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Abbreviations : NR, Nuclear Receptor; PR, Progesterone Receptor; SRC-1, Steroid Receptor Coactivator-1; bHLH, basic Helix-Loop-Helix; LB, leptomycin B; NES, Nuclear export signal; PRE, Progesterone Response Element; SERM: Selective Estrogen Receptor Modulator.

ABSTRACT

The progesterone receptor (PR), a ligand-activated transcription factor, recruits the primary coactivator SRC-1/NCoA-1 to target gene promoters. It is known that PR transcriptional activity is paradoxically coupled to its ligand-dependent down-regulation. However, despite its importance in PR function, the regulation of SRC-1 expression level during hormonal exposure is poorly understood. Here we report that SRC-1 expression level (but not other p160 family members) is down-regulated by the agonist ligand R5020 in a PR-dependent manner. In contrast, the antagonist RU486 fails to induce down-regulation of the coactivator and impairs PR agonist-dependent degradation of SRC-1. We show that SRC-1 proteolysis is a proteasome- and ubiquitin-mediated process that, predominantly but not exclusively, occurs in the cytoplasmic compartment where SRC-1 colocalizes with proteasome antigens as demonstrated by confocal imaging. Moreover, SRC-1 was stabilized in the presence of leptomycin B or several proteasomal inhibitors. Two degradation motifs, amino-acids 2-16 corresponding to a PEST motif and amino-acids 41-136 located in the bHLH domain of the coactivator, were identified and shown to control the stability as well as the hormone-dependent down-regulation of the coactivator. SRC-1 degradation is of physiological importance since the two non-degradable mutants that still interacted with PR as demonstrated by co-immunoprecipitation, failed to stimulate transcription of exogenous and endogenous target genes, suggesting that concomitant PR/SRC-1 ligand-dependent degradation is a necessary step for PR transactivation activity. Collectively, our findings are consistent with the emerging role of proteasome-mediated proteolysis in the gene regulating process and indicate that the ligand-dependent down-regulation of SRC-1 is critical for PR transcriptional activity.

INTRODUCTION

The progesterone receptor (PR), also known as NR3C3, plays a crucial role in the coordination of several aspects of female reproductive development and function (1). Invalidation of the PR gene in mice leads to pleiotropic reproductive abnormalities and demonstrates that PR orchestrates key events associated with the establishment and maintenance of pregnancy. From a pathophysiological perspective, accumulating evidence indicates that PR is involved in breast cancer cells proliferation and is implicated in the development and progression of breast cancer (2). Coregulators (coactivators or corepressors) are important Nuclear Receptor (NR)-recruited cofactors modulating NR-mediated transcription leading to activation or repression of target specific genes (3). SRC-1 is a PR coactivator belonging to the p160 gene family which contains three homologous members (SRC-1, -2, and -3) serving as NR transcriptional coactivators (4). This family of coactivators is characterized by the presence of several conserved functional domains: a bHLH-PAS N-terminal domain, a CBP interacting domain (AD1), a glutamine-rich region, a C-terminal activation domain (AD2), and several L*XX*LL boxes involved in NR binding. The p160 coactivators are defined as “primary coactivators” whose activity is regulated by posttranslational modifications (5-10). The current models indicate that p160 coactivators serve as a recruitment platform for other coactivator complexes carrying intrinsic enzymatic activities to specific enhancers/promoters leading to the covalent modification of specific histones and/or other coregulators involved in the transcriptional machinery (11, 12).

Several experiments have revealed a tight association between the turnover rate of several NR and their transcriptional activity, showing that both aspects of NR function appear to be inversely related (13-18). Among the factors regulating PR levels are its ligands. It was initially shown that administration of progesterone to ovariectomized guinea pigs provoked a rapid fall in uterine receptor concentration (19). Hormone-dependent down-regulation of PR has been finally confirmed by several groups (20-22) but its biological significance is still unclear. Phosphorylation of PR on a key serine residue (Ser294) by MAPKs was shown to couple multiple receptor functions, including ligand-dependent PR down-regulation by the ubiquitin-proteasome pathway (13). The concept that transcriptional activation and ubiquitin-mediated proteolysis are interdependent processes is emerging as a potentially important control mechanism of transcription (16, 23). Although their significance

remains to be defined, it appears that complex interactions between regulatory molecules governing both transcription and ubiquitination/degradation exist (24-26). However, little is known concerning the fate of coregulators during ligand-dependent NR down-regulation (27, 28).

In a previous study, we have shown that SRC-1 is exported from the nucleus to the cytoplasm and speculated that this export might be a regulatory mechanism controlling the termination of hormone action possibly through its degradation (29). In order to establish a link between SRC-1 proteolysis and the PR-mediated transcription process, we studied the mechanism governing SRC-1 proteolysis at the steady-state level and questioned whether the ligand could modulate its turn-over. In this study, we demonstrate that SRC-1 undergoes covalent modifications by ubiquitin which targets the coactivator to the proteasome at the steady-state level. We identify two critical degron domains directly linked to the coactivator proteolysis. Aside from this ligand-independent stability regulation, we show that SRC-1 undergoes accelerated agonist-dependent and PR-mediated down-regulation via the ubiquitin–proteasome pathway. SRC-1 proteolysis occurs concomitantly of ligand-dependent PR degradation. Of note, the nature of the ligand is shown to be critical for this process since both PR and SRC-1 ligand-dependent proteolysis was inhibited in the presence of RU486, leading to dramatic loss of PR transactivating capability.

RESULTS

SRC-1 mainly colocalizes with cytoplasmic proteasome antigens

In our previous report about the regulatory mechanisms of SRC-1 subcellular trafficking, we have shown that SRC-1 localizes both in nuclear and cytoplasmic corpuscular structures (29). Several studies have reported coregulators localization in organelles (30, 31). We tried to identify the nature of these cytoplasmic and nuclear speckles by colocalization studies with various antigens and with fluorescent organelles markers. Since several nuclear receptors (NR) and coactivators such as SRC-3 have been shown to interact with the proteasome (32, 33), we used confocal microscopy to investigate whether proteasome components might also accumulate in SRC-1 speckles. By using antibodies directed against the human S7 subunit of the 19S (Rpt1) and the α/β subunits of the 20S proteasome, we found that SRC-1 colocalized with both 26S proteasome antigens (Fig 1A and Supplemental Fig S1). The fluorescence intensity profile indicates that colocalization was predominant in SRC-1 speckles: simultaneous fluorescence intensity increase was observed in cytoplasmic speckles but also in lesser extent in nuclear speckles (Fig 1B), suggesting that SRC-1 is mainly but not exclusively proteolyzed in the cytoplasm. Similar intensity profiles were obtained for cells immunolabeled for SRC-1 and the 20S proteasome (data not shown). A partial colocalization of SRC-1 was also found with the Promyelocytic Leukemia Protein (PML) in the typical nuclear domain (ND10) (Supplemental Fig S2 A). Such an association has been already described (34). In contrast, nuclear speckles did not overlap with transcription sites as evidenced by the absence of colocalization with the SC-35/SRp30 spliceosome component (Supplemental Fig S2 B). Similarly, no colocalization of SRC-1 with organelles like mitochondria, lysosomes, peroxisomes or the Golgi apparatus could be observed (Supplemental Fig S2 C-E and data not shown).

SRC-1 is ubiquitinylated in vivo and is degraded by the proteasome

We next studied the mechanism of SRC-1 down-regulation. First we investigated whether the coactivator was ubiquitinylated and targeted to the proteasome. COS-7 cells were transfected with the expression vector encoding the full-length SRC-1 and incubated in the presence of proteasome inhibitors, MG132 or epoxomicin. Consistent with previous reports (14, 35), both inhibitors increased

SRC-1 protein level in comparison to cells treated with vehicule (Fig 2A and Supplemental Fig S3). To demonstrate that SRC-1 is poly-ubiquitinylated, COS-7 cells were transfected with SRC-1 expression vector in the presence or absence of a vector encoding His-tagged ubiquitin (His 6-Ub) and analyzed by Western Blot. In the absence of His 6-Ub, the anti-SRC-1 antibody detected a major band of ~160 kDa (Fig 2B, left panel, lane 1). In cells cotransfected with His 6-Ub expression vector, a moderate decrease in band intensity was observed with a slightly higher molecular weight smear, indicative of ubiquitinylated moieties (Fig 2B, left panel, lane 2). His-tagged proteins were purified by chromatography on nickel-charged agarose beads (Ni-NTA) and analyzed by Western Blot with an anti-SRC-1 antibody to show that these bands correspond to ubiquitinylated SRC-1 (Fig 2B, right panel, lane 2).

Several cytoplasmic proteasome substrates have been shown to relocalize into the nucleus upon stabilization by proteasome inhibitors (36-38). We thus examined whether SRC-1 subcellular distribution was similarly modified in such conditions. Indeed, overnight treatment of cells with MG132 induces an obvious shift of the coactivator into the nucleus (Fig 2C). This result suggests that escape from cytoplasmic proteolytic degradation stimulates the nuclear accumulation of SRC-1 (36). If our hypothesis is true, then inhibition of SRC-1 nuclear export should induce SRC-1 expression level stabilization. To verify this point, we followed the turnover rate of SRC-1Δ(NES), a mutant deleted of its nuclear export signal (NES) (29). The result shows a better stability of this mutant compared to the wt SRC-1 (Supplemental Fig. S4). In a similar approach, we used the nuclear export inhibitor leptomycin B (LB) to impede wt SRC-1 access to cytoplasm. In presence of LB, SRC-1 not only relocalized into the nucleus [data not shown and (29)] but its expression level also increased ~2.5 fold (Fig 2D). However, SRC-1 stabilization with LB did not reach the level obtained with MG132 (data not shown; and compare quantification Fig 2A to Fig 2D). Thus, the nuclear accumulation of the coactivator indicates a possibility of a partial degradation of SRC-1 in the nuclear compartment. Interestingly, similar experiments with the p160 coactivator SRC-3, which has been shown to be degraded mainly in the nucleus (39) showed no significant increase of SRC-3 expression level under LB treatment (Fig 2D). Overall, our data show that SRC-1 turnover is a proteasome- and ubiquitin-mediated process that takes place, predominantly but not exclusively, in the cytoplasm.

Agonist ligand enhances concomitant proteolysis of PR and SRC-1

We next studied SRC-1 degradation in the context of PR activation. Progestins are known to induce PR proteolysis by the proteasome (22, 40). In addition, Li et al have shown that upon ligand treatment, progesterone receptor (PR) preferentially interacts with SRC-1 (41). We thus investigated whether SRC-1 down-regulation might be also modulated by PR ligands. As previously reported (22), immunocytochemical studies (Fig 3A) and Western Blot experiments (Supplemental Fig S5) showed that the agonist ligand R5020 stimulates stably expressed endogenous PR proteolysis after 24 h treatment while the antagonist ligand RU486 prevents PR proteolysis in Ishikawa cells stably expressing PR-B (Ishi-PR-B). To test the impact of ligands on SRC-1 expression level, Ishi-PR-B cells were transiently transfected with a SRC-1 expression vector and incubated overnight with R5020 or RU486. Western Blot analyses revealed that SRC-1 and PR are concomitantly degraded in the presence of agonist R5020 and that RU486 prevents the degradation of both proteins (Fig 3B). Similar results were obtained using different Ishi-PR-B subclones (data not shown). Real time quantitative RT-PCR excluded the possibility of any ligand-dependent down-regulation of SRC-1 mRNA levels (Supplemental Fig S6). MG132 exposure inhibited the agonist-dependent proteolysis of SRC-1 (Fig 3B, lane 4), indicating that this stimulated down-regulation is mediated by the proteasome. Importantly, using antibodies specifically detecting endogenous SRC-1, we similarly observed agonist-dependent degradation of endogenous SRC-1 in Ishi-PR-B cells (Fig 3C and Fig 3D). Of note, a 10-fold excess of antiprogestin RU486 abrogated the R5020-dependent degradation of endogenous SRC-1 and PR as shown in Fig 3D (third lane), suggesting that SRC-1 degradation is tightly linked to the ligand-dependent PR activation. To further verify this hypothesis, we tested if SRC-1 proteolysis could be stimulated in the absence of PR. We used the Ishikawa parental cell line (Ishi-PR-0) initially used to establish the Ishi-PR-B cell line and that lacks PR-B expression (42). Ishi-PR-0 cells were transfected with SRC-1 expression vector and incubated 24 h with R5020 or RU486. Under these conditions, both ligands did not affect SRC-1 expression level, indicating that SRC-1 down-regulation requires the presence of PR-B (Fig 3E). Finally, we determined whether other p160 coactivators such as SRC-2/TIF2/GRIP-1 or SRC-3/AIB1, which are also known proteasome targets (14), could be degraded in response to R5020. None of these coactivators was significantly degraded under similar

experimental conditions (Supplemental Fig S7), suggesting a target-specific coactivator effect of PR.

It has been initially proposed that antiprogestins are capable of inducing PR down-regulation but with much slower kinetics than agonists (22). We therefore tried a longer time point to check if SRC-1 degradation was occurring in presence of RU486. The result shows that, in contrast to 24 h incubation (Fig 3F, lane 3), 48 h treatment with RU486 induced a significant reduction of both SRC-1 and PR (Fig 3F, lane 4). More importantly, in presence MG132, RU486 treatment resulted in a dramatic accumulation of PR and SRC-1 (Fig 3F, lane 5), showing that RU486-induced down regulation is mediated by the proteasome. Thus, these results not only indicate that RU486 impairs the ligand-dependent down-regulation of PR and SRC-1 by slowing down their degradation, but also confirm the concomitance of their ligand-dependent proteolysis. Collectively, our results indicate that specific SRC-1 turn-over is modulated in a ligand-dependent manner and requires PR expression.

Identification of SRC-1 domains involved in its degradation

In order to elucidate the mechanisms driving SRC-1 to the proteasome under basal conditions, we identified the domains involved in SRC-1 turn-over. *In silico* analysis of SRC-1 primary sequence was carried out in search for putative PEST degradation motifs. The result indicated that amino-acids 2 to 16 of SRC-1 had a high score (+9.63) for this type of motif. We therefore focused our investigation on the N-terminal subdomain of the coactivator. A critical importance of the bHLH domain for AIB1/SRC-3 mediated proteolysis has been previously reported by Li and collegues (39). Thus, we also explored the role of this domain in SRC-1 down-regulation. Two deletion mutants were generated lacking either the PEST sequence, or the bHLH domain, encompassing amino-acids 2 to 16 [Δ(PEST)] and amino-acids 41 to 136 [Δ(bHLH)], respectively (Fig 4A).

In order to investigate if these two motifs were involved in SRC-1 degradation, wt SRC-1, Δ(PEST) or Δ(bHLH) mutants were expressed in COS-7 cells, and cycloheximide was added to block protein neosynthesis. The decay of wt SRC-1 and mutant proteins was monitored and quantified by western blot as a function of time. SRC-1 expression levels decreased after 1 h and almost disappeared after 6 h (Fig 4B, left panel), indicating of a half-life of approximately 3 h. In contrast, both Δ(PEST) and

Δ(bHLH) expression levels showed no decrease under the same experimental conditions (Fig 4B, middle and right panel), showing that Δ(PEST) and Δ(bHLH) mutants are more stable than wt SRC-1.

To confirm that these motifs were involved in proteasome-mediated SRC-1 degradation, we compared both mutants and wt SRC-1 localization by immunocytochemistry and found that in contrast to the wild-type coactivator (Fig 1) and the Δ(PEST) mutant, the Δ(bHLH) mutant localized predominantly in the nucleus (Fig 4C). In contrast to the wild-type coactivator (Fig 1), colocalization studies of both mutants with 19S proteasome antigens S7/Rpt1 and with the α/β proteasome 20S subunits showed no significant overlap (Fig 4C and data not shown).

Moreover, to investigate the involvement of these domains on SRC-1 protein stability, we compared the impact of MG132 on both mutants with wt SRC-1. While SRC-1 protein levels were increased ~3 fold under 15 h MG132 treatment (Fig 4D, left panel), the expression level of either Δ(PEST) or Δ(bHLH) remained unchanged under the same conditions (Fig 4D, middle and right panels). Similarly, expression levels of both mutants were not increased in presence of epoxomicin (Supplemental Fig S8). Of note, quantification comparison of band intensity (Fig 4D, histograms) showed that both mutants were expressed to a greater extent than the wild-type coactivator, suggesting that the deletions may have indeed a stabilizing effect on these mutants. Taken together, our observations show that amino-acids 2-16 and 41-136 are involved in SRC-1 down-regulation by targeting SRC-1 to proteasome degradation at the steady-state.

N-terminal degradation motifs of SRC-1 are necessary for its ligand-dependent down-regulation

In order to evaluate the contribution of the two degradation domains in the context of the hormonal activation, we transiently transfected Ishi-PR-B cells with either wt SRC-1, Δ(PEST) or Δ(bHLH) mutants. We hypothesized that if the two degradation motifs are also involved in hormone-stimulated down-regulation of SRC-1, then both mutants should not undergo proteolysis under hormone stimulation. As expected, after 24 h of R5020 treatment, wt SRC-1 was significantly down-regulated, while the expression level of both mutants showed no significant variation (Fig 5A). Interestingly, the ligand-dependent down-regulation of PR still occurred in each condition, showing that the receptor

down-regulation does not require SRC-1 degradation (Fig 5A). To exclude the possibility that the two deletions may have impaired the interaction between the SRC-1 and PR, we conducted reciprocal co-immunoprecipitation experiments in cells transiently expressing PR and either wt SRC-1 or the deletion mutants. The result shown in Fig 5B indicates that PR reciprocally co-immunoprecipitates with wt SRC-1 as well as with Δ(PEST) and Δ(bHLH) mutants. Taken together, these results indicate that under hormonal stimulation, SRC-1 ligand-dependent proteolysis requires both degradation signals.

Since we showed that SRC-1 could be partially proteolyzed in the cytoplasm where it colocalized in speckles with the proteasome (Fig 1), we next wondered if PR will colocalize in the same cytoplasmic speckles. This may specially be the case if we consider the work of Qiu et al. who have shown that PR down-regulation under hormone treatment occurs in the cytoplasm (43). Our result shows that in the absence of hormone, SRC-1 is expectedly cyto-nuclear and does not colocalize with PR (Fig 6A). Eight hours of hormonal treatment (in the presence of cycloheximide) induces the nuclear accumulation of both PR and SRC-1, indicative of their interaction during the nuclear import (29). Interestingly, the ligand also induces the colocalization of PR and its coactivator in cytoplasmic speckles (Fig 6A), suggesting that PR/SRC-1 complexes might be exported back to the cytoplasm. In contrast, in the presence of R5020, Δ(PEST) and Δ(bHLH) mutants were efficiently accumulated in the nucleus, consistent with our coimmunoprecipitation data showing that they do interact with PR in the presence of ligand, but did not colocalize with PR in cytoplasmic speckles (Fig 6B-D). Overall, this experiment suggests that PR and SRC-1 could be proteolyzed as a PR/SRC-1 complex through the same proteasome.

Ligand-dependent proteolysis of SRC-1 is necessary for PR-mediated gene transactivation

To examine the functional link between SRC-1 degradation and its coactivating function, we investigated the impact of coactivator proteolysis on PR-mediated transcription. To this aim, we first analyzed if the proteasome function was required for efficient PR transcriptional activation. Cotransfection of PRE2-TATA-luc reporter gene with the PR encoding vector was performed in parental Ishi-PR-0 cells (devoid of PR), either alone or in combination with the vector encoding SRC-

1. Twenty-four hours after transfection, cells were treated for 24 h with R5020 alone or in combination with MG132. To exclude the possibility that the cellular toxicity of MG132 might affect general transcription in Ishikawa cells, we used a 500 nM concentration of the inhibitor, a dose compatible with cell survival of endometrial carcinoma cell lines (44). We show that MG132 drastically attenuates ligand-dependent PR transactivation (Fig 7A), confirming previous observations made by Dennis et al (45). Interestingly, SRC-1-potentiated PR-mediated transcription was also abolished by the proteasome inhibitor (Fig 7A). This result suggests that the proteasome-mediated degradation is required not only for PR transcriptional activity but also for SRC-1-potentiation of PR. To further explore the relationship between coactivator degradation and the functional consequences on PR-mediated transcription, we used the two non degradable mutants Δ(PEST) and Δ(bHLH) in cotransfection experiments with PR (Fig 7B). Since these 2 mutants are not efficiently degraded by the proteasome (see Fig 4B and 4D), we predicted that they might not exert efficient potentiation of PR transactivation. Indeed, in the presence of R5020, SRC-1 strongly coactivated PR while both Δ(PEST) and Δ(bHLH) mutants were unable to enhance PR-mediated transactivation as compared to wt SRC-1 (Fig 7B). These results suggest that the concomitant degradation of SRC-1 and PR is necessary for efficient transcriptional activity of the receptor. Finally, to determine whether the functional link between SRC-1 proteolysis and its coactivating properties were also relevant for human endogenous gene activation, we quantified the level of the progesterone-induced amphiregulin gene that we have previously studied (46). Parental Ishi-PR-0 cells were transfected with PR alone or in combination with wt or SRC-1 mutants. Amphiregulin mRNA levels were significantly increased upon R5020-dependent PR activation and were further enhanced in the presence of SRC-1 (Fig 7C). Conversely, coexpression of PR with either Δ(PEST) or Δ(bHLH) mutant significantly reduced amphiregulin expression (P <0.001). Taken together, our results demonstrate that hormone-induced degradation of SRC-1 is physiologically relevant for potentiation of PR-mediated transcriptional events.

DISCUSSION

In this study, we investigated the impact of SRC-1 proteolysis on PR-mediated transcription. We provided evidence that the agonist-dependent degradation of SRC-1 is pivotal for PR-mediated transcription. We have established that agonist ligand R5020, but not antagonist RU486, induces the concomitant degradation of endogenous or ectopic PR and SRC-1. Interestingly, SRC-1 turn-over requires the presence of PR. Both basal and induced SRC-1 down-regulation are mediated through the proteasome pathway and seem to occur at least in part, in the cytoplasmic compartment. Two regions located in the N-terminal part of SRC-1 (i.e., a PEST motif and amino-acids 41-136 of the bHLH domain) were identified as two degron motifs. Both signals were shown to be responsible for basal- and hormone induced-degradation of SRC-1. Deletion of each of these domains [Δ(PEST) and Δ(bHLH) mutants] leads to non-degradable SRC-1 mutants insensitive to proteasome inhibitors. By comparing the biological functions of these two mutants with wt SRC-1, we found that they were incapable of potentiating PR-mediated transactivation on a synthetic PR response-element but also on amphiregulin, an endogenous PR target gene. The HAT motif and the CBP interacting domain of SRC-1 are known to regulate the transcriptional activity of SRC-1 (47, 48). Both regions are present in Δ(PEST) and Δ(bHLH) mutants (Fig 4A), and therefore the reduced PR-dependent transactivation of the mutants is not due to an alteration of these regulatory domains but rather to a defect in down-regulation. Thus, our results are indicative of a functional link between proteasome-mediated down-regulation of SRC-1 and its coactivating property.

We have previously shown that SRC-1 is a transcriptional coactivator whose localization is hormonally regulated in the presence of PR (29). Mainly functioning in the nuclear compartment, this coactivator may also be present in the cytoplasm, predominantly concentrated in cytoplasmic speckles (29). Several studies have also demonstrated that p160 coregulators might be localized in the cytoplasm (7, 30, 31). Although the concentration of SRC-1 in cytoplasmic speckles was initially reported to be linked to overexpression (49), it has been also observed for endogenous p160 coactivators (50) and, more importantly, a recent study correlated this archetypical distribution with the cytoplasmic sequestration of SRC-1 by SIP (SRC-Interacting Protein) (51). During our primary

search to identify the nature of these speckles, we initially observed a colocalization between SRC-1 and proteasome antigens, indicating that SRC-1 cytoplasmic speckles are enriched of proteasome components (Fig 1A). Similar subcellular distribution studies already reported SRC-2 colocalization with proteasome antigens but specifically at the nuclear level (34, 52). Coactivator/proteasome interaction have been also described at the biochemical level for the p160 coactivators (33, 53), as well as NR such as the thyroid receptor, the retinoic acid receptors RARα and RXR, the estrogen receptor ERα, or the vitamin D receptor (32). We detected a strong colocalization in the cytoplasmic compartment although a weaker colocalization in speckles was also observed in the nuclear compartment (Fig 1B) indicative of a predominant but not exclusive proteolysis of the coactivator in the cytoplasmic compartment. Interestingly, nuclear export of SRC-3 has been shown to be required for its proteasomal degradation (54). However, our finding is not consistent with the work of Li et al. who recently showed that proteasome-dependent turnover of SRC-3 occurs specifically in the nucleus (39). Although we could not completely exclude that nuclear degradation also occurs for SRC-1 (see colocalization profiles Fig 1B), this discrepancy between SRC-1 and SRC-3 argues for the fact that each SRC family member has different and specific physiological functions (55).

We have shown that the ubiquitin-proteasome pathway mediates selective degradation of SRC-1 and regulates the steady-state expression level of the coactivator. Similarly, Yan et al have shown that several SR coactivators were degraded through the ubiquitin-proteasome dependent pathway and that SRC-1 proteolysis occurs specifically through the Ubiquitin-Conjugating Enzyme 2 (35). The half-life regulation of p160 coactivators has been extensively investigated since the discovery of their prototype SRC-1 and several studies have demonstrated the physiological and pathophysiological importance of regulating SRC-1 expression levels (56-58). SRC-1 is an important modulator of PR-mediated gene transcription and in order to accurately exert its physiological function its level must be therefore tightly regulated *in vivo*. In this context, Han et al used an original transgenic mouse model in which SRC-1 levels were shown to influence the compartment specific corepressor-to-coactivator ratio in order to modulate PR activity in uterus (59). Cell regulation of SRC-1 levels seems to be also critical for tumorigenesis and studies have demonstrated that SRC-1 expression is significantly increased in breast tumors and positively correlates with disease recurrence and poor disease-free

survival (55). Consistent with this finding, SRC-1 level is up-regulated during mammary tumor progression (60) and the role of this coactivator in promoting mammary tumor cell invasion was recently demonstrated *in vivo* (57, 58).

Beside the regulation of SRC-1 proteolysis at basal level, the present study also analyzes ligand-stimulated down-regulation of the coactivator. Similarly to other rapidly turned over transcription factors, engagement of PR in transactivation has been shown to be coupled to PR degradation by the ubiquitin-proteasome pathway (13). However, the functional impact of the SRC-1 coactivator on PR-mediated transactivation has never been clearly established. We demonstrate for the first time that concomitantly to PR degradation, SRC-1 proteolysis is dramatically increased in the presence of the agonist ligand R5020 and that this process is mediated through the proteasome. Similarly to PR (22), this down-regulation is necessary for PR-mediated transcription. Recent advances in molecular biology have redefined the role of proteasome as a regulatory system that influences the fate of many cellular processes, such as cell proliferation, apoptosis, and more recently gene transcription. Despite the disparate nature of the later process, a growing body of evidences indicates that ubiquitin and the proteasome are intimately involved in NR-mediated gene control (45, 61, 62). Steroid hormone receptors and their coactivators cycle onto and off steroid-responsive promoters in a ligand-dependent manner and it is now believed that the ubiquitin–proteasome functions in promoting the turnover of transcription complexes, thereby facilitating proper gene transcription (16, 63). Dennis et al. have proposed the existence of a transcriptional mechanism that link the proteasome function with the continued recruitment of RNAPII to sustain the transcriptional response (45). Consistent with these observations are the fact that (i) a number of ubiquitin pathway enzymes and components of the proteasome have been found to act as modulators of NR function (24, 26, 64) and that (ii) enzymes and components of the proteasome are recruited to the promoters of NR-responsive genes (16, 63).

In spite of this, it is difficult to conceive how a coactivator will be paradoxically part of a coactivating complex positively modulating gene activation and at the same time a specific target of the ubiquitin-proteasome pathway. Thus, the coupling of PR/SRC-1 proteolysis and efficient transcriptional activation is counterintuitive and rather puzzling but could be a general phenomenon occurring during transcription (65). Consistent with this, is the fact that neither PR nor its coactivator were down-

regulated in presence of the antagonist RU486. This result may suggest that RU486 indirectly prevents recruitment of the proteasome machinery, thereby inhibiting transcription. The same observation was made with ERα and the partial antagonist Tamoxifen though it may not be considered as a general phenomenon for Steroid Receptor since the pure antagonist Faslodex dramatically stabilize ER in similar conditions (49). It is not the first example of a hormonal regulatory mechanism implicated in specific coregulators proteolysis : indeed, SRC-2 is down-regulated through the activation of the cAMP dependent protein kinase pathway (52). More importantly, Gianni et al showed that SRC-3, but not SRC-1 or SRC-2, is phosphorylated by p38MAPK in a Retinoic Acid-dependent manner and then degraded by the proteasome pathway (27). In this case, phosphorylation of SRC-3 has a biphasic effect on RARα transactivation with facilitation followed by restriction of transcription.

Since the presence of PR is required for SRC-1 degradation, two important remaining questions concern the identification of the key-player responsible for SRC-1 degradation and whether this factor is involved in both basal and ligand-induced SRC-1 down-regulation. Shao et al used RNA interference to knock-down SRC-3 that consequently abolishes ERα ligand-dependent degradation, suggesting that the coactivator itself regulates ERα degradation (66). Conversely, since the two non-degradable mutants did not impede the ligand-induce PR down-regulation (Fig 5A), our results do not converge towards a link between the recruitment of a common E3-ligase by SRC-1 which will in turn induce the ligand-dependent degradation of the PR/SRC-1 complex. The signal that targets PR and SRC-1 to progress from transcription to degradation may also involve post-translational modifications operating like a molecular signature such as phosphorylation, ubiquitinylation or sumoylation (9, 67, 68). Alternatively, direct recruitment of ligase in the vicinity of the coactivator complex or directly at the enhancer level may be also implicated in SRC-1 turnover along with PR. A good candidate would be the PR-B coactivator/ubiquitin ligase E6-AP since this coactivator plays a major role in controlling the regulated degradation of SRC-3 and PR-B isoform (54, 69). Alternatively, the colocalization with proteasome antigens observed in our study (Fig 1) might also be linked to the direct interaction observed between SRC-1 and the proteasome through the Low Molecular mass Polypeptide 2 proteasome subunit (LMP2) (53). Such a direct ligand-dependent interaction may drive the coactivator to proteolysis. Another potential candidate for PR and SRC-1 degradation might be Jab1, a coactivator

involved in ER degradation (70). We are currently investigating this hypothesis, since we have shown in a previous study that Jab1 is a coactivator of PR, inducing the formation of a PR/SRC-1/Jab1 ternary complex during the transcription process (71).

In summary, we demonstrate in the present study that SRC-1 expression level is hormonally regulated by the ligand. While, in presence of an agonist the PR/SRC-1 complex is proteolyzed in order to achieve transcription, an antagonist as RU486 impairs the ligand-dependent degradation of PR/SRC-1 and consequently the transactivation process. Our data indicate that the expression level of SRC-1 coactivator is critical for PR transcriptional activity. These findings are consistent with the emerging role of the 26S proteasome in the gene regulation process (72). P160 family members are certainly not the only coactivators implicated in such processes and it will be interesting to elucidate the sequential progression of each coregulator degradation during gene regulation.

MATERIALS AND METHODS

Hormone and inhibitors

Cycloheximide, Epoxomicin (Epoxo), MG132, Leptomycin B (LB) were purchased from Sigma (St Louis, MO). Agonist R5020 (17,21-dimethyl-19-norpregna-4,9-dien-3,20-dione) and antagonist RU486 (Sigma, St Louis, MO) were used at a concentration of 10nM, except where indicated.

Plasmids

Nomenclature: derivatives denoted with a Δ lack the protein segment delineated by the numbered amino-acids. Plasmids encoding the wild-type human progesterone receptor (pSG5-PR) and coactivator SRC-1 (pSG5-SRC-1, pSG5-HA-SRC-1, pSG5-HA-GFP-SRC-1) have previously been described (29). PCR-based site-directed mutagenesis of pSG5-HA-SRC-1 was used to create deletion mutants: pSG5-HA-SRC-1-Δ(2-16) [named "Δ(PEST)"], pSG5-SRC-1-Δ(41-136) [named "Δ(bHLH)"] and pSG5-HA-SRC-1Δ(990–1060) [named "Δ(NES)", (29)]. The plasmid pPRE2-TATA-Luc has been previously described (71). Plasmid pSG5-His6-Ub is a gift of D. Bohmann (Laboratory EMBL, Heidelberg). Plasmids pSG5-SRC-2 and pCR3.1-SRC-3 have been described previously described (7, 73) and GFP-Peroxisome targeting signal expression vector was purchased (Clontech, Mountain View, CA).

Cell culture and DNA transfection

Human endometrial Ishikawa cells (parental cell line "Ishi-PR-0" and stable "Ishi-PR-B") were provided by Dr LJ. Blok (Erasmus University, Rotterdam, Netherland) (74). COS-7, HEK293, Ishi-PR-0 and Ishi-PR-B were grown in DMEM containing 10% fetal bovine serum (Biowest, Miami, FL) and supplemented with L-glutamine and antibiotics (penicillin / streptomycin, PAA Laboratories GmbH, Austria). For hormonal regulation experiments, cells were grown in the presence of 10% steroid-depleted FBS prior (24h) and during transfection experiments. Transfections were performed with the indicated expression vectors using LipofectAMINE 2000 according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA).

Antibodies

Monoclonal anti-PR antibodies used in the study were the Let126 (0.5µg/mL) (75), the monoclonal anti-PR from Novocastra (NCL-L-PGR-312/2) or the rabbit polyclonal anti-PR (sc-538) from Santa Cruz Biotechnology, used for immunoprecipitaion. Anti-SRC-1 mouse monoclonal antibody (Millipore, Billerica, MA) was used for Western Blot and immunocytochemistry (1µg/mL). Endogenous SRC-1 was detected with anti-SRC-1 (sc-6096) purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA 3F10 (200ng/mL) was from Roche Applied Science (Indianapolis, IN). Rabbit polyclonal antibody directed against human S7/Rpt1 and 20S proteasome subunits, and KAT13C/NCOA2/SRC-2 were purchased from Abcam (Cambridge, MA) and used at 1:1000 dilution. Anti-α-tubulin (1:10000) and anti SC-35 (1µg/mL) were purchased from Sigma (St Louis, MO). Anti-PML was provided by H de Thé (IUH, Paris, France). Anti-SRC3/AIB1 antibody was purchased from BD Biosciences (San Diego, CA) and was used at 0.5µg/ml. Secondary antibodies (1:4000) : anti-mouse, anti-rat, anti-rabbit antibodies conjugated to alexa 488 (green) or 595 (red) or Dylight 549 (red) were from Invitrogen (Carlsbad, CA) and Jackson ImmunoResearch Laboratories (West Grove, PA). Secondary peroxydase-conjugated anti-mouse (Calbiochem, San Diego, CA) and anti-rabbit (Vector laboratories Inc., Burlingame, CA) antibodies were used at 1:15000 dilution.

Luciferase reporter gene assays

COS-7 cells were cultured in free steroid medium and reverse transfected in 96-well plates with 4ng PR, 100ng PRE2-TATA-Luciferase, 100ng SRC-1 (wild-type or mutants), and 5ng β-galactosidase (internal control). The pBlue-Script plasmid was used to equally adjust DNA quantity. After 24h transfection, cells were incubated with or without 10nM R5020 for 24h. Cells were collected with the Passive Lysis Buffer (Promega, Madison, WI) and luciferase activity was measured with a luminometer (Victor, Perkin Elmer, Waltham, MA). Luciferase activity was normalized with β-galactosidase activity. The results are means ± S.E. of four independent experiments.

Immunocytochemistry

Cells were seeded in 24-well plates and processed as previously described (7). Briefly, cells were fixed with 4% paraformaldehyde and permeabilized for 30 min with a 0.5% solution of Triton X100 diluted in PBS. Cells were then incubated with primary antibody overnight at 4°C, followed by the appropriate fluorochrome-coupled secondary antibody (alexa 488 or 595, Invitrogen; or Dylight 549, Jackson ImmunoResearch Laboratories) for 30 min. Nuclear counterstaining was performed with 0.5 µg/mL DAPI (4′,6′-diamidino-2-phenylindole) and coverslips were mounted on slides with ProLong Gold mounting medium (Invitrogen, Carlsbad, CA). For standard microscopy (Fig 2 and 3), fluorescent cells were observed with an Olympus Provis AX70 and images were acquired with Qcapture Pro version 5.1 (Q Imaging Inc., Surrey, BC) using an Evolution VF Monochrome camera (Media Cybernetics Inc., Bethesda, MD).

Confocal Microscopy

For Fig 1, 4 and 6, a Zeiss LSM-510 confocal laser scanning microscope (Carl Zeiss, Thornwood, NY) was used for fluorescence acquisition. Images of fixed cells were collected from equatorial planes of cells with a pinhole setting of ~1.0 airy unit (AU) (optical thickness of 0.8 µm) using a x63:1.4NA oil immersion plan-apochromat objective with X8 frame averaging accumulation. In order to exclude crosstalk artifacts, both red and green fluorescence emission were acquired sequentially in separated channels. The confocal microscope settings were kept the same for all scans. To validate colocalization of proteins (Fig 1 and 4), line scans of intensity profiles across the cells were generated with the LSM browser software (76). This function associates the merge images with an intensity profile of each channel, measured along a freely positioned line. To obtain an average representative intensity profile expressed as arbitrary units (AU), lines were drawn through the middle of each cell images in a distance covering the cytosol and the nucleus. Green lines represent the intensity profile for the proteasome antigen S7/Rpt1 signal and the red lines represent the intensity profile for SRC-1 signal.

Western Blot and Immunoprecipitation

Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.2 mM NaF, 0.2 mM Na_3VO_4, protease inhibitor cocktail) for 15 min, and the debris were cleared by centrifugation at 14000 × *g* for 15 min at 4°C. Samples were resolved by 7.5% SDS gel electrophoresis and transferred onto nitrocellulose membranes. The indicated antibodies were diluted in TBST buffer supplemented with 5% non fat milk and added to the membranes for 1h30 at room temperature (RT) or overnight at 4°C followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies for 45 min at RT. All proteins were detected with ECL Plus detection reagents (Amersham Biosciences Corp, Piscataway, NJ) and visualized by chemiluminescence. For the normalization, the membrane was stripped, probed with anti-α-tubulin antibody diluted to 1:1000 (Sigma, St Louis, MO). The bands were quantified after digitalization on a gel scanner using Image J software. Results, mean of 3 independent experiments (except Fig 5A), are presented as the ratio SRC-1(or PR)/α-Tubulin and are expressed as fold induction above the value measured for wild-type SRC1 in the absence of MG132 arbitrary set at 1. For coimmunoprecipitation, HEK 293 cells were transfected in 100mm plate with either wt SRC-1, Δ(PEST) or Δ(bHLH) plasmids, and cultured in presence of 10^{-8}M R5020 for 24h. Cells were lysed at 4°C in 500µl lysis buffer and cell debris were pelleted by centrifugation (14.000 rpm, 15 min, 4°C). Immunoprecipitation of the supernatant with anti-SRC-1 or with the rabbit polyclonal anti-PR or with IgG control were performed with Protein G Magnetics Beads (Millipore, Billerica, MA) according to the manufacturer instructions. Bound immunocomplexes were boiled in Laemmli buffer, separated by 7.5% SDS-PAGE, blotted nitrocellulose membranes with anti 1µg/mL SRC-1 (Millipore, Billerica, MA) and anti PR-B (Let 126, 0.5µg/mL) antibodies, detected with ECL Plus detection reagents (Amersham, Biosciences Corp, Piscataway, NJ), and visualized by chemiluminescence.

Real Time RT-PCR

The Ishikawa cell line expressing PR-B or not was transfected by the indicated plasmids by Polyfect reagent (QIAGEN, Valencia, CA) in six-well plates (six wells per condition). After a 2 h-treatment by R5020 10nM, cells were washed and lysed by Trizol reagent (Life Technologies, Gaithersburg, MD).

Total RNA were extracted as described by the manufacturer. One microgram of each sample was treated by DNase I and was reverse transcribed using random primers as previously described (77). Real-time quantitative PCR of amphiregulin gene was performed as described (46) using the Power SYBR Green master mix (Applied Biosystem, Carlsbad, CA) in duplicate with 1:20 fraction of each cDNA sample and the corresponding primers, using an ABI Prism 7300 apparatus. For each sample, the mRNA concentration was extrapolated from standard curve and averaged Ct value was divided by that of the corresponding reverse-transcribed 18S RNA (relative mRNA).

Statistical analysis

Data are expressed as the mean ± SEM. Mann Whitney U-test was used to determine significant differences between two groups. For multiple comparisons, Kruskal-Wallis test followed by Dunn's post-test was performed using the computer software Prism 4 (GraphPad Software, San Diego, CA). Statistical significance is indicated at P values < 0.05, 0.01 and 0.001.

FIGURES LEGENDS

Fig.1. Colocalization of SRC-1 with the 26S proteasome by confocal microscopy.

A, Colocalization analysis between HA-SRC-1 and endogenous proteasome antigens S7/Rpt1 and 20S subunits. COS-7 cells were transiently transfected with the expression vector encoding HA-SRC-1. Cells were fixed after 40 h, immunolabeled with anti-HA and either anti-S7/Rpt1 or anti-20S antibodies, and then observed by confocal microscopy.

B, Validation of colocalization by scan of intensity profiles of a representative cell (expressed as arbitrary units, AU). Fluorescence intensity was calculated and plotted by drawing a line through the middle of the cell image in a distance covering several cytosolic and nuclear foci. *Green lines* represent the intensity profile for the proteasome antigen S7/Rpt1 signal and the *red lines* represent the intensity profile for SRC-1 signal. Indicated numbers refer to identified speckles: cytoplasmic (1 to 9), nuclear (8 to 11). Note that although the fluorescence intensity from the two channels is different, the peaks of both signals are overlaping.

Fig.2. SRC-1 is proteolyzed by the 26S proteasome in a ubiquitin-dependent manner.

A, COS-7 cells were transfected with the expression vector encoding SRC-1 and incubated in the absence or presence of MG132 (5 µM) during 15 h. Expression of SRC-1 was analyzed by Western blot using anti-SRC-1 and anti-α-tubulin antibodies. Bands intensity corresponding to SRC-1 were quantified as described in “Materials and Methods”.

B, CV-1 cells were transfected with the expression vector encoding HA-SRC-1 in the presence of the His6-tagged ubiquitin expression vector (His 6-Ub). Whole cell extracts were analyzed by electrophoresis on 6.4% SDS-PAGE and immunoblotted with anti-HA monoclonal antibody. Alternatively, the same co-transfected CV-1 cells were lyzed in buffer containing guanidium-HCl (Ni-NTA). The ubiquitin-modified proteins were purified using Ni-NTA agarose beads as described under “Materials and Methods.” Affinity purified proteins were separated by electrophoresis, and His6-SRC-1 conjugates were detected by Western blot using the anti-HA monoclonal antibody. The ubiquitin conjugates of SRC-1 are indicated with brackets.

C, COS-7 cells were transfected with the expression vector encoding HA-SRC-1. Twenty hours post-

transfection, cells were incubated during 24 h with MG132 (1 µM) or treated with vehicle (DMSO). Cells were then fixed and immunolabeled with anti-HA antibody.

D, COS-7 cells were transfected with the expression vector encoding SRC-1 or SRC-3 and treated similarly than in C except that MG132 was replaced by Leptomycin B treatment (LB, 20 ng/ml). Expression levels of SRC-1 and SRC-3 were analyzed by Western blot using anti-SRC-1 or anti-SRC-3 monoclonal antibodies as indicated. Bands intensity representing the mean of at least 2 independent experiments were quantified as described in “Material and Methods”.

Fig.3. Ligand- and PR-dependent SRC-1 proteolysis.

A, Ishi PR-B cells, a cell line stably expressing PR-B, were cultured 24 h in the absence or in the presence of either the agonist R5020 (10 nM) or the antagonist RU486 (10 nM). Cells were then treated for immunocytochemistry with anti-PR antibody (Let 126) and observed by fluorescence microscopy.

B, Ishi PR-B cells were transfected with the SRC-1 encoding vector. After 48 h, cells were cultured 15h as indicated, either in the absence of ligand (control vehicule, -H), in the presence of R5020 (10 nM), RU486 (10 nM), or in the presence of both R5020 (10 nM) and MG132 (5 µM). Whole cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies.

C, Non-transfected Ishi PR-B cells were treated as in B. Cells were immunolabeled for endogenous SRC-1 using an anti-SRC-1 antibody. Note the agonist-ligand-dependent down-regulation of endogenous SRC-1.

D, Non-transfected Ishi PR-B cells were cultured 24 h in the absence of ligand (vehicule, -H) or in the presence of either the agonist R5020 (10 nM) alone or in combination with a 100x excess of the antagonist RU486 (1 µM). Whole cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted to detect endogenous SRC-1 and PR with the indicated antibodies.

E, Ishi PR-0 cells (parental cell line, devoid of PR) were treated as in Fig 3A. Cells were then analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies.

F, Ishi PR-B cells were transfected with the SRC-1 encoding vector. After 24h, cells were cultured

either in the absence of ligand (vehicule, -H), treated with R5020 (10 nM, 24h), RU486 (10 nM, 24 h or 48 h), or RU486 (10 nM, 24 h) along with MG132 (1 µM). Whole cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies.

Fig.4. The N-terminal Region of SRC-1 targets the coactivator to degradation.

A, Schematic representation of the wild-type coactivator SRC-1 (1441 amino-acids in length) with boxes corresponding to major functional domains: bHLH: basic Helix Loop Helix domain, PAS: Per-ARNT-Sim motif, NR1 and NR2: Nuclear Receptor-Interacting Domains 1 and 2, CBP/p300 interacting domain, Q: glutamine-rich domain. SRC-1 deletion mutants Δ(PEST) and Δ(bHLH) are represented below with a thick line interrupted by a gap corresponding to the deleted amino-acids.

B, COS-7 cells were transfected as indicated with SRC-1, Δ(PEST) or Δ(bHLH) encoding vectors. Seventy-two hours after transfection, cells were treated with cycloheximide (100 µg/ml) during 1, 4 or 6 h. Whole cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies. Bands intensities (right panel) representing the mean of at least 2 independent experiments were quantified as described in “Material and Methods”.

C, Upper panel: Colocalization analysis of SRC-1 deletion mutants and S7/Rpt1. COS-7 cells were transiently transfected with Δ(PEST) or Δ(bHLH) encoding vectors. Cells were fixed after 40 h and immunolabeled with anti-HA and anti-Rpt1/S7 antibodies prior analysis by confocal microscopy.

Lower panel : scan of intensity profiles expressed as arbitrary units, AU. Fluorescence intensity was calculated and plotted by drawing a line through the middle of the cell image in a distance covering several cytosolic and nuclear foci. *Green lines* represent the intensity profile for the proteasome antigen S7/Rpt1 signal and the *red lines* represent the intensity profile for Δ(PEST) or Δ(bHLH) signals. Note the absence of significant peaks with overlapping signals.

D, COS-7 cells were transfected with HA-SRC-1, Δ(PEST) or Δ(bHLH) encoding vectors. After 48 h, cells were incubated during 15 h with MG132 (5 µM) or vehicule. Whole cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies. The band intensities (right panel) were quantified as described in “Materials and Methods”.

Fig 5. Ligand-dependent down-regulation of SRC-1 requires both degradation motifs of the coactivator.

A, Ishi PR-B cells were transfected as indicated with HA-SRC-1, Δ(PEST) or Δ(bHLH) encoding vectors. After 48 h, cells were cultured in the absence of ligand (vehicule, -), or in the presence of the agonist R5020 (10 nM) during 24h. The corresponding whole cell extracts were analyzed by electrophoresis on 7.5% SDS-PAGE and immunoblotted with the indicated antibodies. The band intensities (lower panel) were quantified as described in "Materials and Methods".

B, HEK293 cells were cotransfected with PR and either the SRC-1, Δ(PEST), or Δ(bHLH) encoding vectors. Twenty four hours after transfection, cells were treated during 24 h with the agonist R5020 (10 nM). A coimmunoprecipitation assay was performed using either the anti-SRC-1, the anti-PR, or the IgG1 control antibodies (IgG1). Purified proteins were separated on 7.5% SDS-PAGE. Co-precipitated complexes were identified with the indicated antibodies.

Fig. 6. Colocalization of PR and SRC-1 in cytoplasmic speckles.

A, COS-7 cells were transiently transfected with the expression vector encoding HA-SRC-1 and PR. Twenty-four hours after transfection, cells were incubated or not for 8 h with R5020 in presence of cycloheximide (100 µg/ml) prior fixation. Cells were immunolabeled with anti-PR (Let 126) and anti-HA antibodies.

B and C, cells were treated as in A, except that PR was transfected as indicated with Δ(PEST) and Δ(bHLH), respectively.

D, Quantification of cells treated as described in A, B and C. Percent of cells treated with R5020 showing nuclear localization with or without cytoplasmic speckles. At least 100 cells were counted.

Fig.7. SRC-1 degradation is necessary for PR transcriptional activity.

A, Ishi PR-0 cells were cotransfected as indicated with expression vectors encoding PR and SRC-1 together with the reporter gene PRE2-TATA-luc and the internal control pRS-β-gal. Cells were incubated with R5020 (10 nM) and treated or not with MG132 (500 nM) during 24 h. Luciferase

activity was quantified and normalized by β-galactosidase activity. Data represent means ± SEM of at least three independent determinations.

B, COS-7 cells were cotransfected as indicated with HA-SRC1, Δ(PEST) or Δ(bHLH) encoding vectors, together with expression vector encoding PR, the reporter gene PRE2-TATA-luc and the internal control pRS-β-gal. Cells where treated during 24 h with R5020 (10 nM) or vehicule (control, -). Luciferase activity was quantified and normalized by β-galactosidase activity. Data represent means ± SEM of four independent determinations performed in triplicate.

C, Ishi PR-0 cells were cotransfected as indicated with HA-SRC-1, Δ(PEST) or Δ(bHLH) encoding vectors together with PR encoding vector and were treated with the agonist R5020 10 nM for 3 h. Total RNAs were extracted and relative expression of amphiregulin gene was quantified by qRT-PCR. Results, normalized by the amplification of 18S RNA, are mean ± SEM of three independent determinations. Statistical significance *** $P<0.001$ vs wild-type SRC-1 used as reference.

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REFERENCES

1. **Li X, O'Malley BW**. (2003). Unfolding the action of progesterone receptors. J Biol Chem 278:39261-39264

2. **Lange CA**. (2008). Challenges to defining a role for progesterone in breast cancer. Steroids 73:914-921

3. **Lonard DM, O'Malley BW**. (2006). The expanding cosmos of nuclear receptor coactivators. Cell 125:411-414

4. **Xu J, Li Q**. (2003). Review of the in vivo functions of the p160 steroid receptor coactivator family. Mol Endocrinol 17:1681-1692

5. **Chen D, Ma H, Hong H, Koh SS, Huang SM, Schurter BT, Aswad DW, Stallcup MR**. (1999). Regulation of transcription by a protein methyltransferase. Science 284:2174-2177

6. **Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfeld MG**. (1997). The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature 387:677-684

7. **Amazit L, Pasini L, Szafran AT, Berno V, Wu RC, Mielke M, Jones ED, Mancini MG, Hinojos CA, O'Malley BW, Mancini MA**. (2007). Regulation of SRC-3 intercompartmental dynamics by estrogen receptor and phosphorylation. Mol Cell Biol 27:6913-6932

8. **Naeem H, Cheng D, Zhao Q, Underhill C, Tini M, Bedford MT, Torchia J**. (2007). The activity and stability of the transcriptional coactivator p/CIP/SRC-3 are regulated by CARM1-dependent methylation. Mol Cell Biol 27:120-134

9. **Rowan BG, Weigel NL, O'Malley BW**. (2000). Phosphorylation of steroid receptor coactivator-1. Identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway. J Biol Chem 275:4475-4483

10. **Wu RC, Qin J, Hashimoto Y, Wong J, Xu J, Tsai SY, Tsai MJ, O'Malley BW**. (2002). Regulation of SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) Coactivator activity by I kappa B kinase. Mol Cell Biol 22:3549-3561

11. **McKenna NJ, O'Malley BW**. (2002). Minireview: nuclear receptor coactivators--an update. Endocrinology 143:2461-2465

12. **Rosenfeld MG, Lunyak VV, Glass CK**. (2006). Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes Dev 20:1405-1428

13. **Shen T, Horwitz KB, Lange CA**. (2001). Transcriptional hyperactivity of human progesterone receptors is coupled to their ligand-dependent down-regulation by mitogen-activated protein kinase-dependent phosphorylation of serine 294. Mol Cell Biol 21:6122-6131

14. **Lonard DM, Nawaz Z, Smith CL, O'Malley BW**. (2000). The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. Mol Cell 5:939-948

15. **Wijayaratne AL, McDonnell DP**. (2001). The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. J Biol Chem 276:35684-35692

16. **Reid G, Hubner MR, Metivier R, Brand H, Denger S, Manu D, Beaudouin J, Ellenberg J, Gannon F**. (2003). Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. Mol Cell 11:695-707

17. **Yokota K, Shibata H, Kobayashi S, Suda N, Murai A, Kurihara I, Saito I, Saruta T**. (2004). Proteasome-mediated mineralocorticoid receptor degradation attenuates transcriptional response to aldosterone. Endocr Res 30:611-616

18. **Tirard M, Almeida OF, Hutzler P, Melchior F, Michaelidis TM**. (2007). Sumoylation and proteasomal activity determine the transactivation properties of the mineralocorticoid receptor. Mol Cell Endocrinol 268:20-29

19. **Milgrom E, Thi L, Atger M, Baulieu EE**. (1973). Mechanisms regulating the concentration and the conformation of progesterone receptor(s) in the uterus. J Biol Chem 248:6366-6374

20. **Mullick A, Katzenellenbogen BS**. (1986). Progesterone receptor synthesis and degradation in MCF-7 human breast cancer cells as studied by dense amino acid incorporation. Evidence for a non-hormone binding receptor precursor. J Biol Chem 261:13236-13246

21. **Wei LL, Krett NL, Francis MD, Gordon DF, Wood WM, O'Malley BW, Horwitz KB**. (1988). Multiple human progesterone receptor messenger ribonucleic acids and their autoregulation by progestin agonists and antagonists in breast cancer cells. Mol Endocrinol 2:62-72

22. **Lange CA, Shen T, Horwitz KB**. (2000). Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc Natl Acad Sci U S A 97:1032-1037

23. **Lipford JR, Deshaies RJ**. (2003). Diverse roles for ubiquitin-dependent proteolysis in transcriptional activation. Nat Cell Biol 5:845-850

24. **Masuyama H, MacDonald PN**. (1998). Proteasome-mediated degradation of the vitamin D receptor (VDR) and a putative role for SUG1 interaction with the AF-2 domain of VDR. J Cell Biochem 71:429-440

25. **Nawaz Z, Lonard DM, Dennis AP, Smith CL, O'Malley BW**. (1999). Proteasome-dependent degradation of the human estrogen receptor. Proc Natl Acad Sci U S A 96:1858-1862

26. **Verma S, Ismail A, Gao X, Fu G, Li X, O'Malley BW, Nawaz Z**. (2004). The ubiquitin-conjugating enzyme UBCH7 acts as a coactivator for steroid hormone receptors. Mol Cell Biol 24:8716-8726

27. **Gianni M, Parrella E, Raska I, Jr., Gaillard E, Nigro EA, Gaudon C, Garattini E, Rochette-Egly C**. (2006). P38MAPK-dependent phosphorylation and degradation of SRC-3/AIB1 and RARalpha-mediated transcription. Embo J 25:739-751

28. **Han SJ, Tsai SY, Tsai MJ, O'Malley BW**. (2007). Distinct temporal and spatial activities of RU486 on progesterone receptor function in reproductive organs of ovariectomized mice. Endocrinology 148:2471-2486

29. **Amazit L, Alj Y, Tyagi RK, Chauchereau A, Loosfelt H, Pichon C, Pantel J, Foulon-Guinchard E, Leclerc P, Milgrom E, Guiochon-Mantel A**. (2003). Subcellular localization and mechanisms of nucleocytoplasmic trafficking of steroid receptor coactivator-1. J Biol Chem 278:32195-32203

30. **Chen Y, Chen PL, Chen CF, Sharp ZD, Lee WH**. (1999). Thyroid hormone, T3-dependent phosphorylation and translocation of Trip230 from the Golgi complex to the nucleus. Proc Natl Acad Sci U S A 96:4443-4448

719 31. **Grenier J, Trousson A, Chauchereau A, Cartaud J, Schumacher M, Massaad C**. (2006).
720 Differential recruitment of p160 coactivators by glucocorticoid receptor between Schwann cells and
721 astrocytes. Mol Endocrinol 20:254-267
722 32. **Ferrell K, Wilkinson CR, Dubiel W, Gordon C**. (2000). Regulatory subunit interactions of the 26S
723 proteasome, a complex problem. Trends Biochem Sci 25:83-88
724 33. **Yi P, Feng Q, Amazit L, Lonard DM, Tsai SY, Tsai MJ, O'Malley BW**. (2008). Atypical protein
725 kinase C regulates dual pathways for degradation of the oncogenic coactivator SRC-3/AIB1. Mol Cell
726 29:465-476
727 34. **Baumann CT, Ma H, Wolford R, Reyes JC, Maruvada P, Lim C, Yen PM, Stallcup MR, Hager**
728 **GL**. (2001). The glucocorticoid receptor interacting protein 1 (GRIP1) localizes in discrete nuclear foci
729 that associate with ND10 bodies and are enriched in components of the 26S proteasome. Mol
730 Endocrinol 15:485-500
731 35. **Yan F, Gao X, Lonard DM, Nawaz Z**. (2003). Specific ubiquitin-conjugating enzymes promote
732 degradation of specific nuclear receptor coactivators. Mol Endocrinol 17:1315-1331
733 36. **Chen F, Chang D, Goh M, Klibanov SA, Ljungman M**. (2000). Role of p53 in cell cycle regulation
734 and apoptosis following exposure to proteasome inhibitors. Cell Growth Differ 11:239-246
735 37. **Davarinos NA, Pollenz RS**. (1999). Aryl hydrocarbon receptor imported into the nucleus following
736 ligand binding is rapidly degraded via the cytosplasmic proteasome following nuclear export. J Biol
737 Chem 274:28708-28715
738 38. **Tomoda K, Kubota Y, Kato J**. (1999). Degradation of the cyclin-dependent-kinase inhibitor p27Kip1
739 is instigated by Jab1. Nature 398:160-165
740 39. **Li C, Wu RC, Amazit L, Tsai SY, Tsai MJ, O'Malley BW**. (2007). Specific amino acid residues in
741 the basic helix-loop-helix domain of SRC-3 are essential for its nuclear localization and proteasome-
742 dependent turnover. Mol Cell Biol 27:1296-1308
743 40. **Syvala H, Vienonen A, Zhuang YH, Kivineva M, Ylikomi T, Tuohimaa P**. (1998). Evidence for
744 enhanced ubiquitin-mediated proteolysis of the chicken progesterone receptor by progesterone. Life Sci
745 63:1505-1512

41. **Li X, Wong J, Tsai SY, Tsai MJ, O'Malley BW**. (2003). Progesterone and glucocorticoid receptors recruit distinct coactivator complexes and promote distinct patterns of local chromatin modification. Mol Cell Biol 23:3763-3773

42. **Smid-Koopman E, Blok LJ, Kuhne LC, Burger CW, Helmerhorst TJ, Brinkmann AO, Huikeshoven FJ**. (2003). Distinct functional differences of human progesterone receptors A and B on gene expression and growth regulation in two endometrial carcinoma cell lines. J Soc Gynecol Investig 10:49-57

43. **Qiu M, Olsen A, Faivre E, Horwitz KB, Lange CA**. (2003). Mitogen-activated protein kinase regulates nuclear association of human progesterone receptors. Mol Endocrinol 17:628-642

44. **Dolcet X, Llobet D, Encinas M, Pallares J, Cabero A, Schoenenberger JA, Comella JX, Matias-Guiu X**. (2006). Proteasome inhibitors induce death but activate NF-kappaB on endometrial carcinoma cell lines and primary culture explants. J Biol Chem 281:22118-22130

45. **Dennis AP, Lonard DM, Nawaz Z, O'Malley BW**. (2005). Inhibition of the 26S proteasome blocks progesterone receptor-dependent transcription through failed recruitment of RNA polymerase II. J Steroid Biochem Mol Biol 94:337-346

46. **Georgiakaki M, Chabbert-Buffet N, Dasen B, Meduri G, Wenk S, Rajhi L, Amazit L, Chauchereau A, Burger CW, Blok LJ, Milgrom E, Lombes M, Guiochon-Mantel A, Loosfelt H**. (2006). Ligand-controlled interaction of histone acetyltransferase binding to ORC-1 (HBO1) with the N-terminal transactivating domain of progesterone receptor induces steroid receptor coactivator 1-dependent coactivation of transcription. Mol Endocrinol 20:2122-2140

47. **Liu Z, Wong J, Tsai SY, Tsai MJ, O'Malley BW**. (1999). Steroid receptor coactivator-1 (SRC-1) enhances ligand-dependent and receptor-dependent cell-free transcription of chromatin. Proc Natl Acad Sci U S A 96:9485-9490

48. **Smith CL, Onate SA, Tsai MJ, O'Malley BW**. (1996). CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. Proc Natl Acad Sci U S A 93:8884-8888

49. **Stenoien DL, Patel K, Mancini MG, Dutertre M, Smith CL, O'Malley BW, Mancini MA**. (2001). FRAP reveals that mobility of oestrogen receptor-alpha is ligand- and proteasome-dependent. Nat Cell Biol 3:15-23

50. **Chen SL, Wang SC, Hosking B, Muscat GE**. (2001). Subcellular localization of the steroid receptor coactivators (SRCs) and MEF2 in muscle and rhabdomyosarcoma cells. Mol Endocrinol 15:783-796

51. **Zhang Y, Zhang H, Liang J, Yu W, Shang Y**. (2007). SIP, a novel ankyrin repeat containing protein, sequesters steroid receptor coactivators in the cytoplasm. Embo J 26:2645-2657

52. **Hoang T, Fenne IS, Cook C, Borud B, Bakke M, Lien EA, Mellgren G**. (2004). cAMP-dependent protein kinase regulates ubiquitin-proteasome-mediated degradation and subcellular localization of the nuclear receptor coactivator GRIP1. J Biol Chem 279:49120-49130

53. **Zhang H, Sun L, Liang J, Yu W, Zhang Y, Wang Y, Chen Y, Li R, Sun X, Shang Y**. (2006). The catalytic subunit of the proteasome is engaged in the entire process of estrogen receptor-regulated transcription. Embo J 25:4223-4233

54. **Mani A, Oh AS, Bowden ET, Lahusen T, Lorick KL, Weissman AM, Schlegel R, Wellstein A, Riegel AT**. (2006). E6AP mediates regulated proteasomal degradation of the nuclear receptor coactivator amplified in breast cancer 1 in immortalized cells. Cancer Res 66:8680-8686

55. **Xu J, Wu RC, O'Malley BW**. (2009). Normal and cancer-related functions of the p160 steroid receptor co-activator (SRC) family. Nat Rev Cancer 9:615-630

56. **Fleming FJ, Myers E, Kelly G, Crotty TB, McDermott EW, O'Higgins NJ, Hill AD, Young LS**. (2004). Expression of SRC-1, AIB1, and PEA3 in HER2 mediated endocrine resistant breast cancer; a predictive role for SRC-1. J Clin Pathol 57:1069-1074

57. **Wang S, Yuan Y, Liao L, Kuang SQ, Tien JC, O'Malley BW, Xu J**. (2009). Disruption of the SRC-1 gene in mice suppresses breast cancer metastasis without affecting primary tumor formation. Proc Natl Acad Sci U S A 106:151-156

58. **Qin L, Liu Z, Chen H, Xu J**. (2009). The steroid receptor coactivator-1 regulates twist expression and promotes breast cancer metastasis. Cancer Res 69:3819-3827

59. **Han SJ, Jeong J, Demayo FJ, Xu J, Tsai SY, Tsai MJ, O'Malley BW**. (2005). Dynamic cell type specificity of SRC-1 coactivator in modulating uterine progesterone receptor function in mice. Mol Cell Biol 25:8150-8165

60. **Hudelist G, Czerwenka K, Kubista E, Marton E, Pischinger K, Singer CF**. (2003). Expression of sex steroid receptors and their co-factors in normal and malignant breast tissue: AIB1 is a carcinoma-specific co-activator. Breast Cancer Res Treat 78:193-204

61. **Muratani M, Tansey WP**. (2003). How the ubiquitin-proteasome system controls transcription. Nat Rev Mol Cell Biol 4:192-201

62. **Collins GA, Tansey WP**. (2006). The proteasome: a utility tool for transcription? Curr Opin Genet Dev 16:197-202

63. **Perissi V, Aggarwal A, Glass CK, Rose DW, Rosenfeld MG**. (2004). A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. Cell 116:511-526

64. **Nawaz Z, Lonard DM, Smith CL, Lev-Lehman E, Tsai SY, Tsai MJ, O'Malley BW**. (1999). The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. Mol Cell Biol 19:1182-1189

65. **Molinari E, Gilman M, Natesan S**. (1999). Proteasome-mediated degradation of transcriptional activators correlates with activation domain potency in vivo. Embo J 18:6439-6447

66. **Shao W, Keeton EK, McDonnell DP, Brown M**. (2004). Coactivator AIB1 links estrogen receptor transcriptional activity and stability. Proc Natl Acad Sci U S A 101:11599-11604

67. **Salghetti SE, Caudy AA, Chenoweth JG, Tansey WP**. (2001). Regulation of transcriptional activation domain function by ubiquitin. Science 293:1651-1653

68. **Chauchereau A, Amazit L, Quesne M, Guiochon-Mantel A, Milgrom E**. (2003). Sumoylation of the progesterone receptor and of the steroid receptor coactivator SRC-1. J Biol Chem 278:12335-12343

69. **Ramamoorthy S, Dhananjayan SC, Demayo FJ, Nawaz Z**. Isoform-specific degradation of PR-B by E6-AP is critical for normal mammary gland development. Mol Endocrinol 24:2099-2113

70. **Callige M, Kieffer I, Richard-Foy H**. (2005). CSN5/Jab1 is involved in ligand-dependent degradation of estrogen receptor {alpha} by the proteasome. Mol Cell Biol 25:4349-4358

71. **Chauchereau A, Georgiakaki M, Perrin-Wolff M, Milgrom E, Loosfelt H**. (2000). JAB1 interacts with both the progesterone receptor and SRC-1. J Biol Chem 275:8540-8548

72. **Kwak J, Workman JL, Lee D**. The proteasome and its regulatory roles in gene expression. Biochim Biophys Acta

73. **Grenier J, Trousson A, Chauchereau A, Amazit L, Lamirand A, Leclerc P, Guiochon-Mantel A, Schumacher M, Massaad C**. (2004). Selective recruitment of p160 coactivators on glucocorticoid-regulated promoters in Schwann cells. Mol Endocrinol 18:2866-2879

74. **Smid-Koopman E, Kuhne LC, Hanekamp EE, Gielen SC, De Ruiter PE, Grootegoed JA, Helmerhorst TJ, Burger CW, Brinkmann AO, Huikeshoven FJ, Blok LJ**. (2005). Progesterone-induced inhibition of growth and differential regulation of gene expression in PRA- and/or PRB-expressing endometrial cancer cell lines. J Soc Gynecol Investig 12:285-292

75. **Lorenzo F, Jolivet A, Loosfelt H, Thu vu Hai M, Brailly S, Perrot-Applanat M, Milgrom E**. (1988). A rapid method of epitope mapping. Application to the study of immunogenic domains and to the characterization of various forms of rabbit progesterone receptor. Eur J Biochem 176:53-60

76. **Potokar M, Kreft M, Chowdhury HH, Vardjan N, Zorec R**. (2006). Subcellular localization of Apaf-1 in apoptotic rat pituitary cells. Am J Physiol Cell Physiol 290:C672-677

77. **Viengchareun S, Kamenicky P, Teixeira M, Butlen D, Meduri G, Blanchard-Gutton N, Kurschat C, Lanel A, Martinerie L, Sztal-Mazer S, Blot-Chabaud M, Ferrary E, Cherradi N, Lombes M**. (2009). Osmotic stress regulates mineralocorticoid receptor expression in a novel aldosterone-sensitive cortical collecting duct cell line. Mol Endocrinol 23:1948-1962

Fig. 1

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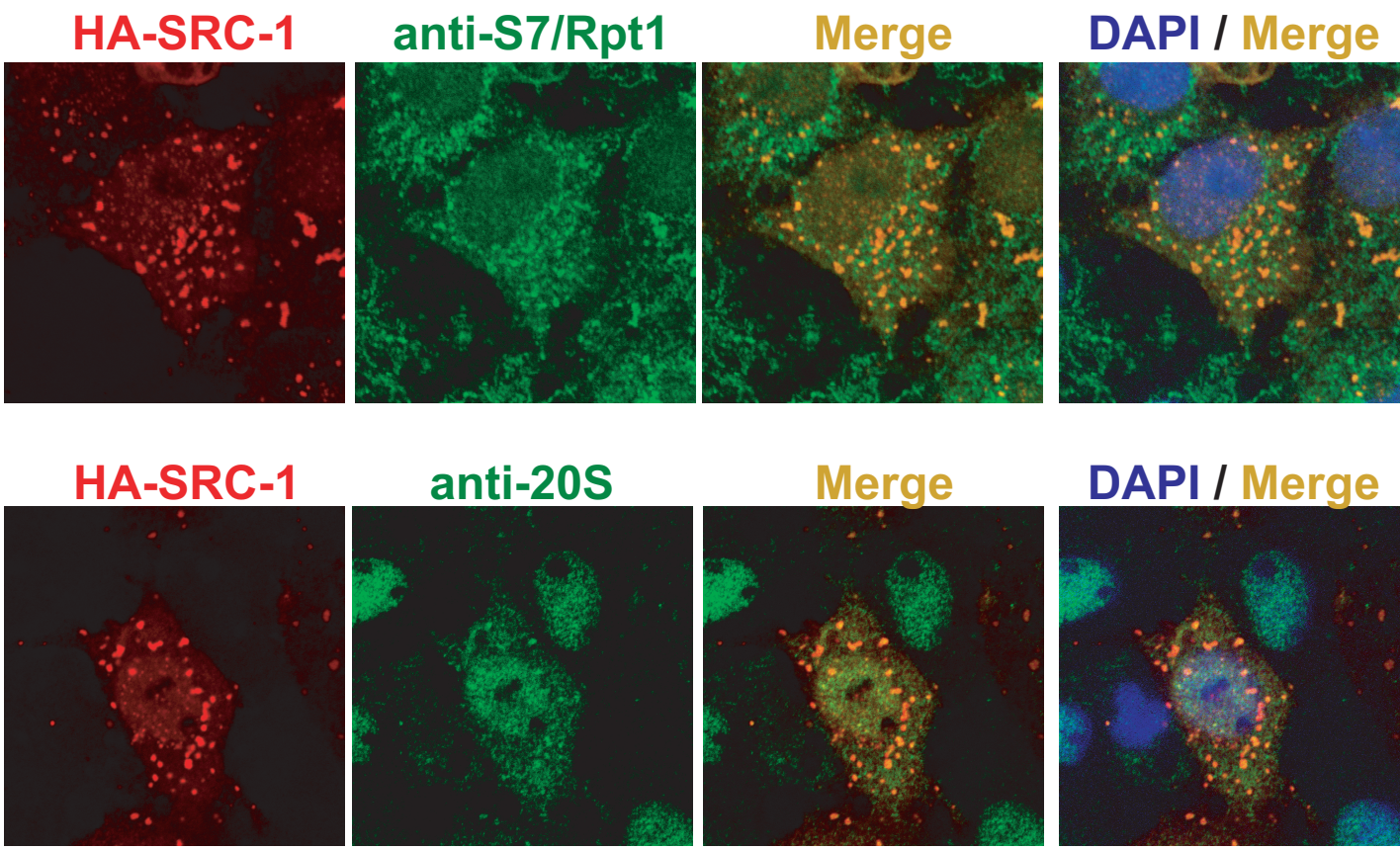
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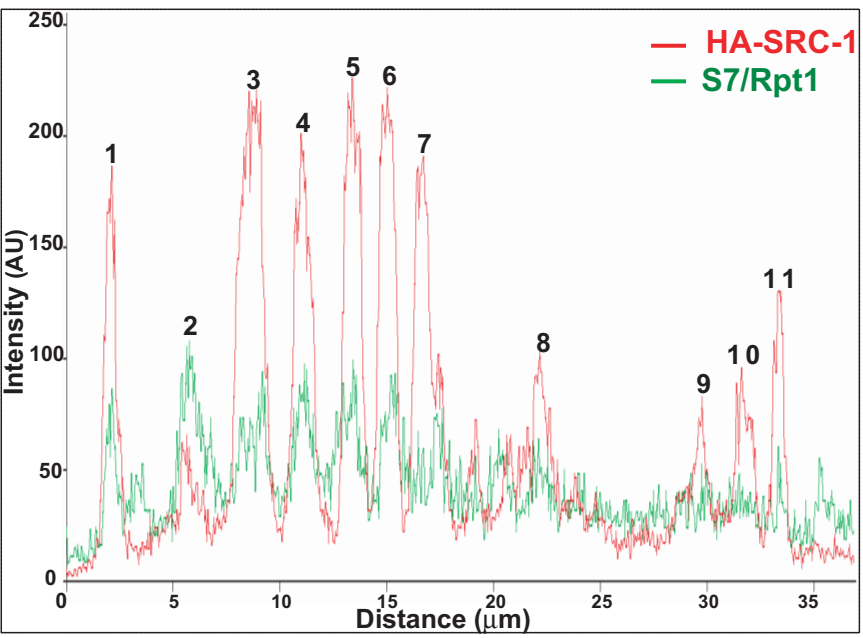
anti-S7/Rpt1
Merge
DAPI / Merge
HA-SRC-1
anti-20S
Merge
DAPI / Merge

B


HA-SRC-1
S7/Rpt1
Intensity (AU)
Distance (μm)
0
50
100
150
200
250
0
5
10
15
20
25
30
35
1
2
3
4
5
6
7
8
9
10
11

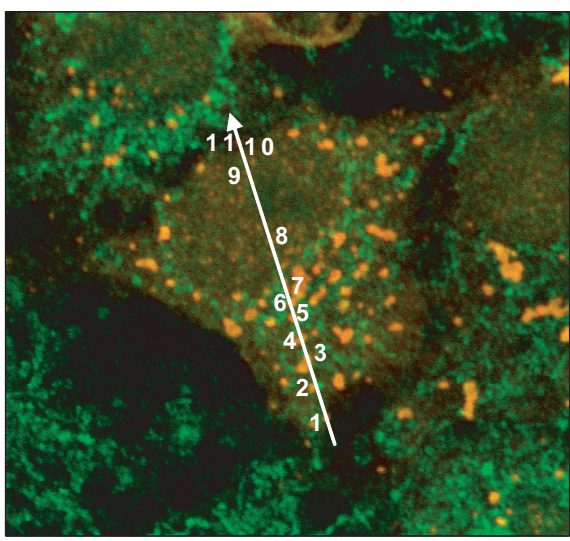

1
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Fig. 2

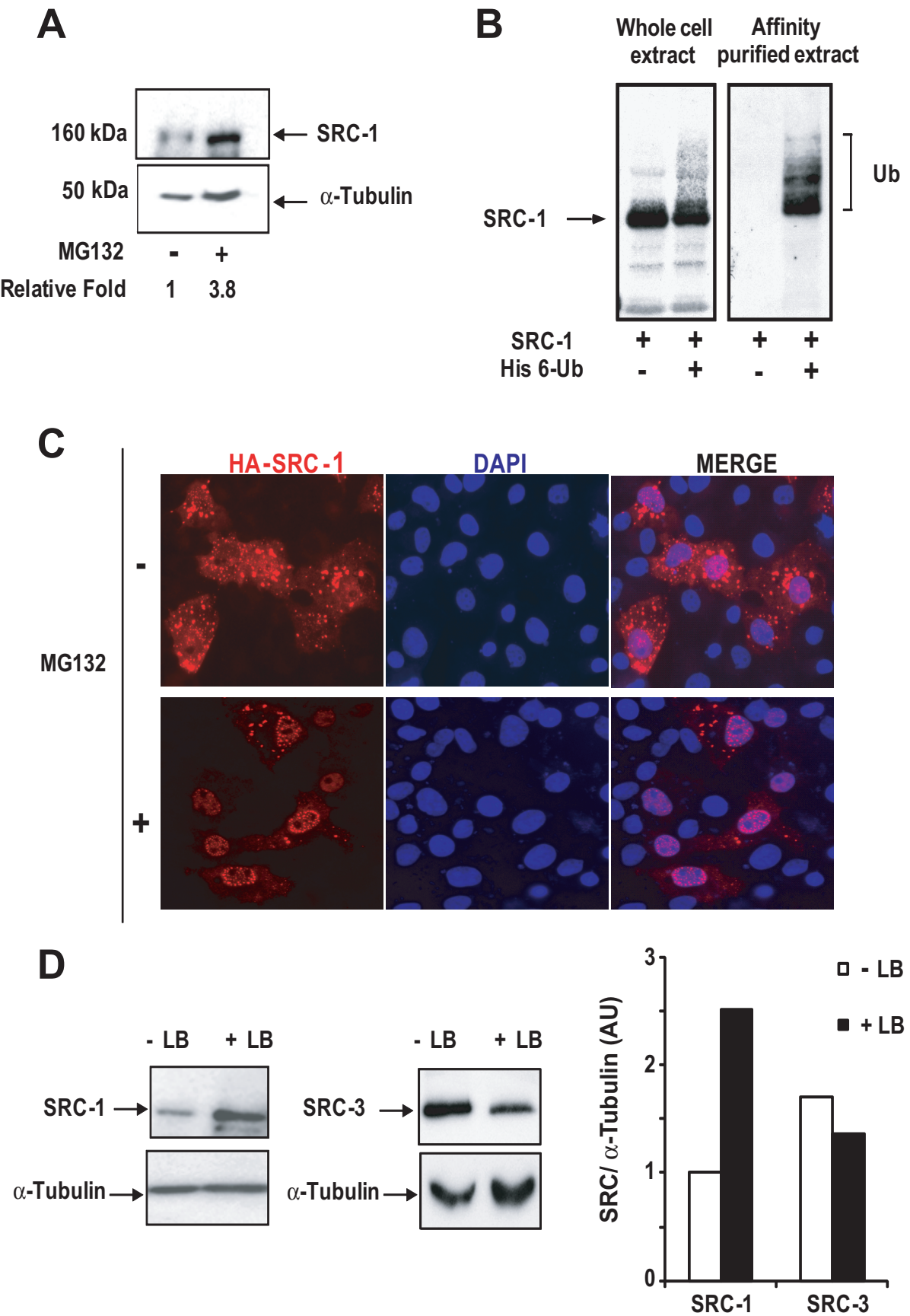
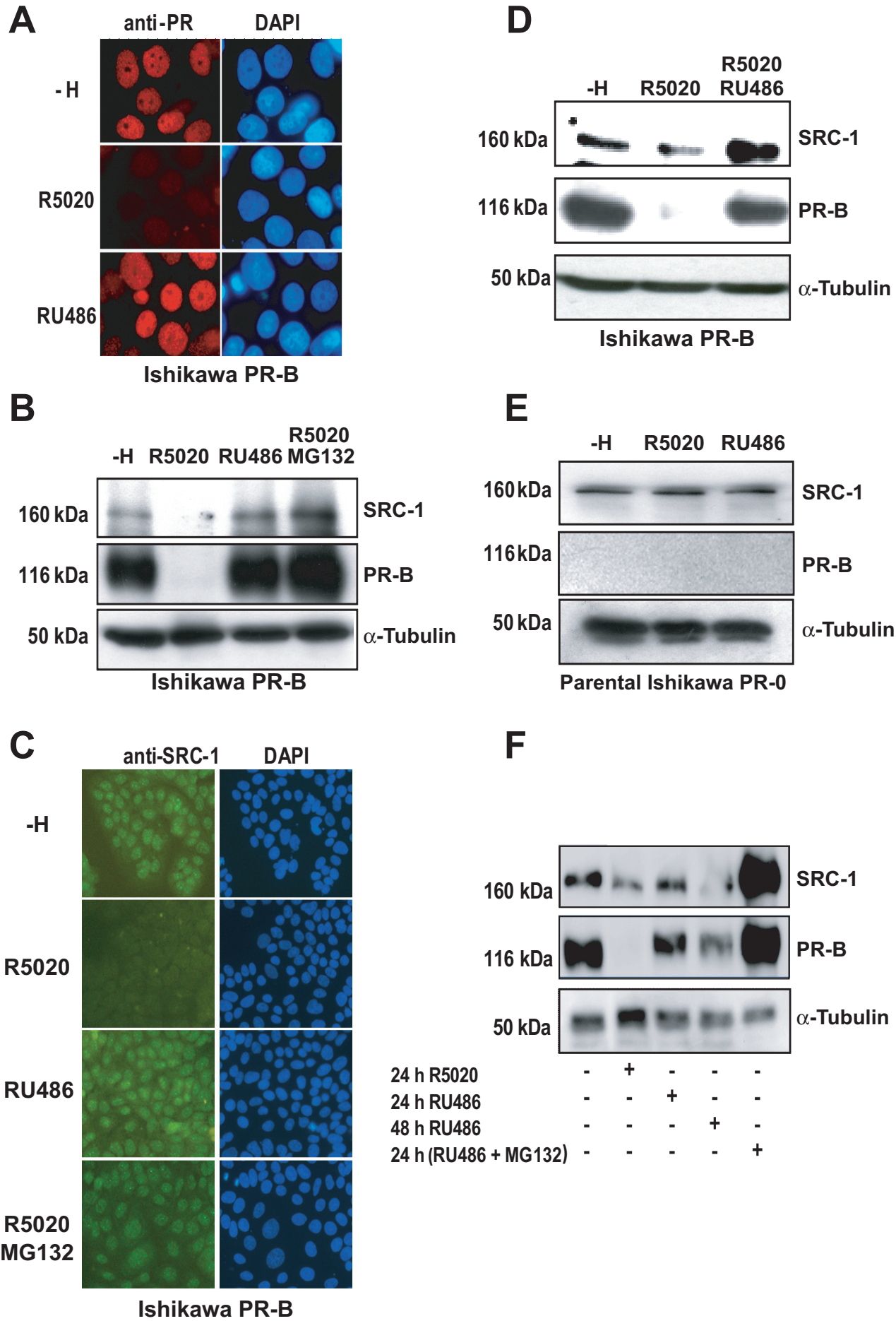

A
160 kDa
SRC-1
50 kDa
α-Tubulin
MG132 - +
Relative Fold 1 3.8
B
Whole cell extract
Affinity purified extract
SRC-1
Ub
SRC-1 + + + +
His 6-Ub - + - +
C
HA-SRC-1
DAPI
MERGE
MG132
-
+
D
- LB + LB
SRC-1
α-Tubulin
- LB + LB
SRC-3
α-Tubulin
SRC/ α-Tubulin (AU)
- LB
+ LB
SRC-1
SRC-3

Fig. 3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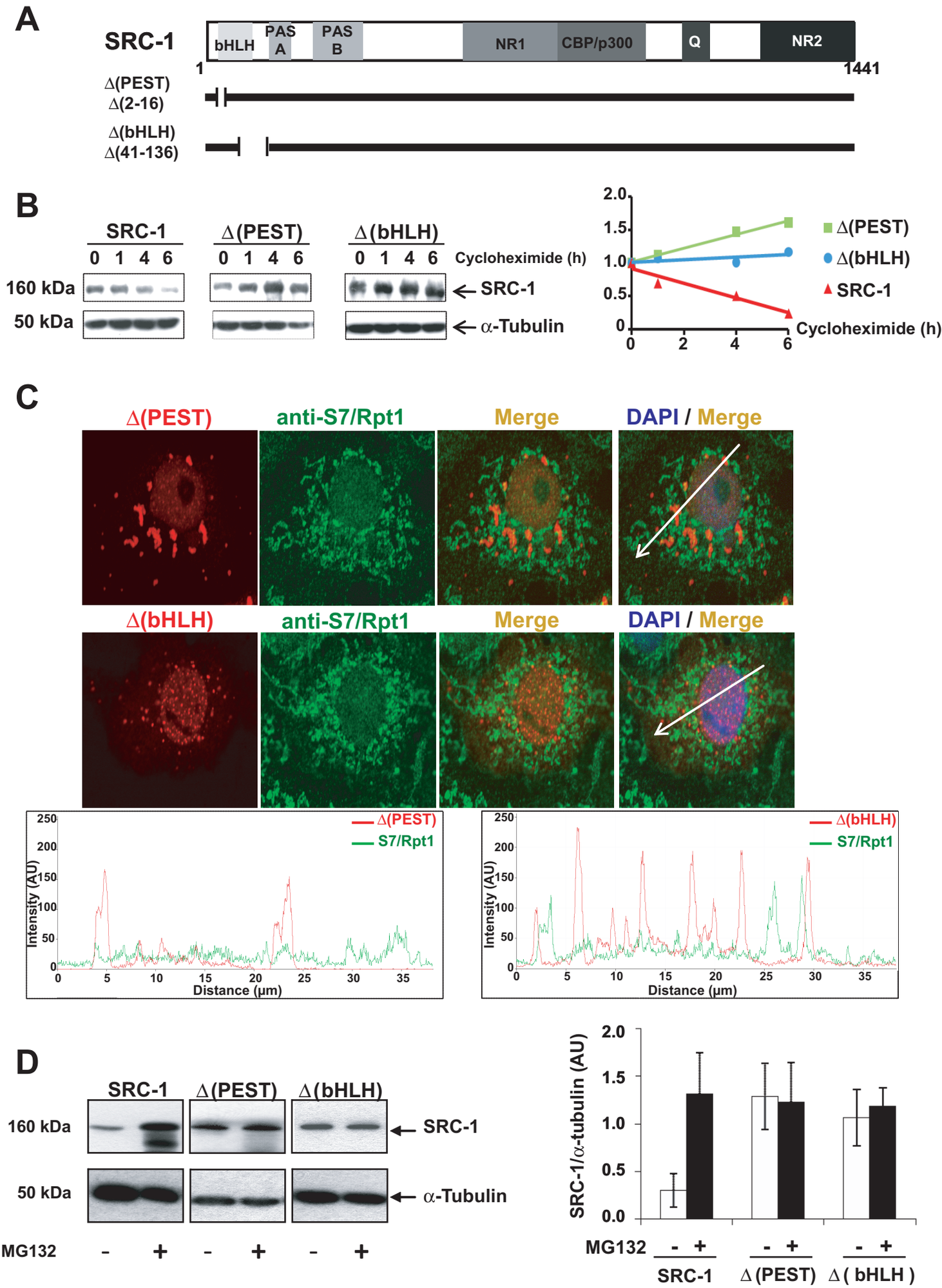
anti-PR
DAPI
- H
R5020
RU486
Ishikawa PR-B
B
-H
R5020
RU486
R5020
MG132
160 kDa
116 kDa
50 kDa
SRC-1
PR-B
α-Tubulin
Ishikawa PR-B
C
anti-SRC-1
DAPI
-H
R5020
RU486
R5020
MG132
Ishikawa PR-B
D
-H
R5020
R5020
RU486
160 kDa
116 kDa
50 kDa
SRC-1
PR-B
α-Tubulin
Ishikawa PR-B
E
-H
R5020
RU486
160 kDa
116 kDa
50 kDa
SRC-1
PR-B
α-Tubulin
Parental Ishikawa PR-0
F
160 kDa
116 kDa
50 kDa
SRC-1
PR-B
α-Tubulin
24 h R5020 - + - - -
24 h RU486 - - + - -
48 h RU486 - - - + -
24 h (RU486 + MG132) - - - - +

Fig. 4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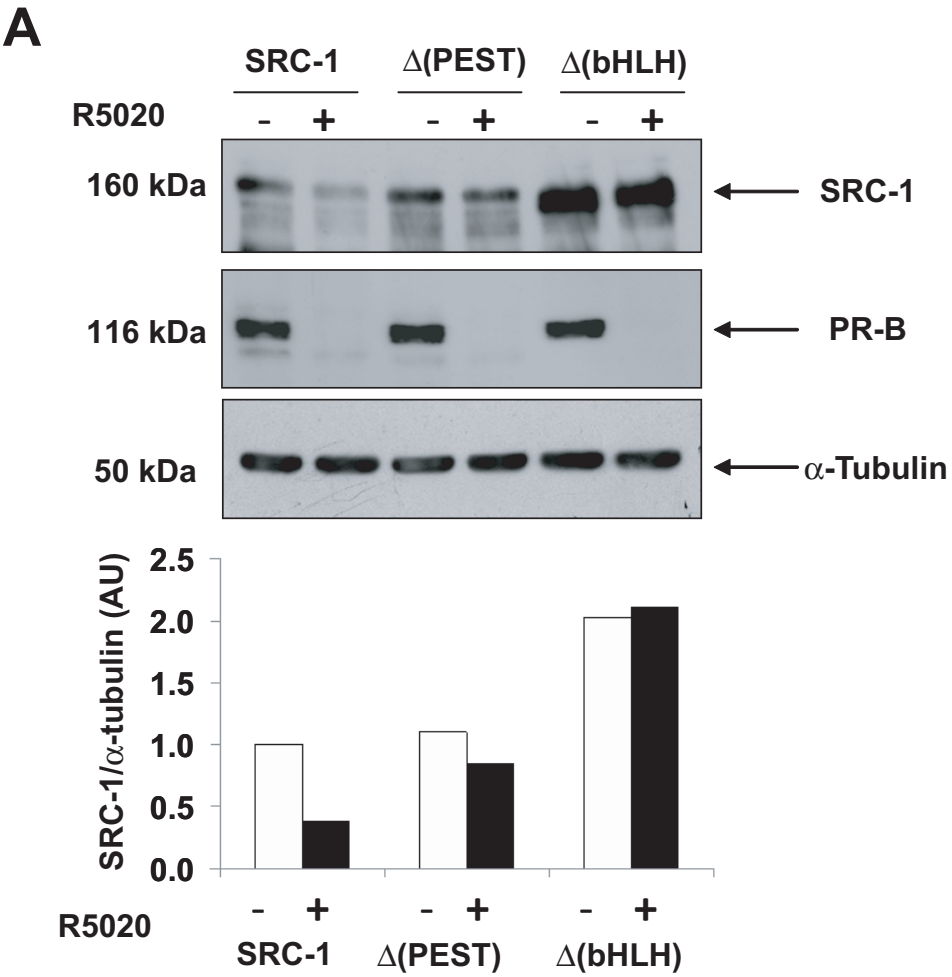
SRC-1
bHLH
PAS A
PAS B
NR1
CBP/p300
Q
NR2
1
1441
Δ(PEST)
Δ(2-16)
Δ(bHLH)
Δ(41-136)
B
SRC-1
Δ(PEST)
Δ(bHLH)
0 1 4 6
Cycloheximide (h)
160 kDa
50 kDa
SRC-1
α-Tubulin
2.0
1.5
1.0
0.5
0
Δ(PEST)
Δ(bHLH)
SRC-1
Cycloheximide (h)
C
Δ(PEST)
anti-S7/Rpt1
Merge
DAPI / Merge
Δ(bHLH)
Intensity (AU)
Distance (μm)
S7/Rpt1
D
SRC-1
Δ(PEST)
Δ(bHLH)
160 kDa
50 kDa
SRC-1
α-Tubulin
MG132
SRC-1/α-tubulin (AU)
Δ(PEST)
Δ(bHLH)

Fig. 5


A
SRC-1
Δ(PEST)
Δ(bHLH)
R5020
- + - + - +
160 kDa
SRC-1
116 kDa
PR-B
50 kDa
α-Tubulin
SRC-1/α-tubulin (AU)
2.5
2.0
1.5
1.0
0.5
0.0
R5020
- + - + - +
SRC-1
Δ(PEST)
Δ(bHLH)

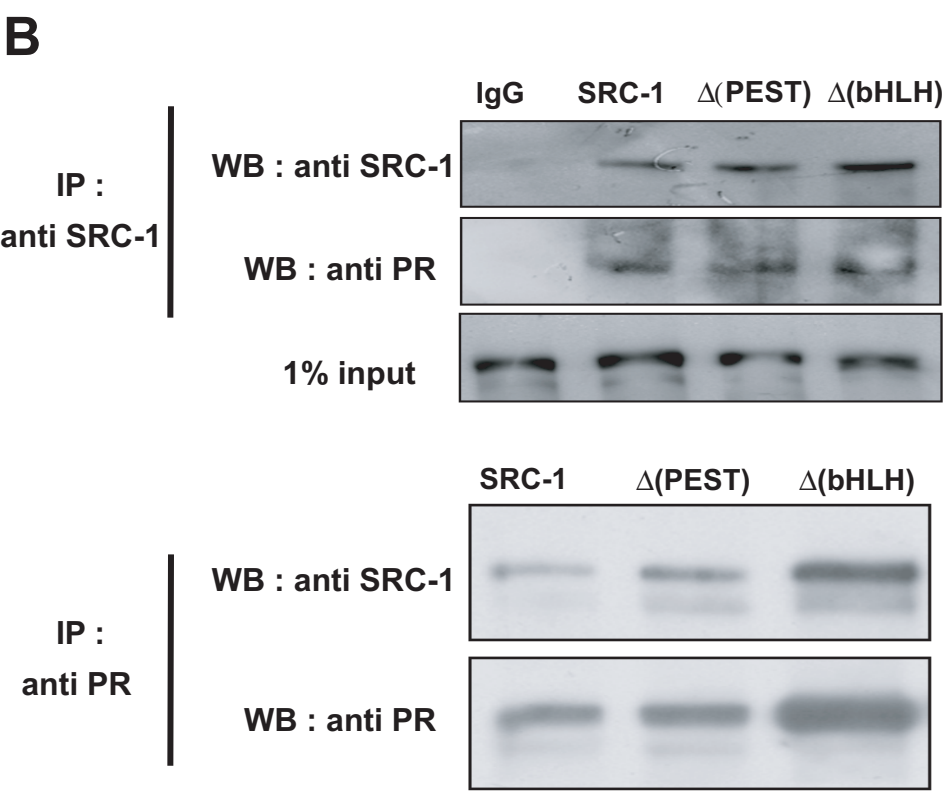
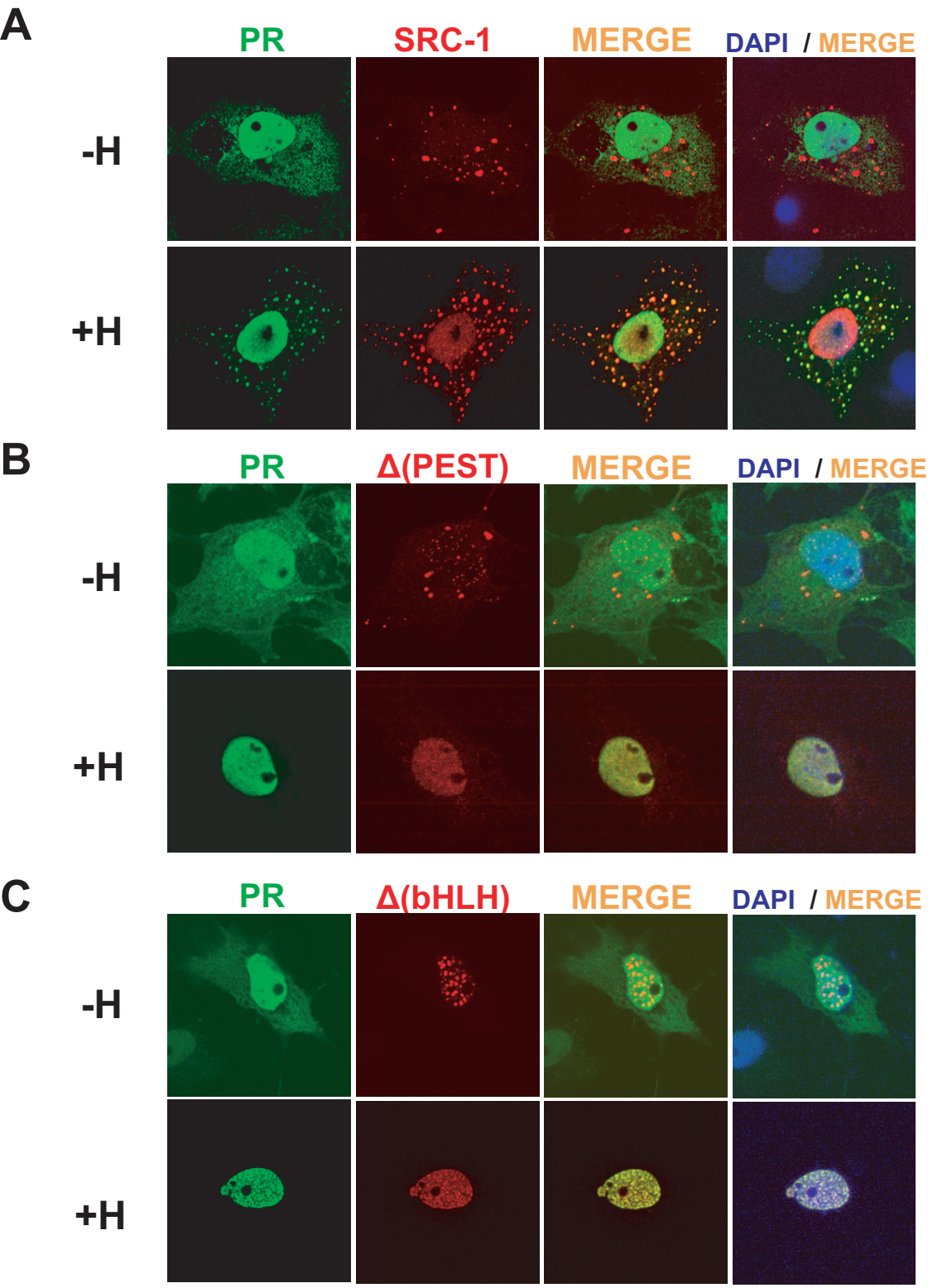

B
IgG
SRC-1
Δ(PEST)
Δ(bHLH)
IP :
anti SRC-1
WB : anti SRC-1
WB : anti PR
1% input
SRC-1
Δ(PEST)
Δ(bHLH)
IP :
anti PR
WB : anti SRC-1
WB : anti PR

Fig. 6


A
PR
SRC-1
MERGE
DAPI / MERGE
-H
+H
B
PR
Δ(PEST)
MERGE
DAPI / MERGE
-H
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C
PR
Δ(bHLH)
MERGE
DAPI / MERGE
-H
+H

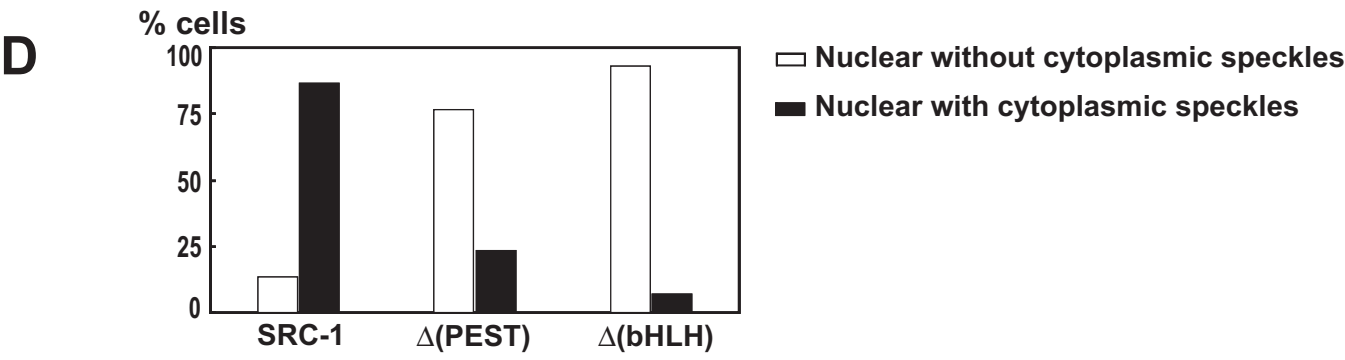
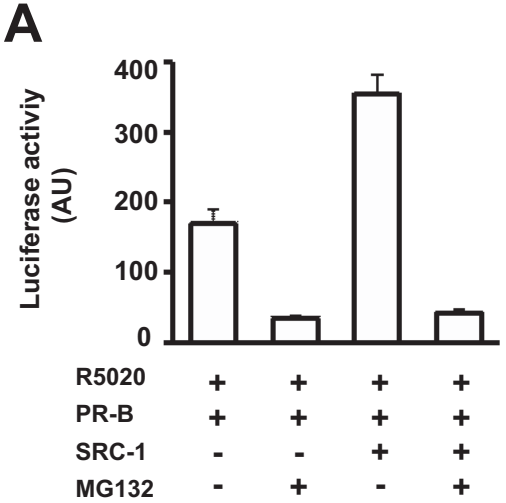
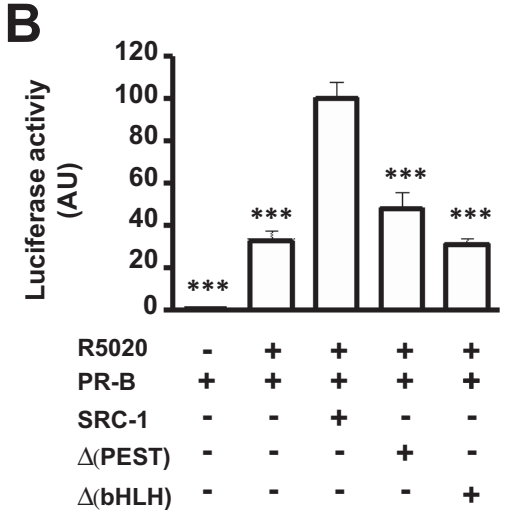
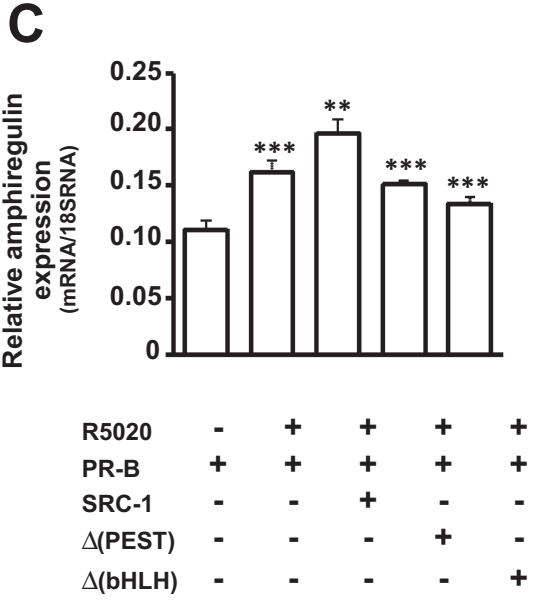

D
% cells
100
75
50
25
0
SRC-1
Δ(PEST)
Δ(bHLH)
Nuclear without cytoplasmic speckles
Nuclear with cytoplasmic speckles

Fig. 7


A
Luciferase activiy (AU)
400
300
200
100
0
R5020 + + + +
PR-B + + + +
SRC-1 - - + +
MG132 - + - +


B
Luciferase activiy (AU)
120
100
80
60
40
20
0
***
***
***
***
R5020 - + + + +
PR-B + + + + +
SRC-1 - - + - -
Δ(PEST) - - - + -
Δ(bHLH) - - - - +


C
Relative amphiregulin expression (mRNA/18SRNA)
0.25
0.20
0.15
0.10
0.05
0
***
**
***
***
R5020 - + + + +
PR-B + + + + +
SRC-1 - - + - -
Δ(PEST) - - - + -
Δ(bHLH) - - - - +