11-HSD2 SUMOylation Modulates Cortisol-induced Mineralocorticoid Receptor Nuclear Translocation Independently of Effects on Transactivation

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Associate Editor's comments

The reviewer recommend some rewording of your manuscript. The most important editing concerns the cause and effect relationship and the conclusions drawn from your data. Given the limitations of your experiments and the mysterious nature of some results, you must qualify and not over-state your conclusions. We would allow you to speculate what types of additional experiments are important for a better understanding of the mechanisms for the observed effects of K266 mutation.

Thank you for the opportunity to send a revised version of our manuscript.
Following the reviewers suggestions we have modified the title of the manuscript and introduced some changes in the Abstract and Discussion with the goal of avoiding overstating our conclusions. Changes are detailed below. Also, in answering comments by reviewer #2, we have tried to clarify that at no point in the manuscript do we propose that HSD2 SUMOylation could be the main protective mechanism limiting MR activation by glucocorticoids. Our data does not contradict all the data showing that impaired cortisol to cortisone conversion is what causes the syndrome of Apparent Mineralocorticoid Excess.
Finally, we believe that our Discussion already points towards specific lines of research that could lead to a better understanding of the observed effects of mutant K266. Specifically: a) detailed study of MR and HSD2 interactome (lines 410-423); b) determining whether HSD2 is situated near the nuclear pore and in that case disrupting that specific localization (lines 424-431); c) structural analysis of differential MR conformational changes in response to different ligands and in the presence or absence of SUMOylated HSD2 (lines 432-439).

Reviewers' comments

Reviewer #1*: The authors should be commended for their careful revision and inclusion of additional experimental data. The new Figure 3 lends much stronger support for SUMOylation of HSD2.*
The manuscript is well written and provides novel information. The revision has improved the manuscript significantly and strengthened it.
There are just a few suggestions to alter the text to make the manuscript fully consistent with the revised focus on localization.

We would like to thank the reviewer for these comments. Please see our answers to your specific points below.

1) I would suggest modifying the title to remove indications of 11β-HSD2 SUMOylation dependent changes in MR transactivation.

Some possibilities could be:

11β-HSD2 SUMOylation Modulates Cortisol-induced Mineralocorticoid Receptor Nuclear Translocation Independently of Effects on Transactivation

11β-HSD2 SUMOylation Modulates Cortisol-induced Mineralocorticoid Receptor Nuclear Translocation but not its ability to limit Transactivation

11β-HSD2 SUMOylation Dissociates Cortisol-induced Mineralocorticoid Receptor Nuclear Translocation from Transactivation

We have modified the title using the first possibility suggested by the reviewer.

2) the following two changes are strongly suggested:

Line 385: change:

" only 11β-HSD2 that has been SUMOylated appears to be"
to:

"only 11β-HSD2 that can be SUMOylated appears to be"

(There is no evidence that it is only the SUMOylated fraction that is responsible for the effect. The modified text keeps options open)

This line of text has been modified as suggested.

Lines 443 to 446:

Should be replaced with something similar to:

"Interestingly, although impairing HSD2 SUMOylation enhances MR nuclear localization, it does not alter its ability to limit cortisol mediated cofactor recruitment to the receptor or to modulate its transcriptional activity. This phenomenon uncovers a complex and SUMOylation-regulated functional role of 11β-HSD2 that dissociates glucocorticoid-dependent MR subcellular localization from transcriptional activity."

We have replaced the text in lines 443-446 with a paragraph similar to the one suggested by the reviewer. The text now reads "Interestingly, although impairing 11β-HSD2 SUMOylation enhances cortisol-dependent MR nuclear translocation, the amount of MR/co-activator complexes formed remains unaltered, resulting in the same transcriptional activity. This phenomenon uncovers a complex and SUMOylation-regulated functional role of 11β-HSD2 that dissociates glucocorticoid-dependent MR subcellular localization from transcriptional activity".

***Reviewer #2**: It is clear that the authors have worked hard to respond to the comments of the referees. However I remain concerned at the statement that 'taken together, our data demonstrate that SUMOylation of 11β-HSD2 at residue K266 controls cortisol mediated MR nuclear translocation'.*

We have modified this statement, which now reads, "Taken together, our data suggests that SUMOylation of 11β-HSD2 at residue K266 modulates cortisol-mediated MR nuclear translocation independently of effects on transactivation".

As the authors indicate in their response 'so far no syndrome of mineralocorticoid excess-associated mutation affecting residue K266 has been described.' This must be one of the acid tests of their hypothesis. By way of contrast a series of mutations of 11β-HSD2 have been described producing the Syndrome of Mineralocorticoid Excess (SAME) and are closely linked to the failure of inactivation of cortisol.

We are fully convinced that failure of inactivation of cortisol is the basis of SAME, as indicated in the Introduction. Our data shows that lack of HSD2 SUMOylation (either by mutation of K266 residue or by enzymatic deSUMOylation) facilitates cortisol-induced MR nuclear translocation. However, this does not translate in increased transcriptional activity. Therefore, we do not expect this increased nuclear MR abundance would produce SAME. However, we do point out (Discussion, lines 378-382) that mutation K266R produces a mild change in HSD2 enzymatic activity, reminiscent of the effect of mutation R279C found in mild cases of SAME in humans. Based on this, we cannot fully discard the possibility that mutations in residue K266 could theoretically produce some form of SAME. Since this is not supported by data from clinical genetics, we would like to avoid overstating this possibility.

The authors go on to state:
'With the current data we cannot predict whether a defect in SUMOylation would produce SAME.' If SUMOylation is the mechanism that controls cortisol-mediated MR nuclear translocation then this would be expected.

See answer to the point above.

There appears to be an error on page 4. The authors state:

'We do not believe that SUMOylation and not cortisol to cortisone conversion is the main protective mechanism.' I presume they mean 'We believe that SUMOylation and not cortisol to cortisone conversion is the main protective mechanism.'

We apologize for not being sufficiently clear in our previous answer to the reviewer´s comments. As we have indicated in the manuscript and also in the current rebuttal, it is clear to us that SAME arises from lack of efficient conversion of cortisol to cortisone by HSD2. As explained above, we now show that HSD2 SUMOylation is important to control cortisol-induced MR nuclear translocation, but this does not result in increased receptor activity. Therefore, we conclude that SUMOylation per se is not the main protective mechanism. However, since mutant K266R shows a mild change in enzymatic activity, it is still possible that this could produce a slightly impaired cortisol to cortisone conversion in vivo. Our current data does not allow to test this possibility.

In summary I believe that it is reasonable for the authors to state that lack of SUMOylation of 11β-HSD2 at residue K266 results in cortisol mediated MR nuclear translocation. The importance of this mechanism in controlling the normal specificity of the MR remains to be determined. It is also unclear if defects of SUMOylation can produce a syndrome of apparent mineralocorticoid excess.

We fully agree with this summary. We believe that in its current form the manuscript presents this view of the data.

11β-HSD2 SUMOylation Modulates Cortisol-induced Mineralocorticoid Receptor Nuclear Translocation Independently of Effects on Transactivation

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Disclosure summary: The authors have nothing to disclose.

Abstract

The enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) has an essential role in aldosterone target tissues, conferring aldosterone selectivity for the mineralocorticoid receptor (MR) by converting 11β-hydroxyglucocorticoids to inactive 11-ketosteroids. Congenital deficiency of 11β-HSD2 causes a form of salt-sensitive hypertension known as the syndrome of apparent mineralocorticoid excess. The disease phenotype, which ranges from mild to severe, correlates well with reduction in enzyme activity. Furthermore, polymorphisms in the 11β-HSD2 coding gene (*HSD11B2*) have been linked to high blood pressure and salt-sensitivity, major cardiovascular risk factors. 11β-HSD2 expression is controlled by different factors such as cytokines, sex steroids or vasopressin, but post-translational modulation of its activity has not been explored. Analysis of 11β-HSD2 sequence revealed a consensus site for conjugation of small ubiquitin-related modifier (SUMO) peptide, a major post-translational regulatory event in several cellular processes. Our results demonstrate that 11β-HSD2 is SUMOylated at lysine 266. Non-SUMOylatable mutant K266R showed slightly higher substrate affinity and decreased Vmax, but no effects on protein stability or subcellular localization. Despite mild changes in enzyme activity, mutant K266R was unable to prevent cortisol-dependent MR nuclear translocation. The same effect was achieved by co-expression of wild-type 11β-HSD2 with SENP1, a protease that catalyzes SUMO deconjugation. In the presence of 11β-HSD2-K266R increased nuclear MR localization did not correlate with increased response to cortisol or increased recruitment of transcriptional co-regulators. Taken together, our data suggests that SUMOylation of 11β-HSD2 at residue K266 modulates cortisol-mediated MR nuclear translocation independently of effects on transactivation.

Précis

Post-translational modification of 11β-hydroxysteroid dehydrogenase type 2 by SUMOylation at residue K266R has a key role in controlling MR subcellular localization in the presence of cortisol.

Introduction

11β-hydroxysteroid dehydrogenase (11β-HSD)[1] isozymes catalyze the interconversion between biologically active 11β-hydroxyglucocorticoids and inactive 11-ketosteroids (1). The isoform 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) catalyzes the NAD-dependent reaction to convert cortisol to inactive cortisone (2). The reverse reaction is NADPH-dependent and is catalyzed by the isozyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), leading to increased local cortisol concentration in tissues such as liver and adipose (3,4).

11β-HSD2 is specifically expressed in aldosterone target tissues, such as epithelial cells from distal colon and principal cells of the distal nephron, and serves to protect the non-selective mineralocorticoid receptor (MR) from activation by glucocorticoids, which circulate at much higher concentration than aldosterone. 11β-HSD2 confers aldosterone-specificity on MR and brings key physiological functions of electrolyte and volume homeostasis under the control of the renin-angiotensin system (RAS) (4).

11β-HSD2 can be saturated by high cortisol levels due to cortisol hypersecretion, resulting in inappropriate MR activation (5,6). Similarly, decreased 11β-HSD2 activity also leads to inappropriate activation of MR by endogenous glucocorticoids. In both situations, MR-target proteins, such as the epithelial sodium channel (ENaC) are uncoupled from the RAS leading to renal sodium retention and a salt-sensitive increase in blood pressure, both in humans (7) and in rodent models (8-10). This concept is illustrated by the syndrome of apparent mineralocorticoid excess (AME), characterized by hypertension, hypokalemia and low renin and aldosterone levels (11). AME can be congenital, due to loss-of-function mutations of the 11β-HSD2 coding gene (*HSD11B2*) (12), or acquired, most commonly due to excessive consumption of natural licorice, which contains glycyrrhetinic acid that together with its derivative, carbenoxolone, inhibit 11β-HSD2 (13). In patients, the severity of AME symptoms correlates well with the underlying enzyme activity (14). Several polymorphisms in *HSD11B2* correlate with slightly decreased 11β-HSD2 activity and associate to salt-sensitive increase of blood pressure both in healthy volunteers and patients with essential hypertension (15-21). This evidence indicates that appropriate

control of 11β-HSD2 expression and activity is essential for blood pressure regulation (22). Factors controlling 11β-HSD2 expression are not fully defined, although there is evidence indicating transcriptional regulation by cytokines, sex steroids, vasopressin and microRNAs (1,23). However, cellular mechanisms that dynamically regulate 11β-HSD2, such as post-translational modifications have not been explored.

Small ubiquitin-related modifiers (SUMO) are ~11 kDa polypeptides that are post-translationally conjugated to other proteins. This reversible post-translational modification is typically observed in nuclear proteins, related to transcription factor regulation (24), DNA damage response (25), mitosis or cell cycle progression (26). However, regulatory SUMOylation has also been described in cytosolic or plasma membrane proteins (27). Analysis of the human 11β-HSD2 sequence uncovered a canonical SUMOylation consensus motif around lysine 266 (Fig. 1). Based on that, we hypothesized that SUMOylation of K266 in 11β-HSD2 may be involved in regulating enzyme activity and therefore could affect MR glucocorticoid-dependent transcriptional activity. Our results show that 11β-HSD2 is SUMOylated at residue K266. Non-SUMOylatable mutant 11β-HSD2, K266R, displays mild changes in enzymatic activity with slightly higher affinity but decreased Vmax and is unable to prevent cortisol-dependent MR nuclear translocation. Paradoxically, increased MR nuclear localization in the presence of 11β-HSD2-K266R does not correlate with enhanced activity in the presence of cortisol. Our results uncover a prominent role of 11β-HSD2 SUMOylation in controlling cortisol-dependent MR subcellular localization.

Experimental procedures

Sequence analysis - We used the prediction algorithm GPS-SUMO (28) to search for consensus SUMOylation motifs (ψ-K-x-D/E, where ψ is a large hydrophobic residue, K is the lysine conjugated to SUMO, x is any amino acid, and D or E is an acidic residue -Asp or Glu-) in 11β-HSD2 from different species. Additionally, GPS-SUMO was used to predict putative SIMs in the same sequences. Multiple sequence comparison was performed with Clustal Omega (European Bioinformatics Institute).

Plasmid constructs - Generation and use of functional fluorescent derivatives of MR with insertion of YFP or GFP after amino acid 147 (MR-147-YFP/GFP) has been previously described (29-31). Plasmid pcDNA3.1-MR, expressing hMR-WT was previously described (32). Plasmid expressing wild-type human 11β-HSD2 fused to FLAG epitope (11β-HSD2-FLAG) cloned in pcDNA3 (Invitrogen) has been previously described (33). 11β-HSD2 coding sequence was amplified by PCR and subcloned in peCFP-N1 vector (Clontech) to produce an in-frame fusion with the cyan fluorescent protein (CFP) (11β-HSD2-CFP). 11β-HSD2 non-SUMOylatable mutant K266R and AME mutants R337C (34) or R213C (35) were obtained upon introducing point mutations by site-directed mutagenesis using the Quickchange Lightning Kit (Agilent Technologies). Human SUMO1 cloned in pEYFP-C1 (Clontech) to express YFP-SUMO1 was a gift from Dr. Edward Yeh (Addgene plasmid 13380) (36). Sequence of human deSUMOylase SENP1 cloned in pFlag-CMV (Sigma) to express epitope-tagged FLAG-SENP1 was obtained from Addgene collection (plasmid 17357) (37). SENP1 sequence was amplified by PCR and subcloned in pcDNA 3.1 (Invitrogen) to remove the FLAG epitope from the original construct in order to prevent cross-reactions PLA. Generation of a plasmid expressing HA-tagged steroid receptor co-activator 1 (SRC-1) has been previously described (29). All constructs and mutations were confirmed by DNA sequencing.

Cell culture, transfection and hormone treatment - We used COS-7 cells, which lack endogenous MR and GR expression (38). COS-7 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% FBS. Cells were regularly tested to ensure absence of

mycoplasma infection. Cells were transfected with Jetprime (Polyplus Transfection, Illkirch, France) as described before (29,30). pcDNA3.1 (Invitrogen) was used as a control in transfections. At the time of transfection cells were washed and transferred to growth medium supplemented with charcoal-stripped FBS (Lonza) to eliminate steroids. Twenty-four hours after transfection, cells were washed and treated with the indicated amounts of aldosterone or cortisol. Aldosterone and cortisol were obtained from Sigma, dissolved in ethanol, and added to cells to the final concentration indicated for each experiment. Control cells were treated with ethanol at the same dilution used for treatments (1:1000).

Immunoprecipitation and western blot analysis - Immunoprecipitation of 11β-HSD2 was performed using a mouse monoclonal antibody against FLAG epitope (Sigma) at 1:150 dilution in lysis buffer (in mM: NaCl, 170; EDTA, 1; DTT, 1; Tris-HCl, 20; pH 7.6) supplemented with 0.5% Nonidet^{TM}-P40 and a protease inhibitor cocktail (Roche). Antibody-antigen complex capture was performed using Proteome Protein A and Protein G Magnetic Beads kit (Millipore). Western blot analysis was performed as previously described (29,30). Human 11β-HSD2 was detected with rabbit polyclonal antibody (H-145, Santa Cruz Biotechnology; epitope corresponding to amino acids 261-405). MR was detected with mouse monoclonal antibody rMR365-4D6, developed by Dr. Celso Gomez-Sanchez et al. (39) and obtained from the Developmental Studies Hybridoma Bank (The University of Iowa, Department of Biology, Iowa City, IA). When indicated, fluorescent fusion proteins were detected using a polyclonal antibody raised in rabbit (Abcam), kindly provided by Dr. Raimundo Freire (40). To control for total protein loading we used monoclonal antibodies against GAPDH (Abcam) or β-actin (Sigma). Secondary antibodies conjugated with peroxidase (GE Healthcare) were used at 1:10000 dilution. Western blots were developed with Immun-Star WesternC kit (Bio-Rad) and signals were detected with a Chemidoc imaging system (Bio-Rad) and quantified with the software provided by the manufacturer (Image Lab, Bio-Rad).

Cell imaging and kinetic analysis of nuclear translocation - Semiquantitative analysis of subcellular distribution in the absence of aldosterone was performed as previously described (41). Briefly, cells were transfected with the indicated combination of plasmids, grown for 48 hours in culture medium supplemented with charcoal-stripped serum. Cells were then fixed, mounted, and images were taken

under a confocal microscope. At least 75 cells per condition were scored into five categories (N, exclusive nuclear localization; N > C, predominant nuclear localization; N = C, even distribution throughout cytosol and nucleus; N < C, predominant cytosolic localization; C, exclusive cytosolic localization). Data are shown as the percentage of cells in each category from the total amount of cells scored. Images were collected using a Fluoview 1000 confocal microscope (Olympus, Barcelona, Spain). Kinetic analysis of cortisol-induced MR nuclear translocation was performed as previously described (30,42). Briefly, cells were transfected with MR-147-GFP and grown for 48 hours in DMEM supplemented with charcoal-stripped FBS. Cells were then transferred to extracellular saline (in mM: NaCl, 137; KCl, 4; $CaCl_2$, 1.8; $MgCl_2$, 1; glucose, 10; HEPES,10; pH 7.4), placed under a Fluoview 1000 confocal microscope (Olympus) in a temperature-controlled environmental chamber set at 37°C and treated by adding 10 nM aldosterone to the medium. Images were collected for 60 minutes at a sampling rate of one every 2 minutes. Quantitative analysis of MR-GFP distribution was performed frame-by-frame using the manufacturer´s software (Olympus). Recordings in the absence of aldosterone were performed to control for photobleaching of GFP. Data processing and sigmoid curve-fitting were performed using Prism 5 (GraphPad) according to the following equation:

$$F = F_0 + \left[\frac{F_{max} - F_0}{1+\exp\left(\frac{t_{1/2}-t}{V_n}\right)}\right] \qquad \text{Equation 1}$$

where F_0 is the initial nuclear fluorescence, F_{max} is the maximal nuclear fluorescence reached, $t_{1/2}$ is the time (min) at which fluorescence is halfway between F_0 and F_{max}, and V_n is a factor determining how steeply nuclear accumulation changes with time.

In situ *proximity ligation assay (PLA)* - PLA was performed using a commercially available kit (Duolink, Olink Biosciences, Uppsala, Sweden) as described (29,30). 11β-HSD2 SUMOylation was detected in COS-7 cells transfected with WT or K266R 11β-HSD2-FLAG and YFP-SUMO1 by using a mouse monoclonal anti-FLAG antibody (clone M2, Sigma) and a rabbit polyclonal anti-GFP (Abcam). MR

nuclear interaction with SRC-1 coactivator was detected in COS-7 cells transfected with WT MR and SRC-1-HA by using a mouse monoclonal anti-HA antibody (clone HA.11, Covance) and a rabbit polyclonal anti-MR (MR-H300, Santa Cruz Biotechnology). The antibodies used in the assay were previously validated by immunocytochemistry using previously described procedures (31,41). Specificity controls consisted of non-transfected cells, cells where one of the transfected plasmids was omitted or cells not treated with MR ligand. Results were quantified using the software provided by the manufacturer (Duolink Image Tool) and are expressed as average number of puncta per cell area.

Transactivation function assays - MR transcriptional activity was assayed by co-transfecting a plasmid encoding MR with a plasmid containing a synthetic promoter containing two copies of the basic glucocorticoid-response element (GRE) fused to the firefly luciferase gene (GRE2X-luc; kindly provided by Dr. Rainer Lanz), and a third plasmid containing Renilla luciferase under the control of a cytomegalovirus promoter (pSG5-ren; kindly provided by Dr. Fátima Gebauer), as previously described (29,30). 11β-HSD2 constructs were included in the transfection mix as indicated in each experiment. Total amounts of transfected DNA were kept constant. Cotransfected *Renilla* and firefly luciferase activities were measured sequentially using a commercially available kit (Dual-Glo, Promega). MR-dependent transcriptional activity was calculated as the ratio firefly luciferase/*Renilla* luciferase. Results are given as normalized average ± SE. EC_{50} values were calculated from normalized data fitted to a log(agonist) versus response equation with variable slope using Prism 5 (GraphPad Software).

11β-HSD2 activity assays - 11β-HSD2 enzyme activity was determined using COS-7 cells transfected with WT or non-SUMOylatable mutant K266R cDNAs. Cells were washed 24 h after transfection and then transferred to charcoal-stripped serum-supplemented DMEM. Cells were treated with the indicated time and cortisol concentration. Cells were collected for protein assay by the method of Bradford and medium was used to extract steroids in ethyl acetate (HPLC grade) for analysis by liquid chromatography and tandem mass spectrometry (LC-MS/MS). The organic layer was dried by heating at 60ºC under a nitrogen gas current. Steroids were then dissolved in mobile phase (30% acetonitrile) and analyzed by LC-MS/MS as per (43) to obtain the absolute amount of cortisol and cortisone present in the medium.

Negative control consisted on samples from non-transfected cells run in parallel. Enzyme activity was calculated as pmol of cortisone accumulation normalized by time and total amount of protein. In certain experiments we expressed the data as percentage conversion of cortisol to cortisone. Data were fitted to a Michaelis-Menten equation using Prism 5 software (GraphPad Software).

Independently, 11β-HSD2 activity was analyzed by measuring ^{3}H-corticosterone conversion into ^{3}H-11-dehydrocorticosterone by HPLC as described (44). Data were analyzed by measuring the percentage conversion of cortisol to cortisone.

Statistical analysis - Statistical analysis was performed using Prism 5 software (GraphPad Software). Unpaired Student *t* test, one-way ANOVA followed by Dunn's multiple comparison test as indicated in each Figure.

Results

11β-HSD2 is SUMOylated at lysine 266

We used the prediction algorithm GPS-SUMO (28) to investigate whether the human 11β-HSD2 sequence displays any consensus SUMOylation sites. Results showed that lysine 266 is part of a sequence matching the core SUMOylation consensus motif ψ-K-x-D/E flanked by P and G residues (Fig. 1A), which conform a high probability SUMOylation site (45). This lysine and the SUMO consensus motif are highly conserved in other mammals including primates, ruminants and rodents, but not pigs (Fig. 1A). Mapping of this residue to a structural homology model of 11β-HSD2, based on the crystal structure of 11β-HSD1 (46), shows that it lies on a β-sheet exposed to the solution and away from the core of the enzyme containing the catalytic and co-factor binding sites (Fig. 1B). In addition to the canonical high probability covalent SUMOylation site surrounding K266, the algorithm predicted additional lower probability SUMOylation sites and SUMO interaction motifs (SIM), which may mediate non-covalent interaction with SUMO peptides (on-line supplemental information, Table S1).

To establish experimentally whether 11β-HSD2 is SUMOylated at residue K266, we co-transfected FLAG-tagged WT or K266R mutant 11β-HSD2 with YFP-SUMO1 to perform intramolecular *in situ* proximity ligation assay (PLA) in COS-7 cells. Immunostaining of transfected WT or mutant 11β-HSD2 shows similar distribution patterns consistent with the expected ER-associated localization (6), indicating that mutation K266R does not alter subcellular sorting of the enzyme (Fig. 2A). PLA results using anti-FLAG and anti-YFP antibodies show a prominent signal suggesting WT 11β-HSD2 SUMOylation in the cytosolic, ER-associated region (Fig. 2B), even though most of YFP-SUMO-1 protein localizes in the nucleus, as expected (36). PLA signal of non-SUMOylatable mutant K266R was strongly reduced to approximately 25% of the WT signal (Fig. 2B and *2*C). Further support for 11β-HSD2 SUMOylation was obtained by co-transfecting the established deSUMOylase Sentrin-specific protease 1 (SENP1) (47). PLA signal was drastically reduced by co-expression of SENP1 to approximately 20% of the signal (Fig. 2B and 2C). Non-transfected cells or omission of either one of the transfected plasmids resulted in the

absence of signal (Fig. 2B and 2C). These results are consistent with 11β-HSD2 SUMOylation, predominantly at K266 residue.

To confirm 11β-HSD2 SUMOylation at residue K266 and quantify the steady-state fraction of total enzyme showing this post-translational modification, we co-transfected COS-7 cells with either FLAG-tagged, WT 11β-HSD2 or non-SUMOylatable mutant K266R with a plasmid expressing YFP-SUMO1. Western blot analysis of cell lysates demonstrates comparable expression levels for WT and mutant 11β-HSD2 as well as for YFP-SUMO1 (Fig. 3A). After immunoprecipitation of 11β-HSD2 with an anti-FLAG antibody and electrophoresis under denaturing conditions, we performed western blots with anti-YFP (to detect YFP-SUMO1) and anti-11β-HSD2. Results obtained with anti-YFP antibody show bands migrating at approximately 80 kDa (Fig. 3B), which would be consistent with the predicted molecular mass of approximately 82.5 kDa resulting from the mass of 11β-HSD2, YFP and SUMO1 (44, 27 and 11.5 kDa, respectively). The signal was notably decreased in the case of K266R mutant, despite equal levels of total protein expression (Fig. 3A and 3B). Probing immunoprecipitation products with anti-11β-HSD2 recognized bands at the predicted 11β-HSD2 molecular mass (44 kDa), indicating that both the WT and mutant forms were immunoprecipitated to comparable extents (Fig. 3C). In addition, longer exposure tf the blot allowed detecting a slower-migrating band (Fig. 3D), which coincides with the band detected by anti-YFP at approximately 80 kDa (Fig. 3B). Taken together, these results further support that 11β-HSD2 is SUMOylated and that the majority of the signal arises from modification of residue K266 residue, consistently with results from PLA (Fig. 2). Interestingly, the larger, SUMOylated form of 11β-HSD2 is not detected when total protein is analyzed by western blot using the input sample (Fig. 3A). Also, when probing immunoprecipitation products with anti-11β-HSD2 antibody the larger form of 11β-HSD2 requires longer exposure to be detected than the unmodified form (Fig. 3C and 3D) suggesting that a low proportion of the protein is SUMOylated in our experimental conditions. This is consistent with the general finding that the proportion of proteins modified by SUMO *in vivo* is very low, however the

modification usually has a high functional impact, a phenomenon that has been termed the "SUMO paradox" (48).

SUMOylation alters 11β-HSD2 enzyme kinetics but not protein abundance

To assess whether SUMOylation at residue K266 alters 11β-HSD2 enzyme abundance or activity, we transiently transfected WT or mutant 11β-HSD2 in COS-7 cells, quantifying protein expression by western blot. Mutants R213C and R337C, which induce AME in humans, were included as controls. R213C is a loss-of-function mutation (12), while R337C is a destabilized variant of the protein (34,49). Quantification of 11β-HSD2 expression in transfected cells showed that non-SUMOylatable mutant K266R or mutation R213C did not produce any significant change in protein abundance when compared to WT 11β-HSD2 (Fig. 4). As expected, mutant R337C showed clearly diminished expression to approximately 20% of WT level (Fig. 4).

In order to test whether mutation K266R alters 11β-HSD2 activity, we measured conversion of cortisol to cortisone in intact COS-7 cells transfected or not with 11β-HSD2 variants by performing liquid chromatography coupled to mass spectrometry (LC-MS/MS) on cell extracts. First, we compared the time-dependent conversion of 300 nM cortisol to cortisone in non-transfected or WT 11β-HSD2 transfected cells (Fig. 5A). Results show a linear time-dependent cortisol conversion in cells transfected with WT 11β-HSD2 and a very slow conversion in non-transfected cells (lower than 1% in up to 4 hours incubation; Fig. 5A). We then compared WT 11β-HSD2 and non-SUMOylatable mutant K266R activity by incubating 30 minutes with increasing cortisol concentration and fitting the data to a Michaelis-Menten curve (Fig. 5B). We obtained a Km of 565 ± 18 nM for WT and 377 ± 13 for K266R mutant and a Vmax of 574 ± 19 pmol/hour/mg of protein for WT and 395 ± 4 for K266R mutant. The parameters calculated for WT 11β-HSD2 are consistent with previously published data (35,49). Differences in enzyme parameters did not reflect differential expression of the 11β-HSD2 constructs (Fig. 4).

We used an independent method to assess 11β-HSD2 activity by measuring ^{3}H-corticosterone conversion to ^{3}H-11-dehydrocorticosterone by HPLC. COS-7 cells were treated with 10 nM ^{3}H-corticosterone for 60

min and conversion to ^{3}H-11-dehydrocorticosterone was measured and normalized to total protein content. WT 11β-HSD2 showed a 12.24% ± 0.55 conversion (mean ± SE, n=6), while mutant K266R showed a 12.72% ± 0.43 conversion (n=6).

Lack of SUMOylation at residue K266 allows cortisol-dependent MR nuclear translocation

To investigate whether K266 SUMOylation has a role on 11β-HSD2 function in diminishing MR activation by glucocorticoids, we first asked whether mutant K266R affects 11β-HSD2 ability to prevent cortisol-induced MR nuclear translocation. To that end we studied nuclear translocation of our fully functional fluorescent derivative MR, with YFP inserted after amino acid 147 (29-31), cotransfected or not with different 11β-HSD2 constructs fused to CFP (WT; AME loss-of-function mutant R337C; and non SUMOylatable mutant K266R). The assay was performed in COS-7 cells, which lack detectable endogenous expression of 11β-HSD2 (Figs. 2, 4 and 5). Transfected cells were left untreated or exposed overnight to two different physiological cortisol concentrations (100 or 500 nM). In the absence of ligand, MR localization is predominantly cytosolic in most (> 80%) of the cells (Fig. 6A and 6B) as previously described in the same cell line (50). Cotransfection with WT 11β-HSD2, K266R or R337C did not alter naïve MR subcellular localization in the absence of cortisol (Fig. 6A and 6B). Overnight treatment with 100 nM cortisol stimulated full MR translocation to the nucleus (> 90% of cells). WT 11β-HSD2 coexpression prevented MR translocation, keeping a predominantly cytosolic MR localization, consistent with the cortisol-inactivation function of the enzyme (Fig. 6C and 6D). Cotransfection of 11β-HSD2-R337C construct failed to prevent cortisol-induced MR translocation (Fig. 6C and 6D), consistent with the loss-of-function effect previously described for this AME mutant (34). Non-SUMOylatable mutant 11β-HSD2-K266R coexpression was also unable to prevent MR nuclear translocation induced by 100 nM cortisol, behaving like the loss-of-function mutant R337C (Fig. 6C and 6D). Overnight exposure to 500nM cortisol resulted in MR full nuclear translocation in all conditions (Fig. 6E and 6F). This demonstrates that 11β-HSD2 capacity to inactivate cortisol can be exceeded by high cortisol levels at the high end of the physiological range, consistent with previous data (6,44).

Since lysine residues are the target of other post-translational modifications in addition to SUMOylation, the effect of the K266R mutant on cortisol-induced MR translocation could reflect other actions, including acetylation or ubiquitination. To assess this, we co-expressed WT 11β-HSD2 with the established deSUMOylase SENP1. This experiment also resulted in full cortisol-induced MR translocation, consistent with a SUMOylation-mediated effect on the ability of 11β-HSD2 to prevent cortisol access to MR (Fig. 6G and 6H). It is worth noting that MR has also been described to be SUMOylated in cells (51). Our experiment indicates that if MR is SUMOylated under our experimental conditions, SENP1-mediated removal of SUMO does not prevent cortisol-induced receptor nuclear translocation.

We next investigated the kinetics of cortisol-induced nuclear translocation in living COS-7 cells by imaging MR subcellular dynamics for the first hour after 100 nM cortisol addition. We ran time-lapse experiments in cells co-transfected with MR-147-GFP with or without WT 11β-HSD2, AME mutant R337C or non-SUMOylatable mutant K266R. All 11β-HSD2 constructs used were CFP-tagged. Images were taken every two minutes up to 60 minutes after ligand addition and the ratio of nuclear MR was calculated for every frame. Full cortisol-dependent MR nuclear translocation was achieved approximately 50 minutes after ligand addition (Fig. 7). As expected, WT HSD2 prevented nuclear import (< 10% of the receptor translocated over the time period). MR translocation in the presence of AME mutant R337C or non-SUMOylatable mutant K266R, was indistinguishable from the control MR condition (Fig. 7). These data are consistent with subcellular localization after overnight treatment (Fig. 6C and 6D), suggesting that 11β-HSD2 ability to prevent cortisol-induced MR translocation is regulated by SUMOylation of lysine 266.

11β-HSD2-K266R decreases cortisol- but not aldosterone-dependent MR transcriptional response

We then tested whether MR-mediated gene transactivation is affected by 11β-HSD2 SUMOylation. MR activity was assessed in COS-7 cells by cotransfecting the receptor with a luciferase reporter gene under the control of a promoter with two glucocorticoid-response elements (GRE2X). We treated cells

overnight with increasing doses of cortisol in the presence or absence of the different 11β-HSD2 constructs. MR cortisol-dependent transactivation curve showed an EC_{50} of 40 nM (Fig. 8A). AME mutants 11β-HSD2-R337C or R213C did not alter cortisol potency to elicit MR-dependent transactivation, presenting an EC_{50} of 32-44 nM, as expected for loss-of-function constructs. The presence of WT 11β-HSD2 produced the expected decrease in cortisol potency and MR maximal response, with an EC_{50} of approximately 2 μM, two orders of magnitude higher than cortisol EC_{50} for MR. Surprisingly, non-SUMOylatable mutant 11β-HSD2-K266R also decreased cortisol EC_{50} (1.4 μM) and MR maximal response, behaving like WT 11β-HSD2 (Fig. 8A). Western blot analysis showed that results of activity assays are not due to impaired MR expression (Fig. 8C and 8D).

Taking into consideration the experiments described above, it is clear that 11β-HSD2-K266R promotes nuclear MR translocation by cortisol but does not alter its dose-dependence for transactivation (compare Fig. 6C and 8A). This may suggest that MR is functionally impaired in the presence of 11β-HSD2-K266R. Therefore, we tested aldosterone-dependent MR transactivation with the different 11β-HSD2 constructs (Fig. 8B). All dose-response curves were superimposed, with an EC_{50} of approximately 0.1-0.2 nM, as expected for aldosterone (32). This result demonstrates that 11β-HSD2-K266R does not impede *per se* MR activation.

11β-HSD2 SUMOylation affects cortisol-dependent recruitment of co-activators to MR

To gain insight into the mechanism by which 11β-HSD2-K266R prevents cortisol-induced MR transactivation, we tested the interaction between MR and SRC-1, a well-known co-activator of MR (52). We cotransfected COS-7 cells with WT MR and SRC1 tagged with an HA epitope. SRC-1 displays a predominantly nuclear localization both in control and cortisol-stimulated conditions, whereas MR shows the expected ligand-induced trafficking from cytosol to nucleus (Fig. 9A). To quantitatively assess MR-SRC-1 interaction we used PLA in cells co-expressing these two proteins with CFP-tagged WT 11β-HSD2 or K266R mutant. Results show a prominent PLA signal in the nucleus when MR is cotransfected with SRC-1 and nuclear translocation is promoted by cortisol (Fig. 9B). PLA signal was reduced to ~

40% by co-expression of either WT or K266R 11β-HSD2 (Fig. 9B and 9C). Non-transfected cells, omission of either one of the transfected plasmids or no ligand addition resulted in the absence of signal (Fig. 9B and 9C). To ensure that decreased PLA signals did not arise from altered protein expression levels we quantified MR, SRC-1 and 11β-HSD2 abundance by western blot. This experiment showed that co-expression of the three different proteins did not affect their relative levels (Fig. 9D and 9E). These results demonstrate that efficient interaction of MR with SRC-1 is impaired not only by the presence of active 11β-HSD2 (which decreases MR nuclear translocation through cortisol inactivation) but also by the non-SUMOylatable mutant K266R.

Discussion

Our results show that 11β-HSD2 is modified by SUMOylation, with a key conjugation site at residue K266, which is part of a canonical consensus SUMO-conjugation site. The post-translational modification likely affects a small percentage of the total cellular protein. This does not preclude an effect on the biology of 11β-HSD2, since it is a common observation that a small fraction of steady-state SUMOylation frequently has important consequences on the total cellular pool of the modified protein (48). This idea is reinforced by the fact that SENP1 co-expression with WT 11β-HSD2 reproduces the effect seen with mutant K266R. The implication is that SUMO modification likely alters substrate proteins long after de-conjugation, perhaps by modifying the protein environment where they reside. This may have implications for the functional observations reported in this work, as we shall discuss below.

The effect of K266 modification on 11β-HSD2 enzymatic activity appears to be mild, with a 30% decrease in Vmax that is not attributable to decreased protein expression. According to structural homology modeling using as template the crystal structure of 11β-HSD1 (46) or 17β-HSD1 (53), residue K266 lies exposed to the surface of the enzyme (Fig 1B). This area is not directly involved in ligand or cofactor binding regions and does not form part of the enzyme dimerization interphase. Therefore, it is not surprising that, unlike the AME mutations affecting those regions (53), SUMOylation at residue K266 does not dramatically alter enzyme activity. On the other hand, the effect of mutant K226R is reminiscent of a mutation in a nearby residue, R279C, which produces a mild form of AME and displays a reduction of approximately 33% in Vmax without changes in Km (54). Therefore, it is clear that even a rather small change in enzyme kinetics not affecting Km is able to diminish the efficiency of 11β-HSD2 to prevent glucocorticoid-induced MR activation.

Despite only mild effect on enzyme kinetics, the consequence of K266R mutation or enzymatic SUMO de-conjugation on the ability of cortisol to translocate MR to the nucleus is striking. It is clear that regardless of measurable enzymatic activity, only 11β-HSD2 that can be SUMOylated appears to be able to prevent cortisol from inducing MR translocation to the nucleus. However, what is even more surprising

is that MR translocated under those conditions (lack of 11β-HSD2 SUMOylation at residue K266) does not increase transactivation of a target promoter, indicating that there is a strong dissociation between MR subcellular localization and activity. To better understand the relationship between MR nuclear translocation and activity, we generated combined plots where both parameters can be visualized together (Fig. 10). This analysis clearly shows that MR ligand-dependence for nuclear translocation is left-shifted when compared to ligand-dependence for transactivation, particularly in the case of cortisol, both in the absence or presence of 11β-HSD2 (compare Fig. 10B and 10C). Lack of SUMOylation in 11β-HSD2-K266R makes the difference in ligand-dependence of both processes even more prominent (Fig. 10D). In fact, data obtained *in vivo* already suggests this difference. In a detailed study examining subcellular localization of MR and GR in rat kidney under different corticosteroid circulating levels, Ackermann et al. (55) provided evidence to suggest that 11β-HSD2 is sufficient to prevent glucocorticoid-induced GR translocation to the nucleus, but not MR. Therefore, under physiological conditions, enough glucocorticoids escape 11β-HSD2 action to translocate MR to the nucleus, but not to overly activate the receptor and produce excessive Na^+ reabsorption. This fits well with data showing that under physiological conditions 11β-HSD2 activity cannot prevent most of epithelial MR being occupied by glucocorticoids (56), but somehow is able to lock glucocorticoid/MR complexes in an inactive state (57). It has been proposed that 11β-HSD2-dependent production of NADH could be involved in this phenomenon. According to this hypothesis, decreased 11β-HSD2 activity would reduce NADH cellular levels and consequently unlock glucocorticoid/MR activity. It may be that 11β-HSD2 SUMOylation alters the coupling of metabolic processes with glucocorticoid-dependent MR translocation, although our data does not allow assessing this possibility.

Taken together, our data suggests that lack of 11β-HSD2 SUMOylation dissociates MR subcellular localization and activity, allowing cortisol-induced nuclear translocation without generating the conformation necessary to recruit transcriptional co-activators. We speculate that 11β-HSD2 may be interacting directly or indirectly with MR, allowing a cortisol-mediated conformational change of the

receptor that participates in its activation and depends on previous 11β-HSD2 modification by SUMOylation. This would be in agreement with the proposed function of SUMOylation of altering the environment where the modified protein interacts even after removal of SUMO (48). Could SUMOylation alter the proposed physical interaction between MR and 11β-HSD2, previously suggested in the literature (6)? This hypothesis implies that adequate interaction between MR and 11β-HSD2 is necessary for the correct cortisol-induced conformational change of the receptor required to recruit transcriptional co-activators. In the absence of SUMOylation, this interaction would be altered, decreasing MR transcriptional efficiency, but increasing its ability to translocate to the nucleus. Based on this idea, we checked whether we could detect direct interaction between MR with 11β-HSD2 by PLA, but the results were negative (data not shown[1]). This suggests that MR and 11β-HSD2 may not closely interact in the cell, although the result does not preclude the possibility that both proteins are part of a larger complex and that SUMOylation of 11β-HSD2 alters the nature of it.

A different explanation for our data could be based on a compartmentalization of the effects of 11β-HSD2 on cortisol concentration in the cell. Under this scenario, lack of 11β-HSD2 SUMOylation would change MR subcellular localization, shifting it towards the nucleus, but nuclear MR would still be depleted of cortisol because of the enzymatic activity of 11β-HSD2. For instance, it is conceivable that 11β-HSD2 SUMOylation could place the enzyme near the nuclear pore, creating a low-cortisol nanodomain. SUMO-dependent targeting of proteins to the nuclear pore localization has been previously described (58). Disruption of this localization could favor MR nuclear localization without changing the bulk concentration of cortisol in the cell. Further work will be needed to address this hypothesis.

It remains to be explained why the dissociation between cortisol-dependent nuclear localization and transcriptional activity is larger than that detected with aldosterone (Fig. 10A and 10B). It has been

[1] In addition, one of us (A.O.) has also performed two-hybrid assays and co-IP experiments that failed to see a direct interaction between MR and 11ß-HSD2. A putative bridging protein may exist but has not been identified yet.

demonstrated that cortisol and aldosterone induce differential conformational changes in MR upon binding. For instance, both agonist differ in their ability to induce interdomain interactions between the NH_2- and COOH-terminal domains of MR (59). We suggest that aldosterone ability to stabilize the ligand binding domain of the receptor is enough to allow the correct conformational change, regardless of previous functional or physical interaction with 11β-HSD2, whereas cortisol is unable to do so, due in part to the higher off-rate previously reported (44) and to the influence of non-SUMOylated 11β-HSD2.

In summary, we have shown that 11β-HSD2 is SUMOylated and that this modification mainly takes place at residue K266. Mutation of this residue mildly affects enzyme activity by slightly enhancing substrate affinity and lowering Vmax, but dramatically alters 11β-HSD2 ability to prevent cortisol-induced MR nuclear translocation. Interestingly, although impairing 11β-HSD2 SUMOylation enhances cortisol-dependent MR nuclear translocation, the amount of MR/co-activator complexes formed remains unaltered, resulting in the same transcriptional activity. This phenomenon uncovers a complex and SUMOylation-regulated functional role of 11β-HSD2 that dissociates glucocorticoid-dependent MR subcellular localization from transcriptional activity.

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Figure legends

Figure 1. Prediction of SUMOylation sites in 11β-HSD2 by *in silico* analysis. *A*, Sequence alignment around the highly conserved lysine residue (shaded) that forms part of a canonical SUMOylation consensus sequence, ψ-K-x-D/E (where ψ is a large hydrophobic residue and x is any amino acid), which is indicated above the alignment. The box highlights those species where the SUMOylation consensus sequence is found. *, Residues conserved in every species examined; :, positions with conservative amino acid substitutions; ., partially conserved residues. *B*, Homology model of mouse 11β-HSD2 based on the crystal structure of 11β-HSD1 (46) showing the predicted position of K266. K266 is located in a β-sheet and is facing the aqueous solution. The model shows the cofactor bound to the structure. The area formed by parallel β-sheets defines a core region conserved among all short-chain dehydrogenase reductases. K266 is not within this core and unlikely interferes with catalytic activity.

Figure 2. 11β-HSD2 is SUMOylated mainly at residue K266. *A*, subcellular localization of FLAG-tagged WT 11β-HSD2 and non-SUMOylatable mutant K266R. *B*, representative images of proximity ligation assay (PLA) results examining the interaction of FLAG-tagged 11β-HSD2-WT or non-SUMOylatable mutant K266R with YFP-SUMO1 in the absence or presence of deSUMOylate SENP1. C) Quantitative analysis of PLA signals. Bars represent the average number of puncta/cell area ± S.E. (n = 20 cells per condition). Negative controls consisted on analysis of non-transfected cells (*N.T.*) or leaving out either 11β-HSD2-WT or YFP-SUMO1 from the transfection mix. *n.s.*, no significant difference; ***, $p < 0.001$; one-way ANOVA followed by Dunn's multiple comparisons test.

Figure 3. 11β-HSD2 SUMOylation analyzed by pull-down followed by western blot. Cells were transfected with the indicated combinations of FLAG-tagged WT or mutant 11β-HSD2 and YFP-SUMO. Negative control consisted on omission of 11β-HSD2 in the transfection mix or transfection with empty plasmids (N.T.). Cell lysates were analyzed with anti-β-actin (αβ-actin), anti-11β-HSD2 and anti-YFP antibodies (panel *A*). Immunoprecipitation was performed with an anti-FLAG antibody and products were analyzed with anti-GFP antibody to detect YFP-SUMO (panel *B*) or anti-11β-HSD2 antibody (panels *C*

and *D*). Note that panels *C* and *D* correspond to different portions of the same blot with different exposure times to obtain optimal signals. *Arrowheads* mark the migration of molecular mass markers (values in kDa).

Figure 4. 11β-HSD2-K266R mutant displays normal protein abundance. *A*, representative western blot of 11β-HSD2 WT and mutant expression in COS-7 cells. *NT*, non transfected cells. The same blot was consecutively probed with anti-11β-HSD2 and anti-GAPDH antibodies. *Arrowheads* mark the migration of molecular mass markers (values in kDa). *B*, quantitative analysis of western blots detecting expression of 11β-HSD2 variants. Bars represent the average ± SE of 3-4 independent experiments. *n.s.*, no significant difference; *, $p< 0.05$; one-way ANOVA followed by Dunn's multiple comparisons test.

Figure 5. Non-SUMOylatable mutation K266R alters 11β-HSD2 enzyme kinetics. COS-7 cells were transfected with WT 11β-HSD2 or non-SUMOylatable mutant K266R. After 24h in charcoal-stripped serum-supplemented DMEM, cells were treated with indicated time and cortisol concentration. Steroids in culture medium were quantified by LC-MS/MS. A) Percentage conversion of 300 nM cortisol to cortisone in non-transfected cells (N.T.) or in cells transfected with WT 11β-HSD2. Linear regression was used to fit data points to a linear equation. B) WT and mutant11β-HSD2 enzyme kinetics. Transfected cells were treated with increasing cortisol concentrations for 30 min. Data points represent average values ± SE (N=3 independent experiments, conducted in duplicate) and were fitted to the Michaelis-Menten equation.

Figure 6. 11β-HSD2 SUMOylation is essential for the ability of the enzyme to prevent cortisol-induced MR nuclear translocation. Quantitative analysis and representative images of YFP-tagged MR subcellular localization in COS-7 cells expressed alone or cotransfected with CFP-tagged 11β-HSD2 variants (WT, non-SUMOylatable mutant K266R or AME mutant R337C) in the absence of ligand (*A* and *B*), in cells treated with 100 nM cortisol (*C* and *D*) or in cells treated with 500 nM cortisol (*E* and *F*). Values represent the average percentage of cells in each category from the total amount of cells scored in three independent experiments (N, exclusive nuclear localization; N C, predominant nuclear localization;

N C, even distribution throughout cytosol and nucleus; N C, predominant cytosolic localization; C, exclusive cytosolic localization). MR distribution was also tested in cells cotransfected with 11β-HSD2 in the presence or absence of the deSUMOylase SENP1 and treated with 100 nM cortisol (*G* and *H*).

Figure 7. Cortisol-induced MR nuclear translocation kinetic in the presence of 11β-HSD2 variants. COS-7 cells were co-transfected with the indicated combinations of GFP-tagged MR and CFP-tagged WT or 11β-HSD2 mutants. Untreated cells were placed under the confocal microscope in Ringer´s medium and treated with 100 nM cortisol. *A*, time course analysis of MR nuclear translocation after cortisol addition (time 0). Images were recorded every two minutes. Values represent average ± SE (N = 7-9) percentage nuclear fluorescence intensity *versus* total cellular fluorescence (*F*). Data points were fitted to Boltzmann sigmoidal curves. *B*, representative images of cortisol-induced MR nuclear translocation by 100 nM cortisol. T, time in minutes after cortisol addition.

Figure 8. Cortisol-induced MR nuclear translocation in the presence of non-SUMOylatable 11β-HSD2 results in reduced receptor activity. COS-7 cells were co-transfected with the indicated combinations of YFP-tagged MR and CFP-tagged WT or mutant 11β-HSD2, GRE2X-luciferase and CMV-*Renilla* reporters. After 24h in charcoal-stripped serum-supplemented DMEM, cells were treated with the indicated dose of cortisol or aldosterone overnight and firefly and *Renilla* luciferase activities were determined using the Dual-Glo kit (Promega). Individual points represent the average ± SE ($N = 3$) firefly/*Renilla* values normalized to the maximum activity for each construct in cells stimulated with cortisol (*A*) or aldosterone (*B*). Data points were fitted to a variable slope model (four parameters). *C*, representative western blots of MR expression in COS-7 cells. *NT*, non-transfected cells. The same blot was consecutively probed with anti-MR and anti-GAPDH antibodies. *Arrowheads* mark the migration of molecular mass markers (values in kDa). *D*, quantitative analysis of western blots detecting expression of MR. Bars represent the average ± SE of three independent experiments. *, $p< 0.05$, one-way ANOVA followed by Dunn's multiple comparisons test.

Figure 9. 11β-HSD2- K266R expression impairs MR interaction with SRC-1 coactivator after cortisol treatment. COS-7 cells were co-transfected with the indicated combinations of WT MR, HA-tagged SRC-1 and CFP-tagged 11β-HSD2 constructs. *A,* subcellular localization of MR and SRC-1 before and after 100 nM cortisol treatment. *B,* representative images of proximity ligation assay (PLA) results examining the nuclear interaction between MR and SCR-1 after 100 nM cortisol treatment in the absence or presence of 11β-HSD2-WT or -K266R mutant. PLA was performed with monoclonal anti-HA and polyclonal anti-MR antibodies. *C,* quantitative analysis of PLA signals. Bars represent the average number of puncta/cell area ± S.E. (n = 20-25 cells per condition). Negative controls consisted on analysis of non-transfected cells, leaving out either MR-WT or SRC-1 plasmids from the transfection mix or omission of cortisol treatment. *N.T.*, non-transfected cells. ***, $p < 0.001$, one-way ANOVA followed by Dunn's multiple comparisons test. *D,* representative western blot of MR, SRC-1 and 11β-HSD2 WT and mutant expression when co-transfected in COS-7 cells in the indicated combinations. *NT*, non transfected cells. *Arrowheads* mark the migration of molecular mass markers (values in kDa). *E,* quantitative analysis of western blots detecting expression of MR, SRC-1 and 11β-HSD2 variants. Bars represent the average ± SE of 3 independent experiments.

Figure 10. Ligand dose-dependence regulation of MR nuclear translocation and activity. Plots simultaneously represent relative luciferase activity and percentage nuclear MR localization for the indicated ligand concentration and in the presence or absence of WT or mutant 11β-HSD2. Data points were fitted to a variable slope model (four parameters). Individual data points or bars represent mean ± SE. Nuclear localization data at 0, 100 and 500 nM cortisol comes from Fig. 6. Luciferase activity data comes from Fig. 8.

Figure 1

A

```
                                        ψKx[E/D]
HUMAN ..... AAVALLMDTFSCELLPWGVKVSIIQPGCFKTESVRNVGQWEKRKQLLLANLPQELLQAYG 296
CHIMPANZEE ... AAVALLMDTFSCELLPWGVKVSIIQPGCFKTESVRNVGQWEKRKQLLLANLPQELLQAYG 296
MACAQUE ..... AAVALLMDTFSCELLPWGVKVSIIQPGCFKTESVRNVGQWEKRKQLLLANLPQELLQAYG 296
SHEEP ..... AALALLMGNFSCELLPWGVKVSIILPACFKTESVKDVHQWEERKQQLLATLPQELLQAYG 296
COW ..... AALALLMGNFSCELLPWGVKVSIIQPACFKTESVKDVHQWEERKQQLLATLPQELLQAYG 296
MOUSE ..... AAIALLMDTFGCELLPWGIKVSIIKPGCFKTDAVTNVNLWEKRKQLLLANIPRELLQAYG 296
RAT ..... AAIALLMDTFSCELLPWGIKVSIIQPGCFKTEAVTNVNLWEKRKQLLLANLPRELLQAYG 296
GUINEA PIG ... AAIVLLTDLFGSELIPWGIKVSVIQPGCFKTESVMNVKLWEQRKQLLLANLPRELLQAYG 296
PIG ..... AAVALLMDSFSCELQPWGVKVSVIQPACFRTEAVKNVDQWEERKRQLLATLPQELLQAYG 296
CHICKEN ..... AALSLLMDTFRSELQPWGVKVSLILPGYFKTAT-CDPDFWKLQKEQLVARLPRELLQAYG 244
FROG ..... AALNRVMDIFRHELMPWGVKVILILPASYKTGAHDNHIHWENQHKKLLANLPIELLQEYG 300
ZEBRAFISH .... AALNLFINTLRHELDPWGVKVSTILPSAYKTGQSSNAEYWEKQYKSLLQGLSPNLLEEYG 285
               **:  . . :  ** ***:**  * *. ::*    :   *: : . *:  :  :**: **
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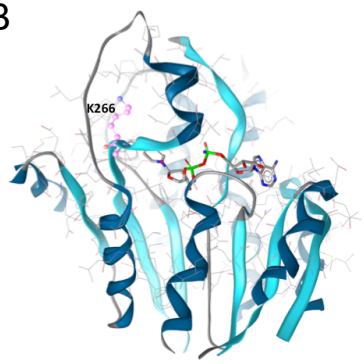
B



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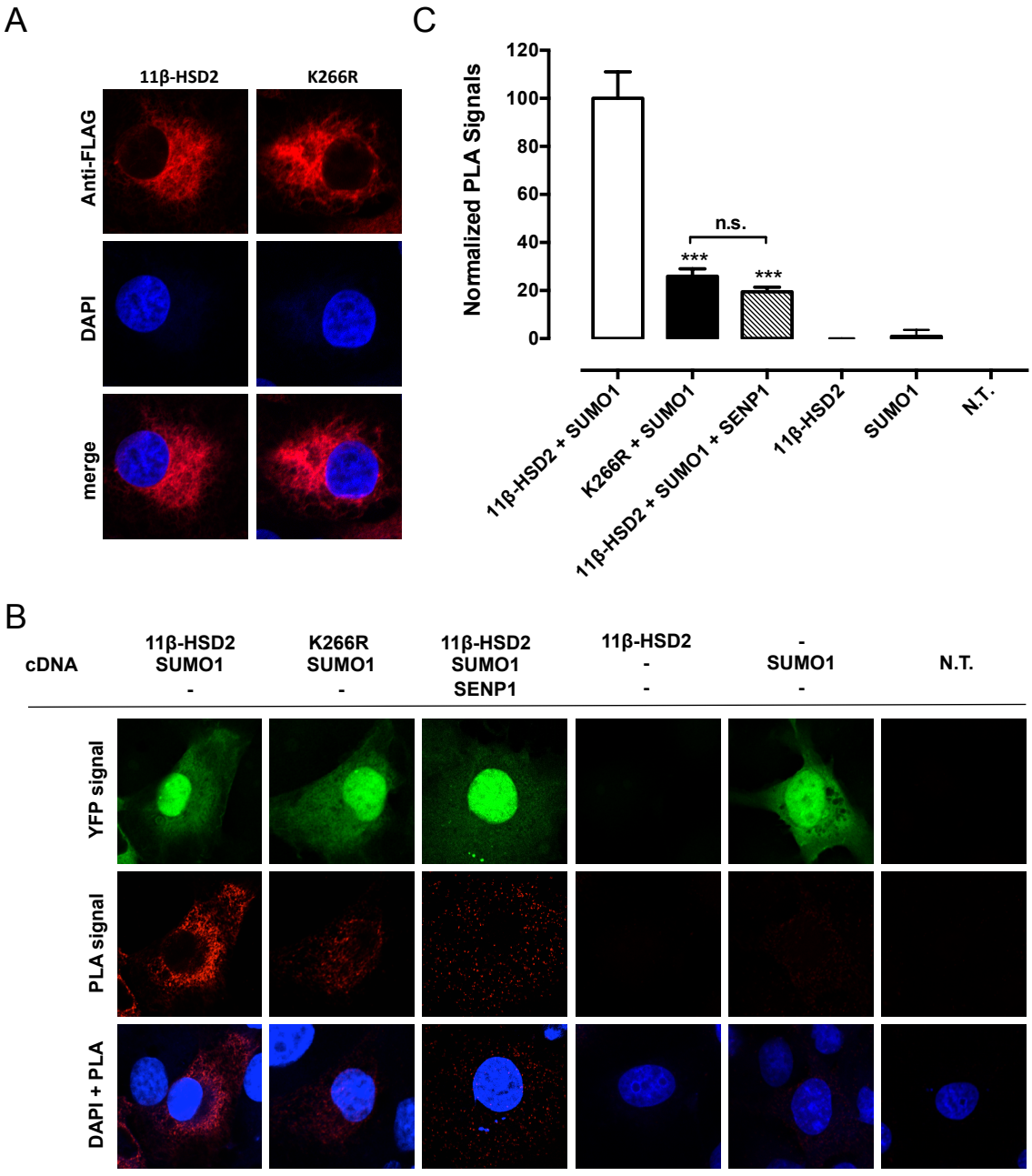

Figure 3

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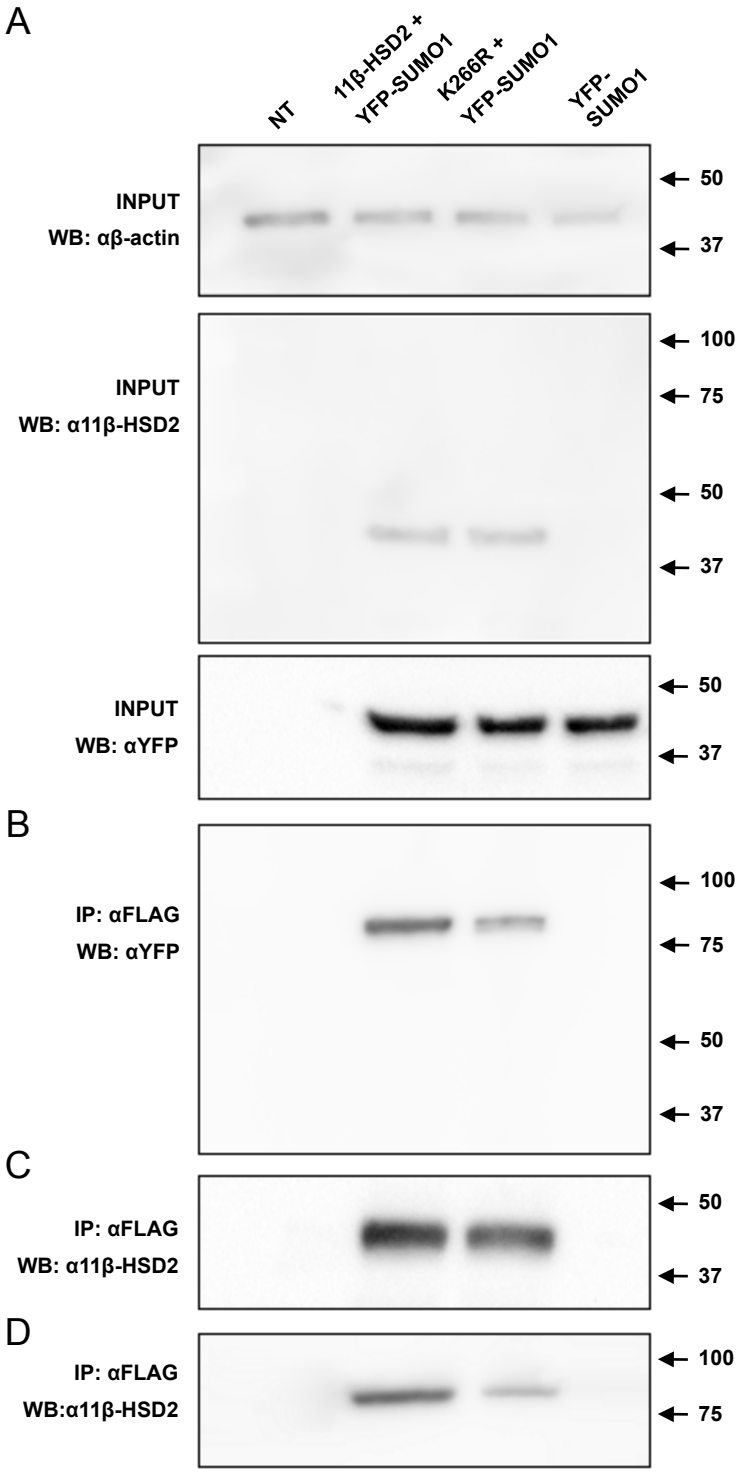

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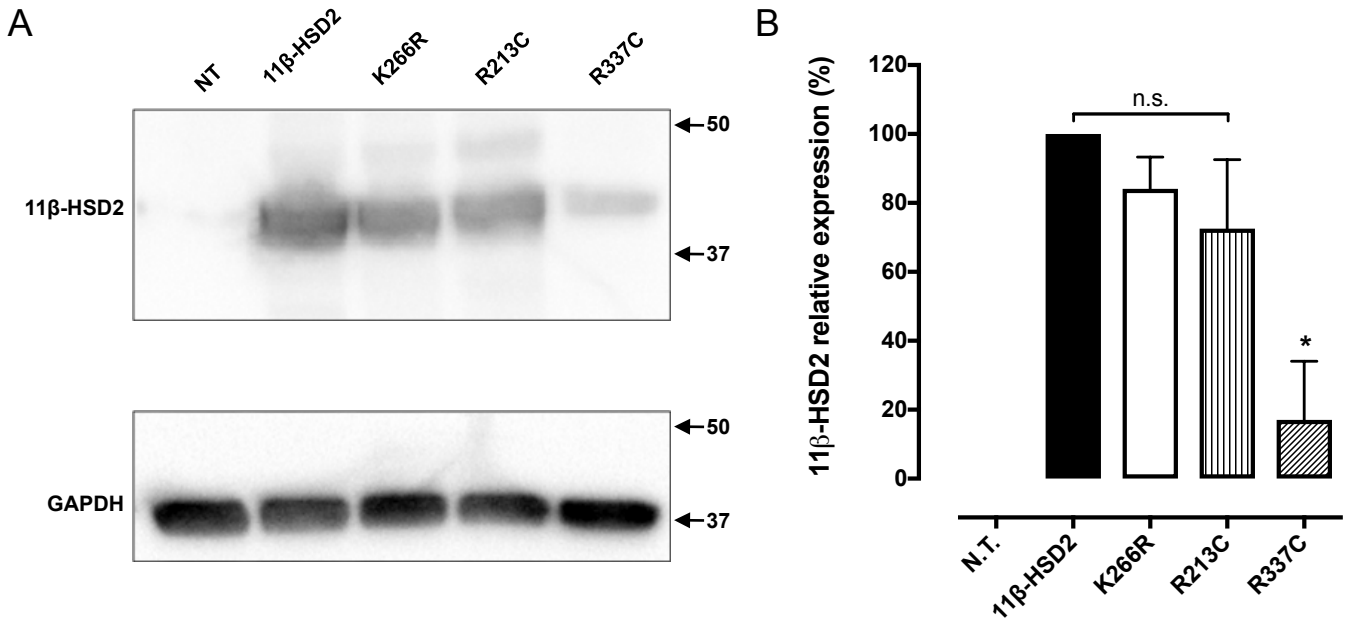

Figure 5

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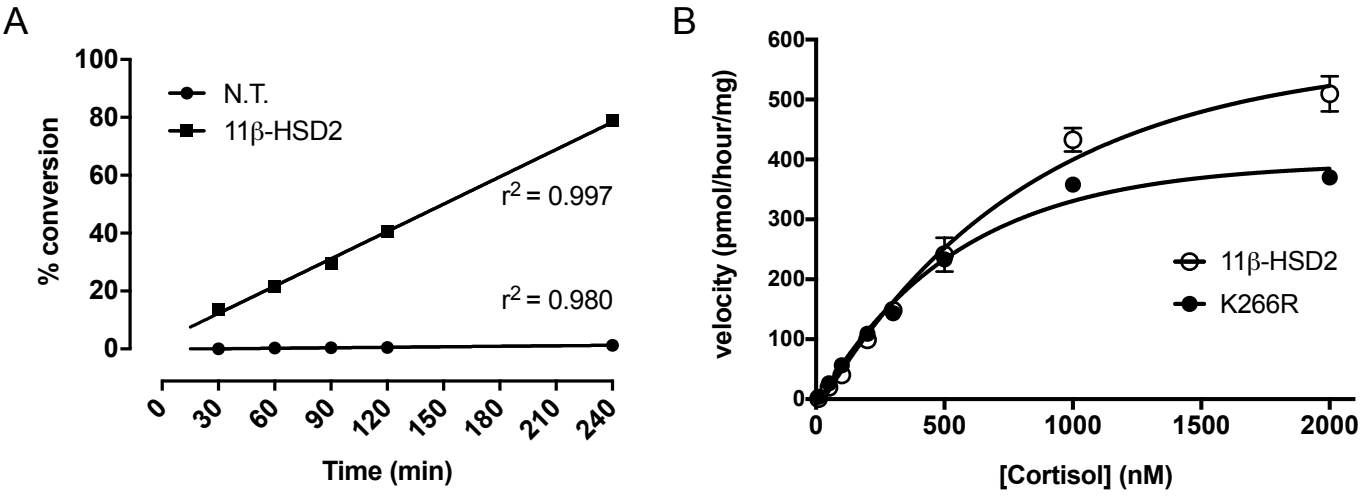

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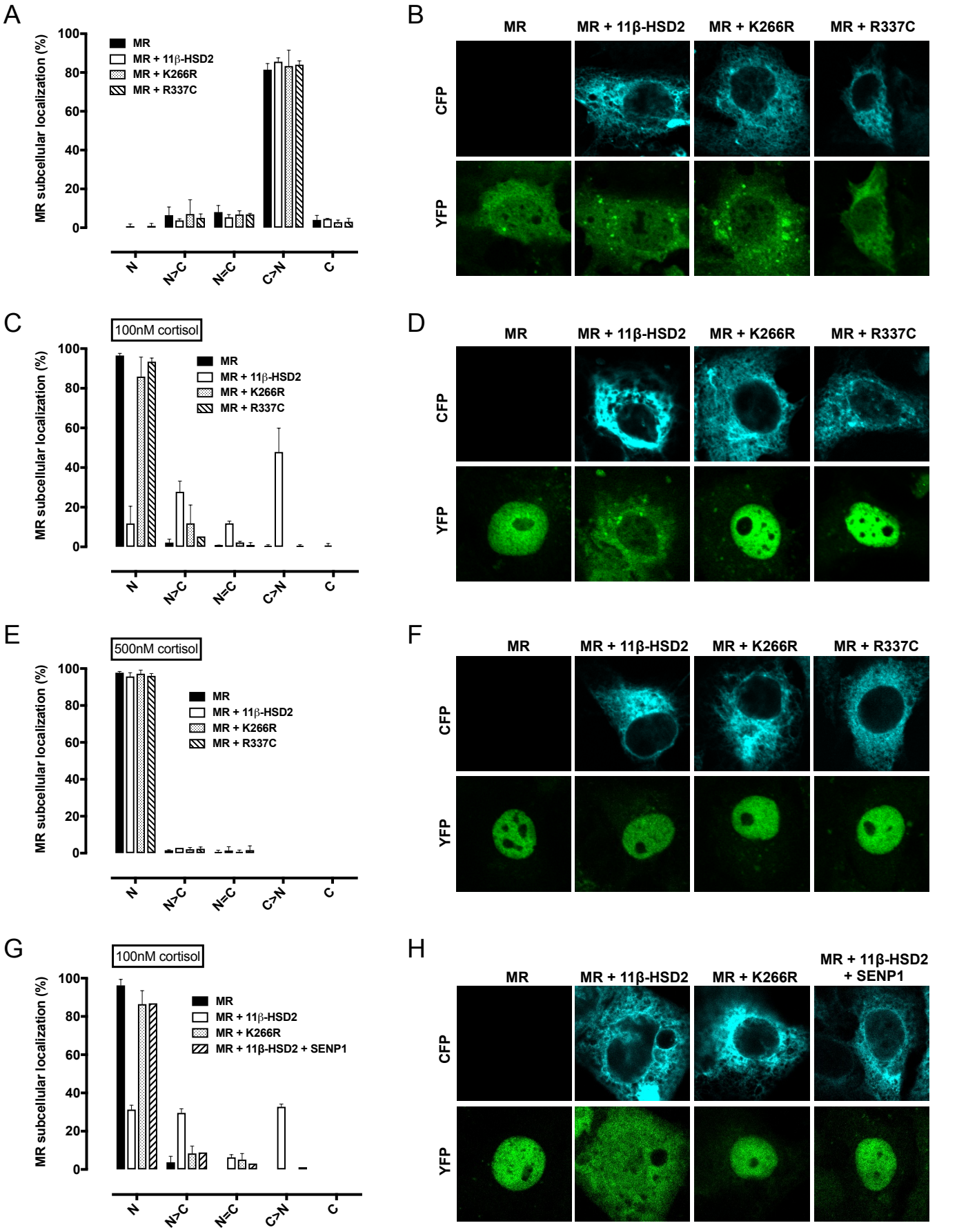

Figure 7

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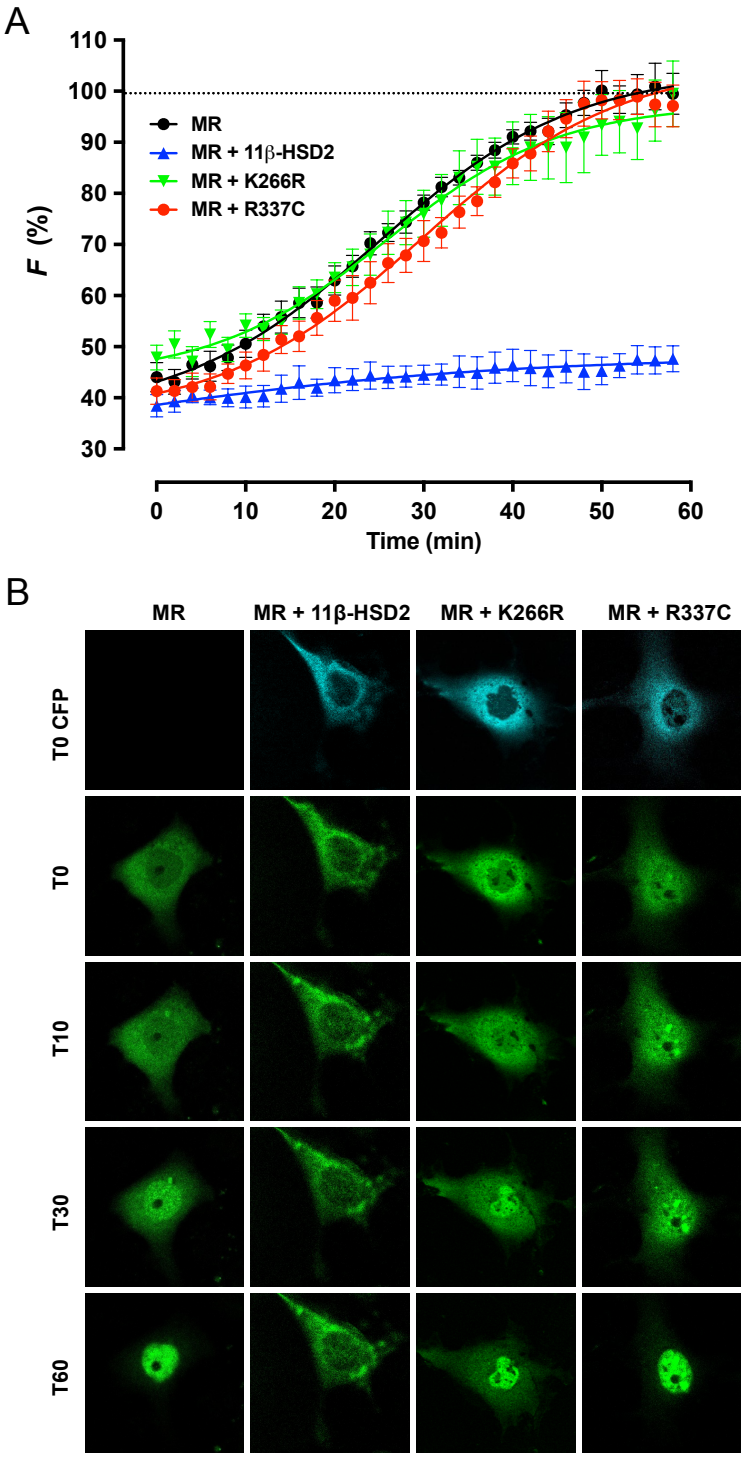

Figure 8

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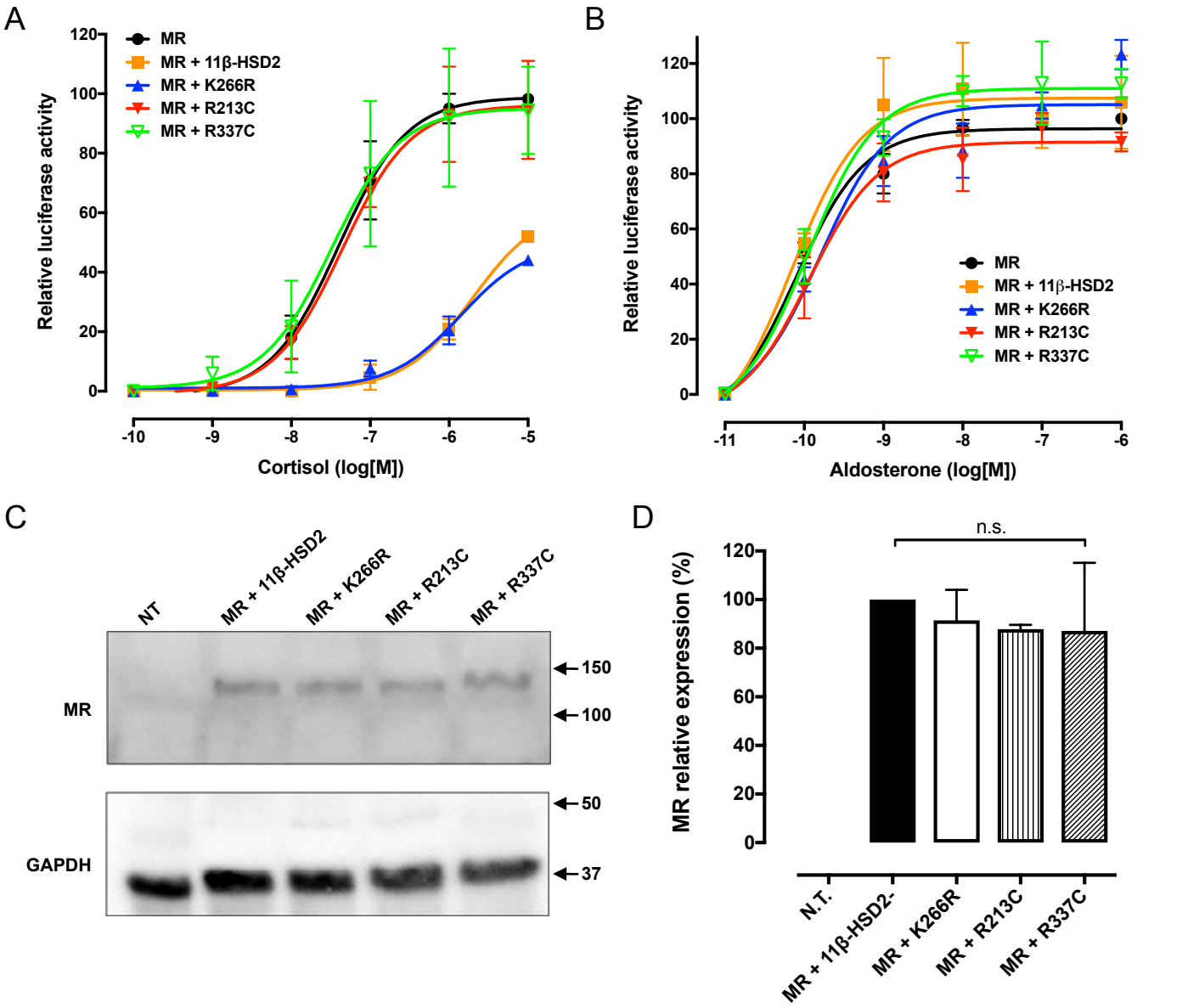

Figure 9

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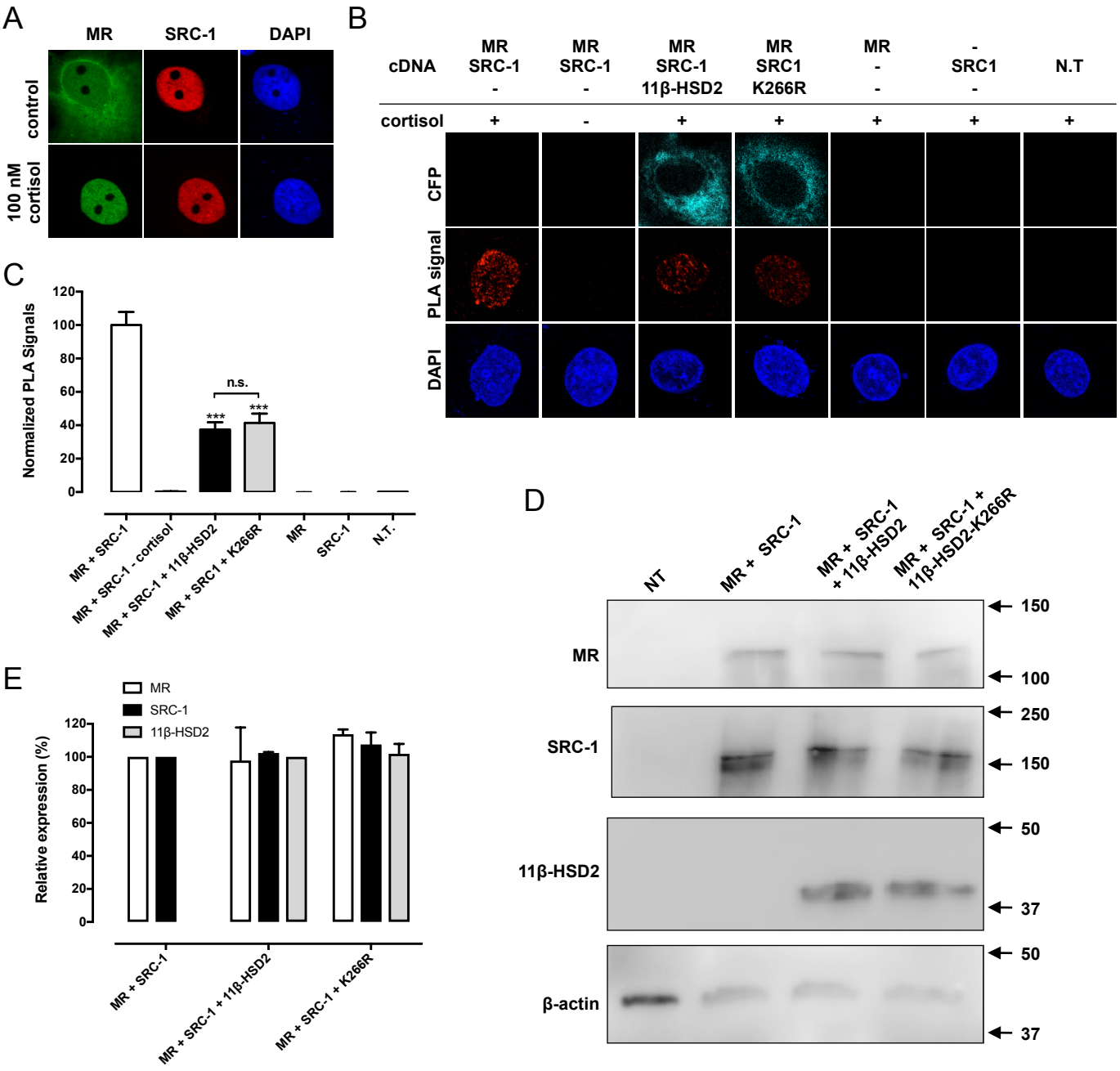

Figure 10

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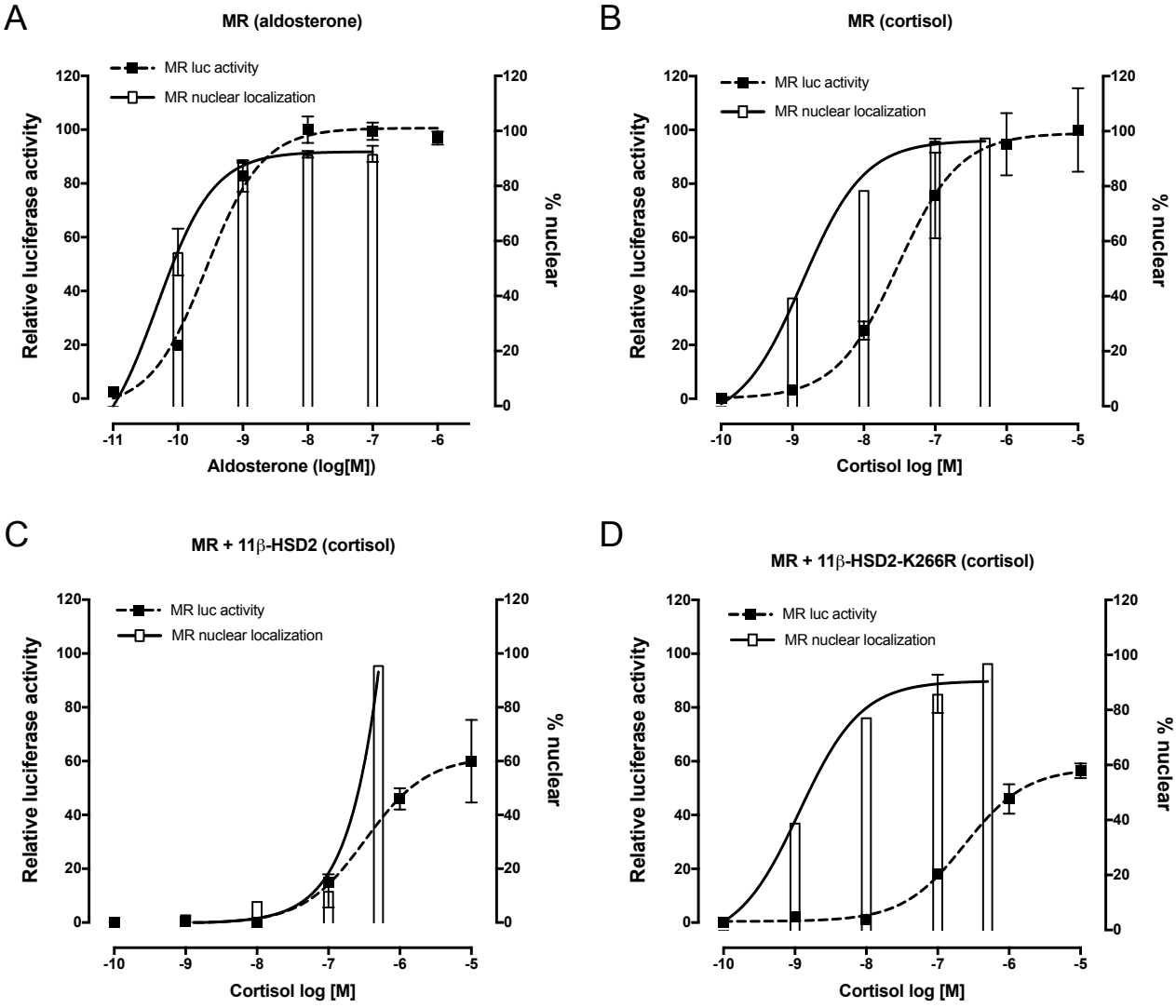

Antibody Table

Peptide/protein target
MR
MR
11b-HSD2
GFP
GAPDH
b-Actin
HA epitope
FLAG epitope
IgG heavy chains and all classes of immunoglobulin light chains from rabbit
IgG heavy chains and all classes of immunoglobulin light chains from mouse
Purified immunoglobulin fractions from normal rabbit serum
Purified immunoglobulin fractions from normal mouse serum

Antigen sequence (if known)	Name of Antibody
Rat mineralocorticoid receptor amino acids 365-381	rMR365 4D6
Human mineralocorticoid receptor amino acids 1-300	H-300
Human 11b-HSD2 amino acids 261-405	H-145
Recombinant full length GFP protein	Anti-GFP
Full length native protein from human erythrocytes	Anti-GAPDH
SGPSIVHRKCF	AC-40
CYPYDVPDYASL	HA.11 clone 16B12
DYKDDDDK	Anti-FLAG® M2
	Alexa Fluor® 594 Goat
	Alexa Fluor® 594 Goat
	Amersham ECL Mouse
	Amersham ECL Rabbit

Manufacturer, catalog #, and/or name of individual providing the antibody
Dr. Celso Gomez-Sanchez (antibody available through the Developmental Studies Hybridoma Bank, product # rMR365 4D6)
Santa Cruz Biotechnology, catalog # sc-11412
Santa Cruz Biotechnology, catalog # sc-20176
Abcam, catalog # 290
Abcam, catalog # 9484
Sigma-Aldrich, catalog # A3853
Covance, catalog # MMS-101R
Sigma Aldrich, catalog #F-1804
Life Technologies (Molecular Probes), catalog # A-11037
Life Technologies (Molecular Probes), catalog # A-11005
GE Healthcare (Amersham), catalog # NA931
GE Healthcare (Amersham), catalog # NA934

Species raised in; monoclonal or polyclonal	Dilution used
Mouse monoclonal	Undiluted hydridoma supernatant (WB)
Rabbit polyclonal	1:200 (IF and PLA)
Rabbit polyclonal	1:600 (WB)
Rabbit polyclonal	1:3000 (WB); 1:1000 (IF); 1:200 (PLA)
Mouse monoclonal	1:10000 (WB)
Mouse monoclonal	1:1000 (WB)
Mouse monoclonal	1:1000 (WB); 1:1000 (IF); 1:200 (PLA)
Mouse monoclonal	1:150 (IP)
Goat polyclonal	1:500 (IF)
Goat polyclonal	1:500 (IF)
Donkey polyclonal	1:20000 (WB)
Sheep polyclonal	1:20000 (WB)

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