gamma$  rescue cell lines (and not in the WT cells), some genes, exemplified by *Cyp26a1*, were not or less efficiently activated in the cell lines expressing the RAR $\gamma$  and RAR $\alpha$

phospho-mutants, respectively (Fig. 6D). Reciprocally, other genes such as *Dlx3* were activated only in the RAR $\gamma$  S79A rescue cell line and not in the WT RAR $\gamma$  one (Fig. 6D). Note that *Dlx3* was also activated by RAR $\alpha$  but independently of its phosphorylation.

Altogether these results indicate that, depending on the gene, the phosphorylation of RARs can have either positive or negative effects on their activation by RA, increasing the complexity of the transcriptional process.

## DISCUSSION

Here we have described a genome-wide analysis of the genes that are transcriptionally regulated by RARs in mouse embryonic fibroblasts, both in the absence and presence of RA. Several studies have reported that expression of certain genes is activated by RA in MEFs (Delacroix et al., 2010) as well as in other cell types (Hua et al., 2009; Mahony et al., 2011; Mendoza-Parra et al., 2011; Ross-Innes et al., 2010; Su and Gudas, 2008). However, the originality of the present study resides in the comparison of WT MEFs with MEFs in which all three RARs or

**Table 1. Recapitulation of the genes that are RA-activated in MEFs and their regulation by RAR $\gamma$  and RAR $\alpha$  and their phosphorylated forms**

Gene name	MEF lines: response to RA					
	WT	KO	RAR $\gamma$ WT	RAR $\gamma$ S79A	RAR $\alpha$ WT	RAR $\alpha$ S77A
<b>Metabolism</b>						
<i>Cyp26b1</i>	++	–	++	++	++	++
<i>Dhrs3</i>	++	–	++	++	++	++
<i>Cyp26a1</i>	±	–	+	–	+	±
<b>Signaling</b>						
<i>Tnfrsf1b</i>	+	–	+	+	+	–
<i>Cxcr7</i>	+	–	+	+	+	+
<i>Stab1</i>	+	–	–	+	+	+
<i>Rab20</i>	+	–	+	–	–	–
<i>Rhpn2</i>	+	–	+	–	–	–
<i>Wnt7b</i>	–	–	++	++	++	++
<i>Nrip1</i>	–	–	+	+	+	+
<i>Dlx3</i>	–	–	±	+	++	++

one or two individual RAR were knocked out in the triple RAR null background. Such a strategy has the advantage of allowing the characterization of all the RAR-target genes and the analysis of receptor subtypes requirement, without artefactual and redundant RAR subtype responses (Plassat et al., 2000; Rochette-Egly and Chambon, 2001; Rochette-Egly et al., 2000; Taneja et al., 1996). Therefore it allowed us to identify that unliganded and liganded RARs control the expression of different sets of genes: genes involved in adhesion in the absence of ligand and genes involved in signaling in the presence of ligand. In addition, the generation of rescue cell lines expressing RAR $\alpha$  or RAR $\gamma$  mutated at their N-terminal phosphorylation site allowed the selection of genes that require the phosphorylation of the receptors for their regulation by RA. Finally, the comparison of the WT and triple knockout cells with the different rescue lines highlighted the complexity and the diversity of the transcriptional programs regulated by RARs both in the absence and presence of RA.

#### A novel ligand-independent function of RARs in the regulation of adhesion genes

We identified several transcripts that were differentially expressed between wild-type and RAR $\alpha$ , - $\beta$ , - $\gamma$  knockout MEFs in the absence of ligand. Indeed, we have shown that the inactivation of the three RARs is associated with the downregulation of transcripts associated with genes involved in cell adhesion, such as integrins, laminins, cadherins and collagen. Consequently, the triple RAR knockout cells lost their ability to adhere and migrate on specific substrates. Moreover, given that the adhesion proteins are linked to the cellular cytoskeleton (Miranti and Brugge, 2002; Zamir and Geiger, 2001), they remained compact round cells and displayed a disrupted network of actin filaments, compared to the WT cells.

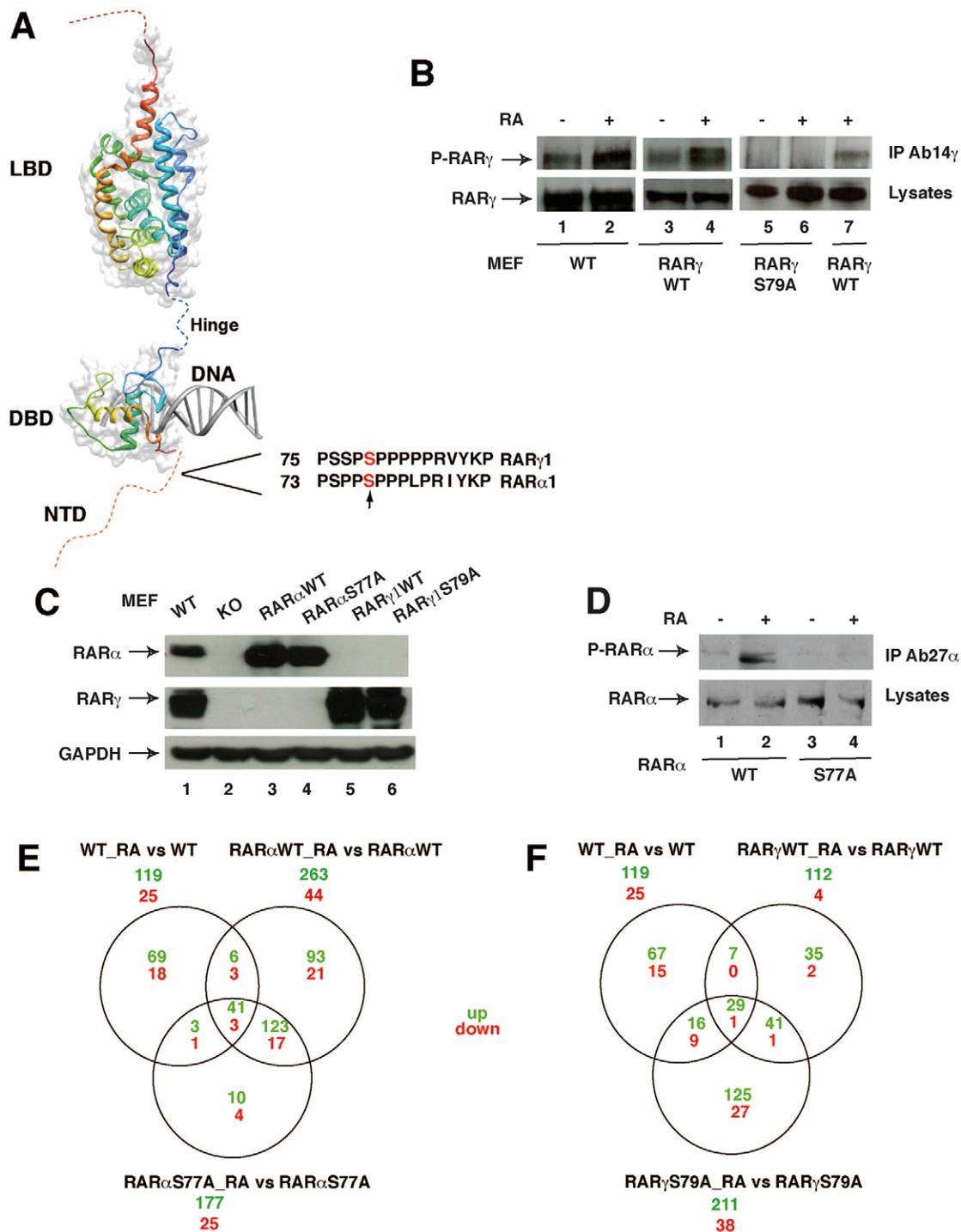
Another important finding of this study is that, among the adhesion genes that are downregulated in the triple RAR knockout cells, 40% are restored in the RAR $\alpha$  and/or RAR $\gamma$  rescue cell lines and thus are controlled by a single specific RAR (RAR $\alpha$  or RAR $\gamma$ ) in the absence of ligand. In contrast, 60% are not restored. Remarkably, expression of both RAR $\alpha$  and RAR $\gamma$  does not restore either the adhesion, migration and morphology of MEFs to WT levels. In fact our results suggest that RAR $\alpha$  and RAR $\gamma$  cooperate with RAR $\beta$ , despite this latter RAR being expressed at hardly detectable levels. Consequently, the right adhesion properties of MEFs require the expression of subsets of genes, which are controlled by diverse combinations of RARs.

Remarkably, all the adhesion genes regulated by RARs in the absence of ligand, contain several potential RAR-binding sites in their promoters. However, which sites do RAR $\alpha$  and/or RAR $\gamma$  occupy and how unliganded RARs maintain the expression of these genes for the proper adhesion and spreading of the cells, will require further investigations. According to the classical model (Dilworth and Chambon, 2001), unliganded and DNA-bound RAR $\alpha$  represses transcription through the recruitment of corepressor complexes that maintain chromatin in a condensed and repressed state. However, recent studies reported that RAR $\alpha$  can mediate substantial levels of transcriptional activation in the absence of ligand through the recruitment of complexes that regulate the methylation of specific promoter regions (Delacroix et al., 2010; Laursen et al., 2012). RAR $\gamma$  has been also shown to control gene transcription in the absence of ligand (Su and Gudas, 2008) and it has been proposed that this property would reflect its weak ability to bind corepressors (Farboud et al., 2003; Hauksdottir et al., 2003).

#### In the presence of ligand, RARs control the expression of genes encoding essential proteins involved in cell signaling

We also profiled the genes that are regulated by RA in MEFs. Gene ontology analysis showed that, in these cells, the largest class of target genes are involved in metabolism (*Cyp26b1* and *Dhrs3*) and in signal transduction (*Pfkfb3*, *Tnfrsf1b*, *Cxcr7*, *Adora2b*, *Rab20* and *Rhpn2*). It must be stressed that this repertoire of RA-regulated genes in MEFs is significantly different from that observed in other cell types such as F9 or ES cells (Mahony et al., 2011; Mendoza-Parra et al., 2011; Moutier et al., 2012; Su and Gudas, 2008) (Z.A.T., S.G., A.P., T.Y., Sylvia Urban, B.J., C.K., Irwin Davidson, Andree Dierich, C.R.E., unpublished data). Indeed, most of the developmental genes such as the Hox genes, which are strongly activated in F9 and ES cells, were not activated in MEFs. Such a difference has been attributed to the fact that MEFs and ES cells share only a small subset of RAR-bound genes (Delacroix et al., 2010) because of a silenced chromatin signature in fibroblasts, which correspond to a more differentiated cell type (Delacroix et al., 2010; Kashyap and Gudas, 2010).

The originality of the present study resides in the comparison of WT MEFs with MEFs in which all three RARs or one or two individual RAR were knocked out in the triple RAR null background. Such a strategy provides a means of analyzing each receptor requirement, without redundancies. Consequently, we found that the activation of all the RA-regulated genes was



**Fig. 8. Influence of RAR phosphorylation on the regulation of their target genes by RA.** (A) Schematic representation of RARs showing the phosphorylation site located in the NTD. (B) In WT MEFs, RA induces the phosphorylation of RAR $\gamma$  as shown by immunoprecipitation with a monoclonal antibody specifically recognizing RAR $\gamma$  phosphorylated at S79 (P-RAR $\gamma$ ). RAR $\gamma$  is also phosphorylated in MEFs expressing RAR $\gamma$  WT but not in MEFs expressing RAR $\gamma$  S79A. (C) Expression of RAR $\alpha$  (WT and S77A) and RAR $\gamma$ 1 (WT and S79A) to WT levels in the triple RAR knockout background. (D) RAR $\alpha$  is phosphorylated in MEFs expressing RAR $\alpha$  WT but not in MEFs expressing RAR $\alpha$  S77A, as shown by immunoprecipitation with a monoclonal antibody specifically recognizing RAR $\gamma$  phosphorylated at S79. (E) Venn diagram comparing the RA-regulated genes in WT MEFs and in MEFs expressing RAR $\alpha$  WT or RAR $\alpha$  S77A in the triple RAR knockout background. (F) Venn diagram comparing the RA-regulated genes in WT MEFs and in MEFs expressing RAR $\gamma$  WT or RAR $\gamma$  S79A.

abolished in the triple RAR knockout MEFs, validating that they are RAR-target genes. Taking advantage of cells expressing RAR $\alpha$  or RAR $\gamma$  in the triple RAR knockout background, we also found that, depending on the gene, their RA regulation can be

mediated by either RAR $\alpha$  or RAR $\gamma$  (each RAR being able to substitute for the other) or by a single RAR (RAR $\alpha$  or RAR $\gamma$ ). The actual role of RAR $\beta$ 2 is still unclear since this receptor is also under the control of both RAR $\alpha$  and RAR $\gamma$ .

Taking into account that the majority of the RA-target genes contain several RAR-binding sites, one can speculate that the activation of the RAR-target genes requires the binding of one or several RAR subtypes at different sites and/or their dynamic and sequential recruitment at a same site (Mendoza-Parra et al., 2011). Overall, these observations complicate the models of gene regulation by RARs and highlight the diversity of the regulatory programs by RARs, depending on the gene and on the feature of the cell type.

#### In MEFs, phosphorylation of RARs is required for RA activation of specific genes

The fact that RAR $\alpha$  and RAR $\gamma$  are rapidly phosphorylated in response to RA, brought into question the repertoire of the RA target genes that are regulated by their phosphorylated forms. The comparison of WT MEFs with MEFs expressing the WT RARs or the phospho-mutants in the triple RAR knockout background, allowed us to identify two genes (*Rab20* and *Rhpn2*) that are activated only by the phosphorylated form of RAR $\gamma$ , and one (*Tnfrsf1b*), which is controlled by the phosphorylated form of RAR $\alpha$ . All these genes encode key proteins involved in cell signaling. Indeed, *Rab20* encodes a GTPase, which associates with and dissociates from membranes and which like all the GTPases of the Rab family is a key regulator of all transport steps between intracellular compartments (Pylypenko and Goud, 2012). *Rhpn2* encodes rhophilin-2, which is a RhoA-GTPase-binding protein and which regulates actin cytoskeleton organization (Peck et al., 2002). *Tnfrsf1b* belongs to the tumor necrosis factor receptor super family and is linked to pro- and anti-apoptotic pathways (Aggarwal, 2003). In contrast, other genes are activated only by the non-phosphorylated form of RAR $\alpha$  or RAR $\gamma$  and not by the WT receptors, indicating that RAR phosphorylation can also have negative effects on gene expression. These genes, exemplified by *Stab1*, also encode proteins involved in cell signaling.

Thus one can speculate that, in MEFs, the phosphorylated forms of RAR $\alpha$  and RAR $\gamma$  control the activation of genes that are essential for cell proliferation and/or survival. In contrast, other results from our laboratory indicate that in other cell types such as ES cells, the phosphorylated form of RAR $\gamma$  regulates other genes, which are involved in development (Z.A.T., S.G., A.P., T.Y., Sylvia Urban, B.J., C.K., Irwin Davidson, Andree Dierich, C.R.E., unpublished data). Thus not only the repertoire of the RA-activated genes, but also their dependency on RAR phosphorylation depends on the cell type.

According to our recent studies, phosphorylation controls the association–dissociation of coregulators involved in transcription, through allosteric control (Chebaro et al., 2013; Samarut et al., 2011). Moreover, we have shown that phosphorylation of the N-terminal serine residue, which is located in the vicinity of the DBD, controls the association–dissociation of coregulators (Lalève et al., 2010) and therefore the recruitment of RARs to some promoters (Bruck et al., 2009; Lalève et al., 2010). Further work is in progress in order to decipher how phosphorylation controls the binding of RARs to specific gene promoters.

In conclusion, the current study increased the repertoire of RAR-target genes and revealed that in the absence of ligand, RARs control the expression of genes associated with cell adhesion and migration. Such findings may have important biological implications during embryonic development (Samarut and Rochette-Egly, 2012). It may also have implications in the migration and invasion properties of carcinoma-associated fibroblasts, especially in the context of breast

cancers characterized by aberrant RA signaling (Goetz et al., 2011; Siletz et al., 2013; Tari et al., 2002). It also revealed new genes that depend on the phosphorylation state of RARs for their activation by RA. Finally it shed light on the diversity and complexity of the regulatory programs by RARs, depending on the gene and on the feature of the cell type.

## MATERIALS AND METHODS

### Antibodies and reagents

Rabbit polyclonal antibodies against RAR $\alpha$  [RP $\alpha$  (F)], RAR $\gamma$  [RP $\gamma$  (F)] and RAR $\beta$  [RP $\beta$  (F)] were previously described (Bruck et al., 2009; Lalève et al., 2010; Rochette-Egly et al., 1992). RP $\alpha$  (F) and RP $\gamma$  (F) were purified on Sulfolink gel columns (Pierce Chemical, Rockford, IL, USA) coupled to the corresponding immunizing peptide (Buchanan et al., 2011; Lalève et al., 2010; Vernet et al., 2006). Mouse monoclonal antibodies against RAR $\gamma$ 1 [Ab1 $\gamma$  (A1)] and RAR $\gamma$ 2 [Ab10 $\gamma$  (A2)] were previously described (Bastien et al., 2000) as well as mouse monoclonal antibodies recognizing specifically RAR $\alpha$  phosphorylated at S77 (RAR $\alpha$ -P-S77) (Bruck et al., 2009) or RAR $\gamma$  phosphorylated at S79 (RAR $\gamma$ -P-S79) (Lalève et al., 2010). The other mouse monoclonal antibodies used were against  $\beta$ -actin (Santa Cruz Biotechnology Inc.), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Millipore) and vinculin (Sigma Aldrich). All-trans retinoic acid was from Sigma Aldrich, and BMS 195614 (RAR $\alpha$  antagonist), BMS 493 (pan RAR reverse agonist), BMS753 (RAR $\alpha$  agonist), BMS961 (RAR $\gamma$  agonist), MM11253 (RAR $\gamma$  antagonist) and LE135 (RAR $\beta$  antagonist) were from Tocris Bioscience.

### Cell lines, immunoblotting and immunoprecipitation

Mouse embryo fibroblasts (MEFs) with all three RARs deleted, RAR( $\alpha,\beta,\gamma$ )-/-, were as described previously (Altucci et al., 2005; Chapellier et al., 2002) as well as MEFs expressing WT RAR $\alpha$  or RAR $\alpha$  S77A in a RAR( $\alpha,\beta,\gamma$ ) null background (Bruck et al., 2009). WT RAR $\gamma$ 1 and RAR $\gamma$ 1 S79A were subcloned from the pSG5 constructs (Bastien et al., 2000) into a pCA vector with a CAG early promoter coupled to *PGK*/neomycin and reintroduced in the triple RAR knockout cells using Lipofectamine<sup>®</sup> 2000 transfection (Invitrogen). RAR $\alpha$  subcloned into a pCA vector with a CAG early promoter coupled to *PGK*/hygromycin was also introduced into the RAR $\gamma$ 1 rescue line the X-tremeGENE HP DNA Transfection reagent (Roche). The stable rescue lines were selected with G418 for 10 days and analyzed for the presence of the transgene by qPCR and immunoblotting.

When 80–90% confluent, cells were treated with RA ( $10^{-7}$  M; Sigma Aldrich Corporation) after 24 hours in medium containing 1% dextran-charcoal-treated fetal calf serum. Extracts, preparation and immunoblotting were described previously (Bour et al., 2005). Immunoprecipitation was performed with mouse monoclonal antibodies immobilized on Dynabeads<sup>®</sup> Protein G (Invitrogen).

### High throughput mRNA sequencing (RNA-seq)

After isolation of total RNA, a library of template molecules suitable for high throughput DNA sequencing was created following the Illumina ‘Truseq RNA sample prep v2’ protocol with some modifications. Briefly, mRNA was purified from 2 mg total RNA using oligo(dT) magnetic beads and fragmented using divalent cations at 94°C for 8 minutes. The cleaved mRNA fragments were reverse transcribed to cDNA using random primers and then the second strand of the cDNA was synthesized using polymerase I and RNase H. The next steps of RNA-Seq library preparation were performed in a fully automated system using an SPRIworks Fragment Library System I kit (ref A84803, Beckman Coulter, Inc.) with the SPRI-TE instrument (Beckman Coulter, Inc.). Briefly, in this system double stranded cDNA fragments were blunted, phosphorylated and ligated to indexed adapter dimers, and fragments in the range of ~200–400 bp were size selected. The automated steps were followed by PCR amplification [30 seconds at 98°C; (10 seconds at 98°C, 30 seconds at 60°C, 30 seconds at 72°C)×12 cycles; 5 minutes at 72°C]. Then surplus PCR primers were removed by purification using AMPure beads (Agencourt Biosciences Corporation) with a Biomek 3000

instrument (Beckman Coulter, Inc.). DNA libraries were checked for quality and quantified using 2100 Bioanalyzer (Agilent). The libraries were loaded in the flow cell at 11 pM concentration and clusters generated and sequenced in the Illumina HiSeq2000 as single-end 50 base reads.

Image analysis and base calling were performed using CASAVA v1.8.2. Sequence Reads were mapped onto the mm9 assembly of the mouse genome by using Tophat v1.4.1 (Trapnell et al., 2009) and the bowtie v0.12.7 aligner. Only uniquely aligned reads were retained for further analyses. Gene expression was quantified using HTSeq v0.5.3p3 (Anders and Huber, 2010) and gene annotations from Ensembl release 66. Read counts were normalized across libraries with the method proposed by Anders and Huber (Anders and Huber, 2010). Comparisons of interest were performed using the statistical method proposed by Anders and Huber (Anders and Huber, 2010) implemented in the DESeq v1.6.1 Bioconductor package. *P*-values were adjusted for multiple testing by using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Only genes with  $|\log_2 \text{fold-change}| > 1$  or  $< -1$  and an adjusted *P*-value  $< 0.05$  were considered. Functional analyses of these genes were performed using the Manteia program (<http://manteia.igbmc.fr>).

### Motif research

The gene regions located  $\pm 10$  kb from the gene limits (Ensembl release 63) were analyzed using regular expression searches to detect perfect consensus 5'-RGKTS-3' half sites with the different spacings. The potential RAR binding elements were aligned on the same strand to ensure the sense and antisense matches gave homogeneous positions.

### Cell adhesion and migration assays

Cell adhesion on ECM proteins was carried out using the CytoSelect 48-well cell adhesion assay (ECM array colorimetric assay; Cell Biolabs). MEFs (60,000 cells) were seeded onto the wells pre-coated with a single ECM protein (collagen I, collagen IV, laminin, fibrinogen, fibronectin and BSA as a negative control). After 1 hour at 37°C, the non-adherent cells are washed away and the attached cells are quantified by colorimetry.

Cell migration was analyzed using the Radius™ CytoSelect 24-Well Cell Migration Assay kit according to the manufacturer's instructions (Cell Biolabs). MEFs were seeded in the wells of the Radius plate, pre-coated with collagen I, laminin or fibrinogen. Once cells formed a monolayer, the assay was initiated by removing the hydrogel spot located in the center of the well, and cell migration was evaluated for 16 hours by phase-contrast time-lapse video microscopy.

### Phase-contrast time-lapse video microscopy

MEFs (200,000 cells) were seeded in 12-well cell culture microplates pre-coated with laminin (5  $\mu\text{g}/\text{cm}^2$ ; Roche Applied Science) and rapidly analyzed with a Leica DM IRE2 inverted microscope equipped with phase-contrast optics, a thermostatically controlled heating stage and a Plexiglas incubator (37°C, 5% CO<sub>2</sub>). A computer-controlled movable stage was mounted on the microscope, allowing simultaneous recordings from several microscopic fields. Images were obtained sequentially at 2 minute intervals, for a total time of observation of 6 hours. The initial focus (*z*-position) of the individual fields did not change during the recording time. Thus, no auto focusing of the individual fields was necessary. Images were stored using MetaMorph software (Universal Imaging). Two to three microscope fields per well were selected and their *x*,*y*,*z*-positions stored in a computer, with 20 $\times$  magnification.

### Immunofluorescence

Cells grown on Lab-Tek® glass chamber slides (Thermoscientific) were fixed in 4% paraformaldehyde (PFA)–PBS (20 minutes), permeabilized with 0.1% Triton X-100 (15 minutes) and blocked with 1% BSA in PBS (30 minutes). Then the cells were incubated with the primary antibodies, followed by Alexa-Fluor™-448 or -555-conjugated secondary antibodies (Invitrogen). When mentioned, cells were also incubated with fluorescent green CF488 phalloidin (Biotium, Inc. Hayward USA). Nuclei were counterstained with DAPI (Sigma-Aldrich). Cells were analyzed by

fluorescence microscopy using a LEICA upright DM4000B microscope equipped with a Photometrics CoolSnap® camera and software. The objective used was Leica HCX PL APO 63 $\times$  1.40 LBL.

### RNA isolation and RT-qPCR

RNA isolation and RT-qPCR were performed as described previously (Bour et al., 2005). Primer sequences are available upon request.

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### Competing interests

The authors declare no competing interests.

### Author contributions

Z.A.T., A.P. and D.A. devised and undertook all experimental work and analyzed the data. S.G. performed the RT-qPCR experiments and R.L. generated the rescue cell lines. B.J. performed the RNA-seq experiments and T.Y. and C.K. performed the bioinformatic analysis of the results. C.R.E. analyzed the data and wrote the paper.

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### Supplementary material

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