# GEMIN4 functions as a coregulator of the mineralocorticoid receptor

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## Abstract

The mineralocorticoid receptor (MR) is a member of the nuclear receptor superfamily. Pathological activation of the MR causes cardiac fibrosis and heart failure, but clinical use of MR antagonists is limited by the renal side effect of hyperkalemia. Coregulator proteins are known to be critical for nuclear receptor-mediated gene expression. Identification of coregulators, which mediate MR activity in a tissue-specific manner, may allow for the development of novel tissue-selective MR modulators that confer cardiac protection without adverse renal effects. Our earlier studies identified a consensus motif among MR-interacting peptides, MPxLxxLL. Gem (nuclear organelle)-associated protein 4 (GEMIN4) is one of the proteins that contain this motif. Transient transfection experiments in HEK293 and H9c2 cells demonstrated that GEMIN4 repressed agonist-induced MR transactivation in a cell-specific manner. Furthermore, overexpression of GEMIN4 significantly decreased, while knockdown of GEMIN4 increased, the mRNA expression of specific endogenous MR target genes. A physical interaction between GEMIN4 and MR is suggested by their nuclear co-localization upon agonist treatment. These findings indicate that GEMIN4 functions as a novel coregulator of the MR.

## Introduction

The mineralocorticoid receptor (MR) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors. It belongs to the subgroup of steroid hormone receptors, which also includes the glucocorticoid receptor (GR), progesterone receptor (PR), androgen receptor (AR), and estrogen receptor (ER) (Lu *et al.* 2006, Griekspoor *et al.* 2007). It is unique in its ability to bind several classes of steroid hormones including the mineralocorticoid aldosterone, and glucocorticoids (cortisol in humans and corticosterone in rodents; Arriza *et al.* 1987, Sutanto & de Kloet 1991). The MR plays a critical role in controlling sodium and potassium transport in epithelial

cells, most notably in the kidneys and colon (Rogerson & Fuller 2000). It is also present in non-epithelial cells in the heart, blood vessel walls, hippocampus, and adipose tissue (Lombes *et al.* 1992, 2000, Meijer 2002, Caprio *et al.* 2007). While MR activation by aldosterone in the kidney serves to maintain extracellular fluid volume and normal cardiovascular function, MR activation in the heart appears to have limited physiological roles (Lalevé *et al.* 2005). However, inappropriate MR activation in the heart, by either aldosterone or cortisol in the setting of oxidative stress, plays a key role in the pathogenesis of cardiac inflammation, vascular dysfunction, remodeling, fibrosis,

and hypertrophy, which are precursors to cardiac failure (Qin *et al.* 2003, Young & Funder 2004, Rickard *et al.* 2009, Young & Rickard 2012). Large clinical trials have all demonstrated a significant reduction in the morbidity and mortality of patients with congestive heart failure on MR antagonist treatment (Pitt *et al.* 1999, 2003, Zannad *et al.* 2010). However, the widespread use of MR antagonists is limited by the adverse effect of hyperkalemia due to their effect at the renal epithelia.

An ideal therapeutic agent would be a selective MR modulator that antagonizes the cardiac MR without affecting the renal MR. Tissue selectivity has been achieved with other nuclear receptors, for example, the selective ER modulator raloxifene antagonizes ER activity in the breast to combat breast cancer but activates ER in the bone to prevent osteoporosis (Turner *et al.* 1987, Jordan 1992, Group EBCTC 1998). One of the mechanisms underlying the tissue-specific actions of selective nuclear receptor modulators is their differential recruitment of coregulators (Smith & O'Malley 2004, McDonnell & Wardell 2010).

Coregulators, composed of coactivators and co-repressors, are cellular factors that interact with nuclear receptors to potentiate or attenuate transactivation (McKenna & O'Malley 2000). Since the identification, over a decade ago, of steroid receptor coactivator 1 (SRC1) as a nuclear receptor coregulator (Onate *et al.* 1995), there has been an expanding literature in this field with the discovery of over 300 coregulators (Malovannaya *et al.* 2011, McKenna 2011). Unlike nuclear receptors, which are structurally conserved, coregulators are structurally and functionally diverse, and are often recruited in a ligand- and cell type-specific manner as demonstrated for the ER, AR, peroxisome proliferator-activated receptor gamma (PPARγ), and a range of other receptors (Kodera *et al.* 2000, Kraichely *et al.* 2000, Bramlett *et al.* 2001, Klokk *et al.* 2007, McKenna 2011). They have been recognized to play a central role in modulating gene expression mediated by nuclear receptors and are thought to impart tissue and ligand specificity to receptor activity (Lonard & O'Malley 2006).

An understanding of MR coregulator interactions is relatively limited in comparison with other members of the steroid hormone receptor family. Progress in the search for MR-interacting coregulators has been hampered by technical issues such as limited MR protein stability, lack of suitable antibodies, and lack of endogenous MR-expressing cell lines (Galigniana 1996, Gomez-Sanchez *et al.* 2011, Cato & Fuller 2012). Furthermore, full-length MR has been particularly difficult to purify due to its inherent instability

and the lack of a suitable host to express the active receptor at a high level (Clyne *et al.* 2009). At present, only a handful of coregulators have been identified for the MR (reviewed by Yang & Young (2009)) and only one, Tesmin, has been reported to be a ligand discriminatory coactivator (Rogerson *et al.* 2014). None of these have been examined for cell or tissue specificity.

Of the various technologies used to study nuclear receptor structure and for coregulator discovery, combinatorial phage display is a rapid and sensitive method to probe receptor structure and has been used to screen for peptides that interact with the ER, AR, PPARγ, and liver receptor homolog 1 (LRH1; Hall *et al.* 2000, Chang *et al.* 2003, 2005, Safi *et al.* 2005, Mettu *et al.* 2007). We have previously used M13 phage display to probe MR structure and isolated ligand-selective peptides that interact with the MR (Yang *et al.* 2011). Furthermore, we identified a unique consensus-binding motif MPxLxxLL among peptides that demonstrated a robust interaction with the MR in a mammalian two-hybrid assay. We performed sequence alignment of the consensus motif within a protein database and identified Gem (nuclear organelle)-associated protein 4 (GEMIN4) as a potential molecular partner of the MR. We therefore hypothesize that GEMIN4 is a novel MR coregulator. In the current study, we aim to investigate the effect of GEMIN4 on MR-mediated gene transcription.

## Materials and methods

### Plasmids

Full-length GEMIN4 in pBIND vector (pBIND-GEMIN4) was used for transactivation assays, gene expression analysis, and immunofluorescence studies. It was constructed from the cDNA clone of full-length GEMIN4, PBSR–GEMIN4 (Bioscience GeneService, Source BioScience Nottingham, UK, clone #5272653). For MR transactivation assays, full-length MR in an expression vector (pRShMR) and *MMTV* promoter fused to a luciferase reporter gene (MMTV-Luc) were used. Alternative MR-responsive promoters, pGL4.23-CNKSR3-Luc and pGL4.23-GILZ-Luc, were kindly provided by Dr Tim Ziera (Ziera *et al.* 2009). Expression plasmids of GR (PRshGR), PR (pSG5-hPR1), AR (pCMV-AR3.1), and ER (pCMV-ERα) and the reporter constructs for PR (pA3-PRE2-Luc), AR (pGL4.14-PB3-Luc, also known as ARR3-tk-Luc), and ER (pERE-tk-Luc) were generously provided by Professors R M Evans, S Nordeen, C Clarke, B Katzenellenbogen, and W Tilley.

## Mammalian cell culture and transient transfection

Human embryonic kidney 293 (HEK293) and H9c2 (rat cardiac myoblast) cells sourced from American Type Culture Collection (ATCC), Manassas, VA, USA were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 1 mM non-essential amino acid, 1 mM L-glutamate, and 1 mM penicillin (10 U/l) in a humidified 37 °C incubator with 5% $CO_2$. HEK293 cells stably transfected with MR (MR+ HEK293 cells) (Kurihara *et al*. 2005) were maintained in DMEM supplemented with 0.7 mg/ml G418 (Geneticin selective antibiotic, Invitrogen). For transactivation assays, cells were seeded at a density of $1 \times 10^5$ (HEK293) or $5 \times 10^4$ (H9c2) cells/well in 24-well plates 1 day before transfection. For gene expression assays, cells were seeded at a density of $5 \times 10^5$ (MR+HEK293) or $1 \times 10^5$ (H9c2) cells/well in six-well plates. Transfections were performed using Fugene 6 (Roche Molecular Biochemicals) following the manufacturer's protocol and the medium was changed to DMEM plus 5% charcoal-stripped fetal bovine serum. After 24 h, the medium was refreshed with DMEM plus 5% charcoal-stripped fetal bovine serum and the appropriate hormones added. Cells were harvested 3 h after treatment for RNA extraction or 24 h after treatment for luciferase assays. Luciferase activity was measured using the EnVision multilabel plate reader (Perkin-Elmer, Waltham, MA, USA). All measurements were performed in triplicate in at least two independent experiments.

For transactivation assays, the following plasmids (and quantities per well in a 24-well plate) were used: MR, GR, PR, AR, or ER (200 ng), corresponding reporter (250 ng), pBIND vector or pBIND-GEMIN4 (50–200 ng), and PBSK (to normalize total DNA to 650 ng/well).

For gene expression assays, the following plasmids (and quantities per well in a six-well plate) were transfected: pBIND vector or pBIND-GEMIN4 (1000 ng).

## Western blot analysis

The expression of MR protein in HEK293 cells in the presence of GEMIN4 was assessed by western blotting. HEK293 cells ($5 \times 10^5$ cells/well in six-well plates) were transfected with PRshMR (200 ng) and varying amounts of GEMIN4 (0, 50, or 200 ng). The cells were incubated for 48 h before harvest. The cells were washed with PBS, and then lysed by the addition of 200 μl of gel loading buffer (10 mM Tris, pH 6.8, containing 2% SDS, 10% glycerol, 4% β-mercaptoethanol, and 1% bromophenol blue). The lysates were collected and boiled for 5 min. Ten microliters of lysate were run on a 10% polyacrylamide gel and then electroblotted onto Hybond P membranes. The expression of MR protein was assessed using the MAB MR1–18 (Gomez-Sanchez *et al*. 2011). Non-specific binding was blocked by incubating the membranes for 1 h at room temperature in 20 mM Tris (pH 7.6), 0.14 M NaCl, and 0.1% Tween 20 (TBST) containing 5% skim milk powder. The membranes were then incubated with a primary MR1–18 antibody overnight at 4 °C, followed by incubation with a secondary HRP-linked anti-mouse antibody (1:1000; Dako, Carpinteria, CA, USA) for 1 h at room temperature. Antibody binding was visualized by chemiluminescence using the ECL Plus Kit (Amersham-Pharmacia Biotech AB). Loading control was assessed using an antibody to β-actin.

## RNA interference

MR+HEK293 cells were seeded in six-well plates, grown to 60% confluence, and transfected with scrambled siRNA or GEMIN4-specific siRNA (Trilencer-27 siRNA to GEMIN4, Origene, Rockville, MD, USA) using siTran reagent (Origene) as per the manufacturer's protocol. Subsequent hormone treatment, RNA extraction, and mRNA analysis proceeded as described in previous sections.

## Gene expression analysis using quantitative real-time RT-PCR

Total RNA from MR+HEK293 cells or H9c2 cells grown in six-well plates was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA cleanup was performed using Ambion DNA-free DNase treatment (Applied Biosystems). cDNA was synthesized from 500 ng of total RNA using SuperScript III reverse transcriptase enzyme (Invitrogen).

Quantitative real-time RT-PCR analysis was conducted using a 7900HT Fast Realtime PCR System (Applied Biosystems, ABI). Each 10 μl reaction in a 384-well plate format included 5 μl SYBR Green Master Mix, 0.25 μl of the forward primer (10 μM), 0.25 μl of the reverse primer (10 μM), 0.5 μl of water, and 4 μl of the diluted cDNA (diluted 1:4). GAPDH was chosen as the internal standard to control for RT efficiency and loading accuracy. PCR set up in 384-well plates was performed using a robotic Qiagen CAS1200 Liquid Handling Instrument. Cycling conditions comprised a 10 min initial denaturation step at 95 °C, 40 cycles of denaturation at 95 °C for 15 s followed by annealing at 60 °C for 1 min. A melting curve analysis was used to assess the specificity of PCR products. The resultant mRNA levels were analyzed using the SDS Automation Controller Software (version 2.3; Applied Biosystems). The data were calculated from the results of at least three independent

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**Table 1** Gene specific primer pairs used for RT-PCR

| | Primer sequences | |
| --- | --- | --- |
| | Sense | Anti-sense |
| **Human** | | |
| *GAPDH* | 5′-CCCATCACCATCTTCCAGGAG-3′ | 5′-GTTGTCATCGATGACCTTGGC-3′ |
| *18S (RNA18S1)* | 5′-CGGCTACCACATCCAAGGAA-3′ | 5′-GCTGGAATTACCGCCGCT-3′ |
| *GILZ* | 5′-TCTGCTTGGAGGGGATGTGG-3′ | 5′-ACTTGTGGGGATTCGGGAGC-3′ |
| *SGK* | 5′-CCCCCTTTTAACCCAAATGT-3′ | 5′-TCAGAGGAAAGAGTCCGTGG-3′ |
| *FKBP5* | 5′-TCTCCTTGCTGCCTTTCTGA-3′ | 5′-GGCCTTGTCACAGCATTCAA-3′ |
| *PER1* | 5′-TCTACATTTCGGAGCAGGCAGCCG-3′ | 5′-CGCTTGCAACGCAGCA-3′ |
| *CNKSR* | 5′-TGGACAGCCTCTTCTCGTTT-3′ | 5′-TAGCGCGTGCTGTCTTCACATTC-3′ |
| *GEMIN4* | 5′-CTCTGTGTGGAACTCGGACA-3′ | 5′-GCCGGTAGCTGTCGTAACTT-3′ |
| *MR (NR3C2)* | 5′-GAGAAAAGCCCGTCTGTTTG-3′ | 5′-AGAGGAGTTCCCTGGGTGAT-3′ |
| **Rat** | | |
| *18S* | 5′-CGGCTACCACATCCAAGGAA-3′ | 5′-GCTGGAATTACCGCCGCT-3′ |
| *Gilz* | 5′-AGTTCCAGCTGGCCAAGGAG-3′ | 5′-TCCCATCTCTGTCCCGTCT-3′ |
| *Sgk* | 5′-CCCCCATTTAACCCAAATGT-3′ | 5′-GAACCATCCTGGGAGCTTTC-3′ |
| *Cnksr* | 5′-TCCTTCTTCCTCAGTGGACA-3′ | 5′-TAGCGCGTGTCTTCACATTC-3′ |
| *Mr* | 5′-GCAGCGAAACAGATGATCCAG-3′ | 5′-TCTCCAACTCAAAGCGAACGA-3′ |

experiments with all reactions performed in triplicate. Gene-specific primer pairs used for RT-PCR are included in Table 1.

## Co-localization of MR and GEMIN4 by immunofluorescence microscopy

HEK293 cells were cultured on glass coverslips coated with poly-L-lysine. Cells were transfected with PRshMR and pBIND-GEMIN4 and incubated in DMEM with charcoal-stripped serum for 24 h before being treated with $10^{-8}$ M aldosterone for 30 min. Cells were rinsed with PBS and fixed at $-20\,^{\circ}$C with ice-cold methanol for 15 min. Samples were blocked with Image-iT FX signal enhancer (I36933, Invitrogen) for 1 h at room temperature and then incubated with primary antibodies at $4\,^{\circ}$C overnight. A rabbit polyclonal primary antibody to GAL4 DNA-binding domain was used to detect pBIND-GEMIN4 (Abcam, Milton, Cambridge, UK, anti-GAL4 antibody, ab1396). The mouse MAB to MR, MR1–18 (1D5), was a gift from the laboratory of Prof. Celso Gomez-Sanchez. Samples were washed three times with PBST and incubated with conjugated secondary antibodies for 1 h. Secondary antibodies were purchased from Invitrogen and include: AlexaFluor 488 goat anti-mouse IgG (A11029) and Alexa-Fluor 546 goat anti-rabbit IgG (A11035). Cells were washed three times with PBST before nuclear staining with DAPI for 1 min followed by another three washes. Coverslips were mounted on slides using FluroSave reagent (Calbiochem, EMD Millipore Headquarters, Billerica, MA, USA, 345789) and images were acquired at $1024\times1024$ pixels (12 bits) using a Nikon C1 confocal laser scanning microscope on a Ti-E base. A $40\times$ 0.75 NA objective was used for detection with DAPI (emission filter 450/35 nm), Alexa488 (emission filter 515/30), and Alexa546 (emission filter 605/75) excited with 405, 488, and 561 nm lasers respectively.

## Statistical analysis

Statistical analysis for each dataset was performed using tools within the GraphPad Prism5 Software package (GraphPad Software, Inc., San Diego, CA, USA). Means were compared using a one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. In transactivation assays, luciferase values obtained using an empty vector treated with hormones were normalized to one with all other luciferase values expressed as fold change over the empty vector. For gene expression assays, relative mRNA expression was calculated using the 2 $(-\Delta\Delta Ct)$ method, whereby comparison was made between treated and untreated samples within each transfection group. The relative mRNA expression in samples transfected with each protein with or without ligand was then compared with those transfected with an empty vector or scrambled siRNA using an unpaired *t*-test. A *P* value of $<0.05$ was considered statistically significant. The data are expressed as means$\pm$s.e.m. The *P* values are annotated as *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

## Results

### Identification of GEMIN4 by sequence alignment of consensus motif within protein database

Combinatorial peptide phage display used to screen for MR-interacting peptides identified 17 LxxLL-constrained

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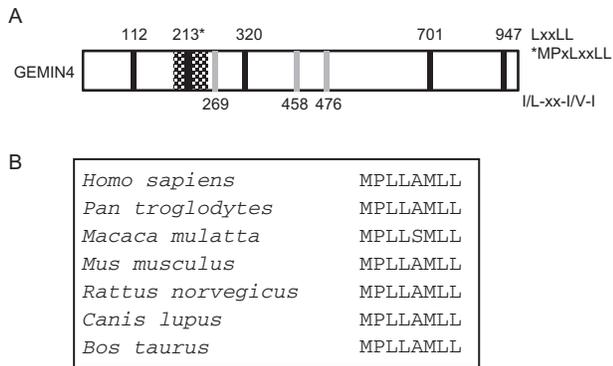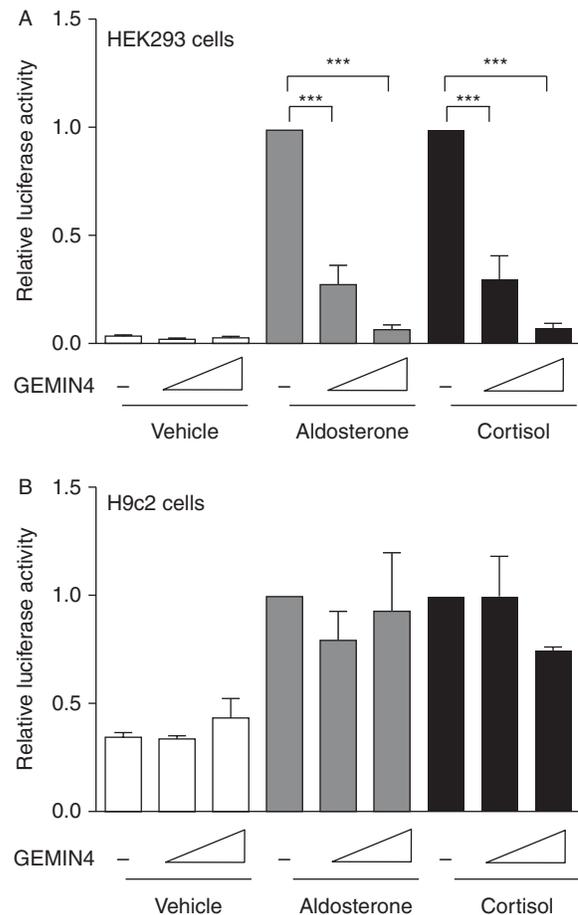
**Figure 1**

A schematic of coactivator and corepressor motifs in GEMIN4 and sequence conservation in mammalian species. (A) The GEMIN4 protein (1058 amino acids) contains five coactivator LxxLL (residues at positions 112–116, 320–324, 701–705, and 947–951), including one MPxLxxLL (residues at positions 213–220), and three corepressor I/L-xx-I/V-I (amino acid residues at positions 269–273, 458–462, and 476–480) motifs. A nuclear localization signal is indicated by the shaded box (residues 194–243). (B) The MPxLxxLL motif is highly conserved across mammalian species.

peptides that interacted with the MR in an aldosterone-dependent manner. Fifty percent contained a unique consensus motif, MPxLxxLL, with a methionine at the −3 position relative to the core LxxLL motif and a proline at the −2 position (Yang et al. 2011). Studies with other nuclear receptors have demonstrated that residues flanking the central LxxLL motif may confer receptor specificity and modulate the interaction of the receptor with the particular coregulator. The presence of a proline residue at the −2 position has been described for a few classes of LxxLL-containing peptides (Chang et al. 1999). For example, PPARγ1 prefers to bind HPLLxxLL, whereas LRH1 prefers PILxxLL (Safi et al. 2005, Mettu et al. 2007). MPxLxxLL has not been previously described for other nuclear receptors and is not found in the sequences of naturally occurring coregulators known to interact with the MR. As it may represent a MR-preferred peptide-binding sequence, it was used to search a non-redundant Homo sapiens protein database within the Basic Local Alignment Search Tool (BLAST). GEMIN4 was selected for further characterization as a potential coregulator on the basis of its nuclear location and possession of five LxxLL coactivator motifs in addition to three I/L-xx-I/V-I corepressor motifs (Fig. 1A). The MPxLxxLL motif within GEMIN4, from amino acid positions 213 to 220, is highly conserved among mammalian species, which suggests an important evolutionary role for this motif in GEMIN4 function (Fig. 1B). Notably, this motif lies within a nuclear localization signal (NLS) of GEMIN4 from amino acid

positions 194 to 243 (Lorson et al. 2008). However, MPxLxxLL is not typically described as a nuclear localization motif and its exact contribution to nuclear localization within the long NLS has not been characterized.

## GEMIN4 represses MR-mediated transactivation in an agonist concentration- and cell-line-dependent manner

To investigate the functional consequences of an interaction between MR and GEMIN4, transactivation assays were performed. As expected, transient transfection of the human MR expression plasmid (PRshMR)



**Figure 2**

GEMIN4 repressed both aldosterone- and cortisol-induced MR-mediated transactivation at the MMTV promoter in HEK293 cells, but not H9c2 cells. (A) HEK293 or (B) H9c2 cells transfected with pRShMR, MMTV-Luc plus empty vector, or GEMIN4 were treated with either vehicle or aldosterone ($10^{-8}$ M) or cortisol ($10^{-8}$ M) for 24 h. Luciferase activity is expressed relative to empty vector plus ligand. Assays were performed three times in triplicate. For each ligand, mean values were compared using one-way ANOVA: ***$P < 0.001$.
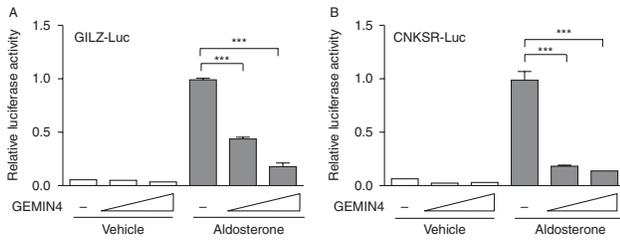
**Figure 3**

GEMIN4 repressed aldosterone-induced MR-mediated transactivation at the *GILZ* and *CNKSR* promoters in HEK293 cells. HEK293 cells transfected with pRShMR, (A) GILZ-Luc, or (B) CNKSR-Luc plus empty vector or GEMIN4 were treated with either vehicle or aldosterone ($10^{-8}$ M) for 24 h. Luciferase activity is expressed relative to empty vector plus ligand. Assays were performed twice in triplicate. For each ligand, mean values were compared using one-way ANOVA: ***$P < 0.001$.

significantly activated a luciferase reporter construct containing the *MMTV* promoter (MMTV-Luc), in an aldosterone- and cortisol-dependent manner in both HEK293 cells (Fig. 2A) and H9c2 cells (Fig. 2B). Co-transfection of GEMIN4 at 50 and 200 ng profoundly repressed both aldosterone- and cortisol-induced MR-mediated transactivation of the reporter construct in HEK293 cells (Fig. 2A). There was no effect on basal MR activity in the absence of hormone (Fig. 2A). However, the inhibitory effect of GEMIN4 was not observed in H9c2 cells in the presence of either aldosterone or cortisol (Fig. 2B). GEMIN4 exhibited a similar repressive effect on other MR-responsive reporters including the glucocorticoid-induced leucine zipper (*GILZ* (*TSC22D3*)) promoter (GILZ-Luc) and connector enhancer of kinase suppressor of ras 3 (*CNKSR*) promoter (CNKSR3-Luc), in HEK293 cells (Fig. 3A and B). Again, the repressive effect was not observed in H9c2 cells (Supplementary Figure 1, see section on supplementary data given at the end of this article). The cell-selective effect may be explained by the greater abundance of GEMIN4 relative to β-actin in HEK293 cells compared with H9c2 cells, both before and after transfection (Supplementary Figure 2). Given that GEMIN4 caused similar effects on MR-mediated transactivation in the presence of either aldosterone or cortisol, and only in the HEK293 cells, further experiments were performed with aldosterone and HEK293 cells only, unless otherwise stated. We performed an aldosterone dose–response study to evaluate the nature of the inhibition by GEMIN4 (Fig. 4). GEMIN4 decreased the magnitude of the aldosterone-induced MR-mediated transactivation rather than inducing a 'right shift' of the dose–response curve. This argues that GEMIN4 does not alter the affinity of binding of the ligand to the MR but rather interferes

with transactivation *per se*. We also evaluated the effect of transfecting 50 or 200 ng of GEMIN4 on MR protein expression (Supplementary Figure 3). Of the two concentrations, 50 ng did not have any impact but 200 ng of GEMIN4 induced a 20% reduction in MR protein expression. This is an interesting phenomenon but not sufficient to explain the 70 or 95% reduction in MR activity associated with the transfection of 50 or 200 ng GEMIN4.

## GEMIN4 also represses transactivation mediated by other steroid hormone receptors

To investigate the effect of GEMIN4 on the function of other steroid hormone receptors, HEK293 and H9c2 cells were transfected with human GR, AR, PR, and ERα expression plasmids and their corresponding luciferase reporter constructs plus empty vector or pBIND-GEMIN4. Cells were treated with the appropriate agonist ligand. Each receptor exhibited the expected ligand-dependent transactivation at their respective promoter constructs (Fig. 5A, B, C and D, Supplementary Figure 4A, B, C and D, see section on supplementary data given at the end of this article). In HEK293 cells, co-transfection of GEMIN4 significantly repressed transactivation mediated by GR in a manner similar to the MR, although the repressive effect on GR was minimal at 50 ng of GEMIN4 (Fig. 5A). GEMIN4 also profoundly repressed transactivation mediated by the AR (Fig. 5B) and PR (Fig. 5C). The degree of repression of PR-mediated transactivation at the 200 ng concentration
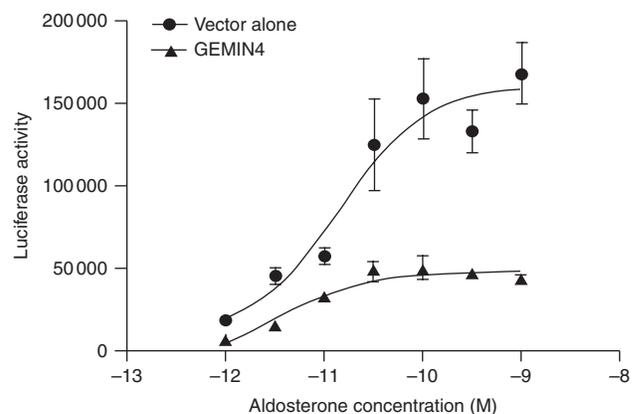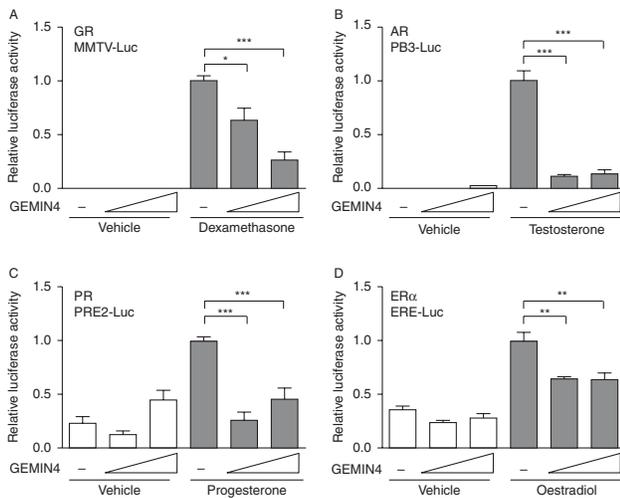


**Figure 4**

GEMIN4 reduced the maximum response of MR to aldosterone at the *MMTV* promoter in HEK293 cells. MR-mediated transactivation of the *MMTV* promoter in response to increasing concentrations of aldosterone in HEK293 cells transfected with pRshMR plus empty vector or GEMIN4 (50 ng; $n = 3$).
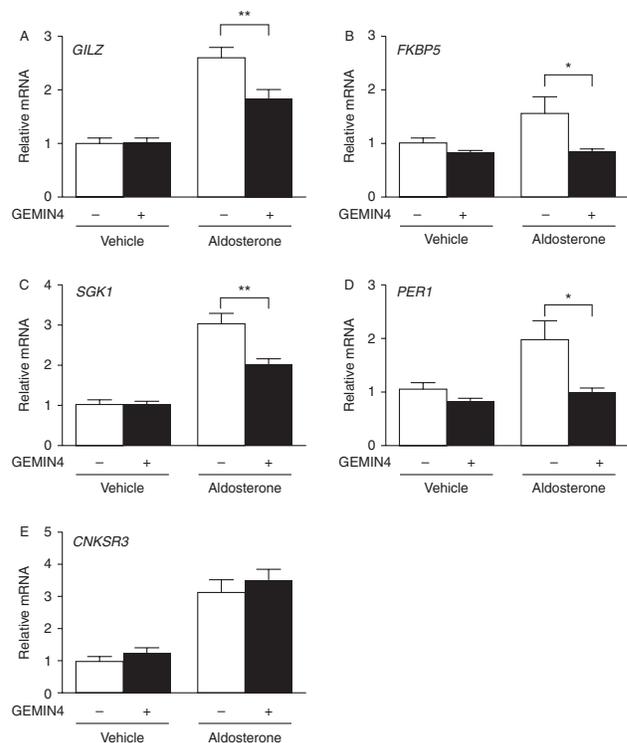
**Figure 5**

GEMIN4 repressed GR-, AR-, and PR-mediated, but not ERα-mediated, transactivation in HEK293 cells. HEK293 cells transfected with expression plasmids for (A) GR, (B) AR, (C) PR, or (D) ERα and their respective reporter constructs, plus empty vector, or GEMIN4 were treated with either vehicle or dexamethasone ($10^{-8}$ M), testosterone ($10^{-7}$ M), progesterone ($10^{-7}$ M), or estradiol ($10^{-7}$ M) respectively for 24 h. Luciferase activity is expressed relative to empty vector plus ligand. Assays were performed twice in triplicate. For each ligand, mean values were compared using one-way ANOVA: *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

of GEMIN4 did not appear as striking as at 50 ng. However, when the basal activity of the PR reporter (in the absence of hormone) was taken into account, GEMIN4 at the concentration of 200 ng completely abolished the progesterone-induced increase in PR activity (Fig. 5C). While ER activity appeared to be reduced by GEMIN4 upon estradiol ($E_2$) treatment, the actual effect was not significant when changes in basal activity were taken into account (Fig. 5D). In contrast, in the H9c2 cells, GEMIN4 only repressed GR and AR transactivation at 200 ng (Supplementary Figure 4A and B) and did not significantly repress PR (Supplementary Figure 4C). In contrast to HEK293 cells, GEMIN4 did significantly repress ER transactivation in H9c2 cells (Supplementary Figure 4D). These results indicate that GEMIN4 is able to repress the activity of other steroid hormone receptors, with the exception of the ER in HEK293 cells and the PR in H9c2 cells.

## Overexpression of GEMIN4 decreases endogenous MR target gene expression in a gene- and cell-specific manner

To address the functional effects of GEMIN4 on endogenous MR target gene expression, we transfected GEMIN4 into stably transfected MR-expressing HEK293 cells

(MR+HEK293). The expression of multiple MR target genes, including *GILZ*, FK506 binding protein 5 (*FKBP5*), serum and glucocorticoid-regulated kinase 1 (*SGK1*), period circadian protein homolog 1 (*PER1*), and *CNKSR3*, was evaluated 3 h after hormone treatment as their expressions have been shown to be either maximal or near-maximal at this time point (Náray-Fejes-Tóth *et al.* 1999, Soundararajan *et al.* 2005, Tanaka *et al.* 2007, Ziera *et al.* 2009, Sekizawa *et al.* 2011). Treatment with aldosterone in MR+HEK293 cells transfected with an empty vector caused a robust induction of *GILZ* mRNA levels by 2.6-fold, indicating that the stably expressed MR protein is physiologically functional (Fig. 6A). Transfection of GEMIN4 reduced mRNA levels of endogenous *GILZ* by 30% (Fig. 6A). A significant repressive effect of GEMIN4 was also observed for aldosterone-induced expression of *FKBP5*, *SGK1*, and *PER1* (Fig. 6B, C and D)



**Figure 6**

GEMIN4 inhibited aldosterone-induced expression of the endogenous MR target genes *GILZ*, *FKBP5*, *SGK1*, and *PER1*, but not *CNKSR3*. mRNA levels of (A) *GILZ*, (B) *FKBP5*, (C) *SGK1*, (D) *PER1*, and (E) *CNKSR3* in response to vehicle or aldosterone ($10^{-8}$ M, 3 h) in MR+HEK293 cells transfected with an empty vector or GEMIN4. All experiments were repeated at least three times in triplicate; *SGK1* results were obtained after four independent experiments. mRNA expression was normalized to *GAPDH*. Mean values were compared by the unpaired *t*-test: *$P<0.05$ and **$P<0.01$ vs empty vector plus aldosterone.
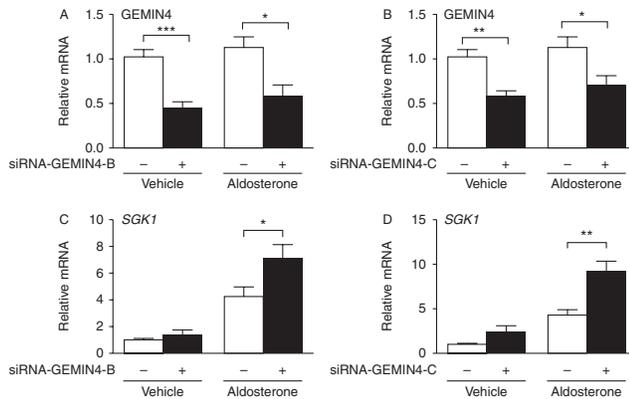
**Figure 7**

GEMIN4 knockdown increased aldosterone-induced expression of the endogenous MR target gene *SGK1*. mRNA levels of (A and B) GEMIN4 and (C and D) *SGK1* in response to vehicle or aldosterone ($10^{-8}$ M, 3 h) in MR+ HEK293 cells transfected with scrambled siRNA, (A and C) siRNA-GEMIN4-B (10 nM) or (B and D) siRNA-GEMIN4-C. Experiments were repeated three times in duplicate. mRNA expression was normalized to *GAPDH*. Mean values were compared by the unpaired *t*-test: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs scrambled siRNA.

but not observed for *CNKSR3* mRNA level (Fig. 6E). H9c2 cells transfected with an empty vector showed similar strong induction of GILZ and SGK upon aldosterone treatment, indicating the presence of endogenous MR. However, GEMIN4 overexpression did not produce any effect on aldosterone-induced gene expression in these cells (data not shown), consistent with the results obtained from earlier transactivation studies. These findings indicate that exogenous GEMIN4 functions as a transcriptional corepressor for endogenous MR-mediated transactivation in a gene- and cell-specific manner.

## Knockdown of endogenous GEMIN4 increases MR target gene expression in a gene-specific manner

To investigate the role of endogenous GEMIN4 in MR-mediated gene transcription, GEMIN4 knockdown was performed in MR+HEK293 cells using siRNA (siRNA-GEMIN4-B and -C) before aldosterone treatment and MR target gene analysis. Transfection of siRNA-GEMIN4-B and -C, compared with scrambled siRNA, effectively reduced the endogenous level of GEMIN4 mRNA by ~50% (Fig. 7A and B). In keeping with its role as a corepressor, decreased GEMIN4 led to a reciprocal twofold increase in the expression of *SGK1* (Fig. 7C and D); however, the effect was not observed for *GILZ*, *FKBP5*, *PER1*, or *CNKSR3* (Supplementary Figure 5, see section on supplementary data given at the end of this article). There was no significant increase in their mRNA expression upon GEMIN4 knockdown. These findings indicate that, similar to exogenous GEMIN4, endogenous GEMIN4 also functions as a gene-specific corepressor for MR-mediated transactivation.

## MR co-localizes with GEMIN4 to the cell nucleus

To determine whether MR and GEMIN4 exhibited subcellular co-localization, HEK293 cells were transfected with PRshMR and pBIND-GEMIN4 and examined by immunofluorescence microscopy. In the absence of aldosterone, MR was localized to the nucleus and cytoplasm, while GEMIN4 was primarily nuclear (Fig. 8, upper panels). Treatment with $10^{-8}$ M aldosterone resulted in the complete nuclear localization of MR,
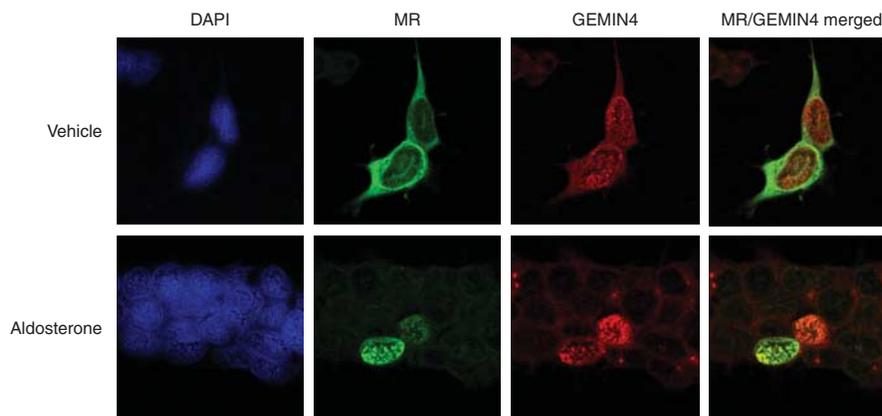


**Figure 8**

GEMIN4 and MR co-localized to the nuclei of HEK293 cells. HEK293 cells transfected with pRShMR and GEMIN4 were treated with either vehicle (upper panel) or aldosterone ($10^{-8}$ M) (lower panel) for 30 min. Immunofluorescence staining from left to right shows: nuclear DAPI (blue), MR (green), GEMIN4 (red), and MR/GEMIN4 merged (yellow).

which merged with GEMIN4 (Fig. 8, lower panels). These findings indicate that MR co-localized with GEMIN4 to the nuclei of HEK293 cells in the presence of aldosterone and supports an anatomical or functional interaction between these two proteins.

## Discussion

In the current study, we explored the consensus MR-binding motif, MPxLxxLL (Yang *et al.* 2011), and identified GEMIN4 as a novel corepressor of the MR, which repressed MR-mediated gene transcription in a gene- and cell-specific manner. The nuclear co-localization of MR with GEMIN4 upon agonist treatment was supportive of its role as an MR-interacting coregulator protein. Furthermore, GEMIN4 also appeared to be a context-dependent corepressor of the other steroid hormone receptors.

GEMIN4 was originally identified as a member of the survival motor neuron (SMN) complex, together with GEMIN2–GEMIN7 and SMN protein (Charroux *et al.* 2000, Todd *et al.* 2010). The SMN complex is ubiquitously expressed and required for the assembly of spliceosomal small nuclear ribonucleoproteins in the cytoplasm and the subsequent pre-mRNA splicing in the nucleus, which is an essential part of eukaryotic mRNA biogenesis (Meister *et al.* 2000, Pellizzoni *et al.* 2002). The SMN complex is localized to the cytoplasm, nucleoli, and discrete nuclear foci known as Cajal bodies (in adult tissues and most cultured cells) or gems (in embryonic cells) (Gall 2003). However, GEMIN4 has not previously been shown to modulate nuclear receptor activity.

The current study demonstrated, for the first time, a functional interaction between GEMIN4 and the MR using transactivation assays, gene expression analysis, and co-localization studies. It revealed that GEMIN4 profoundly repressed agonist-induced, MR-mediated transactivation at three different MR-responsive promoters, namely *MMTV*, *GILZ*, and *CNKSR*. This repressive effect was observed only in the HEK293 cell line but not in the H9c2 cell line, highlighting the importance of cellular context on coregulator function. The repressive effect and cell type specificity of GEMIN4 were confirmed by real-time quantitative RT-PCR, whereby overexpression of GEMIN4 in the HEK293 cells, but not the H9c2 cells, significantly decreased the endogenous expression of four well-characterized MR target genes, including *GILZ*, *FKBP5*, *PER1*, and *SGK1*. The repressive effect of GEMIN4 on transcription was gene specific as the mRNA levels of *CNKSR3* remained unchanged. In keeping with its role as a corepressor, knockdown of GEMIN4 increased the

endogenous expression of *SGK1*. However, mRNA levels of *GILZ*, *FKBP5*, and *PER1* did not change with GEMIN4 knockdown, suggesting either a dose-dependent effect of GEMIN4 or that *GILZ*, *FKBP5*, and *PER1* expression may involve additional factors that compensate for the reduction in GEMIN4.

Gene-specific effects of coregulators at other nuclear receptors have been extensively described in the literature and various mechanisms have been proposed (O'Malley 2003, Won Jeong *et al.* 2012). It is thought that allosteric changes in nuclear receptor conformation resulting from the interaction of the receptor with distinct DNA response elements may alter its binding affinity for coactivators (Wood *et al.* 2001, Klinge *et al.* 2004). Promoter dependence has been demonstrated for the MR corepressor, protein inhibitor of activated STAT1 (PIAS1), which depended on sumoylation of the MR for its repressive effect at the glucocorticoid response element promoter, but was sumoylation independent at the *MMTV* promoter (Pascual-Le Tallec *et al.* 2003). Based on the literature, it seems plausible that the response elements for the various MR target genes in HEK293 cells may each have their own unique structures and induce distinct MR conformations such that GEMIN4 binds differentially to each promoter and produces gene-specific transcriptional modulation.

The gene-specific repressive effect of GEMIN4 was not observed in transactivation assays, where an equally repressive effect was observed on MR-mediated transactivation at the *GILZ* and *CNKSR* promoters. This discordance may be explained by the differences between an endogenous promoter and an artificial promoter construct. It has been clearly demonstrated in recent studies that nuclear receptors are often recruited to non-promoter regions and sites away from the promoter (Jagannathan & Robinson-Rechavi 2011). The dynamic endogenous interactions within a chromatin environment cannot be fully recapitulated with the use of promoter–reporter constructs.

The cell specificity of the ability of GEMIN4 to repress MR-mediated transactivation in HEK293 but not H9c2 cells was striking. Cellular context was also reported as important for the interaction between death-associated protein 6 (DAXX) and MR, where a strong induction of the luciferase reporter was only observed when DAXX was coexpressed with the MR in HN9.10 cells but not in SHY-5Y cells (Obradovic *et al.* 2004). In the literature, there are only limited examples of the ability of the cellular environment to alter coregulator function in a cell-type specific manner. For example, it has been demonstrated that E$_2$-dependent interaction of ERα with CREB-binding

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protein was detected in HeLa cells but not HepG2 cells (Jaber *et al.* 2004). In our study, the cell-specific differences reflect different levels of GEMIN4 expression in the two cell lines, and possibly different concentrations of other modifying proteins in each cellular milieu.

As discussed earlier in this study, GEMIN4 is best known for the role it plays in pre-mRNA splicing. While proteins involved in splicing and RNA metabolism have not been described as coregulators of the MR, they have certainly been described for other nuclear receptors, especially in the capacity of corepressors. For example, RNA splicing factors, PTB-associated splicing factor (PSF) and p54nrb, inhibited the transcriptional activity of AR by interrupting its interaction with the androgen response element (Dong *et al.* 2007). The exact mechanism of transcriptional repression of the MR by GEMIN4 remains yet to be explored.

The repressive action of GEMIN4 was not limited to the MR. It also repressed GR-, AR-, and PR-mediated trans-activation in HEK293 cells, and GR-, AR-, and ER-mediated activity in H9c2 cells. The result indicates cellular differences that cannot be entirely accounted for by differences in endogenous GEMIN4 levels. While it would be useful to identify MR-selective coregulators, nuclear receptor-specific coregulators are rarely reported in the literature. In the case of the AR, of the 167 coregulators identified, only two are qualified as AR specific (Heemers & Tindall 2007). Of all the MR coregulators identified, ELL is the only MR-selective coactivator (Pascual-Le Tallec *et al.* 2005), while NF-YC is the only selective corepressor (Murai-Takeda *et al.* 2010) described to date. Notably, coregulators that bind non-specifically may still display preference for one particular nuclear receptor. SRC1 and SRC2, for example, have been reported to interact with both PR and GR. However, at the *MMTV* promoter in a human breast cancer cell line, PR preferentially recruited SRC1 while GR recruited SRC2 along with a unique set of downstream coregulator molecules (Li *et al.* 2003). In the case of GEMIN4, its repressive effect was more marked at the MR than at the GR. The different degrees of repression may discriminate between MR- and GR-mediated gene transcription in tissues where they are co-expressed. Overall, our results indicate that GEMIN4 is a corepressor of all the steroid hormone receptors with the exception of ER in HEK293 cells and PR in H9c2 cells, and may be further explored in the context of different receptors.

The present data provide compelling evidence for a functional role of GEMIN4 as a corepressor of the MR. This novel finding expands the current cohort of MR corepressors published in the literature and may enhance our understanding of MR-mediated signaling in different tissue contexts. The cell- and gene-specific interaction of GEMIN4 with the MR may also represent suitable targets for the development of selective MR modulators in the future.

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