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ORIGINAL ARTICLE

The E3 ubiquitin ligase complex component COP1 regulates PEA3 group member stability and transcriptional activity

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In this study, we report that the PEA3 group members interact with the mammalian really interesting new gene (RING) E3 ubiquitin ligase constitutive photomorphogenetic 1 (COP1), which mediates ubiquitylation and subsequent proteasome degradation of the p53 and c-Jun transcription factors. This interaction is mediated by the central region of COP1 including the coiled-coil domain and two COP1-interacting consensus motifs localized in the well-conserved N-terminal transactivation domain of the PEA3 group members. At the transcriptional level, COP1 reduces the transcriptional activity of ERM and the two other PEA3 group proteins on Ets-responsive reporter genes; this effect being dependent on the RING domain of COP1 and the two COP1-interacting motifs of ERM. Reduced transcriptional activity was, however, not related to COP1-induced changes in ERM stability. In fact, increased ubiquitylation and subsequent proteasomemediated degradation of ERM is achieved only when COP1 is expressed with DET1, a key COP1 partner within the ubiquitylation complex. Conversely, we show that the depletion of COP1 or DET1 by small interference RNA (siRNA) in U2OS cells stabilizes endogenous ERM whereas only COP1 knockdown enhances expression of ICAM-1, a gene regulated by this transcription factor. These results indicate that COP1 is a complex regulator of ERM and the two other PEA3 group members.

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Introduction

The PEA3 group is composed of three highly conserved Ets transcription factors: ERM, ETV1/ER81, and PEA3, which are often over-expressed in different types of cancers. Experimental regulation of PEA3 group member expression influences the invasive process, suggesting these factors have a key role in metastasis (de Launoit *et al.*, 2006). To regulate their target genes, these transcription factors are subjected to multiple post-translational modifications such as phosphorylation and acetylation (de Launoit *et al.*, 2006). Recently, it has been shown that they can also be post-translationally covalently linked to polypeptides such as ubiquitin (Ub) (Takahashi *et al.*, 2005; Baert *et al.*, 2007) and SUMO (Degerny *et al.*, 2005; Gocke *et al.*, 2005). We thus have shown that SUMO modification of ERM negatively regulates its transcriptional activity without affecting DNA-binding or subcellular localization (Degerny *et al.*, 2005).

Both Ub and SUMO conjugation pathways comprise three enzymatic activities (Gill, 2005): E1 enzyme allowing activation of Ub or SUMO, E2 enzyme responsible for their conjugation and ultimately E3 ligase enzymes that favor conjugation by selecting the substrate and increasing the stability of the E2-target protein complex (Di-Bacco and Gill, 2006). Different E3 ligases for Ub have been currently identified and most use either a homologous to E6-associated protein C terminus (HECT) or a really interesting new gene (RING) domain to catalyze polyubiquitylation. The constitutive photomorphogenetic 1 (COP1) protein, which contains a RING finger, a coiled-coil domain and seven WD repeats, functions in higher plants as an E3 Ub ligase that targets transcription factors for ubiquitylation and degradation during the process of photomorphogenesis (Yi and Deng, 2005). In mammals, COP1 mediates ubiquitylation and degradation of three transcription factors: p53, c-Jun (Yi and Deng, 2005) and FoxO1 (Kato et al., 2008). In fact, over-expression of COP1 counteracts p53 activation and subsequent cellcycle arrest induced by MLF1, an upstream activator of p53 (Dornan et al., 2004). In the case of c-Jun ubiquitylation, COP1 functions rather as an adapter protein recruiting the transcription factor to an E3 complex composed by the DET1, DDB1, cullin4A and Roc1 proteins. In this model, COP1 interacts directly with DET1 (Wertz et al., 2004).

In this study, we report that COP1 interacts with the PEA3 group members. We have identified functional COP1-binding motifs within the ERM acidic transactivation domain highly conserved among the three PEA3 group members. COP1 decreases the transcriptional

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ability of the PEA3 group members and, as shown for ERM, without affecting ubiquitylation and stability of the transcription factor. Increased ubiquitylation, proteasome-mediated degradation and further decrease in transcriptional activity occur only in the presence of both COP1 and DET1. Furthermore, we found that COP1 or DET1 knockdown stabilizes endogenous ERM. However, only depletion of COP1 enhances expression of *ICAM-1*, a gene regulated by this transcription factor.

Results

ERM and the PEA3 group members interact with COP1 through two in-tandem conserved motifs

ERM is subjected to Ub modification (Baert et al., 2007), and possesses in its sequence two consensusbinding motifs (D/ED/EXXXVPD/E) for the E3 Ub ligase COP1 (Yi and Deng, 2005). To test whether this transcription factor can interact with COP1, we first expressed ERM as a glutathione-S-transferase (GST)fusion protein and tested the ability of GST-ERM to bind to COP1. Flag-COP1 produced in transfected cells (Figure 1a, lane 1) was found to bind to GST-ERM (lane 3) but failed to bind to GST alone (lane 2). To confirm this interaction, co-immunoprecipitation experiments were then carried out using cell lysates prepared from COS-7 cells expressing exogenous flag-COP1 and ERM. As shown in Figure 1b, the anti-ERM immunoprecipitate contained flag-COP1, whereas, as control, COP1 was not detected in the anti-ERM immunoprecipitate of cells expressing flag-COP1 or ERM alone. The reciprocal co-immunoprecipitation experiment using the flag antibody gave similar results (Figure 1c). This study was extended to the two other PEA3 group members, that is, ETV1 and PEA3. As shown in Figure 1d, ETV1 and PEA3 co-immunoprecipitated with flag-tagged COP1 as observed for ERM. These results clearly identified COP1 as a binding partner for the PEA3 group members. Finally, to analyze the ERM-COP1 interaction in vivo, we used human osteosarcoma U2OS cells, which express ERM as well as COP1 (Wertz et al., 2004). As shown in Figure 1e, endogenous COP1 was co-precipitated with ERM but was not observed with the use of preimmune serum in place of ERM serum. This finding is consistent with an in vivo interaction between ERM and COP1 and confirms the results obtained with over-expressed proteins.

COP1 possesses three well-characterized domains, that is, the N-terminal RING domain, the coiled-coil central domain and the WD40 domain (Figure 2a). To map the domain responsible for the interaction with ERM, co-immunoprecipitation experiments were performed with deletion mutants of COP1. ERM was coexpressed with the flag-tagged full-length COP1 (1–731) or mutants lacking either the WD40 and the coiled-coil domains (R, 1–214) or the WD40 domain alone (Rcc, 1–344, Figure 2a). Among the three proteins, only the full-length COP1 protein was significantly co-immunoprecipitated with ERM (Figure 2a, compare lanes 1–4), npg

thus suggesting that the WD40 domain is probably necessary for the interaction with ERM. We, therefore, also co-expressed ERM with this COP1 domain extended (ccWD, 208-731) or not (WD, 401-731) with the coiled-coil domain. Surprisingly, WD could not mediate interaction with ERM (Figure 2a, lane 6). In contrast, anti-ERM antibody efficiently co-precipitated ccWD (lane 5) indicating that only COP1 protein containing the coiled-coil domain extended to AA 400 efficiently associates with ERM. To further examine the role of the coiled-coil domain in the ERM-COP1 interaction, we also tested the COP1 spliced variant COP1 Δ 24, which lacks part of the coiled-coil protein interaction domain (AA 277-296, Wertz et al., 2004). Only a very weak binding to ERM was observed in co-immunoprecipitation experiments as compared with wild-type COP1 (Figure 2b). Thus, altogether, these data indicate that the coiled-coil region of COP1 contributes to ERM binding.

We next analyzed which domain(s) of ERM is (are) responsible for this interaction. As indicated above, ERM contains two consensus COP1-binding motifs, one upstream of the ETS DNA-binding domain (at position 341) and the other at position 70 within the N-terminal acidic transactivation domain (Figure 2c, upper panel). To determine whether these consensus motifs are functional for ERM-COP1 interaction, we performed co-immunoprecipitation experiments with deletion mutants of ERM co-expressed in cells with flag-tagged COP1 (Figure 2c, lower panel). Western blot experiments performed with anti-ERM antibody on immunoprecipitated flag-tagged protein complexes revealed that C-terminal deletion of ERM did not affect the COP1-ERM interaction, even when the second consensus-binding motif was removed (ERM_{1-226}) . In contrast, the deletion of the N-terminal domain, which removed the first site (ERM $_{87-510}$), abolished the interaction with COP1 (Figure 2c, lower panel). This suggests that the first consensus site at position 70 of ERM is a functional COP1-interacting site. To confirm its functionality, the conserved VP residues were mutated into AA residues (ERM M1, Figure 2d). We thus performed co-immunoprecipitation experiments and showed that mutation of this site decreased ERM-COP1 interaction as compared with wild-type ERM. As such mutation was found to abolish the interaction of c-Jun with COP1, this result suggested that the N-terminal part of ERM probably contained a second interaction site. Examination of the ERM sequence revealed the presence of a VPD sequence at position 63 which, in contrast to the optimal consensus site at position 70, was not preceded by a stretch of acidic residues. We thus performed mutation of this site $(VP \rightarrow AA; ERM M2)$ and showed that it also decreased the interaction with COP1. However, when both sites were mutated (ERM M1/2), interaction with COP1 was totally abolished (Figure 2d). This clearly indicates that the N-terminal part of ERM contains two functional COP1-interacting sites.

The two functional COP1-interacting sites mapped above are conserved in the two other PEA3 group members (de Launoit *et al.*, 2000). Interestingly, in humans, alternative splicing of the *er81/etv1* gene leads to the absence of exon 5 (54 bp), which encodes the C-terminus of the transcriptional acidic domain and results in a truncated protein called ER81 whereas the full-length protein is called ETV1 (Coutte *et al.*, 1999). In ER81 protein, both functional COP1-interacting sites are absent (Figure 2e). To test whether this naturally existing ER81 protein shows different functional proper-

ties compared with its non-spliced counterpart ETV1 protein, we performed co-immunoprecipitation experiments as in Figures 1 and 2. Our data indicated that ETV1 and ERM interacted with COP1. In contrast, ER81 was not found to be associated with immunoprecipitated COP1 under the same conditions (Figure 2e). In conclusion, this result confirms that COP1 binding involves interaction with the C-terminus of the transcriptional acidic domain of PEA3 group members.



Figure 1 ERM and the two other PEA3 group members interact with COP1. (a) ERM interacts with COP1 *in vitro*. Cellular extract of COS-7 cells transfected with flag-COP1 (lane 1) was incubated with GST (lane 2) or GST–ERM (lane 3) and COP1 was detected by anti-flag immunoblotting. * denotes a nonspecific band. In all, 5% of cellular extract involved in affinity reaction is used as Input (b, c) ERM interacts with COP1 in transfected cells. COS-7 cells were transfected with flag-COP1, ERM or both, immunoprecipitated with anti-ERM antibody (b) or anti-flag antibody (c) and immunoblotted as indicated. (d) The three PEA3 group members interact with COP1. COS-7 cells were transfected with or vithout flag-COP1, immunoprecipitated with anti-flag antibody and immunoblotted as indicated. Anti-ERM antibody cross-reacts with ETV1 and PEA3 (3). (e) Co-immunoprecipitation of ERM and COP1 from U2OS cells. Immunoprecipitation was performed with anti-ERM antibody or preimmune serum (Pre) and proteins were immunoblotted as indicated using antibodies specific for ERM and COP1.

Figure 2 Mapping of the interacting motifs in ERM and COP1 (a) Schematic representation of COP1 and the deletion mutants used in co-immunoprecipitation experiments. R, RING finger domain; cc, coiled-coil domain; WD, WD40 domain. COS-7 cells were transfected with flag-ERM and either flag-COP1, flag-RING, flag-RINGcc, flag-WD40 or flag-ccWD40 (ccWD) and the cellular extracts were immunoprecipitated with anti-ERM antibody (IP ERM) and immunoblotted with anti-flag (IB Flag) and anti-ERM (IB ERM) antibody. Aliquots of the same extracts were analyzed by immunoblotting with the same antibodies to detect exogenous proteins (INPUT). *denotes a nonspecific band. (b) COS-7 cells were transfected with flag-ERM and either flag-COP1 or flag- $COP1\Delta 24$, a splice variant lacking part of the coiled-coil domain, and the cellular extracts were immunoprecipitated with anti-ERM antibody (IP ERM) and immunoblotted with anti-flag (IB Flag) and anti-ERM (IB ERM) antibody. Aliquots of the same extracts were analyzed by immunoblotting with the same antibodies to detect exogenous proteins (INPUT). (c) COS-7 cells were transfected with flag-COP1 and either full-length ERM or the deletion mutants illustrated in the upper panel (1 and 2 indicate the location of the two consensus COP1-interacting motifs). The cellular extracts were immunoprecipitated with anti-flag antibody and immunoblotted as indicated. (d) COS-7 cells were transfected with flag-COP1 and flag-ERM either wild-type (Wt) or mutated (M) as illustrated in the upper panel. The cellular extracts were immunoprecipitated with anti-ERM antibody (IP ERM) or not (INPUT) and immunoblotted as indicated. *denotes a nonspecific band. (e) COS-7 cells were transfected with flag-COP1 and either ERM, ETV1 or ER81, an ETV1 isoform spliced as illustrated in the upper panel. The cellular extracts were immunoprecipitated with anti-flag antibody (IP flag) or not (INPUT) and immunoblotted as indicated. Anti-ETV1 antibody cross-reacts with ERM.

COP1 decreased PEA3 group member-induced transcriptional activity

To analyze the effect of COP1 on ERM-induced transcriptional activity, we performed a luciferase reporter gene assay by using ERM and the ICAM-1 luciferase reporter plasmid previously identified as PEA3 group-responsive (de Launoit *et al.*, 1998). As shown in Figure 3a, COP1 had no important effect on the basal ICAM-1 reporter transcription in RK13

cells. However, increasing amounts of COP1 expression vector decreased ERM-induced transcription on this promoter. This effect was not due to decreased ERM levels because increasing amounts of COP1 did not influence the amount of ERM protein in the transfected cells (Figure 3a, insert). We also examined the effect of COP1 on the activity of ERM mutants. Although mutations of the first (M1) or the second (M2) COP1binding motif reduced the COP1-induced decrease of



ERM transactivation (-55% for M1 and -75% for M2 vs -85% for the wild type), the double mutation (M1/2) totally abolished this effect (Figure 3b) indicating that decreased ERM activity resulted from direct interaction with COP1. Finally, we also tested the effect of COP1 on the transcription of ICAM-1 induced by the other PEA3 group members. Both PEA3- and ETV1-induced transcriptional activities were decreased by COP1, whereas transactivation induced by ER81 was not affected (Figure 3c, upper panel). This latter activity was, however, relatively low. Thus, we also performed experiments on c-fes, another Ets-reporter plasmid (de Launoit et al., 1998). As observed with ICAM-1, only ER81 activity was not significantly affected by COP1 on this promoter (Figure 3e, lower panel). Collectively, these results indicate that COP1 strongly decreases PEA3 group member-dependent transcription through the COP1-interacting motifs.

The effect of different COP1 mutants on ERM activity was also analyzed. We found that the COP1 spliced variant COP1 Δ 24 could not significantly decrease ERM-induced transcription of the ICAM-1 promoter. Similar results were also obtained with ccWD which, in contrast to COP1 Δ 24, efficiently interacts with ERM, thus suggesting the role of the N-terminal RING domain in the COP1-mediated inhibition of ERM activity (Figure 3d). To further analyze this role, we also tested a COP1 RING mutant devoid of ubiquitination activity (Bianchi *et al.*, 2003). Interestingly, the RING mutant did not affect ERM transactivation activity, although it expression was similar to wild-type COP1 (Figure 3d) and still bound to ERM in co-immunoprecipitation assay (see Figure 5c).

COP1 cooperates with DET1 to regulate ubiquitylation and protein level of the PEA3 group members

It has been recently shown in vivo that ERM is subjected to ubiquitylation followed by degradation (Baert et al., 2007). We thus tested whether the expression of COP1 induced a variation of the ubiquitylation level of ERM. For this purpose, we transiently co-transfected COS-7 cells with vectors encoding ERM and His₆-tagged Ub; the cells being treated with the proteasome inhibitor ALLN to block degradation of the Ub-modified proteins. Ub-modified proteins were then isolated on nickel agarose (Ni^{2+}) and analyzed by western blotting with anti-ERM antibody. A major ERM species was observed at approximately 85 kDa in the Ni²⁺-bound protein fraction as a result of Ub overproduction (Figure 4a). As described earlier, it corresponds to a monoubiquitylated form of ERM (Baert et al., 2007). Higher molecular mass species, characteristic of polyubiquitylation (Baert et al., 2007), were also observed (Figure 4a, compare lanes 1 and 2). In the presence of COP1, a slight increase in monoubiquitylation of ERM was observed, but polyubiquitylation was not enhanced (compare lanes 2 and 3). It thus appears that this Ub ligase did not enhance polyubiquitylation of ERM, which agrees with the fact that COP1 did not significantly affect the ERM level in transfected cells (Figure 3a).

It has been reported that the COP1-interacting protein DET1 promotes ubiquitylation and degradation of c-Jun by assembling a multisubunit Ub ligase containing DDB1, CUL4A, ROC1 on the COP-1/ c-Jun complex (Wertz et al., 2004). We thus tested whether expression of DET1 favors ERM ubiquitylation. Using the same approach as above, we showed that the ubiquitylation level of ERM was similar in the presence of COP1 or DET1 alone. However, it was dramatically increased when COP1 and DET1 were co-expressed (Figure 4b, compare lanes 1 and 2 in one hand with lane 3 in the other hand). In contrast, the ubiquitvlation level of ERM mutated on the COP1interacting sites (ERM M1/2) was almost similar in the presence of COP1, DET1 or both proteins (Figure 4b, compare lanes 4, 5 and 6).

As co-expression of COP1 and DET1 dramatically increased **ÊRM** ubiquitylation, we tested the ERM protein level in the presence of both proteins. As shown in Figure 5a, expression of COP1 or DET1 alone did not change the ERM level. In contrast, when COP1 and DET1 were co-expressed, ERM level dramatically decreased (ERM Wt) whereas it was poorly affected when the COP1-interacting sites were mutated (ERM M1/2) or deleted (ERM ΔNt). Similarly, when the assay was performed with ccWD or the COP1 RING mutant instead of wild-type COP1, no important changes in ERM level were detected (Figure 5b). To determine whether the different effects of wild-type COP1 and the two COP1 mutants on ERM levels were related to difference in their capacity to recruit DET1 in ERM complexes, we performed co-immunoprecipitation experiments in transfected cells expressing ERM and DET1 with or without COP1. By co-expressing ERM and Myc-DET1, we could not co-immunoprecipitate DET1 with ERM (Figure 5c, lane 2) suggesting that COP1 is probably the link between ERM and DET1. Accordingly, when COP1 was co-expressed, DET1, as COP1, was associated with ERM (lane 3). However, co-transfection of ccWD or the COP1 RING mutant with ERM and DET1 resulted in the disappearance of DET1 from the ERM complexes (compare lane 3 and 4–5) indicating that in contrast to the wild-type protein, these COP1 mutants could not recruit DET1 to ERM.

To assess whether ERM stability was affected by COP1/DET1, we examined ERM level in transfected cells treated with the protein synthesis inhibitor cycloheximide in the absence or presence of overexpressed COP1 and DET1. As expected, we observed that COP1/ DET1 significantly decreased the half-life of wild-type ERM while that of ERM M1/2 was apparently unaffected under the same conditions (Figure 5d). Transfected cells expressing ERM alone or ERM with both COP1 and DET1 were also treated or not with the proteasome inhibitor ALLN. Clearly, ALLN blocked COP1/DET1-induced ERM degradation (Figure 5e, left panel). This indicated that expression of COP1 and DET1 results in the downregulation of ERM at the protein level through a proteasome-mediated pathway. Similar results were obtained for ETV1 (Figure 5e, middle panel) and PEA3 (not shown). In contrast ER81

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Figure 3 COP1 regulates ERM transcriptional function. (a) RK13 cells were transfected with ERM and an increasing quantity of COP1 together with ICAM-1-Luc reporter. Insert: ERM was transfected with or without COP1 in RK13 cells and the protein levels were assessed by immunoblotting as indicated. (b) RK13 cells were transfected with the ICAM-1-Luc reporter, ERM either wild-type or mutated on the COP1-interacting motifs as described in Figure 2d or empty vector (–) and COP1 as indicated. (c) RK13 cells were transfected with either ICAM-1-Luc reporter or c-fes-Luc reporter, ERM, PEA3, ETV1, ER81 or empty vector (–) and COP1 as indicated. (d) RK13 cells were transfected with the ICAM-1-Luc reporter, or c-fes-Luc reporter, ERM and either flag-COP1, flag-ccWD40, flag-COP1Δ24 or flag-COP1Rmut expressing COP1, which carries two Cys to Ala substitutions in the RING domain. The protein levels were assessed by immunoblotting as indicated.

level was similar in the absence or presence of COP1/ DET1 (Figure 5e, right panel), which agrees with the fact that ER81 lacks the COP1-interacting motifs. At the transcriptional level, DET1 alone or associated with the ccWD COP1 mutant never affected ERM transcriptional activity on the ICAM-1 promoter. However, in combination with COP1, a further decrease in ERM activity was observed as compared with COP1 alone (Figure 5f). This decrease of approximately 70% was significantly reduced when the cells were treated with the proteasome inhibitor ALLN (Figure 5g) suggesting that this effect is probably because of the decreased ERM level observed only in the presence of both COP1 and DET1.

To confirm the functional role of COP1 and DET1 on endogenous ERM, we used small interference RNAs (siRNA) to knockdown their expression. Experiments were performed in U2OS cells and, as shown in Figure 6a, COP1 siRNA caused a significant decrease in COP1 mRNA whereas it had no effect on DET1



Figure 4 COP1 mediates augmented ubiquitylation of ERM only in the presence of DET1. (a) COS-7 cells were transfected with ERM, His-tagged ubiquitin (His-Ub) and flag-COP1 in the indicated combinations. ALLN-treated cells were lysed, Ub-modified proteins were purified by Nickel affinity chromatography (Ni²⁺ purification) and Ub-modified ERM was detected by immunoblotting using anti-ERM antibody. The expression of ERM and COP1 in cell lysate was detected by immunoblotting as indicated. The arrow indicates the position of the monoubiquity-lated form of ERM. *denotes a nonspecific band. (b) COS-7 cells were transfected with ERM wild-type or M1/2 mutant and Histagged Ub together with flag-COP1 and/or Myc-DET1. ALLN-treated cells were lysed and cell lysates and purified Ub-modified proteins were analyzed by immunoblotting as indicated.

mRNA. Similarly, DET1 mRNA was the only one reduced on expression of DET1 siRNA, indicating the specificity of the two siRNA used. Concerning ERM, which was hardly detected in these cells, depletion of COP1 by siRNA resulted in a large increase of its protein level, as revealed by western blot. Similar results were obtained in response to DET1 depletion (Figure 6a), indicating that COP1 or DET1 knockdown both resulted in ERM stabilization. In these cells, COP1 siRNA also induced an increase in the basal activity of the transfected ICAM-1 reporter plasmid (Figure 6b). This effect was, however, not found on the ICAM-1 reporter plasmid mutated on the Ets-binding sites (de Launoit *et al.*, 1998), indicating that it is mediated by an

Ets transcription factor. However, in the same assay, DET1 siRNA did not significantly change the activity of the ICAM-1 reporter plasmids used. Finally, to determine whether COP1 knockdown could also affect the expression of the endogenous *ICAM-1* gene, U2OS cells transfected with COP1 or DET1 siRNA have been tested for *ICAM-1* expression by quantitative reverse transcriptase–PCR. As compared with the control cells, upregulated expression of *ICAM-1* was observed in COP1 siRNA treated cells, this expression being only poorly affected in response to DET1 knockdown (Figure 6c). These results thus strongly suggest that most of the observed ICAM-1 activation resulted from COP1 depletion rather than from increased ERM level in the cells.

Discussion

So far, three COP1 substrates have been identified among mammalian transcription factors: c-Jun, the mammalian homologue of the plant bZIP transcription factor HY5, p53 and FoxO1 (Yi and Deng, 2005; Kato et al., 2008). In this study, we establish that ERM is a substrate for this ligase and that the coiled-coil region of COP1 is required for efficient binding to ERM. COP1 interacts through its WD40 repeats with HY5 and other transcription factors in plant and c-Jun, JunD and FoxO1 in mammals (Yi and Deng, 2005; Kato et al., 2008). However, the coiled-coil domain has not been implicated in these interactions. For c-jun, an additional binding site has nevertheless been described in this region (Bianchi et al., 2003) but, in contrast to ERM, deletion of this domain does not significantly alter the interaction of COP1 with c-jun (Bianchi et al., 2003; Wertz et al., 2004). In fact, the coiled-coil domain of atCOP1 and huCOP1 has been involved in COP1 homodimerization (Yi and Deng, 2005). Interestingly, the N-terminal TAD of ERM was found to contain two functional COP1-interacting motifs with the core sequence VPD/E shared by plant bZIP family COP1 substrates (Holm et al., 2001). It remains to determine whether the presence of two interacting sites might be related to the reported capacity of COP1 to dimerize. COP1-binding sites Indeed. two might favor and stabilize the interaction of ERM with a COP1 homodimer.

The transcriptional activity of the three PEA3 group transcription factors was similarly reduced by COP1 expression. As shown for ERM, this inhibition requires the presence of functional COP1-binding sites but is unrelated to increased ubiquitylation, change in ERM protein levels or COP1-induced change in ERM localization as judged from the results of immunofluorescence studies (data not shown). These data are in good agreement with those obtained on c-Jun transcriptional activity, which is downregulated in the presence of COP1 on AP1-responsive promoters (Bianchi *et al.*, 2003). We also found that activity of the ERM Nterminal TAD fused to Gal4 DBD is dramatically

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Figure 5 In the presence of DET1, COP1 destabilizes ERM and further inhibits its activity. (a) Wild-type ERM, ERM M1/2 mutant or the deletion mutant ERM_{87-510} (ERM ΔNt) were expressed in COS-7 cells with flag-COP1 and/or Myc-DET1 as indicated. ERM, COP1 and DET1 and endogenous actin were detected by immunoblotting with the specific antibodies. Expression levels of COP1, DET1 and actin were determined in the cells cotransfected with wild-type ERM and were similar in the cells cotransfected with the mutated forms of the protein. (b) ERM was expressed with Myc-DET1 and either flag-COP1, flag-ccWD40 or flag-COP1Rmut (Rm) as indicated and proteins were analyzed as in (a). (c) ERM, Myc-DET1 and either flag-COP1, flag-ccWD40 or flag-COP1Rmut (Rm) were expressed as indicated. The proteins in the cell lysates (INPUT) and immunoprecipitated with anti-ERM antibody (IP ERM) were analyzed by immunoblotting as in (a). (d) RK13 cells were transfected with ERM either wild-type or mutated on the two COP1interacting motifs (M1/2) in the absence (control) and presence of COP1 and DET1 and 20h later were treated with cycloheximide (CHX) for the indicated times. The expression of ERM and actin was analyzed by immunoblotting. (e) ERM was expressed in cells either alone or with COP1 and DET1. Six hours before cell lysis, transfected cells were treated with the proteasome inhibitor ALLN or not (-). ERM and actin were detected by immunoblotting with their respective specific antibodies. The same experiments were also performed with ETV1 and ER81 in place of ERM. (f) RK13 cells were transfected with the ICAM-1-Luc reporter together with ERM, DET1 and either COP1 or the C-terminal COP1 deletion mutant ccWD in the indicated combinations. (g) RK13 cells were transfected with the ICAM-Luc reporter together with ERM-COP1 and ERM-COP1-DET1. In total, 18 h before cell lysis, transfected cells were treated with the proteasome inhibitor ALLN (10–40 μ M) or not (–).

decreased in the presence of COP1, suggesting that the COP1-induced effect is independent of the ETS DNAbinding domain (data not shown). Concerning COP1, its ability to decrease ERM-mediated transactivation clearly depends on the presence of an intact RING domain because its deletion or its mutation leading to an E3 ligase defective COP1 mutant abrogates COP1 suppressive activity on transcription without marked

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Figure 6 Consequences of endogenous COP1 and DET1 knockdown in U2OS cells. (a) U2OS cells were transfected with control siRNA (ctrl) or siRNA directed against COP1 (siCOP) or DET1 (siDET). COP1 and DET1 mRNA expression was determined by quantitative RT–PCRs and was set as one in control cells. ERM and actin levels were determined by immunoblot analysis using specific antibodies as indicated. (b) U2OS cells were transfected as above with the ICAM-1-Luc reporter either wild-type (ICAM wt) or mutated on the ETS-binding sites (ICAM mut). (c) COP1 siRNA (siCOP), DET1 siRNA (siDET) and control (ctrl) U2OS cells described in (a) were used to determine the relative mRNA expression levels of *ICAM-1* assessed by quantitative RT–PCR. Results are expressed as ratios of mRNA levels of *ICAM-1* to *GAPDH* (endogenous control standard) (*P<0.01; ANOVA, n=4).

change in ERM binding. It is thus unlikely that COP1. via its RING domain, affects the capacity of the TAD to interact with components necessary for transcriptional activation. A possibility is that, in a context of overexpression, COP1 acts as a transcriptional co-repressor. Such mechanism would be reminiscent of that described for some SUMO E3 ligases of the PIAS family, which can exert SUMO ligase-independent functions in transcriptional regulation (Sharrocks, 2006). Moreover, because the RING finger has been implicated in mediating the interaction of atCOP1 with other proteins (Yi and Deng, 2005), it might be that this COP1 domain could recruit proteins involved in transcriptional repression. However, that may be, the COP1 E3 ligase activity is probably necessary to mediate the COP1 effect and because the RING of huCOP1 has ubiquitylating activity toward COP1 itself (Bianchi *et al.*, 2003), this raises the possibility that ubiquitylation of COP1 may be involved in the transcriptional repression process.

huCOP1 has an intrinsic E3 Ub ligase activity, which is sufficient for the ubiquitylation and subsequent degradation of p53 (Dornan *et al.*, 2004). In contrast, as described for c-Jun (Bianchi *et al.*, 2003), this ligase activity does not promote polyubiquitylation of the PEA3 group members. Concerning c-Jun, it has been suggested that COP1 acts by recruiting a multisubunit E3 complex containing DET1, DDB1, Cul4A and Roc1, through direct interaction with DET1 (Wertz *et al.*, 2004). Accordingly, we show here that, in contrast to the expression of COP1, co-expression of COP1 and DET1 promotes ubiquitylation and proteasomemediated degradation of ERM. This enhanced degradation



Figure 7 Schematic illustration of ERM–COP1 interaction. In presence of COP1 and DET1, COP1 interacts with ERM and functions as a substrate adapter linking ERM to DET1. This leads to ERM ubiquitylation and its proteasome-mediated degradation probably through recruitment of an Ub ligase complex, and thus to reduced ERM target gene activation. When COP1 level exceeds that of DET1, ERM is not destabilized but ERM transactivation is still reduced suggesting a DET1-independent function of COP1 in ERM transcriptional activity regulation (see text for detail). EBS, Ets-binding site.

observed for the three PEA group members requires the presence of the COP1-binding motifs and is counteracted by the downregulation of COP1 or DET1 through siRNA, as shown for endogenous ERM in U2OS cells. As DET1 is not recognized by ERM, it is likely that COP1 and DET1 form a heterodimer serving as a bridge to link the COP1-interacting substrate to an Ub ligase complex in a DET1-dependent manner. It can, however, not be excluded that the binding of DET1 to COP1 might enhance the intrinsic activity of COP1 and promote ubiquitylation of COP1-bound ERM.

We show that in addition to increased ERM levels, knocking down COP1 but not DET1 in U2OS cells results in a significant upregulation of endogenous ICAM-1 gene. This finding and those obtained on the ICAM-1 promoter are consistent with a model whereby COP1 may target ERM to negatively regulate its transcriptional activity in a physiologic context (Figure 7). We thus suggest that the balance between COP1 and DET1 may determine the level of transcriptional activity of ERM and its intracellular concentration. This model can be extended to the two other PEA3 group members ETV1 and PEA3 but not to ER81, a spliced variant of ETV1 lacking a short region containing the COP1binding sites (Coutte et al., 1999). Indeed, as expected, neither ER81-induced transactivation, nor ER81 protein stability was significantly affected by COP1 or COP1-DET1. The regulation by COP1 is thus the first functional difference observed between the two isoforms of the product of the etv1 gene.

The mechanisms by which the Ets transcription factors are regulated by ubiquitylation are currently not described. However, it has recently been shown that the rapid turnover in S phase of the short-lived Ets protein MEF is dependent on the phosphorylation of its C-terminal domain and on the Skp1/Cul1/F-box E3 Ub ligase complex, which targets MEF for ubiquitylation and degradation (Liu et al., 2006). The PEA3 group members are also phosphorylated and it would be interesting to determine whether such modification modulates the association of these transcription factors with COP1. Moreover, in the absence of DET1mediated degradation, COP1 binding could also inhibit activating modifications such as phosphorylation or acetylation (de Launoit et al., 2006) or positively affect sumoylation, which inhibits PEA3 group member activity (Degerny et al., 2005). Further studies are now required to elucidate how COP1 regulates ERM transcriptional activity and turnover.

Materials and methods

Portion of the Materials and methods is presented as Supplementary Information, including plasmid constructs and reverse transcriptase–PCR.

Cell cultures and transfections

RK13, COS-7 and U2OS cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf

serum (Gibco BRL). In all, 1.5×10^5 cells/well were plated in 12-well plates, and the next day transfections were performed using the PEI Exgen 500 procedure (Euromedex, France) with 250 ng total DNA per well, including 25–100 ng reporter plasmid, 10 ng pSG5 or 25 ng pGAP expression vector and 10 ng β-galactosidase expression vector. Activity was determined as described earlier (Degerny *et al.*, 2005). For protein stability and co-immunoprecipitation experiments, cells were transfected in six-well plates with 300 ng of PEA3 group expression plasmid and, when indicated, with 200 ng of COP1 and/or DET1 plasmids. SMART pools (Dharmacon) were used to knockdown COP1 and DET1 in the cells. SiRNA were transfected with Interferin (Polyplus-transfection) according manufacturer's protocol.

Immunoprecipitation and western blot analyses.

Transfected cells were lysed in co-immunoprecipitation buffer (50 mM Tris/HCl, pH 7.5, 125 mM NaCl, 0.2 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 0.5% Triton × 100). Proteins were immunoprecipitated overnight with the anti-FLAG M2 affinity gel (Sigma) at 4 °C or with anti-ERM antibody (anti-ERM₃₅₅₋₅₁₀) followed by incubation with protein A-Sepharose beads for 1 h at 4 °C. Immunoblot analyses were performed with the rabbit anti-ERM₁₂₋₂₂₆ (Baert *et al.*, 1997), anti-flag (Sigma), anti-myc (Invitrogen), anti-ETV1, anti-Gal4, or anti-Actin antibody (Santa Cruz Biotechnology). Co-immunoprecipitation in U2OS cells was performed as described above

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with anti-ERM antibody. Detection of immunoblotted target bands was performed with anti-ERM $_{12-226}$ and anti-COP1 (Bethyl) antibody.

Identification of His-Ub-ERM conjugates

COS-7 cells were transfected with 250 ng/well (six-well plates) of ERM expression plasmid and, when indicated, with 700 ng plasmid coding for His-tagged-Ub and 150 ng of flag-COP1 and Myc–DET1 constructs. After treatment with $50 \,\mu\text{M}$ ALLN for 6 h, the cells were lysed in denaturing buffer containing 6 M guanidium-HCl. His₆–Ub conjugates were purified by metal-chelate affinity chromatography as described earlier (Baert *et al.*, 2007). The proteins were subjected to immunoblotting as described above.

Conflict of interest

The authors declare no conflict of interest.

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