



HAL
open science

Transcriptional ER γ 2-mediated activation is regulated by sentrin-specific proteases

Moritz Hentschke, Ute Süsens, Uwe Borgmeyer

► **To cite this version:**

Moritz Hentschke, Ute Süsens, Uwe Borgmeyer. Transcriptional ER γ 2-mediated activation is regulated by sentrin-specific proteases. *Biochemical Journal*, 2009, 419 (1), pp.167-176. <10.1042/BJ20081556>. <hal-00479078>

HAL Id: hal-00479078

<https://hal.science/hal-00479078v1>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Transcriptional ERR γ 2-mediated activation is regulated by Sentrin-specific proteases

Moritz HENTSCHKE¹, Ute SÜSENS, Uwe BORGMEYER[§]

Zentrum für Molekulare Neurobiologie, Universitätsklinikum Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany

¹ Current address: Institut für Medizinische Mikrobiologie, Virologie und Hygiene, Universitätsklinikum Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany

[§]Corresponding author

Email addresses:

MH: m.hentschke@uke.uni-hamburg.de

US: suesens@zmnh.uni-hamburg.de

UB: uwe.borgmeyer@zmnh.uni-hamburg.de

Accepted Manuscript

Modification with small ubiquitin-like modifiers (SUMOs) has emerged as an important means of regulating the activity of transcription factors, often by repressing their activity. The estrogen receptor-related receptor γ (ERR γ , ERR3, NR3B3) is a constitutively active orphan nuclear receptor. A phosphorylation-dependent sumoylation motif (PDSM) is located in close vicinity of the amino-terminally located ERR γ 2-specific activation function 1 (AF-1). Its function can be substituted by a negatively charged amino acid-dependent sumoylation motif (NDSM). A mutational analysis reveals that ERR γ 2-activity is modulated through sumoylation of a lysine residue at position 40 which in turn is regulated by phosphorylation. Phosphorylation in the +5-position relative to the sumoylation target is directly visualized by a high resolution electrophoretic mobility shift assay (EMSA). Sumoylation represses the activity of ERR γ both, with and without forced expression of the peroxisome proliferator-activated receptor gamma coactivator 1 β (PGC-1 β). Fusion proteins of a heterologous DNA binding domain with the ERR γ 2 amino terminus demonstrate the function of the PDSM as the repression function 1 (RF-1) for the neighboring AF-1. Derepression is achieved by coexpression of Sentrin/SUMO-specific protease (SEN1) family members. Together, our results demonstrate reversible phosphorylation-dependent sumoylation as a means to regulate the activity of an orphan nuclear receptor.

Abbreviations used: AF, activation function; DBD, DNA binding domain; EGFP, enhanced green fluorescent protein; ERR, estrogen receptor-related receptor; HEK, human embryonic kidney; N2A, neuroblastoma-2A; NDSM, negatively charged amino acid-dependent sumoylation motif; PDSM, phosphorylation-dependent sumoylation motif; PGC-1, peroxisome proliferator-activated receptor gamma coactivator; PPAR, peroxisome proliferator-activated receptor; RF, repression function; SEN1, sentrin-specific protease; SUMO, small ubiquitin-like modifier.

Orphan nuclear receptors are a class of endogenous receptors whose cognate ligands are unknown. Although they are often studied to ascertain the identification of physiological ligands, finding additional means to regulate their transcriptional activity is of great interest. Most transcription factors undergo post-translational modification like phosphorylation, acetylation and ubiquitination, which are known to modify their properties [1, 2]. Recently, covalent modification by the small ubiquitin-like protein modifier (SUMO) has emerged as an important mechanism to regulate many protein activities [3, 4]. Similar to ubiquitin SUMO is activated by an E1 enzyme, conjugated by an E2 enzyme (UBC9) and bound to a substrate by a member of the E3 family. Sumoylation usually takes place on a lysine which is part of the core consensus motif ψ KxE, where ψ represents an aliphatic branched amino acid residue and x is any amino acid [5]. Sumoylation is thought to regulate protein functions by altering protein-interactions which in turn affects their subcellular localization and their activities. In case of transcription factors, sumoylation is generally accompanied by repression, suggestive of a promoted interaction with corepressors [6].

Sumoylation is a highly dynamic process regulated by the modulation of expression and activity of SUMO enzymes. In addition there is a crosstalk with other posttranslational modifications, including phosphorylation and acetylation [5]. Phosphorylation-dependent sumoylation is mediated by a composite motif ψ KxE_{xx}S that possesses a serine residue as a potential target for phosphorylation in the +5 position [7-12]. In several SUMO substrates the phospho-sumoyl switch is mediated by the bipartite motif ψ KxE_{xx}SP, termed phosphorylation-dependent sumoylation motif (PDSM) with an SP motif, a proline-directed phosphorylation site [7]. The SP motif corresponds to the ERK consensus site and thus may link the MAPK pathway and sumoylation [13]. A well studied example is the sequential phosphorylation and sumoylation of a PDSM motif in the amino terminus of the peroxisome proliferator-activated receptor γ (PPAR γ) turning off PPAR γ -dependent transcription [14-17]. A PDSM can be viewed as a regulated version of the negatively charged amino acid-dependent sumoylation motif, NDSM, characterized by acidic amino acids within the 10-amino acid downstream region [5, 18].

SUMO-conjugation can be reversed by SUMO-specific proteases, also known as sentrin-specific proteases (SENPs) and as SMT3-specific isopeptidases [19]. Apparently six mammalian SENPs regulate the pool of conjugatable SUMO and the half-life of conjugated proteins, albeit with different specificity towards SUMO paralogues and with different subcellular localization [20]. During deconjugation SENPs can discriminate between SUMO-1 and SUMO-2/3 but the enzymatic efficiency can in addition be target protein-dependent [21]. SENP3 and SENP5 are preferentially active in deconjugating SUMO-2/3-conjugated targets. The recent finding of an unexpected specificity of SENP6 and 7 towards synthetic substrates suggests that SUMOs may not be their physiological substrates at all [22].

SENP1 has been localized in nuclear pores and nucleoplasmic speckles, whereas SENP2 has been mainly detected in nuclear pores. SENP3 and SENP5 are nucleolar and SENP6 and SENP7 are nucleoplasmic family members. The capacity of SENPs to discriminate among substrates together with their distinct subcellular localization and expression pattern argues for non-redundant roles *in vivo* [20].

Hietakangas et al. [7] identified a bipartite PDSM in the amino terminus of ERR γ 2 and demonstrated that Lys⁴⁰ of ERR γ 2 is the main SUMO acceptor. Resulting from different promoter usage the ERR γ gene encodes at least two isoforms with distinct

amino termini, $\gamma 1$ and $\gamma 2$, the latter of which has a 23 residue extension [23]. This extension functions as an autonomous activation function, the AF-1, and thereby contributes to the higher transcriptional activity of ERR $\gamma 2$ [24]. Gal4 fusion proteins with the amino terminus of ERR $\gamma 1$ repress, while those with the amino terminus of ERR $\gamma 2$ activate appropriate reporter genes. Importantly, a GAL4 fusion protein containing only ERR $\gamma 2$ residues 1-23 exhibits higher activity than the whole amino terminal domain [25]. These findings suggest that the repressive function coincides with the sumoylation motif.

Here, we show that the AF-1 of ERR $\gamma 2$ is regulated by the sumoylation site in its vicinity which can be defined as the repression function, RF-1, of ERR γ . Mutagenesis of the RF-1 in the context of a GAL4 fusion protein revealed that all mutants proposed to inhibit sumoylation led to a higher activity of the AF-1. Similarly, a Lys⁴⁰ to arginine mutant, as well as the mutation of the hydrophobic residue Ile³⁹ and of the acidic residue Glu⁴² to alanine led to higher activity of full-length ERR $\gamma 2$. Both, superactivation by replacing the potential phosphorylation acceptor Ser⁴⁵ with alanine and a phosphorylation-dependent mobility shift of protein DNA complexes during gel electrophoresis speak in favor of a functional PDSM. Generating an acidic moiety, by replacing both Ser⁴⁴ and Ser⁴⁵ with glutamic acid, results in down-regulation, supporting the view that the PDSM is the regulated form of an NDSM. This is further corroborated by the repression effect of phosphomimetic mutants in the downstream region.

Furthermore, coexpression of SENPs suggest that regulation by phosphorylation is only one means to regulate the activity of ERR $\gamma 2$. ERR $\gamma 2$ is in addition derepressed by forced expression of desumoylation enzymes SENP1, SENP2, SENP3 and SENP5. Coimmunoprecipitation of ERR $\gamma 2$ and SENP1, as well as the requirement of a functional sumoylation site for SENP-mediated activation speak in favor of a direct mechanism.

EXPERIMENTAL

Plasmids

To express GAL4 fusion proteins Gal4-ERR $\gamma 2/1-38$ and Gal4-ERR $\gamma 2/1-48$, ERR γ fragments were generated with the polymerase chain reactions using $\gamma 2F$ (5'-*GTTCGACATGGATTCCGGTAGAACTTTGCCT*-3' with a *SalI* restriction site shown in italics and the start ATG underlined) and reverse primers ERR γ -48stop (5'-*TCTAGACTAGGAGGCTGGGCTGGAGGGTT*-3') and ERR γ -38stop (5'-*TCTAGACTAGAAGGACGAACAGCTGGAATCA*-3') with *XbaI* and a *BseY1* restriction site in italics and translational stop codons underlined. Mutants of Gal4-ERR $\gamma 2/1-48$ were introduced by generating amplicons with the respective mutated reverse primers. Products were inserted into pGem-Teasy (Promega) and following sequence verification the *SalI/XbaI* inserts were excised and inserted in-frame into pBind3 Δ in which the cytomegalovirus immediate early promoter drives expression of the DBD of the yeast GAL4 protein. The pBind3 Δ vector is a derivative of pBind (Promega) with an altered multiple cloning site and lacking the Renilla luciferase module [25]. To generate mutants of ERR $\gamma 2$, the mutant fragments and the non-mutated 5' end were excised via *SalI* and *BseY1* and inserted into pSPUTK-ERR γ , a plasmid with a newly introduced 5'-located *SalI* restriction site. The insert was then

excised via flanking *HindIII* and *EcoR5* restriction sites and inserted into pCMX. Due to the cloning strategy these constructs code for the full-length ERR γ 2 with an amino terminal tag of three additional amino acids.

The SENP1 expression plasmid pCDNA-FH-Senp1 was provided by Dr. O'Hare of the Marie Curie Research Institute, United Kingdom [26]. To generate non-functional mut-Senp1, Cys⁶⁰³ was mutated to a serine by a PCR approach. Full-length pCMV-SPORT6 expression plasmids (Invitrogen) coding for SENP2 (IMAGE ID 3483046), SENP3 (IMAGE ID 5344274), SENP6 (IMAGE ID 5149995) and SENP7 (IMAGE ID 5364345) were obtained by the RZPD (Deutsches Ressourcenzentrum für Genomforschung GmbH), Berlin, Germany [27]. The Senp5 cDNA obtained by the RZPD in the cloning vector pYX-Asc (IMAGE ID 6812169) was cloned via *SaI*I and *NotI* restriction enzymes into pCMV-SPORT6. The other plasmids have been previously described.

Cell culture, transient transfections, and reporter gene analysis

Human embryonic kidney 293 cells (293) and mouse neuroblastoma Neuro-2a (N2a) cells were grown in Opti-MEM without phenol red (Invitrogen) supplemented with 5% fetal calf serum, 200 units/ml of penicillin G, and 200 μ g/ml streptomycin at 37 °C under a 5% CO₂ atmosphere. For transient transfections cells were seeded in 96-well tissue culture plates (Greiner) and transfected the next day at ~ 50% confluence. 293 were transfected with FuGENE 6 (Roche), using 3 μ l transfection reagent per microgram of DNA, whereas N2a cells were transfected with 2.5 μ l Lipofectamine 2000 (Invitrogen) per microgram of DNA. A CMX expression plasmid coding for β -galactosidase was used as a substitute in the controls of Fig. 1C. EGFP fluorescence generated by pEGFP-C3 (Clontech) was determined the next day (N2a) and two days after transfection (293), respectively, with a Wallac 1420 Multilabel Counter (PerkinElmer). Then light emission generated by luciferase activity with the Bright-Glo Luciferase Assay System according to the manufacturer's instructions (Promega) was measured with the Multilabel Counter. The light emission generated by luciferase activity was normalized to the level of EGFP-fluorescence activity derived from the EGFP control plasmid. Transfections were generally performed in septuplicate. Average relative luciferase light units and standard deviations were calculated using the Prism-4 program (GraphPad.com).

Electrophoretic mobility shift assay

N2A cells (2×10^5) were seeded in 6 well plates and transfected the next day with 2 μ g of expression plasmids as described above. Extracts were prepared after two days by 3 cycles of freeze-thaw in 40 μ l extraction buffer consisting of 20mM HEPES, pH 7.4, 80mM NaCl, 20mM KCl, 2 mM dithiothreitol, 6 % glycerol and 1x Complete protease inhibitor cocktail (Roche) and centrifugation at 16,000 g. The supernatants were stored at -80 °C. For DNA binding, 2.5 μ l of the extracts were diluted in 10 μ l extraction buffer containing 0.5 μ g human Cot-1 DNA and incubated with an excess of radioactively labelled double stranded oligonucleotides. Sequence of the DR0 element, generation of the radioactive probe and electrophoresis have been described [28]. The Gal4 binding element was generated by annealing of the oligonucleotides 5'-AGCTCGGAGGACTGTCCTCCGA-3' and 5'-AGCTTCGGAGGACAGTCCTCCG-3'. To achieve protein charge-dependent resolution, protein DNA complexes were resolved on 5% PAGE in 0.5 x TBE (pH 8.3) at 20V/cm in a cold room at 4 °C until complexes covered a distance of approximately 18 cm. Dephosphorylation was achieved by incubating 8 μ l of the

protein extracts with 1 unit calf intestine alkaline phosphatase (Roche) for 30' at room temperature.

RESULTS

A sumoylation target sequence represses the autonomous AF-1 of ERR γ 2

The 126 residues long N-terminus of ERR γ 2 preceding the DNA binding domain (DBD) functions as a transcriptional activator when fused to the heterologous DBD of the yeast transcriptional activator Gal4 (Gal4-ERR γ 2/1-126). In contrast, an analog fusion with the ERR γ 1 amino terminus starting with the 24th amino acid of ERR γ 2 leads to transcriptional repression [25]. These findings not only argue in favor of a 23 amino acid long AF-1 but also in favor of a repressive function in the remaining 103 amino acids. An amino acid sequence comprising a sumoylation consensus sequence, a PDSM (ϕ KxE_{xx}S), lies in the vicinity of the activation domain (Fig. 1A). The lysine in position 40 (K40) of ERR γ 2 has already been shown to be the major sumoylation target of ERR γ [7].

Therefore, we compared transcriptional activation of the Gal4 DBD with three fusion constructs of the ERR γ 2 amino terminus in human embryonic kidney derived 293 cells. Whereas activation by Gal4-ERR γ 2/1-48 and by Gal4-ERR γ 2/1-126 was similar, a further truncation of ten amino acids caused a fivefold higher activation (Fig. 1B). This fusion protein, Gal4-ERR γ 2/1-38, lacks the sumoylation site, suggesting that sumoylation represses the AF-1. To test whether this effect is specific for human embryonic kidney cells we included mouse neuroblastoma-derived N2a cells in this study. Although overall activation was higher, the effects of the truncations in 293 and N2A cells were similar (Fig. 1C).

In summary, these results suggested that amino acids 39 to 48 of ERR γ 2 constitute an autonomous repression function-1 (RF-1) which regulates the AF-1 of ERR γ 2.

Mutation of the sumoylation target sequence derepresses the autonomous AF-1 of ERR γ 2

To get additional support for a functional sumoylation site, we introduced single amino acid mutations in the Gal4-ERR γ 2/1-48 fusion protein. Neither an F38A nor a T41A mutation in the vicinity of the putative target Lys⁴⁰ did affect the activity of the fusion protein in 293 cells (Fig. 1D). The same holds true for an F38E mutation (not shown). Both amino acids, Phe³⁸ and Thr⁴¹, are not critical residues of the sumoylation consensus sequence. In contrast, the I39A and the E42A mutation, representing the hydrophobic and the acidic residue of the ψ KxE consensus sequence respectively, both caused a fourfold increase in reporter gene activity. A sevenfold increase was observed by the conservative K40R mutation of the putative sumoylation target supporting the hypothesis that a Lys⁴⁰ modification leads to a reduced activity and that the AF-1 is regulated by sumoylation at Lys⁴⁰. Similar results were obtained in N2A cells (Fig. 1E) supporting the above findings and arguing for a general regulatory mechanism.

Mutations of a putative phosphorylation site affect transcriptional activity of the AF-1

Next, we elaborated whether modification of the neighboring phosphorylation site affects the function of the RF-1 as shown for other PDSMs. In contrast to Ser⁴⁴, Ser⁴⁵

is a predicted target of phosphorylation. The S44A and S44E mutations did not significantly affect the transcriptional activity of the fusion proteins in HEK cells (Fig. 1F) or N2A cells (Fig. 1G). However, expression of the S45A mutant enhanced luciferase activity more than twofold in both cell lines, suggesting that phosphorylation of this residue enhances sumoylation. Introducing a negative charge by an S45E mutation diminished the activation effect (Fig. 1F, G). A double mutation of both serines to glutamic acid (S44/45E) was neutral. These findings suggest that a negative charge introduced by phosphorylation of Ser⁴⁵ leads to increased sumoylation and consequently to a reduced transcriptional activity mediated by the AF-1.

To analyze, whether mutations affect protein expression or DNA binding, we performed an EMSA with N2A extracts expressing Gal4-ERR γ 2/1-48 as well as several of its mutants. DNA binding was tested with a radioactive labeled Gal4 DNA binding site. All bands showed similar intensity indicating that differences in activation were neither the result of altered expression nor of altered DNA binding.

An NDSM can substitute a mutated PDSM

The question arose whether an NDSM could substitute a mutated PDSM. To this end, the transcriptional activities of Gal4-ERR γ 2/1-48(S45A) and of the phosphomimetic mutant S45D were compared with S45A mutants with additional single or combinatory P46D, A47D and S48D substitutions. As expected for an NDSM, all negative charges in the +6 to +8 position reduced the transcriptional activity of Gal4-ERR γ 2/1-48(S45A) (Fig. 2). The PDSM was functionally replaced by acidic amino acids in all three positions, as well as only in the +7/+8 position.

Together, these results showed that the AF-1 is regulated by a repressive function, the RF-1, located in its close vicinity and similar results in two distinct cell lines suggest a general regulatory mechanism. The repressive activity which overlaps with a sumoylation site was relieved by mutations predicted to reduce or to abolish sumoylation. The altered activity is not greatly regulated through DNA binding or stability of the proteins. Mutating the putative phosphorylation site suggests that sumoylation is regulated by kinases and phosphatases.

The PDSM is phosphorylated

Indirect evidence suggested that the serine in the +5 position of the sumoylation site is a target of phosphorylation. Since separation of protein-DNA complexes in EMSAs is dependent on the charge of the protein, we investigated protein-DNA complexes at high resolution. The non-mutated fusion protein (Gal4-ERR γ 2(1-48)) has a pI of 6.45 and a net charge of -1. Phosphorylation changes the pI to 6.11 and, depending whether the phosphate group is single or doubly ionized, the net charge to -2 or to -3. In the non-mutated fusion protein as well as in all mutants with an unaffected Ser⁴⁵, three complexes can be distinguished (Fig. 3A). Based on the fact that the Gal4-DBD binds as a dimer to DNA, these bands presumably reflect unmodified, single and double modified dimer-DNA complexes, respectively. The triplet of the E42A mutant ran slower, whereas the K40A mutant ran faster, supporting the interpretation that two charges significantly altered the velocity of the complex. In contrast, the Ser⁴⁵ mutants S45A, S44/45A, and S44/45E exhibited dominant single bands with the expected velocities. Therefore, these data support the assumption that Ser⁴⁵ is the major phosphorylation target. In addition, the analysis of mutants affecting sumoylation

showed that the sumoylation site does not affect protein modification by phosphorylation.

To prove that the occurrence of triplet bands were indeed the result of partial phosphorylation, extracts were pre-incubated with alkaline phosphatase. Dephosphorylation turned the triplet to a single band, reflecting the non-modified protein (Fig. 3B). As expected, Ser⁴⁵ mutants were not affected by dephosphorylation.

Activation of full-length ERR γ 2 by mutation of the amino-terminal sumoylation motif

Given that the sumoylation motif negatively influenced the AF-1 in the context of a fusion protein, we next asked whether this was true in the full-length protein. Therefore, we tested transcriptional activation by ERR γ 2 in N2A cells with pBV-4xDR0, a well established ERR γ reporter with four DR0 elements previously shown to be bound by ERR γ 2 dimers [28, 29]. The I39A, the K40R, and the E42A mutation, all within the sumoylation motif resulted in an approximate four- to six-fold increase in transcription when compared to the wild-type (WT) protein (Fig. 4A). These results support the hypothesis that sumoylation represses the transcriptional activity of ERR γ 2. To question whether sumoylation is regulated by phosphorylation, we tested the S44/45A mutation in the context of the full-length protein. The activity of the mutated protein was about threefold when compared to the wild-type protein (Fig. 4A). To verify that ERR γ and its mutants were properly expressed, cell extracts of transiently transfected N2A cells were tested in an EMSA (Fig. 4B). There were no gross differences in the intensity of protein complexes with the DR0 element. The specificity of the complexes was verified by generating supershift bands with α ERR γ , an ERR γ -specific antiserum [28].

In summary, our results imply that the transcriptional activity of ERR γ 2 is regulated by sumoylation of Lys⁴⁰ which in turn is regulated by phosphorylation.

Sumoylation mutants in the context of a coactivator

Transcriptional activation of ERR γ is greatly enhanced by coactivators, namely by PGC-1 β [30, 31]. Therefore, we tested whether coactivation in N2A cells is affected by the sumoylation site. The activities of the I39A mutant as well as those of the K40R mutant were clearly enhanced by PGC-1 β , indicating that both, the wild-type and the mutants deficient in sumoylation are targets for the coactivator (Fig. 5). In addition, the results revealed that PGC-1 β did not overcome the repressive effect of the RF-1.

AF-1-dependent transcription is enhanced by SENP1 expression

Transcriptional repression by sumoylation can best be verified by a partial relief mediated by SUMO-protease expression. Indeed, forced expression of mammalian sentrin-specific protease SENP1, a widely expressed enzyme clearly enhanced reporter gene activation by Gal4-ERR γ 2/1-48 (Fig. 6A, lanes 1 and 2).

To question whether sumoylation is a prerequisite for the SENP1 effect, various Gal4-ERR γ 2/1-48 mutants were coexpressed with SENP1 in N2a cells (Fig. 6A). Clearly, the SENP1 effect is attenuated or abolished in mutants I39A, K40A and S45A all affecting amino acids of the PDSM consensus. Namely, the K40A mutant is not further activated. The reduced activation by SENP1 of the S45A mutant corroborates the claim that Ser⁴⁵ phosphorylation leads to enhanced sumoylation (Fig. 6A).

To verify that the activation depends on the enzyme activity, we introduced a C603S mutation in the catalytic center of SENP1. This mutation has previously been shown to abolish the activity of the cysteine protease and may function as a dominant negative enzyme [32]. Coexpression of the mutated SENP1 protein (mut-SENP1) did not affect reporter gene expression indicating a direct activation of the ERR γ 2 fusion protein by desumoylation (Fig. 6B). As expected, the K40A mutant was not affected by coexpression of the mutated protein. Sumo proteases bind to the Sumo moiety through salt bridges [21]. The notion that the enzymatic activity rather than binding alone activates ERR γ 2 is underlined by coexpression of a SENP1 fusion protein with the viral VP16 activator domain. Activation by coexpression of SENP1 and SENP1-VP16 were indistinguishable (not shown).

SENP proteins regulate the activity of the AF-1

Mammalian sentrin-specific proteases (SENP1, 2, 3, 5, 6, and 7) differ in their expression pattern, their cellular localization as well as their substrate specificity. Therefore SENP1 to 7 were cotransfected with the AF-1 fusion construct Gal4-ERR γ 2/1-48 and with the respective K40R mutant (Fig. 7A, B). Activation was limited to the non-mutated fusion protein and to SENP1, 2, 3 and 5. The highest activation was observed with SENP2, a protease with a substrate preference for SUMO-2/3. Activation by the SUMO-2/3-specific proteases SENP3 and 5 speaks in favor of ERR γ regulation by SUMO-2/3.

ERR γ 2-mediated gene activation is regulated by SENP

To analyze whether overexpression of SENPs activate ERR γ 2 driving DR0 element-mediated luciferase expression, the full-length protein was transfected along with SENP1 to 7 into N2a cells (Fig. 8A). The results were very similar to those with the AF-1 fusion protein. Activation was observed with SENP1, 2, 3 and 5. However the effects of SENP1 and 2 were indistinguishable. The negative control with a β -galactosidase plasmid as a substitute for the ERR γ plasmid proofed that SENP-mediated activation of DR0 reporter gene expression was ERR γ -dependent (Fig. 8B). To elaborate whether activation by SENP was mediated by the RF-1, we tested the SENP effect with the K40R mutation. SENP-mediated activation was greatly diminished in the presence of the RF-1 mutant (Fig. 8C). This finding supports the biochemical analysis identifying K40 as the major sumoylation target of ERR γ [7]. A spurious activation of the K40R mutant suggests a minor involvement of a sumoylation site in the ligand binding domain. Next, we elaborated whether SENP activation depends on the presence of the AF-1. Compared to ERR γ 2, coexpression of ERR γ 1 with SENP1 and with SENP2 resulted in a weak activation emphasizing the importance of the AF-1 (Fig. 8D).

Together these data reveal the regulation of ERR γ 2 by phosphorylation-dependent sumoylation and underscore the importance of the γ 2-specific AF-1.

DISCUSSION

Small ubiquitin-like modifier conjugation has emerged as an important posttranslational control of protein functions [3, 4]. Here, we provided evidence for the regulation of ERR γ activity by dynamic SUMO conjugation of a lysine in the amino terminal domain. This sumoylation site, apparently regulated by a proline-directed serine phosphorylation site defines an autonomous repression function, the

RF-1. Hietakangas et al. [7] already showed that Lys⁴⁰ of ERR γ 2 is the major target of sumoylation. In addition, the transcriptional activity of the closely related receptor ERR α 1 (NR3B1) is regulated by phosphorylation-dependent sumoylation [33]. While this study was in preparation, Tremblay et al. [34] reported functional sumoylation site in the amino termini of both, ERR α and ERR γ . Their data support the hypothesis that the SUMO moiety conveys a repressive effect. In addition Tremblay et al. showed that sumoylation site mutants display wild-type nuclear localization and DNA binding properties. Our analysis supports their main finding concerning ERR γ , an upregulation in transcriptional activity by the K40R mutation. In contrast to this study, in our experimental settings there was no necessity for a forced expression of coactivator proteins. In addition, we provided evidence that the RF-1 regulates the activity of the neighboring AF-1 independently of additional domains. This goal was achieved by analysis of GAL4-ERR γ 2/1-48 mutants, constructs including the AF-1 (amino acids 1-23) as well as the sumoylation target sequence "IKTE" (amino acids 39-42). F38A and T41A mutations that do not alter the consensus sumoylation motif did not greatly alter the transcriptional activity of the fusion protein. In contrast, GAL4-ERR γ 2/1-38 lacking the motif, as well as the I39A and E42A mutant were derepressed supporting the function of both, the hydrophobic and the acidic residue. In addition, both the K40A and the K40R mutant that preclude sumoylation showed a high constitutive activity.

The mechanisms by which sumoylation interferes with the AF-1 remain unresolved. Based on the study of Tremblay et al. (2008) and our study, a dramatic effect on protein expression and DNA binding can be excluded. Sumoylation of the RF-1 may interfere with coactivator binding or interaction with the transcriptional initiation complex. On the other hand, there may be a gain of function by attracting corepressors. One possible candidate is the corepressor Daxx which has been shown to be actively recruited by sumoylated transcription factors, including the androgen receptor [35, 36]. Alternatively, binding of coactivator and corepressor could be mutually exclusive. However, we can not exclude an additional regulation by acetylation of K40 as for example in the case of the transcription factor MEF2. In MEF2 a phosphorylation-regulated sumoylation-acetylation switch of a PDSM is essential for postsynaptic differentiation [10, 12].

The Gal4 fusion proteins allowed us to explore the ERR γ PDSM with its putative Ser⁴⁵ phosphorylation site at the +5 position by a high resolution EMSA. The non-mutated protein which binds as a dimer gives rise to three complexes. The triplets of the K40A and E42A mutants are shifted up and down by one position, respectively. This suggests that the triplet represents the non-modified, the single and the double phosphorylated dimer, each differing by 2 charges. *In vitro* phosphatase treatment shifts the triplet to the respective slow migrating band, a direct evidence for the phosphorylation status. The high resolution EMSA may be of broader interest as a prototype for studying phosphorylation pathways.

The functional PDSM analysis showed derepression of the S45A mutant and efficient repression of the phosphomimetic S45E and S45D mutants suggesting that the negative charge imparted by phosphorylation is the main trigger for ERR γ sumoylation. Therefore, this orphan receptor belongs to the large group of protein whose activity is regulated by kinase and phosphatase activities. A proline in position 46 points to proline-directed protein kinases as possible regulators of ERR γ . Possible candidates are extracellular signal-regulated kinase (Erk)-1 and -2, MAPKs whose optimum consensus sequence for phosphorylation was defined as "PxSP" where x is a neutral or basic amino acid but ideally not a proline [37, 38]. This motif is in

accordance with the primary sequence "PSSP" directly following the sumoylation motif "IKTE" in ERR γ . The S44/45E mutant being less active than the S45E mutant is in agreement with the finding that acidic residues in NDSMs are more or less overrepresented within the 10-amino acid downstream region [5, 18]. To directly approach the question whether an NDSM can substitute the PDSM, negative charged amino acids in the +5 to +8 position were introduced into the S45A mutant. The activity of Gal4-ERR γ 2/1-48(S45A) was clearly repressed by additional aspartate residues proving that these negative charges functionally substitute serine phosphorylation.

The ERR γ PDSM also modulated the activity of the full-length protein, both in the absence and in the presence of forced coactivator expression. This finding contradicts the recent report by Tremblay et al. [34] who reported the necessity for coactivator expression to achieve activation by a K40R mutation. One plausible cause for this discrepancy could be a higher level of coactivator proteins in our cell lines. This hypothesis is supported by the observation that the mere expression of ERR γ 2 resulted in a more robust activation under our experimental settings. However, our experiments with a heterologous DBD support a direct repression of a construct without involvement of the AF-2. Therefore, it is worthwhile to clarify whether this discrepancy is due to the different cell lines, reporter constructs or other experimental settings.

In addition to a regulation by phosphorylation, our data is suggestive of an ERR γ regulation through control of the SUMO pathway itself. Accumulating evidence shows that target sumoylation is affected by regulated expression of various components of the sumoylation pathway [39]. Namely PIAS [protein inhibitor of activated STAT (signal transducer and activator of transcription)] proteins promote SUMOylation of transcription factors in a manner that resembles the action of RING-type ubiquitin E3 ligases [40]. Since PIAS α /ARIP3 (androgen receptor-interacting protein) [41] [42, 43] is involved in nuclear receptor regulation we studied the effect of a forced expression on ERR γ regulation. Our data do not support a specific effect (data not shown). However, at this point we can not exclude that sumoylation proteins were not limited in the cell lines. Forced expression of PIAS γ has been shown to increase sumoylation of ERR γ [34]. However, pronounced reporter gene repression by forced expression PIAS γ and activation by PIAS γ -VP16 in the absence of ERR γ did not allow us to determine the ERR γ -specific effects (data not shown). In contrast, reporter gene activation by SENPs was strictly ERR γ -dependent. Therefore, we propose ERR γ regulation by the level of SUMO proteases, as well as by their localization and activity. Enzyme activity rather than stable binding as the basis of activation is supported by a fusion with the VP16 domain which did not act as a superactivator. The cysteine mutants of SENP1 and 2 still bind to SUMOs [21, 32] consistent with a weak activation of Gal4-ERR γ 1-48 by mut-SENP1-VP16 constructs (data not shown).

SENPs can be discriminated according to their substrate specificity, their subcellular distribution and their expression pattern. Coexpression of SENP1, 2, 3, and 5 clearly activated full-length ERR γ and fusion proteins in an RF-1-dependent manner. The regulated RF-1 depends on an intact PDSM. The observation of SENP2 being superior to SENP1 when coexpressed with Gal4-ERR γ 1-48 but not with full-length ERR γ may reflect substrate specificity. Coexpression of SENP6, SENP7 and of SENP8 (not shown) did not change ERR γ activity. Whereas SENP8/DEN1

demonstrates activity only against Nedd8-modified proteins [44-46], it is generally assumed that all other SENPs cleave SUMOs. However, this has only been investigated in detail with SENP1, 2, 3 and 5. Sparse data suggesting that SENP6 and 7 are desumoylating enzymes and their unexpected substrate specificity led to the speculation that they either recognize still uncharacterized natural substrates or have evolved a different mechanism of recognition of SUMO substrates from SENP [47]. Indeed, a recent biochemical analysis reveals specificity for SUMO2/3, especially when poly-SUMO2 or poly-SUMO3 chains are used as substrates [48]. Of the three mammalian SUMO paralogues SUMO-1 is \approx 45% identical to SUMO-2 and SUMO-3 which are 96% identical to each other. Studies with SENPs reveal that SENP1 preferentially deconjugates SUMO-1, whereas SENP2 is less efficient at deconjugating SUMO-1 substrates [20]. SENP3 and 5 are SUMO-2/3-specific with a high level of discrimination against SUMO-1. Although SUMO-2 and -3 are nearly identical, SUMO-3 is only inefficiently processed by SENP1 and SENP2. Therefore, activation by overexpression of SENP1 to 5 speaks in favor of ERR γ modification by SUMO-2. Experimental data show that dynamics is much slower for SUMO-1 than for SUMO-2, supporting a regulatory function of SUMO-2.

Studying reversible sumoylation of transcription factors like PPARs and ERRs offers fascinating opportunities to understand the cellular roles of SENPs. In addition, it will be important to decipher the signals and pathways for turning the phosphorylation switch. PPARs and ERRs together with their coregulators have been implicated in the regulation of energy the metabolism [49]. In addition accumulating evidence points the implication of ERR γ as a therapeutic for cancer [50, 51]. Therefore, these receptors attract attention as potential targets for pharmaceutical design. Understanding their coordinated regulation is of general interest and may lead to means of disease prevention or intervention in diseases related to the metabolic syndrome.

Acknowledgements

We are grateful to Peter O'Hare (Marie Curie Research Institute) and Jorma J. Palvimo (University of Helsinki) for supplying expression plasmids for SENP1 and ARIP3, respectively.

References

- 1 Faus, H. and Haendler, B. (2006) Post-translational modifications of steroid receptors. *Biomed. Pharmacother.* **60**, 520-528
- 2 Fu, M., Wang, C., Zhang, X. and Pestell, R. G. (2004) Acetylation of nuclear receptors in cellular growth and apoptosis. *Biochem. Pharmacol.* **68**, 1199-1208
- 3 Geiss-Friedlander, R. and Melchior, F. (2007) Concepts in sumoylation: a decade on. *Nat. Rev. Mol. Cell Biol.* **8**, 947-956
- 4 Meulmeester, E. and Melchior, F. (2008) Cell biology: SUMO. *Nature.* **452**, 709-711
- 5 Anckar, J. and Sistonen, L. (2007) SUMO: getting it on. *Biochem. Soc. Trans.* **35**, 1409-1413
- 6 Lyst, M. J. and Stancheva, I. (2007) A role for SUMO modification in transcriptional repression and activation. *Biochem. Soc. Trans.* **35**, 1389-1392

- 7 Hietakangas, V., Anckar, J., Blomster, H. A., Fujimoto, M., Palvimo, J. J., Nakai, A. and Sistonen, L. (2006) PDSM, a motif for phosphorylation-dependent SUMO modification. *Proc. Natl. Acad. Sci. U S A.* **103**, 45-50
- 8 Stankovic-Valentin, N., Deltour, S., Seeler, J., Pinte, S., Vergoten, G., Guerardel, C., Dejean, A. and Leprince, D. (2007) An acetylation/deacetylation-SUMOylation switch through a phylogenetically conserved psiKXEP motif in the tumor suppressor HIC1 regulates transcriptional repression activity. *Mol. Cell. Biol.* **27**, 2661-2675
- 9 Yang, X. J. and Gregoire, S. (2006) A recurrent phospho-sumoyl switch in transcriptional repression and beyond. *Mol. Cell.* **23**, 779-786
- 10 Gregoire, S., Tremblay, A. M., Xiao, L., Yang, Q., Ma, K., Nie, J., Mao, Z., Wu, Z., Giguere, V. and Yang, X. J. (2006) Control of MEF2 transcriptional activity by coordinated phosphorylation and sumoylation. *J. Biol. Chem.* **281**, 4423-4433
- 11 Hietakangas, V., Ahlskog, J. K., Jakobsson, A. M., Hellesuo, M., Sahlberg, N. M., Holmberg, C. I., Mikhailov, A., Palvimo, J. J., Pirkkala, L. and Sistonen, L. (2003) Phosphorylation of serine 303 is a prerequisite for the stress-inducible SUMO modification of heat shock factor 1. *Mol. Cell. Biol.* **23**, 2953-2968
- 12 Shalizi, A., Gaudilliere, B., Yuan, Z., Stegmuller, J., Shirogane, T., Ge, Q., Tan, Y., Schulman, B., Harper, J. W. and Bonni, A. (2006) A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. *Science (New York, N.Y.)* **311**, 1012-1017
- 13 Guo, B., Yang, S. H., Witty, J. and Sharrocks, A. D. (2007) Signalling pathways and the regulation of SUMO modification. *Biochem. Soc. Trans.* **35**, 1414-1418
- 14 Floyd, Z. E. and Stephens, J. M. (2004) Control of peroxisome proliferator-activated receptor gamma2 stability and activity by SUMOylation. *Obes. Res.* **12**, 921-928
- 15 Ohshima, T., Koga, H. and Shimotohno, K. (2004) Transcriptional activity of peroxisome proliferator-activated receptor gamma is modulated by SUMO-1 modification. *J. Biol. Chem.* **279**, 29551-29557
- 16 Pascual, G., Fong, A. L., Ogawa, S., Gamliel, A., Li, A. C., Perissi, V., Rose, D. W., Willson, T. M., Rosenfeld, M. G. and Glass, C. K. (2005) A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature.* **437**, 759-763
- 17 Yamashita, D., Yamaguchi, T., Shimizu, M., Nakata, N., Hirose, F. and Osumi, T. (2004) The transactivating function of peroxisome proliferator-activated receptor gamma is negatively regulated by SUMO conjugation in the amino-terminal domain. *Genes Cells.* **9**, 1017-1029
- 18 Yang, S. H., Galanis, A., Witty, J. and Sharrocks, A. D. (2006) An extended consensus motif enhances the specificity of substrate modification by SUMO. *EMBO J.* **25**, 5083-5093
- 19 Gong, L., Millas, S., Maul, G. G. and Yeh, E. T. (2000) Differential regulation of sentrinized proteins by a novel sentrin-specific protease. *J. Biol. Chem.* **275**, 3355-3359
- 20 Mukhopadhyay, D. and Dasso, M. (2007) Modification in reverse: the SUMO proteases. *Trends Biochem. Sci.* **32**, 286-295
- 21 Ihara, M., Koyama, H., Uchimura, Y., Saitoh, H. and Kikuchi, A. (2007) Noncovalent binding of small ubiquitin-related modifier (SUMO) protease to SUMO is necessary for enzymatic activities and cell growth. *J. Biol. Chem.* **282**, 16465-16475

- 22 Drag, M., Mikolajczyk, J., Krishnakumar, I. M., Huang, Z. and Salvesen, G. S. (2008) Activity profiling of human deSUMOylating enzymes (SENPs) with synthetic substrates suggests an unexpected specificity of two newly characterized members of the family. *Biochem. J.* **409**, 461-469
- 23 Süssens, U., Hermans-Borgmeyer, I. and Borgmeyer, U. (2000) Alternative splicing and expression of the mouse estrogen receptor-related receptor gamma. *Biochem. Biophys. Res. Commun.* **267**, 532-535
- 24 Hentschke, M. and Borgmeyer, U. (2003) Identification of PNRC2 and TLE1 as activation function-1 cofactors of the orphan nuclear receptor ERR γ . *Biochem. Biophys. Res. Commun.* **312**, 975-982
- 25 Borgmeyer, U., Süssens, U. and Hentschke, M. (2008) An isoform-specific activation function of the orphan nuclear receptor ERR γ . submitted
- 26 Bailey, D. and O'Hare, P. (2002) Herpes simplex virus 1 ICP0 co-localizes with a SUMO-specific protease. *J. Gen. Virol.* **83**, 2951-2964
- 27 Strausberg, R. L., Feingold, E. A., Grouse, L. H., Derge, J. G., Klausner, R. D., Collins, F. S., Wagner, L., Shenmen, C. M., Schuler, G. D., Altschul, S. F., Zeeberg, B., Buetow, K. H., Schaefer, C. F., Bhat, N. K., Hopkins, R. F., Jordan, H., Moore, T., Max, S. I., Wang, J., Hsieh, F., Diatchenko, L., Marusina, K., Farmer, A. A., Rubin, G. M., Hong, L., Stapleton, M., Soares, M. B., Bonaldo, M. F., Casavant, T. L., Scheetz, T. E., Brownstein, M. J., Usdin, T. B., Toshiyuki, S., Carninci, P., Prange, C., Raha, S. S., Loquellano, N. A., Peters, G. J., Abramson, R. D., Mullahy, S. J., Bosak, S. A., McEwan, P. J., McKernan, K. J., Malek, J. A., Gunaratne, P. H., Richards, S., Worley, K. C., Hale, S., Garcia, A. M., Gay, L. J., Hulyk, S. W., Villalon, D. K., Muzny, D. M., Sodergren, E. J., Lu, X., Gibbs, R. A., Fahey, J., Helton, E., Ketteman, M., Madan, A., Rodrigues, S., Sanchez, A., Whiting, M., Madan, A., Young, A. C., Shevchenko, Y., Bouffard, G. G., Blakesley, R. W., Touchman, J. W., Green, E. D., Dickson, M. C., Rodriguez, A. C., Grimwood, J., Schmutz, J., Myers, R. M., Butterfield, Y. S., Krzywinski, M. I., Skalska, U., Smailus, D. E., Schnerch, A., Schein, J. E., Jones, S. J. and Marra, M. A. (2002) Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc. Natl. Acad. Sci. U S A.* **99**, 16899-16903
- 28 Hentschke, M., Süssens, U. and Borgmeyer, U. (2002) Domains of ERRgamma that mediate homodimerization and interaction with factors stimulating DNA binding. *Eur. J. Biochem.* **269**, 4086-4097
- 29 Huppunen, J. and Aarnisalo, P. (2004) Dimerization modulates the activity of the orphan nuclear receptor ERRgamma. *Biochem. Biophys. Res. Commun.* **314**, 964-970
- 30 Hentschke, M., Süssens, U. and Borgmeyer, U. (2002) PGC-1 and PERC, coactivators of the estrogen receptor-related receptor. *Biochem. Biophys. Res. Commun.* **299**, 872-879
- 31 Kamei, Y., Ohizumi, H., Fujitani, Y., Nemoto, T., Tanaka, T., Takahashi, N., Kawada, T., Miyoshi, M., Ezaki, O. and Kakizuka, A. (2003) PPARgamma coactivator 1beta/ERR ligand 1 is an ERR protein ligand, whose expression induces a high-energy expenditure and antagonizes obesity. *Proc. Natl. Acad. Sci. U S A.* **100**, 12378-12383
- 32 Bailey, D. and O'Hare, P. (2004) Characterization of the localization and proteolytic activity of the SUMO-specific protease, SENP1. *J. Biol. Chem.* **279**, 692-703
- 33 Vu, E. H., Kraus, R. J. and Mertz, J. E. (2007) Phosphorylation-dependent sumoylation of estrogen-related receptor alpha1. *Biochemistry.* **46**, 9795-9804

- 34 Tremblay, A. M., Wilson, B. J., Yang, X. J. and Giguère, V. (2008) Phosphorylation-dependent sumoylation regulates estrogen-related receptor-alpha and -gamma transcriptional activity through a synergy control motif. *Mol. Endocrinol.* **22**, 570-584
- 35 Kuo, H. Y., Chang, C. C., Jeng, J. C., Hu, H. M., Lin, D. Y., Maul, G. G., Kwok, R. P. and Shih, H. M. (2005) SUMO modification negatively modulates the transcriptional activity of CREB-binding protein via the recruitment of Daxx. *Proc. Natl. Acad. Sci. U S A.* **102**, 16973-16978
- 36 Lin, D. Y., Huang, Y. S., Jeng, J. C., Kuo, H. Y., Chang, C. C., Chao, T. T., Ho, C. C., Chen, Y. C., Lin, T. P., Fang, H. I., Hung, C. C., Suen, C. S., Hwang, M. J., Chang, K. S., Maul, G. G. and Shih, H. M. (2006) Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. *Mol. Cell.* **24**, 341-354
- 37 Clark-Lewis, I., Sanghera, J. S. and Pelech, S. L. (1991) Definition of a consensus sequence for peptide substrate recognition by p44mpk, the meiosis-activated myelin basic protein kinase. *J. Biol. Chem.* **266**, 15180-15184
- 38 Gonzalez, F. A., Raden, D. L. and Davis, R. J. (1991) Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. *J. Biol. Chem.* **266**, 22159-22163
- 39 Liu, B. and Shuai, K. (2008) Regulation of the sumoylation system in gene expression. *Curr. Opin. Cell Biol.* **20**, 288-293
- 40 Palvimo, J. J. (2007) PIAS proteins as regulators of small ubiquitin-related modifier (SUMO) modifications and transcription. *Biochem. Soc. Trans.* **35**, 1405-1408
- 41 Kotaja, N., Karvonen, U., Janne, O. A. and Palvimo, J. J. (2002) PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. *Mol. Cell. Biol.* **22**, 5222-5234
- 42 Janne, O. A., Moilanen, A. M., Poukka, H., Rouleau, N., Karvonen, U., Kotaja, N., Hakli, M. and Palvimo, J. J. (2000) Androgen-receptor-interacting nuclear proteins. *Biochem. Soc. Trans.* **28**, 401-405
- 43 Kotaja, N., Aittomaki, S., Silvennoinen, O., Palvimo, J. J. and Janne, O. A. (2000) ARIP3 (androgen receptor-interacting protein 3) and other PIAS (protein inhibitor of activated STAT) proteins differ in their ability to modulate steroid receptor-dependent transcriptional activation. *Mol. Endocrinol.* **14**, 1986-2000
- 44 Gan-Erdene, T., Nagamalleswari, K., Yin, L., Wu, K., Pan, Z. Q. and Wilkinson, K. D. (2003) Identification and characterization of DEN1, a deneddylase of the ULP family. *J. Biol. Chem.* **278**, 28892-28900
- 45 Mendoza, H. M., Shen, L. N., Botting, C., Lewis, A., Chen, J., Ink, B. and Hay, R. T. (2003) NEDP1, a highly conserved cysteine protease that deNEDDylates Cullins. *J. Biol. Chem.* **278**, 25637-25643
- 46 Wu, K., Yamoah, K., Dolios, G., Gan-Erdene, T., Tan, P., Chen, A., Lee, C. G., Wei, N., Wilkinson, K. D., Wang, R. and Pan, Z. Q. (2003) DEN1 is a dual function protease capable of processing the C terminus of Nedd8 and deconjugating hyper-neddylated CUL1. *J. Biol. Chem.* **278**, 28882-28891
- 47 Drag, M. and Salvesen, G. S. (2008) DeSUMOylating enzymes--SENPs. *IUBMB life.* **60**, 734-742
- 48 Lima, C. D. and Reverter, D. (2008) Structure of the Human SENP7 Catalytic Domain and Poly-SUMO Deconjugation Activities for SENP6 and SENP7. *The Journal of biological chemistry.* **283**, 32045-32055

- 49 Feige, J. N. and Auwerx, J. (2007) Transcriptional coregulators in the control of energy homeostasis. *Trends Cell Biol.* **17**, 292-301
- 50 Ariazi, E. A. and Jordan, V. C. (2006) Estrogen-related receptors as emerging targets in cancer and metabolic disorders. *Curr. Top. Med. Chem.* **6**, 203-215
- 51 Yu, S., Wang, X., Ng, C. F., Chen, S. and Chan, F. L. (2007) ERR γ suppresses cell proliferation and tumor growth of androgen-sensitive and androgen-insensitive prostate cancer cells and its implication as a therapeutic target for prostate cancer. *Cancer Res.* **67**, 4904-4914

Figure legends

Fig. 1 A sumoylation motif represses the amino terminal ERR γ 2-specific AF-1. **A**) The amino terminus of ERR γ 2 is shown in the one letter code. Residues 1 to 23 corresponding to the AF-1 are shown in italics, amino acid 24 is the putative start methionine of ERR γ 1. Amino acids complying with the sumoylation consensus sequence are underlined, the putative sumoylation target Lys⁴⁰ (K40) and the putative phosphorylation target Ser⁴⁵ (S45) are highlighted. The carboxy termini of the Gal4 fusion proteins ERR γ 2/1-38 and ERR γ 2/1-48 are indicated. **B-G**) 293 cells (**B, D, F**) and N2A cells (**C, E, G**) were transfected with the GAL4 reporter pGL4/5-Luc and expression vectors coding for fusion proteins of the Gal4 DBD with the indicated elements of ERR γ 2. The luciferase activity was normalized to the fluorescence generated by a cotransfected EGFP vector. Relative activity is given with the standard deviation based on a typical experiment performed in septuplicate. **B, C**) A repression function is located between residues 38 and 48 of ERR γ 2. The activity generated by the Gal4 fusion with the amino terminal 126 amino acids of ERR γ 2 was set as 1 and compared with fusions of the first 48 and of the first 38 amino acids, respectively, as well as with the empty Gal4 vector (-). **D, E**) Mutants of the sumoylation motif in the ERR γ 2/1-48 fusion protein lead to an elevated transcriptional activity. Activation by non-mutated Gal4-ERR γ 2/1-48 was arbitrarily set to 1 and compared to its mutants as indicated, as well as to the empty vector. **F, G**) Mutations of a putative phosphorylation site affect transcriptional activation. As in D and E activity of the mutants is shown in relation to the ERR γ 2/1-48 fusion protein as indicated. **H**) Mutations do not affect expression and DNA binding of Gal4 fusion proteins. Extracts of N2A transfected with the Gal4 expression vector (Gal4) or constructs giving rise to the indicated Gal4-ERR γ 2/1-48 fusion proteins were subjected to EMSA with a radioactive labeled Gal4 response element. Only the shifted bands are shown. Cell extracts transfected with a β -Galactosidase plasmid served as a negative control.

Fig. 2 Functional substitution of a PDSM by NDSMs. Transcriptional activation of pGL4/5-Luc, a luciferase reporter with five Gal4 binding sites upstream of a TATA box, by the Gal4 fusion protein Gal4-ERR γ 2/1-48 (nonmut.) was set as 1 and compared to activation by the phosphorylation-deficient mutant S45A and the phosphomimetic mutant S45D. To elaborate whether a negative charge in the downstream region is accompanied by down-regulation of the S45A mutant, the activity of mutants with aspartate residues substituting Pro⁴⁶, Ala⁴⁷, and Ser⁴⁸ was analyzed, as indicated.

Fig. 3 Charge-dependent resolution of protein DNA complexes allows the direct visualization of Ser⁴⁵-phosphorylation. Following electrophoretic separation, only the shifted bands detected by phosphor imaging of a ³²P-labeled Gal4 binding site are shown. **A)** Cellular extracts of N2A cells forced to express Gal4-ERR γ 2/1-48 (nonmut.) and its mutant were analyzed by high-resolution EMSA as indicated. **B)** Before subjecting to an EMSA, cell extracts of transfected cells were preincubated in the absence (-) or in the presence (+) of alkaline phosphatase.

Fig. 4 Mutations of the sumoylation motif led to an enhanced transcriptional activity of ERR γ 2 in N2A cells. **A)** Transcriptional ERR γ 2 (WT) activation of a luciferase reporter with four DR0 response elements was set as 1 and compared to activation by I39A, K40R and E42A mutants, respectively. In addition, the S44/45A mutant affecting the phosphorylation site and a β Gal expression plasmid as negative control (-) were tested. **B)** An EMSA was performed with cellular extracts of N2A cells transfected with expression plasmids coding for wild-type, mutant ERR γ 2 and β Galactosidase (-) as indicated. The addition of α ERR γ , a rabbit antiserum directed against ERR γ is indicated. Only the upper part of the gel is shown.

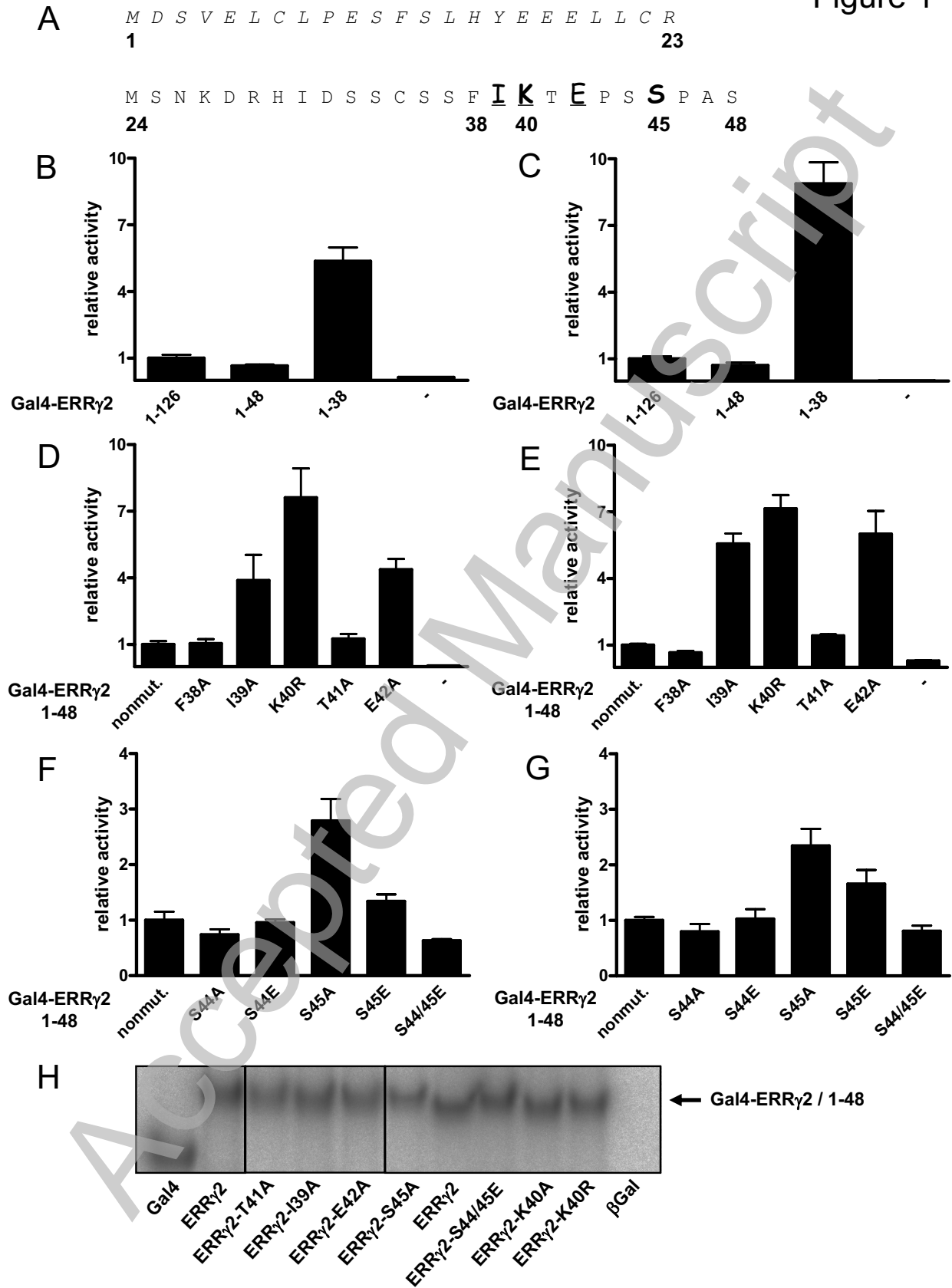
Fig. 5 ERR γ 2 and its sumoylation mutants are activated by the nuclear receptor coactivator PGC1 β . ERR γ 2-dependent activation of pBV-4xDR0-Luc in N2A cells was studied in the absence (-) and in the presence (+) of PGC1 β as indicated. The activation by wild-type ERR γ 2 (WT) was set as 1 and compared with the ERR γ 2 mutants I39A and K40R.

Fig. 6 Enhancement of AF-1 mediated gene activation by SENP1 in the presence of a functional PDSM. **A)** Activation by SENP1 in 293 cells is attenuated or abolished by mutations affecting the sumoylation consensus sequence. Gal4-ERR γ 2/1-48 (nonmut.) and its mutants were coexpressed with either SENP1 (+) or with β Gal expression plasmids (-) as negative controls. Activity in the presence of Gal4-ERR γ 2/1-48 (lane 1) was set as 1. **B)** Activation of Gal4-ERR γ 2/1-48 and of its Lys⁴⁰ to arginine mutant K40R in the presence of either a negative control plasmid (-/-), of a SENP1 expression plasmid (SENP1 +) or of a plasmid driving the expression of a desumoylation deficient SENP1 mutant (mut-SENP1 +).

Fig. 7 AF-1 dependent gene activation in N2A cells is modified by coexpression of desumoylation enzymes. Activation of pGL4/5-Luc in the absence of SENP proteins (-) is set as 1. **A)** AF-1 dependent activation by Gal4-ERR γ 2/1-48 was studied in the absence (-) and in the presence of SENP proteins as indicated. **B)** Activation by the Lys⁴⁰ to arginine mutant K40R of Gal4-ERR γ 2/1-48 in the absence (-) and in the presence of SENP proteins as indicated.

Fig. 8 ERR γ 2 activity is mediated by coexpression of SENP proteins. Plasmids coding for ERR γ 2 (**A**), β -Galactosidase as a negative control (**B**), the Lys⁴⁰ to arginine mutation of ERR γ 2 (**C**) and for ERR γ 1 (**D**) were transfected without (-) or along with one of six SENP family members in N2A cells, as indicated. Luciferase activities generated by pBV-4xDR0-Luc were measured and the activities in the absence of coexpressed proteases were set as 1.

Figure 1



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20081556

Figure 2

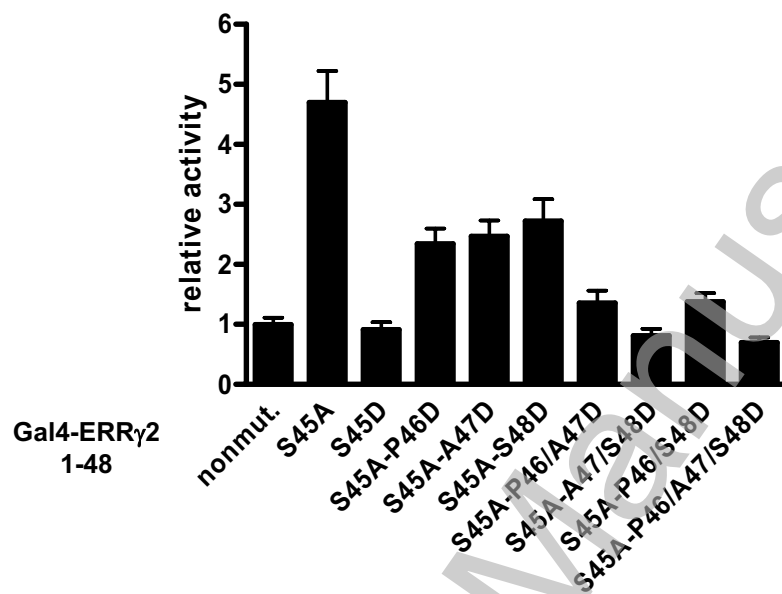
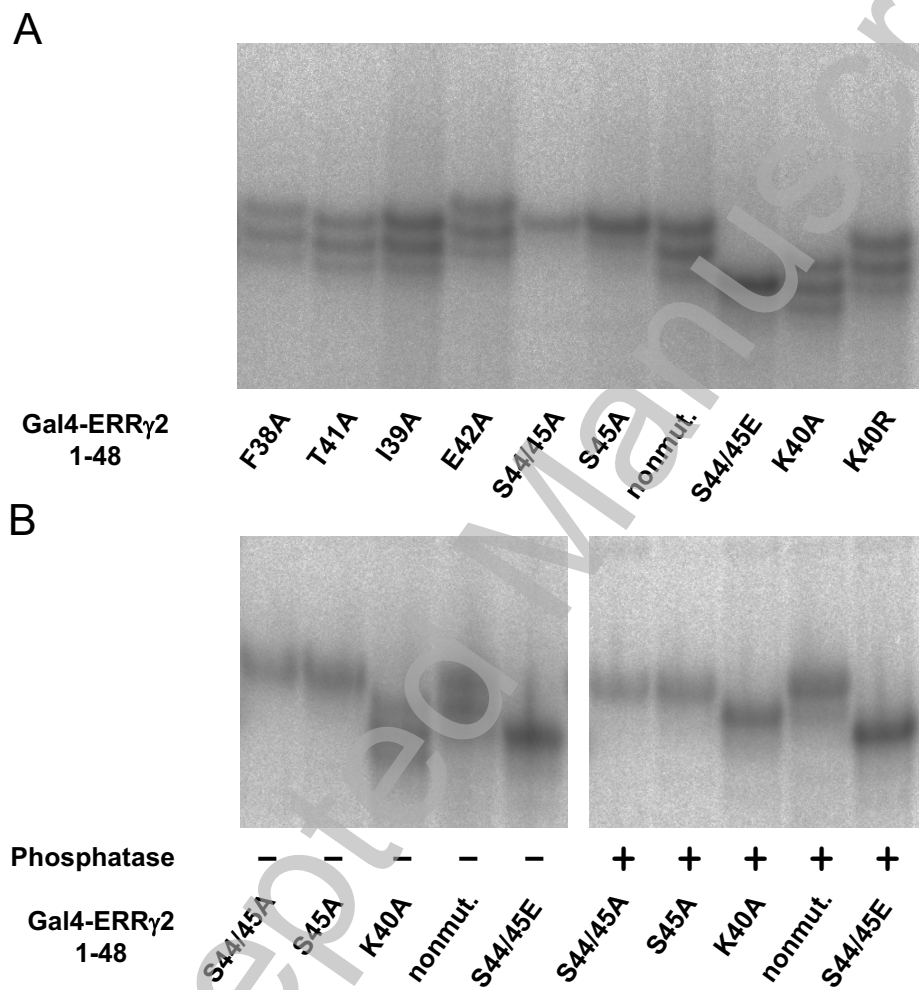
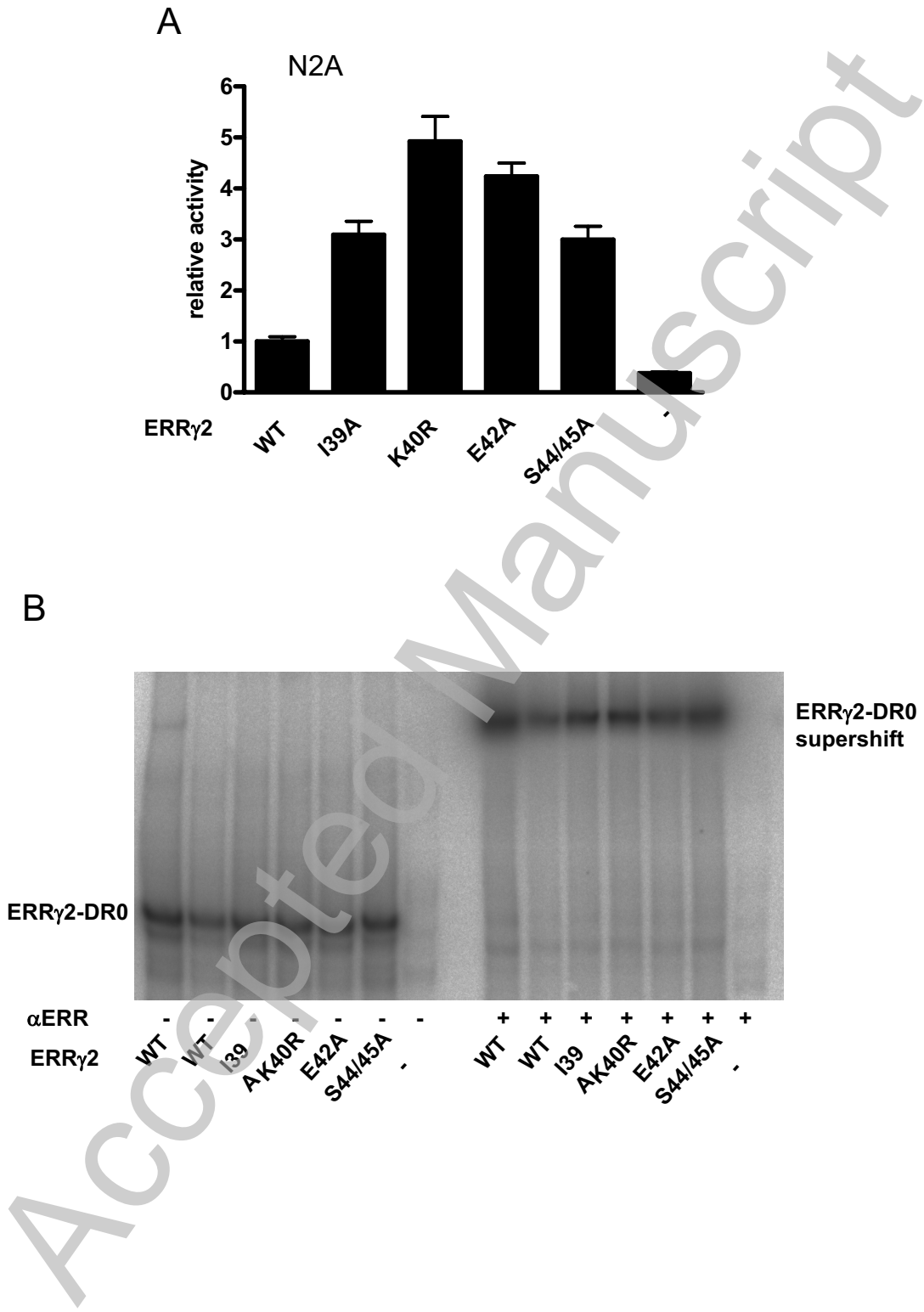


Figure 3



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20081556

Figure 4



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20081556

Figure 5

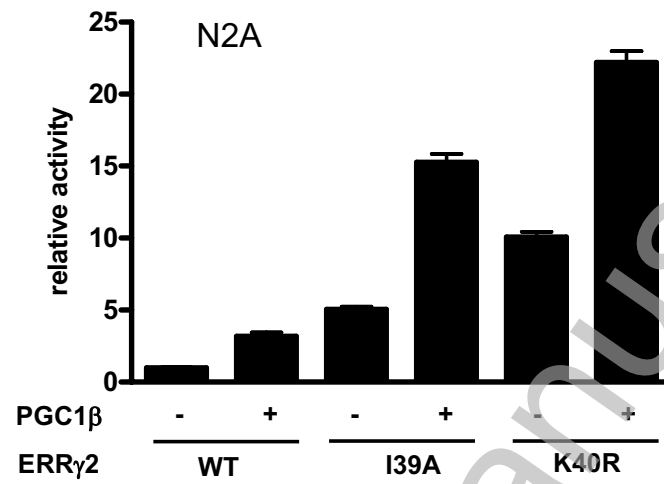
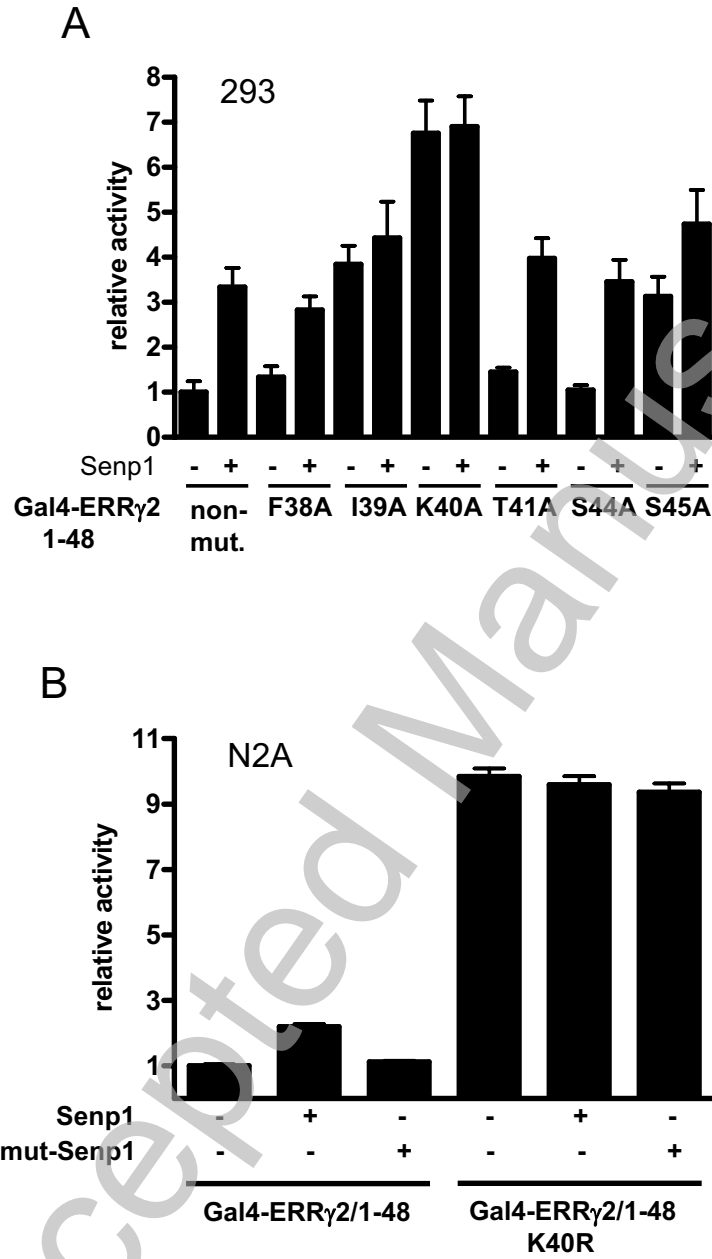


Figure 6



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20081556

Figure 7

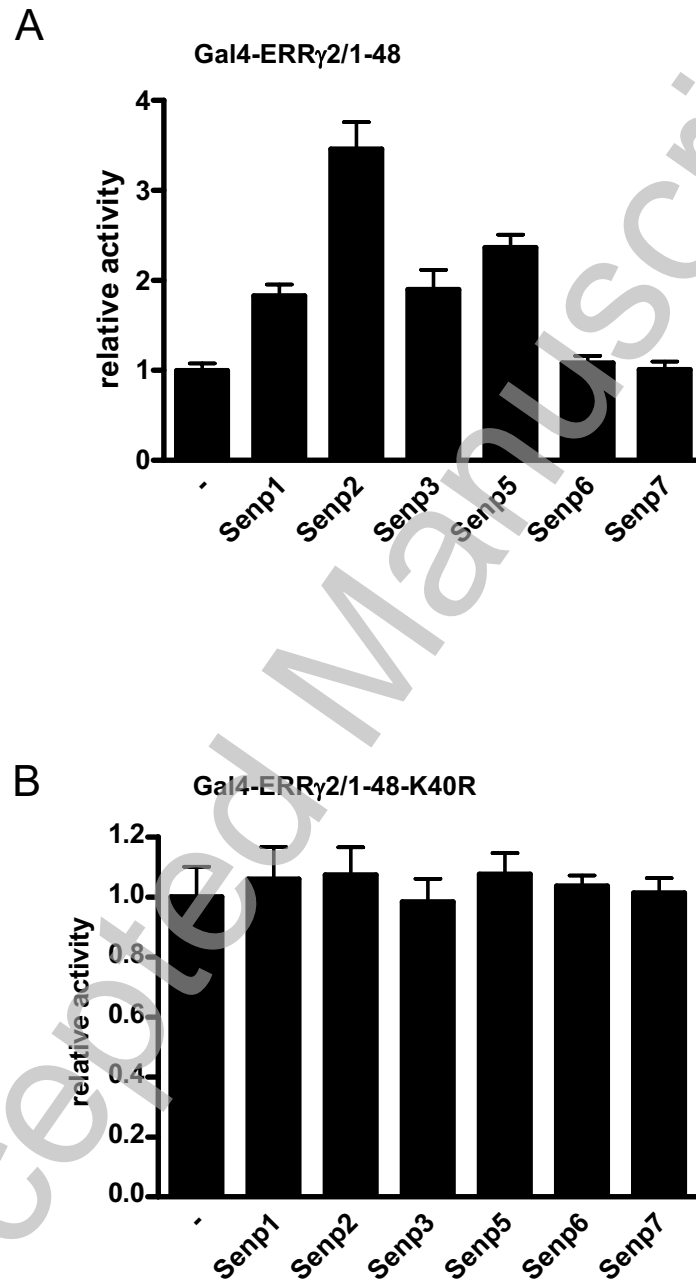


Figure 8

