A coordinated phosphorylation cascade initiated by MSK1 directs RAR alpha recruitment to target gene promoters

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SUMMARY

The nuclear retinoic acid (RA) receptor alpha (RARα) is a transcriptional transregulator that controls the expression of specific gene subsets through binding at response elements and dynamic interactions with coregulators, which are coordinated by the ligand. Here, we highlighted a novel paradigm in which the transcription of RARα-target genes is controlled by phosphorylation cascades initiated by the rapid RA activation of the p38MAPK/MSK1 pathway. We demonstrate that MSK1 phosphorylates RARα at S369 located in the Ligand Binding Domain, allowing the binding of TFIIH and thereby phosphorylation of the N-terminal domain at S77 by cdk7/cyclin H. MSK1 also phosphorylates Histone H3 at S10. Finally, the phosphorylation cascade initiated by MSK1 is required for the recruitment of RARα/TFIIH complexes to response elements and subsequently for RARα target genes activation. Cancer cells characterized by a deregulated p38MAPK/MSK1 pathway, do not respond to RA, outlining the essential contribution of the RA-triggered phosphorylation cascade in RA signaling.

INTRODUCTION

Nuclear Retinoic Acid (RA) receptors (RARs) consist of three subtypes, α (NR1B1), β (NR1B2) and γ (NR1B3) [1-3] which function as ligand-dependent transcriptional regulators heterodimerized with Retinoid X Receptors (RXRs). The basic mechanism for transcriptional regulation by RARs relies on binding to specific response elements (RAREs) located in the promoters of target genes and on ligand-induced structural rearrangements in the Ligand Binding Domain (LBD) that direct the association/dissociation of coregulator complexes with different enzymatic activities including Histone Acetyltransferases/Deacetylases, Histone Methyltransferases/Demethylases, Kinases/Phosphatases, Ubiquitin-ligases/deubiquitinases or DNA-dependent ATPases [4-6]. The exchanges between coregulatory complexes and RARs are dynamic and coordinated [7,8]. *In fine,* they cooperate to alter the chromatin structure surrounding the promoter of target genes, paving the way for the recruitment of the transcription machinery including RNA Polymerase II and the General Transcription Factors [9].

A concept that developed over the last several years is that RARs are subject to phosphorylations [10],which play an important and ever-growing role in the transcription of RA-target genes [11-14]. However, the process appeared to be complex and nothing is known about the upstream kinases that govern RAR phosphorylation in response to RA and how RARs phosphorylation impacts the transcription of target genes. Because p38MAPK is activated in response to RA [15-17], we explored whether there is a connection between this kinase signaling pathway, RARα phosphorylation and the induction of RARα-target genes.

RESULTS

RA activates p38MAPK leading to MSK1 activation.

In Mouse Embryo Fibroblasts (MEF), p38MAPK was activated within minutes following RA treatment. This activation was transient as it peaked at 15 min and was reversed at 60 min (Fig. 1a). P38MAPK was similarly activated by a synthetic RARα agonist but not by RARγ and RARβ agonists nor by RARα antagonists (Fig. 1a). P38MAPK activation was not observed in MEF knockout for the three RARs, MEF (RARα,β,γ)$^{-/-}$ [18,19], but was restored upon reexpression of RARαWT (Fig. 1b), indicating that activation of p38MAPK requires liganded RARα. P38MAPK was also rapidly activated in human breast cancer cell lines, such as MCF7 cells, but not in SKBR3 cells (Fig. 1c).

Downstream of p38MAPK, there is MSK1 [20] which contributes to the regulation of the expression of several genes [21,22]. After RA treatment of MCF7 cells and MEFs, there was an increase in the activated, phosphorylated form of MSK1 which paralleled the increase in phosphorylated p38MAPK (Fig. 1d,e). Activation of MSK1 was inhibited upon knockdown of p38MAPK with specific siRNA (Fig. 1f) or by SB203580, a selective inhibitor of p38MAPK (data not shown), corroborating that activation of MSK1 occurs downstream of p38MAPK in RA signaling. Accordingly, MSK1 was not activated in SKBR3 cells, which are defective in RA induction of p38MAPK (Fig.1e). Other signaling pathways that have been shown to participate in hormone signaling such as Erks and the cAMP/PKA pathways were not activated after RA treatment (data not shown).

RA-activated MSK1 phosphorylates RARα at S369, *in vivo.*

Then we wondered if the rapid and transient RA activation of the p38MAPK/MSK1 pathway was associated with a change in the phosphorylation status of RARα. The RARα protein can be phosphorylated at two serine residues, S77 and S369 located in the N- and C-

terminal domains respectively [10] (Fig. 2a). S77 is phosphorylated by the CAK subcomplex of the general transcription factor TFIIH either *in vitro* (Fig. 2b) or *in vivo* [11,23]. However the kinase that phosphorylates *in vivo* S369, in response to RA, has not yet been identified. S369 belongs to an Arg-Lys rich motif which corresponds to a consensus phosphorylation site for several kinases including MSK1 (Fig. 2a). In *in vitro* phosphorylation experiments, active recombinant MSK1 phosphorylated purified bacterially expressed RARα at S369 (and not at S77), as assessed by immunoblotting with antibodies recognizing specifically RARα phosphorylated at these residues. (Fig. 2b).

We also analyzed the *in vivo* phosphorylation of endogenous RARα in MCF7 cells, after PhosphoProtein affinity purification. The amount of phosphorylated RARα markedky increased at 5-30 min and decreased 60 min after RA treatment (Fig. 2c), in parallel to the activation of p38MAPK/MSK1. This increase in RARα phosphorylation concerned S369 (Fig. 2d). It was inhibited upon knockdown of MSK1 (Fig. 2e) and did not occur in SKBR3 cells, which are defective in MSK1 activation (Fig. 2d), highlighting the importance of the initial RA activation of MSK1 in RARα phosphorylation at S369.

***In vivo*, S369 phosphorylation directs RARα interaction with TFIIH and thereby S77 phosphorylation.**

RARα was also rapidly phosphorylated at S77 after RA addition (Fig. 2d). Unexpectedly, though S77 was not a target for MSK1, phosphorylation of this residue was inhibited by SB203580 and by siRNA against MSK1 (Fig. 2e and data not shown). Therefore, we wondered if phosphorylation of S77 might be controled by that of S369.

MEF expressing RARα either WT, S77A or S369A, as the sole RA-dependent transcriptional activator in a $(RAR\alpha,\beta,\gamma)^{-/-}$ background were generated and compared for RARα phosphorylation at 5 min intervals following RA addition. In each rescue cell line,

MEF(RARαWT), MEF(RARαS369A) and MEF(RARαS77A), the RARα protein was expressed at similar amounts (Fig. 2f) and the p38MAPK/MSK1 pathway was similarly activated in response to RA (Supplemental information, Fig. S1a). In RA-treated MEF(RARαWT), the amount of RARα phosphorylated at S369 and S77 also increased rapidly at 5-15 min (Fig. 2g, lanes 1-4), and was abrogated upon MSK1 knockdown (data not shown). In MEF(RARαS369A) (Fig. 2g, lanes 5-8) and MEF(RARαS77A) (Fig. 2g, lanes 9-12), no signal was obtained by immunoblotting with the antibodies recognizing specifically RARα phosphorylated at S369 and S77 respectively, validating their specificity. In MEF(RARαS77A), the ability of RARα to be phosphorylated at S369 was not affected, while in MEF(RARαS369A), RARα was not phosphorylated at S77. This indicates that phosphorylation of S369 controls that of S77 and not vice-versa.

S369 is located in the LBD, in Loop 9-10 in close proximity to the binding domain of cyclin H (Loop 8-9 and the beginning of H9) [24], which forms with cdk7 and MAT1 the CAK subcomplex of TFIIH that phosphorylates RARα at S77 [11,23]. Therefore we hypothesized that, *in vivo*, the RA-induced phosphorylation of S369 would be the signal allowing the interaction of RARα with TFIIH and subsequently phosphorylation of S77 by cdk7.

With that aim, RARα phosphorylation and interaction with TFIIH were analyzed in ChIP-Western experiments performed with the MEF(RARαWT), MEF(RARαS369A) or MEF(RARαS77A) rescue lines, at 5 min intervals following RA addition (Fig. 2h). In the absence of RA, immunoprecipitated RARαWT was not phosphorylated and did not retain TFIIH (lane 1). As soon as 5 min following RA addition, RARαWT became phosphorylated at S369. Then, at 10 min, RARα interacted with TFIIH and became phosphorylated at S77. RARα phosphorylation and interaction with TFIIH were maintained up to 50 min and then decreased at 60 min. RARαS77A retained the ability to be phosphorylated at S369 and to

recruit TFIIH. In contrast, RARαS369A was unable to interact with TFIIH and was not phosphorylated at S77, indicating that, *in vivo*, phosphorylation of S369 promotes TFIIH binding and thereby S77 phosphorylation. Similar results were obtained in immunoprecipitation experiments performed with cyclin H antibodies (Supplemental information, Fig.S1b).

The integrity of the p38MAPK/MSK1 pathway and of the RARα phosphorylation sites are required for the RA induction of RARα-target genes.

We next asked whether activation of MSK1 is significant for the activation of RARα-target genes. In MCF7 cells and MEFs, RA treatment enhances transcription of the *Cyp26A1* gene which is the paradigm of the RA-target genes, as assessed by qRT-PCR (Fig. 3a,b). No activation of *Cyp26A1* was observed upon knockdown of MSK1 (Fig. 3c,d) or in SKBR3 cells which are refractory to MSK1 activation (Fig. 3b) highlighting the significance of MSK1 activation for RA induction of RARα-target genes.

The RA-induced activation of CYP26A1 was also abrogated in MEFs in which all RARs have been deleted, but was reestablished upon reintroduction of RARαWT (Fig. 3a). However, the RA response was not restored upon reintroduction of the RARαS369A and RARαS77A mutants (Fig.3a), indicating that phosphorylation of S77 and S369 is also required for RARα-mediated transcription.

Upon RA treatment, RARα is recruited with TFIIH to the promoter of the *Cyp26A1* gene.

Next we used chromatin immunoprecipitation (ChIP) experiments to gain mechanistic insight into the role of MSK1 activation and/or RARα phosphorylation in the activation of Cyp26A1 expression. The *CYP26A1* gene promoter contains two functional DR5 RAREs, a

proximal one (R1) and a more distal one (R2) (Fig. 4a), which work synergistically *in vivo*, to provide a maximal response to RA [25,26]. We assessed the RARα occupancy of these promoter regions in ChIP experiments performed at 5 min intervals, up to 1 h, after RA addition to MEF(RARαWT). The specificity of our experimental conditions was checked in the absence of antibodies and with the promoter of the control 36B4 gene, which does not contain any RARE.

The proximal promoter region of Cyp26A1, which encompasses the DR5 R1, the TATA box and the transcription start site, was already occupied to some extent by RARα in the absence of RA (Table 1). Then the level of DR5 R1 bound to RARα started to increase at 30 min after RA addition (Fig. 4c), peaked at 40-55 min, decreased by 60 min and came back close to the initial values at 2h. No additional variations were detected up to 16 h (Supplemental information, Fig. S1c). In contrast, at the distal R2 region, RARα was hardly detected in the absence of RA (Table 1), but was markedly recruited following RA addition with a peak at 40-55 min (Fig. 4b and Supplemental information, Fig. S1c).

It is interesting to note that the corepressor SMRT was bound to R1 in the absence of RA, in line with the presence of RARα in this region, and dissociated rapidly after RA addition (Fig. 4c). However SMRT could not be detected at R2 consistent with the absence of RARα (Fig. 4b).

As RARα binds TFIIH after RA addition, we also examined the recruitment of TFIIH in ChIP experiments performed with antibodies against its cyclin H, cdk7 or XPB subunits. In the absence of RA, the three TFIIH subunits already occupied R1, but could not be significantly detected at R2 (Table 1 and Fig. 4d,e). In response to RA, the levels of R1 and R2 bound to TFIIH increased markeddly and reached a peak at 40-50 min (Fig. 4d,e), which was concomitant with that of RARα. To show that TFIIH and RARα are present in the same complex on the promoter, MEF(RARαWT) were subjected to sequential ChIP (reChIP)

experiments, first with anti-RARα and then with anti-TFIIH antibodies. Both R1 and R2 regions were specifically enriched at 45 min (Fig. 4f,g, lanes 5), demonstrating that TFIIH and RARα form a complex on chromatin in a RA-dependent manner. Finally, ChIP experiments were performed with antibodies recognizing specifically RARα phosphorylated at S77, the cdk7 site. At 45 min following RA addition, RARα recruited to the R1 and R2 regions of the CYP26A1 promoter was phosphorylated at S77 (Fig. 4f,g, lanes 6). Altogether, these results indicate that RARα bound at the promoter is associated with TFIIH and phosphorylated at S77. Note that RNA polII was also recruited to R1 and R2 in parallel to RARα and TFIIH (Fig. 4b,c).

The phosphorylation cascade initiated at S369 and ending at S77 controls the recruitment of both RARα and TFIIH to the Cyp26A1 promoter.

Then we investigated whether phosphorylation is required for promoter recruitment of RARα, in ChIP experiments performed with MEF(RARαS369A) and MEF(RARαS77A) (Fig. 4h,i). RARαS369A which does not interact with TFIIH and therefore cannot be phosphorylated at S77 in response to RA, was not recruited to R1 and R2. RARαS77A which has retained the ability to be phosphorylated at S369 and to interact with TFIIH was also not recruited. In both cases, TFIIH was not recruited either (Fig. 4j,k). From these results and those above, one can conclude that the last step of the phosporylation cascade, i.e. S77 phosphorylation is required for the promoter recruitment of RARα-TFIIH complexes.

Activated MSK1 is also recruited to the Cyp26A1 promoter and phosphorylates Histones H3 at S10.

Next we investigated whether MSK1 is recruited to the Cyp26A1 promoter after RA addition and whether it phosphorylates histones H3 at S10 (H3S10p), a hallmark of gene

transcription activation [22]. ChIP assays performed with antibodies specific for phosphorylated MSK1 showed that, after RA addition to MEF(RARαWT), the activated kinase is recruited to the R1 and R2 regions (Fig. 5b,c), concomitantly with RARα. H3 was phosphorylated at S10 in the same time slot as assessed in ChIPs with antibodies against H3S10p (Fig. 5b,c). Histones H4 were also acetylated at K5, K8 and K12 (Fig. 5b,c). Note that in the context of the R1 region, an additional peak of H4 acetylation was observed at 20-25 min (Fig. 5c), probably reflecting the recruitment of histone acetylases by RARα already bound to this region. Knockdown of MSK1 reduced significantly the accumulation of active MSK1 and H3S10 phosphorylation on both R1 and R2, (Fig. 5d,e), confirming that the RA-induced phosphorylation of H3 is executed by MSK1 and suggesting an additional role for MSK1 in the activation of RARα-target promoters through histone phosphorylation. Interestingly it also blocked the recruitment of RARα and TFIIH (Fig. 5f,g). Moreover, in SKBR3 cells which are defective in MSK1 activation compared to MCF7 cells, RARα and TFIIH recruitment to R2 and R1 remained low and unchanged, irrespective of the RA treatment duration (Fig. 5h,i).

Phosphorylated RARα controls the formation of a bridge between the R1 and R2 regions of the Cyp26A1 promoter.

According to several recent studies, DNA looping may juxtapose promoter DNA with enhancer elements that lie far upstream [27]. Given that, in response to RA, RARα is recruited to both the R1 and R2 regions of the CYP26A1 promoter, in association with TFIIH and RNA PolII, we raised the hypothesis that RARα would bridge R1 and R2 to form an enhanceosome. To demonstrate the existence of such a gene looping, we performed ChIP-loop assays [28], which combine Chromosome Conformation Capture (3C) with chromatin immunoprecipitation (Fig. 6a).

In MEF(RARαWT) RA-treated for 50 min, PCR amplification of non digested immunoprecipitated material with primers corresponding to R1 and R2 (Fig. 6a2), produced a 1930 bp fragment (Fig. 6b, lane 2). Upon digestion with PvuII, which cuts twice between R1 and R2 (Fig. 6a4), a new 1300 bp band was generated (Fig. 6b, lanes 8-15), which represents loss of a 630 bp fragment and religation of the R1 and R2 regions that remained in close proximity due to bridging complexes. Religation was confirmed by PCR amplification with primers on both sides of the junction that generated a 360 bp fragment instead of a 1000 bp one (Fig.6b, lower panel). The 1300 and 360 bp fragments were detected at 30 min up to 60-75 min following RA addition (Fig. 6b,c), when RARα was recruited to both R1 and R2 in ChIP experiments (Fig. 4). Similar results were obtained when immunoprecipitation was performed with TFIIH or RNA polII antibodies (Fig.6c). Collectively, these results indicate that the R1 and R2 regions become juxtaposed in response to RA, through the recruitment of complexes involving RARα as well as TFIIH and RNA PolII.

In contrast, in MEF(RARαS77A), only a transient and delayed bridge was detected when ChIP Loop experiments were performed with RARα or TFIIH antibodies. No bridging occured when immunoprecipitation was performed with RNA polII antibodies (Fig. 6b, lanes 16-28 and Fig. 6c, lanes 10-18). Collectively, such results indicate that efficient looping relies on the promoter recruitment of the phosphorylated form of RARα.

Activated MSK1 and RARα phosphorylation also control the RA induction of other target genes promoters such as the *RARβ2* gene.

In MEFs, other well known RA-target genes such as the *RARβ2* gene are also inhibited upon knockdown of MSK1 or knockout of the three RARs. The RA induction of *RARβ2* was reestablished upon reexpression of RARαWT, but not of RARαS77A or RARαS369A (Supplementary information, Fig. S1a). The *RARβ2* gene promoter contains only one DR5

RARE at position -60 [29] and ChIP experiments (Supplemental information, Supplemental Table 1 and Fig. S1) gave results that were very similar to those obtained above for the proximal R1 region of the Cyp26A1 promoter. Indeed, prior to RA treatment, the DR5 region was already occupied by RARα, TFIIH, and the corepressor SMRT. After RA addition, the corepressor SMRT dissociated rapidly but the levels of DR5 region bound to RARα, TFIIH and RNA polII increased markedly with a peak at 45-55 min. DNA-recruited RARα was associated with TFIIH and phosphorylated at S77, and mutation of the phosphorylation sites abrogated the recruitment of RARα-TFIIH complexes. Phosphorylated MSK1 was also recruited to the promoter which became phosphorylated at H3S10 and acetylated at H4K5;8;12. Finally knockdown of MSK1 abrogated H3S10 phosphorylation and the recruitment of RARα and TFIIH. These results confirm that MSK1 is the upstream signal promoting phosphorylation cascades targeting H3S10 and RARα and subsequently the recruitment of RARα-TFIIH complexes to promoters.

DISCUSSION

In this manuscript, we demonstrated that the activation of the p38MAPK pathway by RA [15,17] is required for the induction of RARα target genes. We outlined a new paradigm in which, *in vivo*, RA induces a phosphorylation cascade, starting with the rapid activation of p38MAPK, leading to MSK1 activation, phosphorylation of RARα and Histone H3 and finally to promoter recruitment of RARα and the transcription machinery (Fig. 7). To our knowledge, this is the first report of a coordinated phosphorylation cascade induced by RA and coupled to promoter activation. It was unexpected and shed light on a new level of complexity in the fine-tuning of RARα-mediated transcription.

The mechanism of activation of p38MAPK/MSK1 is out of the topic of this report, but involves very likely a non-genomic activation event [30], similar to that described for steroid receptors [22,31] and requiring liganded RARα. One of the targets of MSK1 is RARα which becomes rapidly phosphorylated at S369 located in the LBD and we found that phosphorylation of this residue is critical for the recruitment of TFIIH and the subsequent phosphorylation of the NTD at S77 by cdk7. Given that S369 is located in close proximity of the cyclin H binding domain[24], we propose that, *in vivo*, phosphorylation of this residue by MSK1 propagates a signal to the cyclin H binding surface, allowing the recruitment of CAK within TFIIH, in line with our previous *in vitro* results [32]. Ultimately, the consequence of the last step of this cascade, i.e. S77 phosphorylation, is to anchor RARα-TFIIH complexes to target promoters. Additional investigations are required to determine how S77 phosphorylation affects the binding of RARα to response elements. Interestingly, in the context of a promoter with two RAREs, a proximal and a more distal one, as exemplified with the *Cyp26A1* promoter, phosphorylated RARα bridges both RAREs, in association with TFIIH and RNA PolII. Whether other chromatin remodeling and/or transcription factors are also involved to form an enhanceosome [27] awaits further studies.

The other novelty of this study is that MSK1 is recruited to RARα-target promoters in the active phosphorylated form, leading to increased phosphorylation of S10 on H3. A similar mechanism was recently reported for the regulation of other nuclear receptor target genes [22]. Then the question is how MSK1-mediated phosphorylation of H3 contributes to RARα-target genes induction. One possibility is that phosphorylated H3S10 is a chromatin mark accounting in cooperation with other histones modifications for the dissociation of repressive complexes and/or the recruitment of chromatin-remodelling complexes [22]. Finally, how MSK1 is recruited to RA-target promoters and whether MSK1 phosphorylates other relevant targets will require further investigations

An unexpected finding in this study is that RARα-TFIIH complexes started to be recruited to target promoters only at 30 min after RA addition, while phosphorylation and interaction with TFIIH occurred as soon as 5 min. Such a discrepancy suggests that the promoters are not accessible yet and that other RA-dependent events such as chromatin modifications and remodelling are required. In line with such an hypothesis, our results show that histones H4 become acetylated during the first 30 min (Fig. 5). Also unexpected, is that the recruitment of RARα-TFIIH complexes was transient and occurred in a narrow time slot (from 30 to 60-75 min after RA addition). This might be the basis of the absence of variations in the RARα occupancy of the RARβ2 promoter observed by others [33,34]. The RA-induced phosphorylation cascade targeting RARα and H3 was also transient, suggesting the existence of phosphatases that would antagonize the functions of chromatin-tethered kinases [21], MSK1 and cdk7 within TFIIH.

Finally, the present study demonstrated that in ERBB2 positive human breast cancer SKBR3 cells, the p38MAPK/MSK1 pathway is deregulated, compared to the control MCF7 cells. Consequently, the phosphorylation cascade and RARα-mediated transcription are significantly blunted. Whether the deregulation of the p38MAPK/MSK1 pathway reflects the

overexpression of the upstream ERBB2/HER2 oncogene [35,36] awaits further studies. Nevertheless it raises the issue of a contribution of the p38MAPK/MSK1 pathway in RA-induced growth arrest [37] and its specific modulation might be a promising therapeutic strategy in the treatment of RA-resistant cancers. Given that the S369 phosphorylation site is shared by MSK1 and PKA, our data are also particularly attractive in terms of the synergy between RA and cAMP in the treatment of many leukemia cells [38].

METHODS

Materials

RA was from Sigma Aldrich Corporation (USA). The synthetic retinoid agonists and antagonists were as described [12]. Rabbit polyclonal antibodies against RARα, RPα(F), and mouse monoclonal antibodies recognizing cyclin H and RARα phosphorylated at S77 or S369 were previously described [23,24,32]. Monoclonal antibodies against RNA polII phosphorylated at S5 in the carboxy terminal repeat domain, cyclin H and acetylated Histones (H4K5;12 and H4K5;8;12) were produced by the IGBMC facility. Antibodies against MSK1 and β-actin (C11) were purchased from Santa Cruz Biotechnology as well as Polyclonal antibodies against RARα (C-20), SMRT (H-300) and the XPB (S-19) and cdk7 (C-19) subunits of TFIIH used in the ChIP experiments. Antibodies against P-MSK1 and H3S10p were from Cell Signaling and Upstate Biotechnologies Incorporated (Lake Placid, NY, USA), respectively.

Cell lines and immunoblotting.

Mouse embryo fibroblasts with all three RARs deleted, MEF (RARα,β,γ)$^{-/-}$, were as described [18]. RARα WT, S369A or S77A cDNAs sub cloned from the pSG5 constructs [32] into pMSCV Neo plasmid, were reintroduced into these cells by retroviral transfer followed by G418 selection. MCF7 and SKBR3 human breast cancer cells were cultured under standard conditions. All cell lines were treated with RA (10^{-7}M) after 24 hr in medium containing 1% dextran-charcoal-treated FCS. Extracts were prepared and immunoblotted as described [39].

RNA isolation and quantitative RT-PCR (qRT-PCR).

Total RNAs were isolated and subjected to qRT-PCR as described [39]. Primer sequences are as follows. Mouse 36B4, 5'-GAGGTCACTGTGCCAGCTCA-3' and 5'-

GAAGGTGTACTCAGTCTCCA-3'; Mouse CYP26A1, 5'-GGGCTTACTTTGCAAGAGCA-3', and 5'-GAAGGCCTCCTCCAAATGGA-3'; Human 36B4, 5'-GAAGTCACTGTGCCAGCCCA-3' and 5'-GAAGGTGTAATCCGTCTCCA-3'; Human CYP26A1, 5'-TCCAGAAAGTGCGAGAAGAG-3' and 5'-TCTTCAGAGCAACCCGAAAC-3'.

Chromatin immunoprecipitation (ChIPs), reChIPs and ChIP-western experiments

Subconfluent cells were treated with RA (10^{-7}M) and ChIP experiments were performed according to the protocol described by Upstate. Control ChIP was performed without antibody. In Re-ChIP experiments, complexes were eluted by incubation for 30 min at 37°C in 250 µl 95mM NaHCO3 containing 1% SDS, diluted 20 times with Re-ChIP buffer (1% Triton X-100, 50 mM Tris-HCl, [pH 7.5], 2mM EDTA, 150 mM NaCl) and subjected again to the ChIP procedure. Immunoprecipitated DNA was amplified by qPCR using primer pairs designed using the Primer3 software [40]: Mouse 36B4, 5'-TTTGCTGTACT GACTCGGTGA-3' and 5'-CCTCCCACAACAAAACAACC-3'. Mouse Cyp26A1: R2, 5'-AAACAGGAGCAGGCTGAACT-3' and 5'-CGCTGCCACTGTCATATCTT-3'; R1, 5'-GGTAACTCGGAGCTCTGCAC-3' and 5'-CCAGGTTACTGCCCACGTTA-3'; Human 36B4, 5'-AGGACTCCATGTTCCCAAAG-3' and 5'-CGCAGCCAATAGACAGGAG-3'; Human Cyp26A1: R1, 5'-GCGGAACAAACGGTTAAAGA-3' and 5'-GCAGTACAGGTCCCAGAGCTT-3'; R2, 5'-GAGTTCACTCGATGTCACG-3' and 5'- ATCGCGCTGGAGGTAATTCT-3'.

Primers amplification specificity and efficiency were verified on DNA serial dilutions. Occupancy of the promoters was calculated by normalizing the PCR signals from the IP samples to the signals obtained from the input DNA.

For ChIP-Wersten experiments, the precipitated chromatin complexes were proceeded as described [41] and bound proteins were revealed by immunoblotting.

ChIP loop assay

Cells cross-linked with formaldehyde were digested with PvuII (New England Biolabs) (600 U, overnight, 37°C), religated (4 h at 16°C using T4 DNA ligase) as described in [27] and immunoprecipitated with RARα, TFIIH (XPB subunit) or RNA PolII antibodies. Ligation frequencies of restriction fragments were analyzed by qPCR with the following primer pairs. Primers 1 and 2 (R1 & R2), 5'-AGGTCTCACGATCGATCGGC-3' and 5'-ACGCAGTACAGGTCCCAGAG-3'; Primers 3 and 4 (junction), 5'-TATTGCAAGGGTTACCGGAC-3' and 5'-GTAGTAATACCGCCAAGCCG-3'.

***In vitro* RARα phosphorylation and detection of Phosphorylated RARα and p38MAPK.**

In vitro phosphorylation of purified recombinant RARα expressed in *E. coli* by the purified CAK complex [24] or recombinant active MSK1 (Upstate Biotechnology Incorporated, Lake Placid, NY, USA) was performed as previously described [11] followed by immunoblotting with antibodies recognizing RARα or RARα phosphorylated at S77 or S369.

For detection of *in vivo* phosphorylated RARα, cell extracts were prepared and applied to Phosphoprotein Affinity purification columns (PhosphoProtein Purification System, Qiagen S.A.) according to manufacturer-supplied instructions. After washing, column eluates containing protein peaks were concentrated and analyzed by immunoblotting. Phosphorylated $p38^{MAPK}$ was detected by using a Phospho $p38^{MAPK}$ (Thr(P)-180/Tyr(P)-182) ELISA kit (Biosource Invitrogen Corporation).

Short Interfering (si) RNA.

The smartpool small interfering RNA against human MSK1 (M-004665-01), mouse MSK1 (L-040751-00) or mouse p38MAPK (M-040125-00) were purchased from Dharmacon (Lafayette, CO) as well as the control non targeting siRNA pool (D-OO1206-13). SiRNAs (50 nM) were transfected by using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Then cells were vehicle- or RA-treated 48h post transfection.

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FIGURE LEGENDS

Figure 1. *In vivo,* RA activates the p38MAPK/MSK1 pathway.

(a) Analysis of p38MAPK activation in MEF WT treated with RA (10^{-7}M), synthetic retinoids specific for RARα (BMS 753, 10^{-7}M), RARγ (BMS 961, 10^{-7}M), RARβ (BMS 493, 10^{-7}M) or a RARα antagonist (BMS 614, 10^{-6}M). The results are an average of three experiments that agreed within 15%. **(b)** Same experiments with MEFs WT, RAR (α,β,γ)–/– or reexpressing RARαWT in a RAR(α,β,γ) nul background. Western blots show the expression of RARα in the different cell lines (right panel). **(c)** p38MAPK activation in MCF7 and SKBR3 cells treated with RA (10^{-7}M). **(d)** Immunoblotting analysis of active/phosphorylated MSK1 in extracts from RA-treated MCF7 cells applied onto Phosphoprotein Affinity columns. **(e)** Comparison of MSK1 activation/phosphorylation in RA-treated MEFs, MCF7 and SKBR3 cells **(f)** Knockdown of p38MAPK with siRNAs abrogates the activation of MSK1 in RA-treated MEFs.

Figure 2. RA activated MSK1 phosphorylates RARα and induces interaction with and phosphorylation by CAK within TFIIH.

(a) Schematic representation of the RARα1 protein with the functional domains and phosphorylation sites. **(b)** *In vitro* phosphorylation of purified bacterially expressed RARα with highly purified CAK and active recombinant MSK1, was analyzed by immunoblotting with antibodies recognizing RARα phosphorylated at S369 or S77. **(c)** Kinetics of RARα phosphorylation in RA-treated MCF7 cells after Phosphoprotein Affinity purification. **(d)** Immunoblots showing after Phosphoprotein Affinity purification, that RA induces RARα phosphorylation at S369 and S77 in MCF7 cells but not in SKBR3 cells. **(e)** Knockdown of MSK1 abrogates the RA-induced phosphorylation of RARα in MCF7 cells. **(f)** Immunoblots showing the reexpression of RARαWT, S369A and S77A in MEF(RARα,β,γ)$^{-/-}$. **(g)** RARα

phosphorylation in extracts from MEF(RARαWT), (RARαS369A) and (RARαS77A) after Phosphoprotein Affinity purification. **(h)** ChIP-Western experiments performed with MEF(RARαWT), MEF(RARαS77A) and MEF(RARαS369), treated with RA for the indicated times. The complexes immunoprecipitated with RARα antibodies were analyzed by immunoblotting as indicated.

Figure 3. MSK1 is required for the RA-induced activation of the *Cyp26A1* gene.

(a) The RA-induced expression of the *CYP26A1* gene which is abolished in MEF(RARα,β,γ)-/- , is rescued upon reexpression of RARαWT, but not of RARαS369A or S77A, as monitored by qRT-PCR. **(b)** The *CYP26A1* gene is activated in MCF7 but not in SKBR3 cells in response to RA, in qRT-PCR experiments. **(c, d)** Knockdown of MSK1 inhibits the RA-induced expression of CYP26A1 in MEFs and MCF7 cells

Figure 4. Phosphorylation controls the recruitment of RARα and TFIIH to the *CYP26A1* gene promoter.

(a) Schematic representation of the promoter regions of the *CYP26A1* gene, with the primer pairs used for qPCR amplification. **(b, c)** Kinetic ChIP experiments performed with RA-treated MEF(RARαWT) and determining the recruitment of RARα, RNA PolII and SMRT to the R1 and R2 promoter regions. Values correspond to a representative experiment among 5. **(d, e)** Kinetic ChIP experiments determining the recruitment of the cdk7, cyclin H and XPB subunits of TFIIH to R1 and R2. **(f, g)** ChIP and reChIP experiments performed with the indicated antibodies, showing that, at 45 min following RA addition, DNA bound RARα is associated with TFIIH and phosphorylated at S77. Results are an average of 2 experiments that agreed within 20%. **(h-k)** Kinetic ChIP experiments determining the

recruitment of RARα and the XPB subunit of TFIIH at R1 and R2 in MEF(RARαWT), (RARαS369A) and (RARαS77A).

Figure 5. MSK1 is recruited to the Cyp26A1 promoter and phosphorylates histones.

(a) Schematic representation of the CYP26A1 promoter. **(b, c)** Kinetic ChIP experiments showing that after RA addition, P-MSK1 is recruited to R1 and R2 and that H3S10 become phosphorylated and H4K5;8;12 acetylated. **(d-g)** Knockdown of MSK1 with siRNAs abrogates H3S10 phosphorylation and the recruitement of RARα and TFIIH to R1 and R2. **(h, i)** Kinetic ChIP experiments showing the recruitment of RARα and the XPB subunit of TFIIH to R2 and R1 in RA-treated MCF7 and SKBR3 cells. Values correspond to a representative experiment among 3.

Figure 6. ChIP-Loop assay showing that RARα bridges the R1 and R2 regions of the CYP26A1 promoter.

(a) Schematic representation of the CYP26A1 promoter with R1 and R2, the PvuII restriction sites and the primers used to amplify the whole promoter (primers 1 and 2) or the junction (primers 3 and 4). **(b)** ChIP-Loop assay performed with MEF(RARαWT) and MEF(RARαS77A) showing that RARα phosphorylated at S77 controls the formation of a bridge between R1 and R2. Chromatin from control and RA-treated cells was immunoprecipitated with RARα antibodies without or with prior PvuII digestion-ligation of DNA, as indicated. Then qPCR was performed with primer pairs 1 and 2 (upper panels) or 3 and 4 (lower panels). **(c)** Qame as in (b) with RARα, TFIIH and RNA polII antibodies.

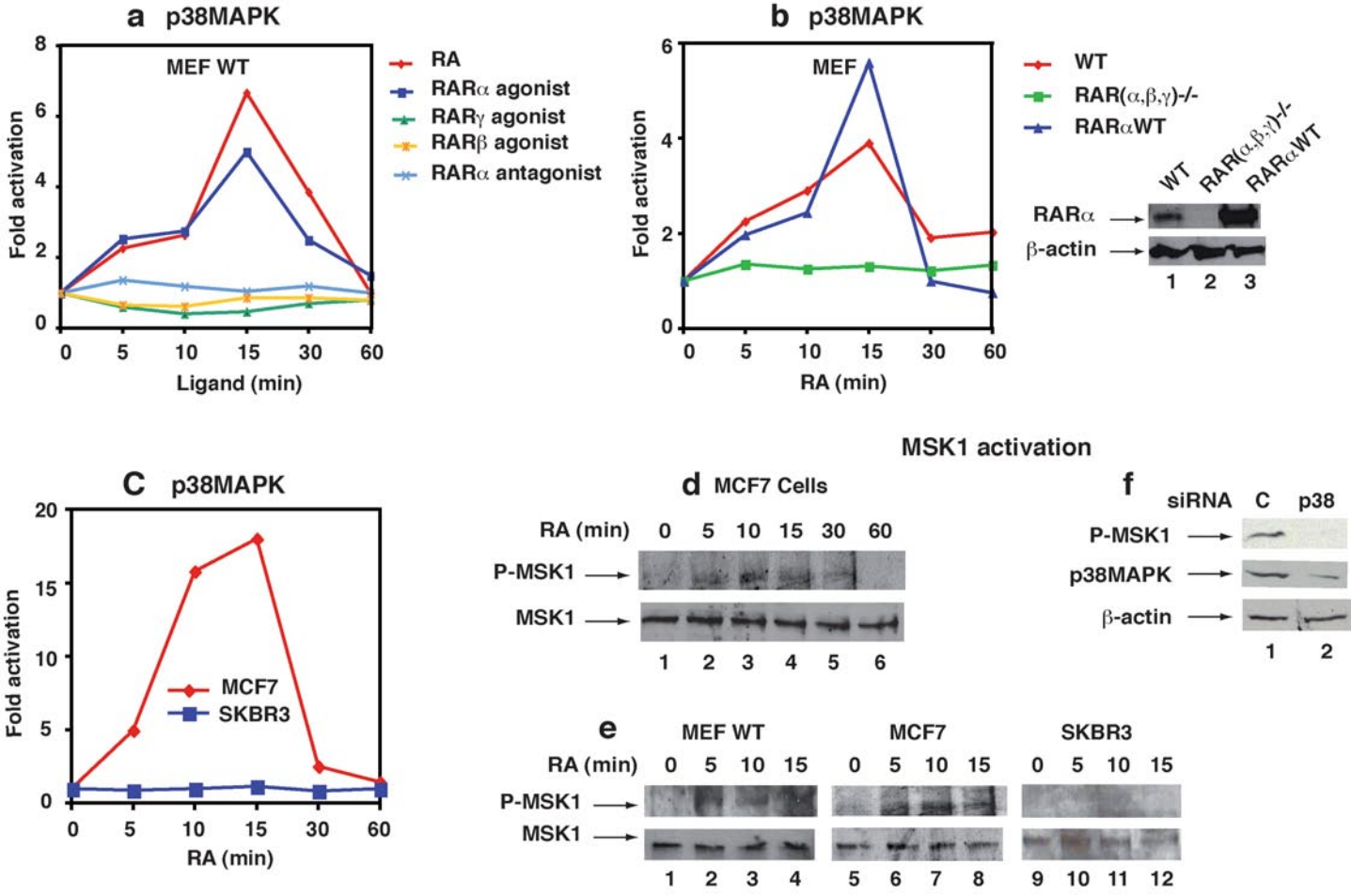
Figure 7. Recapitulation of the phosphorylation cascade induced by RA.

In response to RA, p38MAPK and the downstream protein kinase MSK1 are activated. MSK1 phosphorylates the LBD of RARα at S369, allowing the docking of cyclin H within TFIIH and the formation of a RARα/TFIIH complex. Then the cdk7 subunit of TFIIH phosphorylates the NTD of RARα at S77. MSK1 is also recruited and phosphorylates H3S10. Finally, RARα phosphorylated and associated with TFIIH is recruited to response elements located in the promoter of target genes.

TABLE 1

Chromatin immunoprecipitation experiments (ChIP) determining the basal recruitment of RARα and the XPB subunit of TFIIH to R1 and R2 in the different rescue MEF lines. Values (% of the inputs) are the mean ±SD of triplicates performed on three separate chromatin preparations. Data were analyzed using Microsoft Excel 2002. Unpaired Student's T test for pair-wise comparison was used to determine the statistically significant difference in RARα or TFIIH recruitment versus the 36B4 signals or the signals obtained in the absence of antibody. A p-value less than 0.5 (*) denotes a statistical significance.

MEF	**36B4**		**Cyp26A1-R2**		**Cyp26A1-R1**	
	Control	**Ab RARα**	**Control**	**Ab RARα**	**Control**	**Ab RARα**
RARα WT	0.024±0.003	0.045±0.012	0.036±0.010	0.103±0.021	0.036±0.001	0.807±0.277*****
RARαS369A	ND	ND	0.121±0.015	0.211±0.027	0.121±0.016	0.700±0.317*****
RARαS77A	ND	ND	0.036±0.003	0.075±0.006	0.036±0.003	0.273±0.175
	Control	**AbTFIIH**	**Control**	**AbTFIIH**	**Control**	**AbTFIIH**
RARαWT	0.156±0.012	0.121±0.009	0.146±0.049	0.131±0.032	0.161±0.081	0.659±0.122*****
RARαS369A	ND	ND	0.226±0.051	0.256±0.0339	0.266+0.09	0.831±0.336*****
RARαS77A	ND	ND	0.165±0.057	0.205±0.058	0.176±0.094	1.026+0.049*****


a p38MAPK
MEF WT
Fold activation
Ligand (min)
RA
RARα agonist
RARγ agonist
RARβ agonist
RARα antagonist
b p38MAPK
MEF
Fold activation
RA (min)
WT
RAR(α,β,γ)-/-
RARαWT
WT
RAR(α,β,γ)-/-
RARαWT
RARα
β-actin
1 2 3
C p38MAPK
Fold activation
MCF7
SKBR3
RA (min)
MSK1 activation
d MCF7 Cells
RA (min) 0 5 10 15 30 60
P-MSK1
MSK1
1 2 3 4 5 6
f
siRNA C p38
P-MSK1
p38MAPK
β-actin
1 2
e
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SKBR3
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P-MSK1
MSK1
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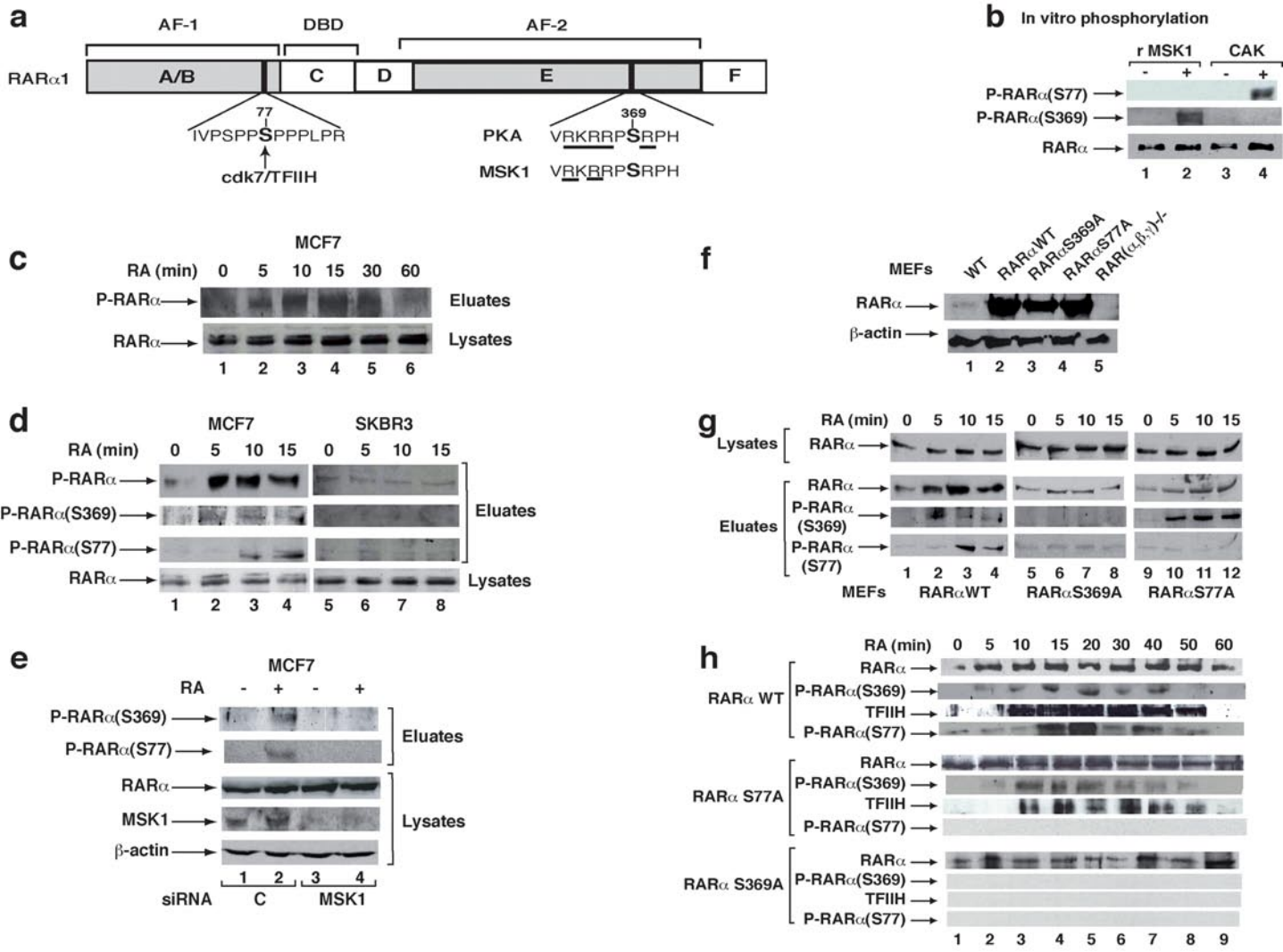
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DBD
AF-2
RARα1
A/B
C
D
E
F
77
IVPSPPSPPPLPR
cdk7/TFIIH
369
PKA
VRKRRPSRPH
MSK1
VRKRRPSRPH
b
In vitro phosphorylation
r MSK1
CAK
P-RARα(S77)
P-RARα(S369)
RARα
1 2 3 4
c
MCF7
RA (min) 0 5 10 15 30 60
P-RARα
RARα
Eluates
Lysates
1 2 3 4 5 6
d
MCF7
SKBR3
RA (min) 0 5 10 15 0 5 10 15
P-RARα
P-RARα(S369)
P-RARα(S77)
RARα
Eluates
Lysates
1 2 3 4 5 6 7 8
e
MCF7
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P-RARα(S369)
P-RARα(S77)
RARα
MSK1
β-actin
Eluates
Lysates
1 2 3 4
siRNA C MSK1
f
MEFs
WT
RARαWT
RARαS369A
RARαS77A
RAR(αβγ)-/-
RARα
β-actin
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RA (min) 0 5 10 15 0 5 10 15 0 5 10 15
Lysates
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P-RARα (S369)
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RA (min) 0 5 10 15 20 30 40 50 60
RARα WT
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P-RARα(S369)
TFIIH
P-RARα(S77)
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TFIIH
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TFIIH
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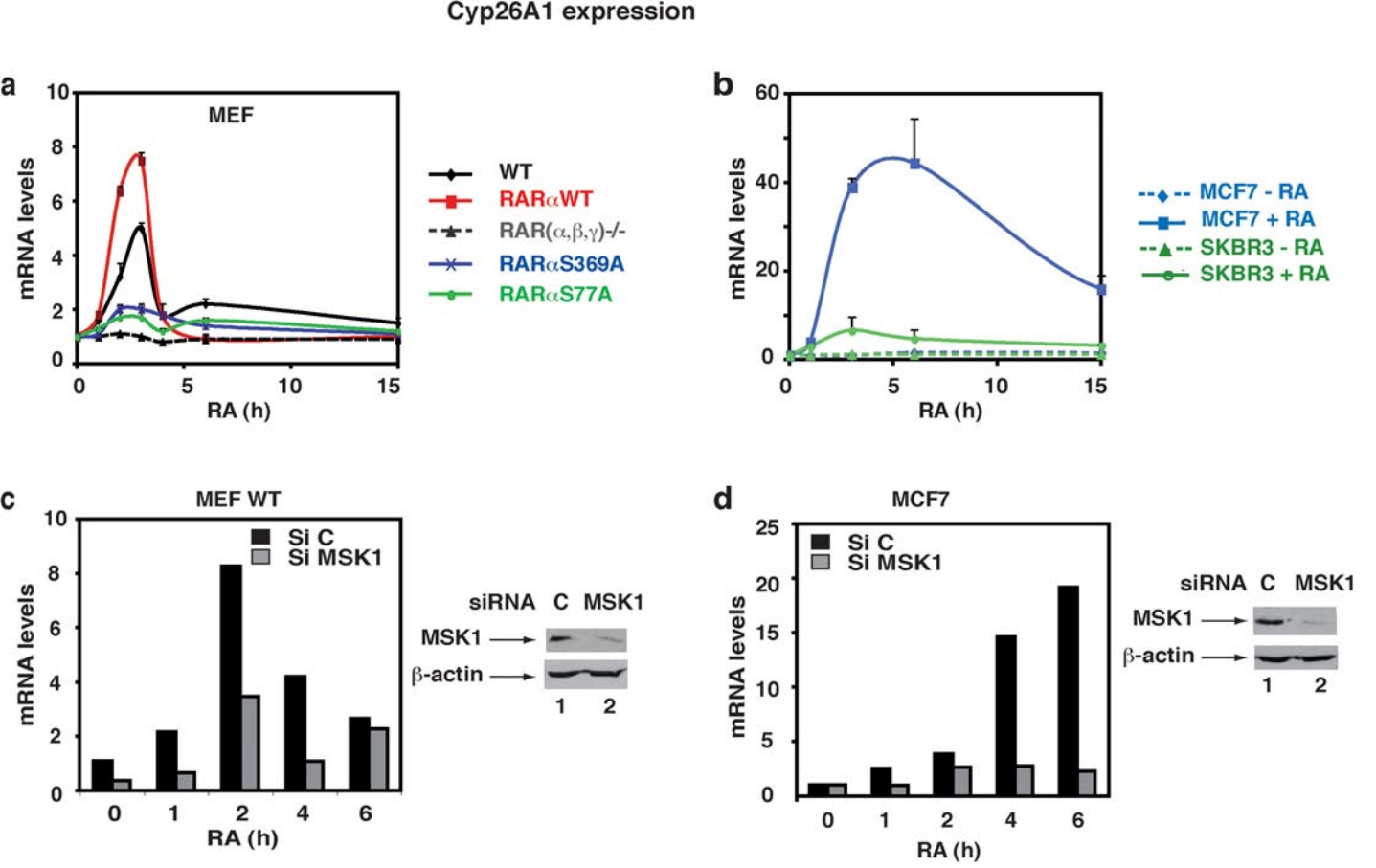
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mRNA levels
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WT
RARαWT
RAR(α,β,γ)-/-
RARαS369A
RARαS77A
b
mRNA levels
RA (h)
MCF7 - RA
MCF7 + RA
SKBR3 - RA
SKBR3 + RA
c
MEF WT
Si C
Si MSK1
mRNA levels
RA (h)
siRNA C MSK1
MSK1
β-actin
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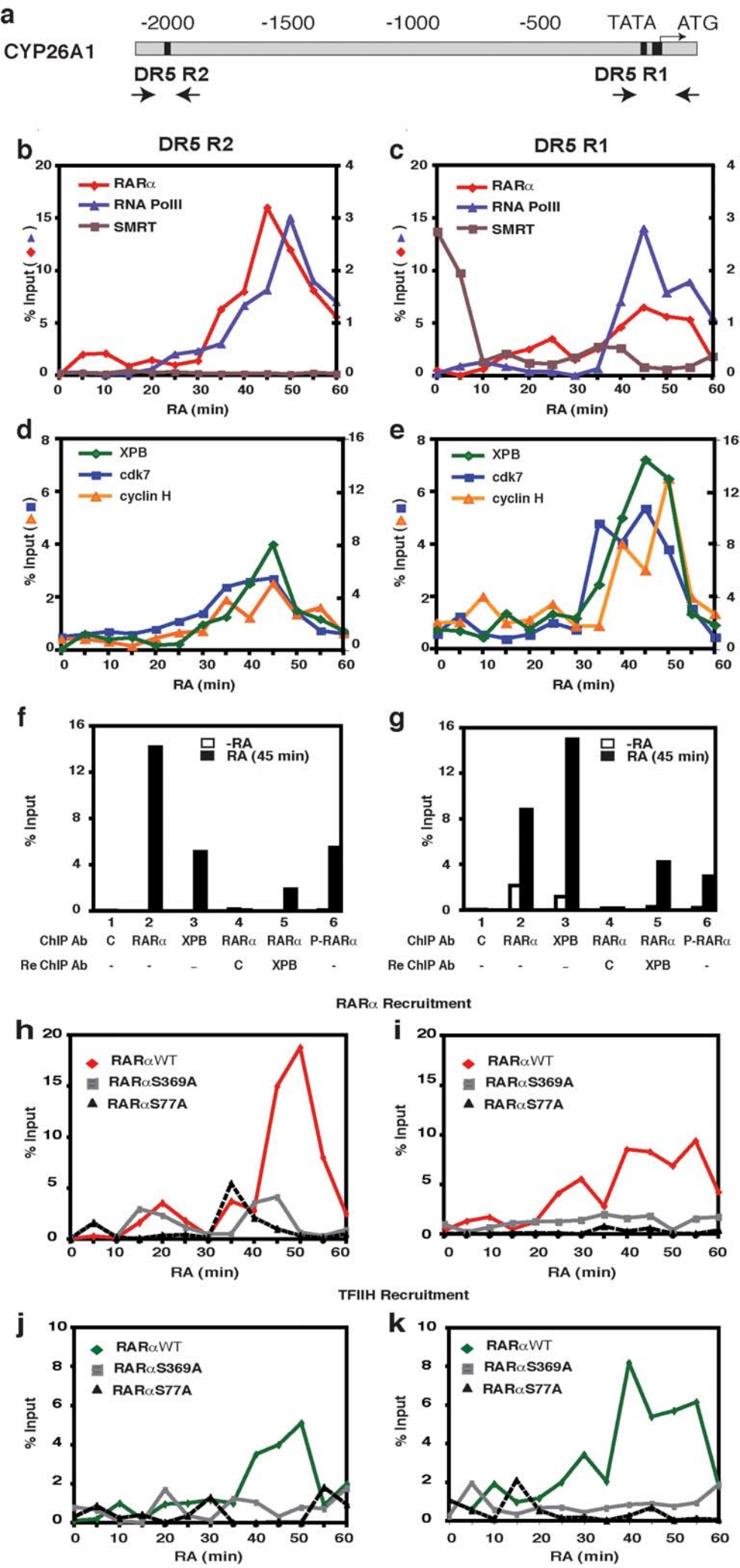
1 2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DR5 R1
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SMRT
% Input
RA (min)
c
DR5 R1
RARα
RNA PolII
SMRT
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XPB
cdk7
cyclin H
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RA (min)
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XPB
cdk7
cyclin H
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RA (min)
f
-RA
RA (45 min)
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Re ChIP Ab - - - C XPB -
g
-RA
RA (45 min)
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RARα Recruitment
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RARαWT
RARαS369A
RARαS77A
% Input
RA (min)
i
RARαWT
RARαS369A
RARαS77A
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RA (min)
TFIIH Recruitment
j
RARαWT
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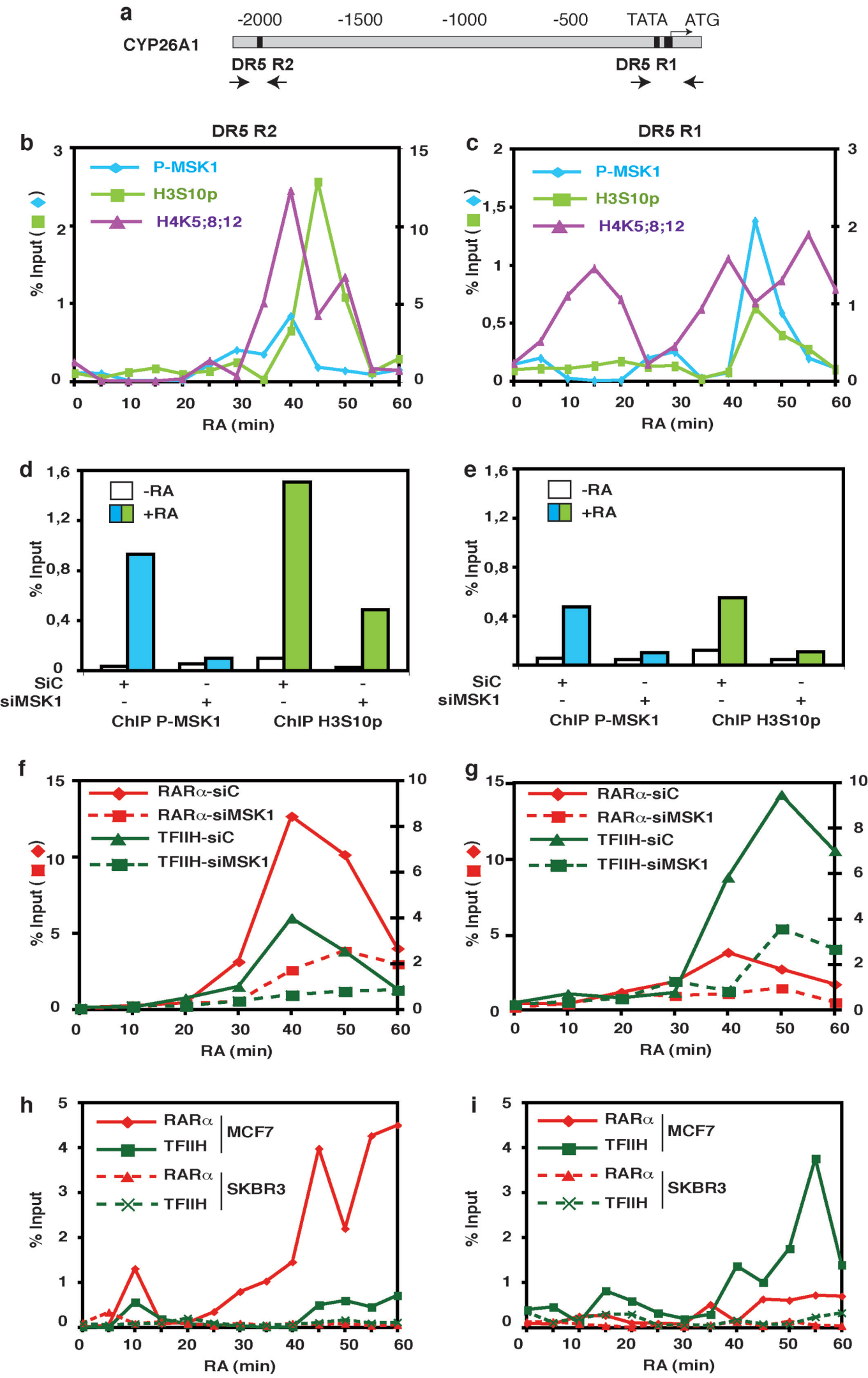
RA (min)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H3S10p
H4K5;8;12
% Input
RA (min)
c
DR5 R1
P-MSK1
H3S10p
H4K5;8;12
% Input
RA (min)
d
-RA
+RA
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SiC
siMSK1
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ChIP H3S10p
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TFIIH-siC
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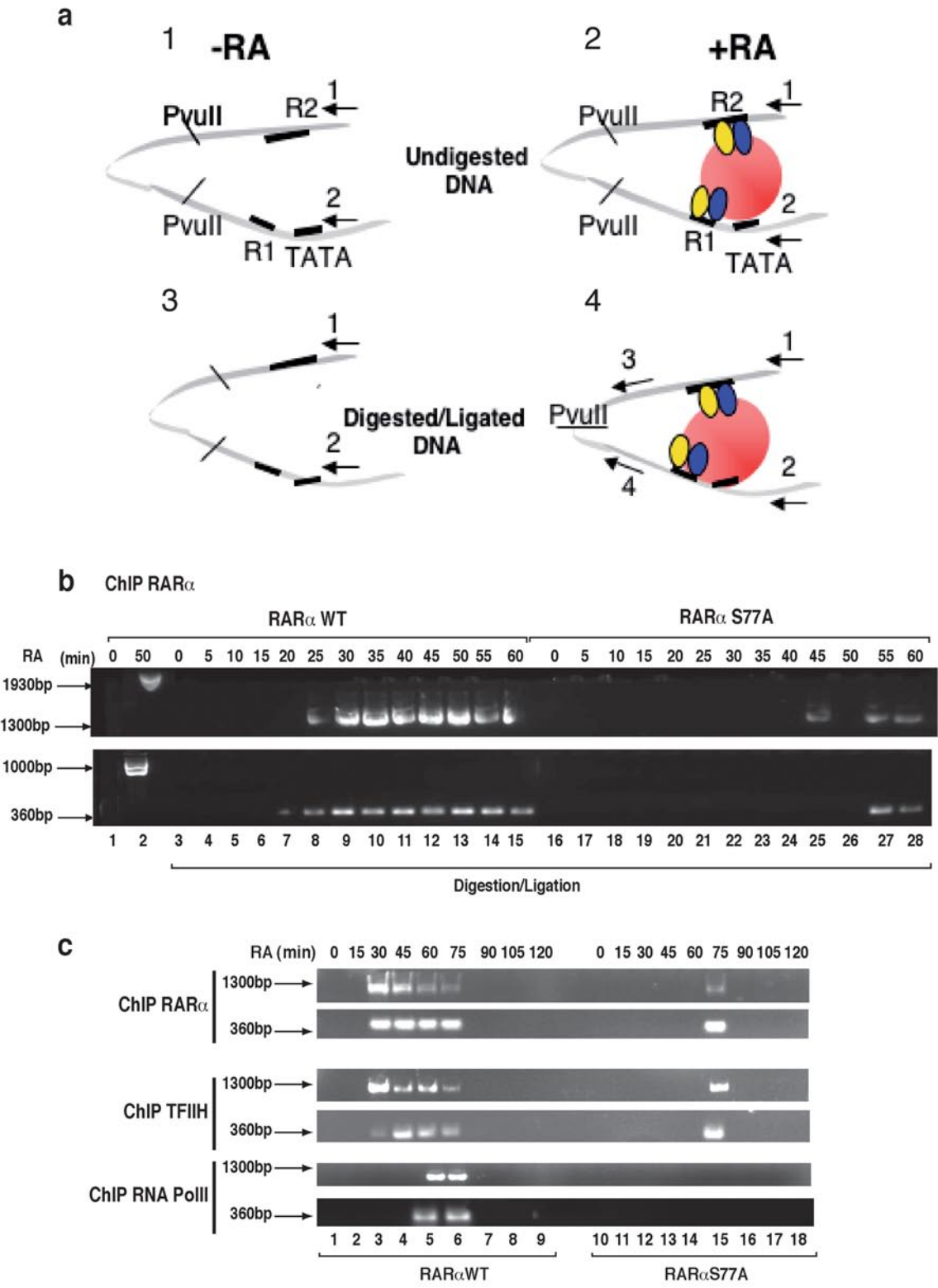
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2 +RA
PvuII
R2
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PvuII
R1 TATA
Undigested DNA
PvuII
R2
1
2
PvuII
R1
TATA
3
4
1
2
Digested/Ligated DNA
3
PvuII
1
2
4
b ChIP RARα
RARα WT
RARα S77A
RA (min) 0 50 0 5 10 15 20 25 30 35 40 45 50 55 60 0 5 10 15 20 25 30 35 40 45 50 55 60
1930bp
1300bp
1000bp
360bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
Digestion/Ligation
c
RA (min) 0 15 30 45 60 75 90 105 120 0 15 30 45 60 75 90 105 120
ChIP RARα
1300bp
360bp
ChIP TFIIH
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RARαWT
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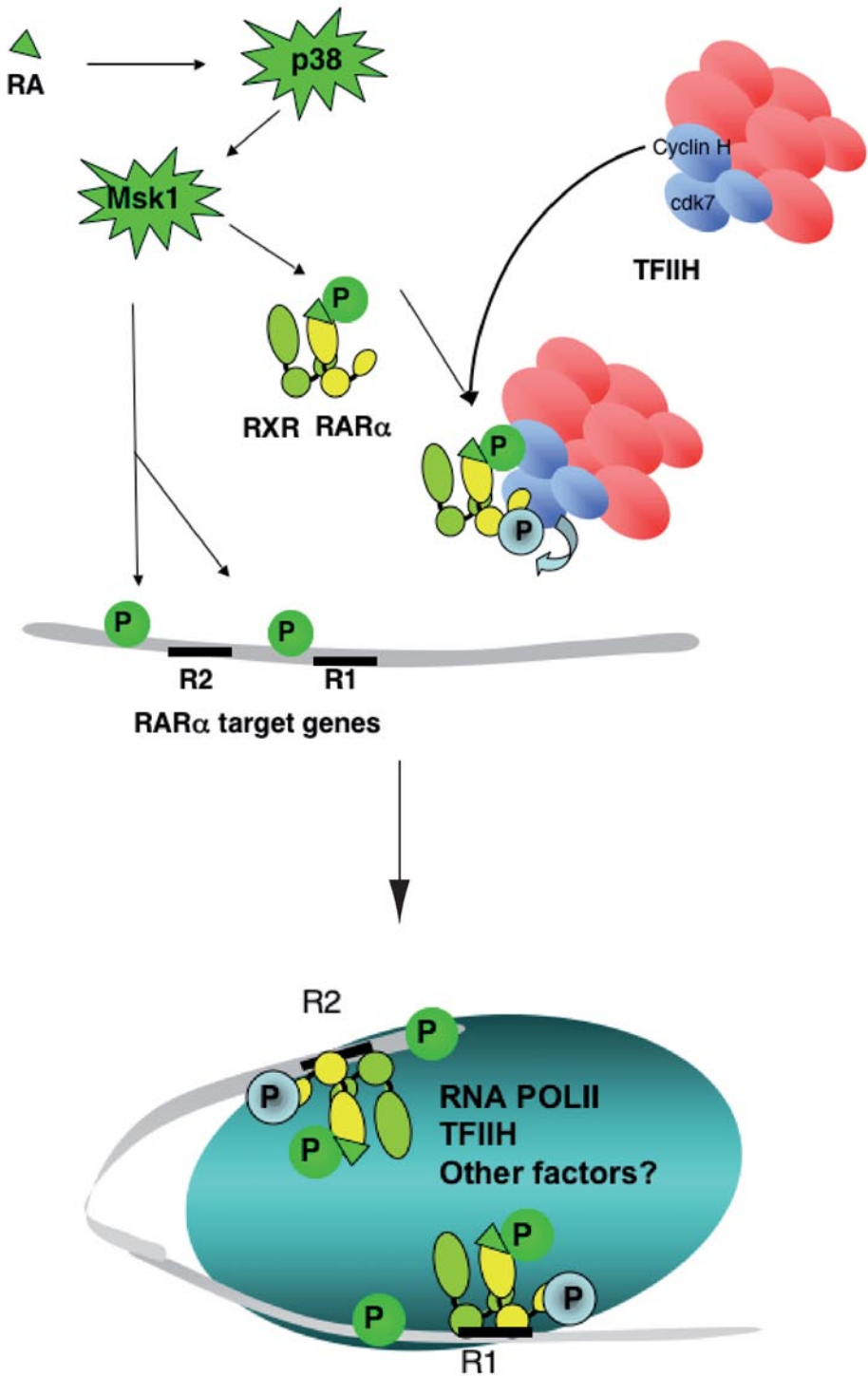
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Msk1
Cyclin H
cdk7
TFIIH
P
RXR RARα
R2
R1
RARα target genes
RNA POLII
TFIIH
Other factors?

TABLE 1

Chromatin immunoprecipitation experiments (ChIP) determining the basal recruitment of RARα and the XPB subunit of TFIIH to R1 and R2 in the different rescue MEF lines. Values (% of the inputs) are the mean ±SD of triplicates performed on three separate chromatin preparations. Data were analyzed using Microsoft Excel 2002. Unpaired Student's T test for pair-wise comparison was used to determine the statistically significant difference in RARα or TFIIH recruitment versus the 36B4 signals or the signals obtained in the absence of antibody. A p-value less than 0.5 (*) denotes a statistical significance.

MEF	**36B4**		**Cyp26A1-R2**		**Cyp26A1-R1**	
	Control	**Ab RARα**	**Control**	**Ab RARα**	**Control**	**Ab RARα**
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RARαS369A	ND	ND	0.121±0.015	0.211±0.027	0.121±0.016	0.700±0.317*
RARαS77A	ND	ND	0.036±0.003	0.075±0.006	0.036±0.003	0.273±0.175
	Control	**AbTFIIH**	**Control**	**AbTFIIH**	**Control**	**AbTFIIH**
RARαWT	0.156±0.012	0.121±0.009	0.146±0.049	0.131±0.032	0.161±0.081	0.659±0.122*
RARαS369A	ND	ND	0.226±0.051	0.256±0.0339	0.266+0.09	0.831±0.336*
RARαS77A	ND	ND	0.165±0.057	0.205±0.058	0.176±0.094	1.026+0.049*