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ORIGINAL ARTICLE

Nuclear localization signal region in nuclear receptor PXR governs the receptor association with mitotic chromatin

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Abstract In recent years, some transcription factors have been observed to remain associated with mitotic chromatin. Based on these observations, it is suggested that these chromatin-bound transcription factors may serve as 'epigenetic marks' for transmission of pattern of gene expression from progenitor to progeny cells. In this context, our laboratory has reported that nuclear receptor PXR, a master regulator of xenobiotic metabolism, remains constitutively associated with mitotic chromatin. However, the region responsible for this interaction with chromatin remained unknown. In this study, we have shown, for the first time, that mitotic chromatin association of this factor is mediated by the combined action of two zinc fingers present in the DNA-binding domain of PXR. Overall, the nuclear localization signal (NLS) region appears to play a major role in this interaction with mitotic chromatin. Also, we have identified a sub-region of 11 amino acid residues within NLS region of PXR (R66-76R) essential for receptor interaction with the mitotic chromatin. Interestingly, this minimal region is sequence-specific and independent of its basic charge. We have termed this minimal sub-region as 'mitotic chromatin binding-determining region' (MCBR). It is suggested that this receptor region is essential for activation of its target genes. Additionally, we have shown that PXR remains associated with the everted repeat (ER6) region of its major target gene, CYP3A4 promoter during mitosis implying its suggested role in 'gene bookmarking'.

Keywords Nuclear receptors · Pregnane and xenobiotic receptor · Mitosis · Zinc fingers · Nuclear localization signal · Gene bookmarking

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Abbreviations

AR	Androgen receptor
BIOPIT	Bimolecular imprints offered to progeny for inheritance of traits
Cdks	Cyclin-dependent kinases
ChIP	Chromatin immunoprecipitation
CTCF	CCCTC-binding factor
DHT	Dihydrotestosterone
ER6	Everted repeat 6
ERα	Estrogen receptor α
Esrrb	Estrogen-related receptor beta
FACS	Fluorescence-activated cell sorting
FLIM	Fluorescence-lifetime imaging microscopy
FOX1	Forkhead box 1
FRET	Fluorescence resonance energy transfer

HMGA1a	High-mobility group proteins A1
HMGB1	High-mobility group box 1
HMGB2	High-mobility group protein B2
hsp70	70 kilodalton heat shock proteins
MCBR	Mitotic chromatin binding-determining region
NLS	Nuclear localization signal
PARP-1	Poly (ADP-ribose) polymerase-1
PcG proteins	Polycomb-group proteins
PXR	Pregnane and xenobiotic receptor
RBPJ	Recombining binding protein suppressor of hairless
Runx3	Runt-related transcription factor 3
TBP	TATA-box-binding protein
TFIID	Transcription factor II D
TFIIH	Transcription factor II Human
XREM	Xenobiotic response element

Introduction

Every cell devotedly replicates its genome and transmits to its progeny by precise distribution of its genomic and molecular belongings. Mitotic phase of the cell cycle is significant in this respect. Mitosis has attracted immense attention in recent years since during this phase of cell cycle, chromatin is highly condensed and transcription is believed to be globally silenced (Spencer et al. 2000; Caravaca et al. 2013; Woodcock and Ghosh 2010; Zaret 2014).

Mitosis is characterized by the disengagement of many basal transcription factors, RNA polymerases, histone modification enzymes, and enhancer-binding factors from the chromatin (Martínez-Balbás et al. 1995; Burke et al. 2005; Egli et al. 2008; Kumar et al. 2008). It has been anticipated that transcriptional silencing and disengagement of factors from the mitotic chromatin are due to high condensation and reduced accessibility of transcription machinery within the condensed space of the chromatin (Gottesfeld and Forbes 1997). In contrast, some of the key proteins are observed to be associated with mitotic chromosomes which include components of initiation complex for RNA pol I, RNA pol II, transcription factor IID, RNA pol III, and transcription factor IIIB (Chen et al. 2002; Christova and Oelgeschläger 2002); chromosome scaffold proteins (Hagstrom and Meyer 2003); the chromosomal passenger proteins (Adams et al. 2001); high-mobility group proteins HMGB1, HMGB2, and HMGA1a (Pallier et al. 2003; Harrer et al. 2004); nuclear receptor PXR (Saradhi et al. 2005); heat shock factor 2 (HSF2) transcription factor (Xing et al. 2005); CTCF insulator protein (Burke et al. 2005); coactivator protein PC4 (Das et al. 2006); transcription factor FOX 1 (Yan et al. 2006) and Runx3 (Young et al. 2007; Pockwinse et al. 2011); hepatocyte nuclear factor-1β (HNF-1β) (Verdeguer et al. 2010); PcG protein (Follmer et al. 2012); PARP-1 (Lodhi et al. 2014); and RBPJ (recombining binding protein suppressor of hairless), the transcriptional effector of the Notch signaling pathway (Lake et al. 2014). These proteins are reported to stay associated with mitotic chromatin, when most other factors are apparently excluded from the chromatin. So, highly condensed mitotic DNA is open for dynamic interactions with some of the key binding factors during mitosis (Chen et al. 2005; Martin and Cardoso 2010; Hsiung et al. 2015).

Constitutive association of a nuclear receptor with mitotic chromatin was reported for the first time by our laboratory (Saradhi et al. 2005). Live cell imaging experiments by transiently expressing GFP-tagged human PXR showed it to be constitutively associated to condensed mitotic chromatin (Saradhi et al. 2005). Similar results were observed with stably expressing untagged human PXR in HepG2 cell lines. The finding that a nuclear receptor associates with mitotic chromatin was a novel and unexpected finding. In addition to this, our laboratory also reported the ligand-dependent association of androgen and estrogen receptors (AR and ERα) to the mitotic chromatin (Kumar et al. 2008; Chaturvedi et al. 2010). The functional significance and molecular basis of such an association are not yet clear but it has been assumed that these associations have significance in gene bookmarking. The biological phenomenon of gene bookmarking during cell cycle is believed to function as an epigenetic mechanism for transmitting the interphase pattern of gene expression from progenitor to progeny cells via mitosis (Saradhi et al. 2005; Kumar et al. 2012; Caravaca et al. 2013; Kadauke and Blobel 2013; Rada-Iglesias 2013; Raccaud and Suter 2018).

Nuclear localization is indispensable for a protein to control the processes of transcription (Spit et al. 1998). Nuclear localization signals (NLSs) are short stretches of mostly basic amino acid sequences (4–30 amino acids). These sequences are too short to form any

independent structure and mostly present inside the nuclear proteins that direct them to the nuclear compartment (LaCasse and Lefebvre 1995). The import of large proteins (> 60 kDa) to the nucleus requires an NLS. Some reports showed that even though a protein contains an NLS, if it does not bind to intra-nuclear components, it is exported out of the nucleus by a default pathway (Schmidt-Zachmann et al. 1993; Laskey and Dingwall 1993). Some studies suggest that to continue residing inside the nucleus, the protein must have a function like DNA- or RNA-binding, since nucleic acid-binding proteins might have several nuclear retention signals facilitating binding to other components in the nucleus which will also determine their nuclear residency. In this context, nuclear receptors are suggested to bind to non-histone proteins and nuclear matrix (Spelsberg et al. 1984; Barrack and Coffey 1980). NLSs and DNA- or RNA-binding motifs directly overlap or are immediately adjacent in more than 80% of nuclear-resident proteins (LaCasse and Lefebvre 1995). The mechanisms and reasons why NLSs and DNA/RNA-binding motifs are closely associated with each other is not yet fully established.

Mitosis is an important event during the cell cycle. For transmission of the 'transcription memory', the transcription factors responsible for one or more particular events need to pass the memory transcript to the progeny cells for gene reactivation after mitosis (Kumar et al. 2012; Raccaud and Suter 2018). Although the binding of PXR and a few other nuclear receptors, on to the mitotic chromatin during cell division, has been well-revealed, but still there are several queries awaiting extensive investigation. For example, the identification of specific domain(s) and/or motifs of PXR responsible for constitutive binding with mitotic chromatin is yet to be revealed. Does PXR bind DNA specifically at the promoter sequences or associates with DNA/chromatin non-specifically remain to be elucidated? Our laboratory previously established the role of NLS region in case of androgen receptor for this transmission (Kumar and Tyagi 2012), but the role of NLS region of the constitutively associated nuclear receptor PXR, which is reported to contain 27 amino acid residues ranging from 66 to 92 amino acids (Kawana et al. 2003), is yet to be studied. In the present detailed study, we have used fluorescent protein tags to elucidate the role(s) of PXR functional domains in living cells during interphase and mitosis. We have shown that DNA-binding domain containing two Zn fingers is essential for association with the mitotic chromatin with NLS region having a decisive role in this interaction.

PXR, the 'master regulator' of xenobiotic metabolism and disposition in the body, regulates the expression of many genes involved in all the three phases of drug metabolism and elimination (Saradhi et al. 2006). One of the most important target genes of PXR is CYP3A4 which is responsible for metabolizing more than 50% of clinical drugs (Mizuno et al. 2009). It has been reported that for activation of CYP3A4 gene, PXR interacts with the ER6 region of CYP3A4 promoter (Blumberg et al. 1998). During mitosis, gene transcription is halted, but several reports suggest that some transcription factors remain bound to their target promoter sites during mitosis in a silenced manner (Yan et al. 2006; Kumar et al. 2012; Festuccia et al. 2016). Whether PXR associates to the promoters of its target genes (like ER6 region of the CYP3A4 promoter) during mitosis is still ambiguous and requires systematic investigation. In this study, we report that PXR association with mitotic chromatin involves sequence-specific binding to the same target sites which are significant for PXR function during interphase. Since PXR is one of the major xenobiotic receptors involved in drug metabolism and disposition, the knowledge on the sequence determinants responsible for the mitotic gene bookmarking will not only pave the way towards the mechanism of action of this xenosensing transcription factor but also provide insight towards future drug development.

Materials and methods

Biochemicals

Escorts III and IV and DNA stain Hoechst were procured from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000 reagent was procured from Invitrogen (Invitrogen, Carlsbad, CA, USA). DMEM, steroid-stripped serum, and foetal bovine serum were purchased from PAN Biotech (Germany). Trypsin-EDTA was procured from Sigma-Aldrich (St. Louis, MO, USA). Plasticwares used in cell culture-based experiments were purchased from Corning Costar Corp (Lowell, MA, USA). Restriction enzymes and chemicals for PCR were obtained from New England

Biolabs (Beverly, MA, USA). Anti-GFP rabbit polyclonal antibody and anti-RFP rabbit polyclonal antibody were procured from Sigma-Aldrich (Cat No. G1544) and abcam (Cat No. ab62341) respectively. Anti-PXR polyclonal antibody and anti-β-actin antibody were developed in our laboratory and described before (Saradhi et al. 2005; Kumar et al. 2008). Horseradish peroxidase-conjugated anti-rabbit secondary antibody for western blot analysis was procured from Sigma-Aldrich (Cat No. A0545). Other general chemicals used were of analytical grade and were procured from different commercial sources.

Cell culture

HepG2 (human hepato-cellular carcinoma cell line), COS-1 (African green monkey kidney fibroblast-like cell line), and HEK-293T (human foetal embryonic kidney cell line) were obtained from the National Cell Repository (NCCS, Pune, India). HepG2, COS-1, and HEK-293T cells were grown as per the recommendation of ATCC (Manassas, USA) in DMEM which was supplemented with 10% foetal bovine serum, 100 μg/ml of penicillin, and 100 μg/ml of streptomycin. The cultures were maintained in a humidified incubator at 5% CO_2 and 95% air atmosphere at 37 °C temperature.

Plasmids

Different GFP-tagged deletion constructs of human PXR were amplified from pSG5-PXR (a gift from S.A. Kliewer, University of Texas, Southwestern Medical Center, Dallas, USA) and subcloned in frame between EcoRI and BamHI restriction sites of pEGFP-C2 (Clonetech, Inc). Constructs having different mutations or truncations of NLS region of PXR were made by either site-directed mutagenesis or subcloning in frame with pDsRedExpress-C1 vector (Clonetech, Inc). Oligonucleotides (oligos) were prepared by Eurofins (Bangalore, India). Oligos used for generating different deletion constructs of PXR or mutants with truncated NLS region of PXR are listed below in Table 1. RFP-tagged SV40 large T antigen NLS (PKKKRKV), nucleoplasmin NLS (KRPAATKKAGQAKKKK), and different subregions of PXR zinc finger and NLS region were made by annealing the respective forward and reverse oligonucleotides (as mentioned in Table 1) and ligating in frame to pDsRedExpress-C1 vector (Clonetech, Inc). RFP-tagged PXR-R66-76RRev mutant which contain a reverse

Table 1 List of oligos used for generating different constructs

Oligos name	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
PXR NTD	GGAATTC CTGGAGGTGAGACCCAAAG	CGGGATCCCGTTGGGGACCTCCGACTTCC
PXR ‘N’ Zn DBD	GGAATTC ATCTGCCGTGTATGTGGGG	CGGGATCCCGGTTGCGTTTCATGGCCCTC
PXR ‘C’ Zn DBD	GGAATTC GCCCGGCTGAGGTGCCC	CGGGATCCCGCATGCCGCTCTCCAGGC
PXR-DBD	GGAATTC ATCTGCCGTGTATGTGGGG	CGGGATCCCGCATGCCGCTCTCCAGGC
PXR-LBD	GGAATTC CAGGGGCTGACAGAGGAGCAG	CGGGATCCCG TCAGCTACCTGTGATGCCG
PXR-NLS (R66-92R)	GGAATTCCAGGAGGGCCATGAAACGC	CGGGATCCCGTCGCCGGGTCTTCCG
PXR K62-76R	AATTCCAAGGGCTTTTTCAGGAGGGC CATGAAACGCAACGCCCGGCTGAGG	GATCCCTCAGCCGGGCGTTGCGTTTC ATGGCCCTCCTGAAAAAGCCCTTGG
PXR K62-67R	AATTCCAAGGGCTTTTTCAGGAGG	GATCCCTCCTGAAAAAGCCCTTGG
PXR A68-76R	AATTCCGCCATGAAACGCAACGCCCG GCTGAGG	GATCCCTCAGCCGGGCGTTGCGTTTC ATGGCGG
PXR R66-76R	AATTCCAGGAGGGCCATGAAACGCAA CGCCCGGCTGAGG	GATCCCTCAGCCGGGCGTTGCGTTTC ATGGCCCTCCTGG
PXR76-66 (66-76 amino acid orientation reversed in complete protein)	TGCAAGGGCTTTTTCAGGCTGCGGGC CAACCGCAAAATGGCCAGGAGGTG CCCCTTCCGGAAGGGC	GCCCTTCCGGAAGGGGCACCTCCTGG CCATTTTGCGGTTGGCCCGCAGCC TGAAAAAGCCCTTGCA
SV40 large T antigen NLS	AATTGCCCCAAGAAGAAGAGGAAGGTG	GATCCACCTTCCTCTTCTTCTTGGGGC
Nucleoplasmin NLS	AATTGCAAACGCCCGGCGGCGACCAA AAAAGCGGGCCAGGCGAAAAAAAA AAAA	GATCTTTTTTTTTTTTCGCCTGGCCC GCTTTTTTGGTCGCCGCCGGGCGTTTGC

sequence of amino acids of R66-76R region of PXR-NLS was made by overlapping PCR using the mutagenic forward and reverse oligoes mentioned in Table 1 and RFP-PXR forward and reverse primers (Dash et al. 2017a) and taking RFP-PXR as the template and finally cloning in frame with pDsRedExpress-C1 vector (Clonetech, Inc). Briefly, first and second PCRs were performed by taking RFP-PXR forward primer and mutagenic reverse primer and RFP-PXR reverse primer with mutagenic forward primer having RFP-PXR as a template. The products of first and second PCRs were mixed in equal molar concentrations and used as a template for the third and final PCR using RFP-PXR forward and reverse primers. The final product was digested with desired restriction enzymes (EcoR1 and BamH1) and ligated in frame with pDsRedExpress-C1 vector (Clonetech, Inc). All the constructs used in this manuscript harboring different mutations, deletions, or reversal were confirmed by sequencing and restriction digestion. RFP-tagged PXR (wild-type), PXR-R66A/R67A, PXR-R91A/R92A and PXR-NLSmut, XREM-Luc (CYP3A4-Luc), and GFP-PXR (wild-type) were described previously (Saradhi et al. 2005; Dash et al. 2017a, 2017b).

Transient transfection and luciferase assay

For luciferase reporter assays, transient DNA transfections in HepG2 cells were performed with Escort III reagent (Sigma) or Lipofectamine 2000 reagent (Invitrogen). Cells were seeded into 24-well cell culture plates and transfected with 300 ng of XREM-Luc promoter-reporter plasmid and 50 ng of either wt-PXR, deletion, or mutational constructs along with 50 ng of β-galactosidase reporter gene construct as per the experimental requirements. Following the transfection period, HepG2 cells were supplemented with fresh DMEM containing 5% steroid-stripped medium without antibiotics and treated with 10 μM rifampicin or DMSO (vehicle) and further incubated at 37 °C for 24 h. After the completion of 24 h of expression period, cells were harvested, lysed, and subjected to luciferase assay as per the manufacturer's protocol mentioned in the kit (Promega, Madison, WI, USA, no. E1501).

Fluorescence microscopy of living cells and quantification

COS-1 or HEK-293T cells were seeded into 35-mm plates and allowed to grow to ~ 70% confluency. The cells were then transfected with 500 ng or 1 μg of plasmids using Escort IV transfection reagent. After 10–12 h of transfection, cells were supplemented with DMEM containing 5% steroid-stripped serum without antibiotics and allowed further 24 h to grow. After 24-h period, subcellular localization during interphase and mitosis was examined with a Nikon fluorescence upright microscope. For visualization of the nucleus, Hoechst was added to living cells at least 2 h before imaging. The fluorescent cells were observed, and images were processed using standard image-processing techniques as described earlier (Chaturvedi et al. 2010; Dash et al. 2017a). To perform statistical analysis, experiments were repeated at least thrice for recording the cells for subcellular localization and mitotic chromatin association of GFP-/RFP-tagged wt-PXR and its deletion or mutational constructs. For statistical score, each time 100 number of cells in interphase and at least 15–20 number of mitotic cells expressing GFP-/RFP-tagged PXR or its deletion or mutational constructs were recorded for subcellular localization and mitotic chromatin association, respectively. For subcellular localization, the receptor distribution pattern was divided into five categories. We considered localization as nuclear (N) when fluorescence was exclusively in the nuclear compartment of the cell. When fluorescence was significantly brighter in the nucleus in comparison to the cytoplasm, we termed it as the predominantly nuclear localization (N>C) and we considered the localization as N=C when fluorescence was distributed uniformly between nucleus and cytoplasm. It was considered as predominantly cytoplasmic (C>N) localization when the receptor expression was brighter in the cytoplasm than in the nucleus. Localization was considered as cytoplasmic (C) when there was presence of fluorescence mostly in the cytoplasmic compartment of the cell and near absent in the nuclear compartment. For easy representation of the results in some cases, we grouped C=N, C>N, and C cells into a single category and termed them as predominantly cytoplasmic (C) (Dash et al. 2017a).

Preparation of whole cell lysate from cultured mammalian cells and western blotting

COS-1 and HEK-293T cells were transiently transfected with about 500 ng–1 μg of desired GFP- or RFP-tagged expression plasmids and allowed to express the particular protein for about 36 h. For preparing whole cell

lysates, cell pellets were washed three times with cold PBS and dissolved in lysis buffer (20 mM Tris pH 7.6, 0.5 mM DTT, 1 mM EDTA, 1 mM PMSF, 0.1% NP-40, and protease inhibitor cocktail) and kept on ice for 30 min with intermittent tapping. After incubation period of 30 min, 5 M NaCl was added to a final concentration of 400 mM and incubated in ice for another 30 min. The clear supernatant (whole cell lysate) was collected by centrifugation at 12,000 rpm for 15 min at 4 °C and was rapidly frozen at − 80 °C till use. For western blotting, whole cell lysates were dissolved in 4× SDS-PAGE sample buffer and boiled at 95 °C for 5 min. Equal amount of proteins (50 μg) was resolved by 10–15% SDS-PAGE. Following resolving, proteins were transferred to polyvinylidene fluoride (PVDF) membrane using a wet transfer unit (Invitrogen). Following transfer, the membrane was blocked with 5% non-fat milk powder dissolved in TBST (TBS with 0.1% Tween-20) for 2 h at room temperature and then incubated with desired antibody in 1% non-fat milk overnight. The PVDF membrane was then washed three times with TBST and incubated with desired secondary antibody. The PVDF membrane was then washed again three times with TBST and the bound antibody complexes were detected using the enhanced chemiluminescence (ECL) system (Rana et al. 2016).

Bioinformatic analysis for studying interaction of NLS region of PXR with DNA

Molecular modelling and molecular dynamic simulations

The complete structure of PXR is not available in the PDB (Protein Data Bank). We have modelled complete structure of PXR using I-Tasser which incorporates a combination of threading, fragment assembly, and ab initio techniques as part of its template-based modelling protocol. Mutant structure is generated using WHATIF server, and to the stable conformation, we subjected the wild and mutant receptor structures for MD simulations. The MD simulations were performed with the help of GROMACS 5.1 package, with the GROMOS 96 force field. The box dimensions ensured that any protein atom was at least 2.5 nm away from the wall of the box with periodic boundary conditions and solvated by simple point charge (spce) water molecules. In order to maintain electro-neutrality condition, $Na^{+}Cl^{-}$ counter ions were added. Steepest descent method was used to minimize energy. In order to keep the system in a stable environment (300 k, 1 bar), Parrinello-Rahman pressure coupling and V-rescale temperature coupling were used and the coupling constant for temperature was set to 0.1, and for pressure, it was set to 2.0 ps. For calculation of electrostatic and van der Waals interactions, the Partial Mesh Ewald (PME) algorithm32 was used and cutoff distance for the short-range vdW (rvdW) was set to 14 Å, where Coulomb cutoff (r coulomb) and neighbor list (rlist) were fixed at 9 Å. Using the LINCS algorithm, all bond lengths were constrained. The time step was set to 0.002 ps. The obtained complexes in a medium were equilibrated for 100 ps in NPT and NVT ensembles. At the end, a 2-ns molecular dynamics simulation was carried out for all the required/studied complexes (Sudhakar et al. 2016).

Protein-DNA docking

Protein-DNA docking was performed using HADDOCK (http://haddock.science.uu.nl/services/HADDOCK/haddock.php). It is an information-driven, flexible docking approach for modelling of biomolecular complexes. Wild and three mutant structures of PXR [PXR-R66A/R67A, PXR-R91A/R92A, and PXR-R66A/R67A/R91A/R92A (NLS mutant)] were subjected for the docking with the DNA (ER6—GATCAATATGAACTCAAAGGA GGTCAGTGAG) which was modelled using 'Discovery Studio'. HADDOCK distinguishes itself from ab initio docking methods as it encodes information from identified or predicted protein interfaces in ambiguous interaction restraints (AIRs) to drive the docking process. In HADDOCK, different web interfaces are available, for example Easy, Expert, Guru, and File upload. For docking, each of these interfaces requires different parameters and requirements. The data we submitted to the server converted to AIRs by HADDOCK and this was used for docking purpose. The molecular topology of the docked molecule is automatically generated. There are three stages of docking, (i) rigid body energy minimization, (ii) semi-flexible refinement in torsion angle space, and (iii) a final refinement in explicit solvent. After completion of each stage, scoring and ranking are done, and the best structures are kept for the next stage. Parameters used such as temperature, number of steps, and force constants can be modified as per the requirement. In its structure calculation engine, HADDOCK also uses the

CNS (crystallographic and NMR) system. Scoring is a weighted sum of van der Waals, electrostatic, desolvation, and restraint violation energies with the buried surface area. By the use of these above parameters, the easy interface mode of docking was performed. The top 10 obtained clusters were viewed only with their top four docked structures. Based on their size, the clusters were numbered, and they were sorted based on HADDOCK score. The top structures were downloaded and viewed by PyMOL (Sudhakar et al. 2016).

Fluorescence-activated cell sorting and chromatin immunoprecipitation assay involving pure mitotic cells

Status of mitotic phase synchronization was analyzed by flow cytometry in HepG2 cell line. For this, 2.0×10^6 cells were seeded in a 100-mm culture plate. At 80% confluency, 100 nm nocodazole was added to the medium and cells were harvested post 16 h of nocodazole treatment. Briefly, cells were harvested by trypsinization and washed thoroughly with ice-cold PBS by centrifuging at 1500 rpm for 5 min. Single-cell suspension was made with cold PBS and fixed in 70% chilled ethanol for at least 2 h at − 20 °C. Then, ethanol was removed, and cells were washed with cold PBS by centrifuging at 1500 rpm for 5 min at 4 °C and incubated with 50 μg/ml of RNase A, 100 μg/ml of propidium iodide, and 0.6% Triton X-100 at 37 °C for 30–60 min in dark and then stored in dark at 4 °C until analysis. A similar 100-mm plate without nocodazole treatment was also processed for the assessment of asynchronous phase. Cell cycle was analyzed to obtain the status of synchronization. The mitotic cells (G2/M) were sorted after passed through a 40-μm filter by the BD FACS Aria III (BD Biosciences) and analyzed using DIVA software. Pre- and post-sorted cell populations were also analyzed to ascertain the purity of the sorted cell population. Before analyzing the post-sorted population, about 50 μg/ml of propidium iodide was added to the sorted cells. A minimum of 10,000 events were recorded for each sample.

For cross-linking proteins to DNA, 1.0×10^6 HepG2 cells containing asynchronous (interphase chromatin) and pure mitotic cells (sorted using FACs) were fixed by adding 1% formaldehyde drop-wise at room temperature with gentle rotation for 10 min. The reaction was stopped by adding 2.5 M glycine with shaking for 5 min at room temperature. The medium was discarded and the cells were rinsed with cold PBS three times. After rinsing, cells were scraped and lysed with the FA lysis buffer (50 mM Tris pH 7.6, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail). The lysate was sonicated on ice to shear the DNA and achieve an average fragment size of 500–1000 bp. One-tenth volume of total lysate was removed and kept as input DNA. The resulting DNA-protein preparation was incubated with protein A agarose beads with PXR antibody or with the control pre-immune for overnight at 4 °C. Next day, the resulting DNA-protein antibody complexes were precipitated with protein A agarose beads, washed, and eluted. After elution, eluted DNA as well as input DNA was treated with proteinase K at 65 °C overnight. The DNA was recovered using phenol/chloroform extraction and ethanol precipitation. Next, the DNA was PCR-amplified in 35 cycles (30 s at 95 °C, 20 s at 65 °C, and 20 s at 72 °C). Products of the reaction were analyzed on 2% agarose gel stained with ethidium bromide. The following promoter-specific primers were used for PCR:

ER6 promoter—FP—5′ ATGCCAATGGCTCC ACTTGAG 3′

ER6 promoter—RP—5′ CTGGAGCTGCAGCC AGTAGCAG 3’

Statistical analysis

All the experiments were done at least three times and the values represent the means ± SD of three or more separate experiments. Statistical analysis was done by using SPSS software. Student's *t* test was used to compare promoter-reporter activity and asterisks (*) signify values that differed significantly from the control experiments with *p* value less than 0.05 ($p < 0.05$).

Results

Both the Zn fingers in DBD of PXR are essential for binding to the mitotic chromatin

PXR resides mainly in the nuclear compartment of the interphase cell and associates constitutively with the mitotic chromatin during all the stages of mitosis (Saradhi et al. 2005). Since PXR is a mitotic chromatin associated protein, we wanted to determine which domain(s) or motifs of the receptor are responsible for mitotic chromatin association. For this purpose,

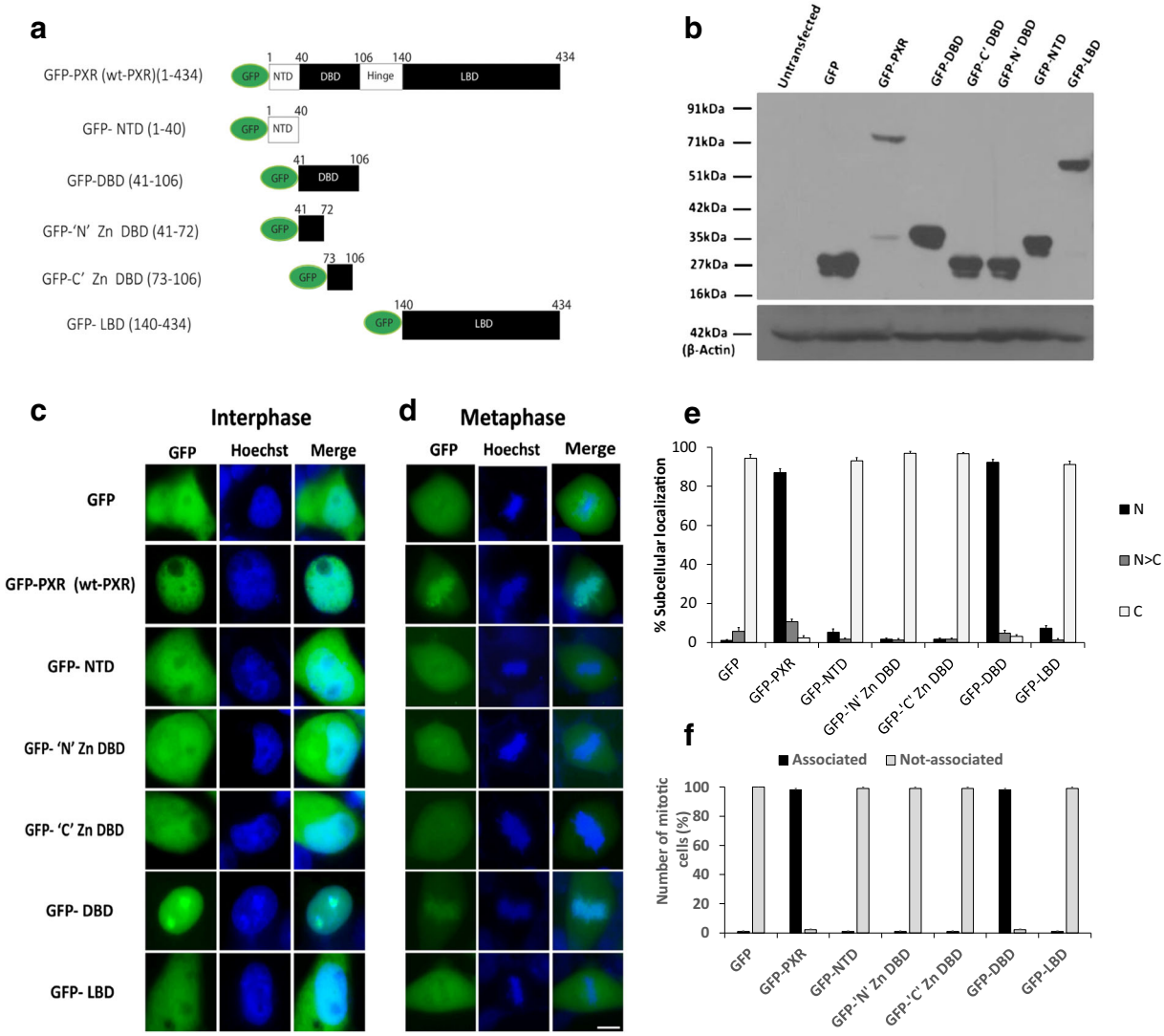

Fig. 1 Both the Zn fingers of DBD are essential for association with mitotic chromatin. **a** Schematic representation of full-length and the deletion constructs of PXR used in the present study. **b** Western blot analysis of different deletion constructs of GFP-tagged PXR showing relative protein expression. Whole cell lysates (50 μg/sample) were loaded in each lane and blotting was performed with anti-GFP antibody (1:5000) as described under the 'Materials and methods' section. β-Actin was used as an internal loading control. COS-1 cells were transiently transfected for the expression of various deletion constructs of GFP-PXR. Cell images for subcellular localization of the receptor during interphase (**c**) and during mitosis (**d**) were recorded using a fluorescence microscope. The GFP fluorescence visualizes the distribution pattern of PXR and its different deletion constructs. Hoechst staining was used as a fluorescent marker for visualizing corresponding nuclei/mitotic chromatin and the merged images for visualizing the corresponding nuclei of GFP-PXR and its deletion constructs. Scale bar = 5 μm. **e** Quantitative data for subcellular localization of PXR and its deletion constructs were observed and classified into groups (N, N>C, and C) by fluorescence microscopy as mentioned in the 'Materials and methods' section. In each case, localization of PXR was recorded in at least 100 transfected cells and average values of three independent experiments were plotted with ± SD and percentage interaction of the receptor with the mitotic chromatin (taking 15–20 mitotic cells in each experiment) **(f)** was assessed and results represented as the mean ± SD of three independent experiments

different GFP-PXR deletion constructs were generated as shown in Fig. 1. Generated constructs included (i) N-terminal domain (NTD), (ii) full-length DBD truncated in the middle creating (a) N-terminal half of DBD containing first Zn finger ('N' Zn DBD) and (b) C-terminal half of DBD containing other Zn finger ('C' Zn DBD), (iii) DNA-binding domain (DBD) containing both the Zn finger domains, and (iv)

ligand-binding domain (LBD). All the deletion constructs showed expected band size in the western blot analysis (Fig. 1b). Also, they showed relatively higher expression in comparison to the wild-type. Only with some exceptions, our general experience with GFP-tagged chimera has been that smaller size proteins in transient transfection experiments are expressed at relatively higher levels as compared to larger ones (Kumar and Tyagi 2012).

To observe the subcellular localization of PXR deletion constructs as shown in Fig. 1, transient expression and live cell imaging were performed with different deletion constructs of GFP-PXR in COS-1 cells. The subcellular localization of the constructs varied widely ranging from nuclear (including N + N>C) to cytoplasmic (N=C + C>N + C) (Fig. 1c). GFP-NTD was found to be uniformly distributed between nucleus and cytoplasm (N=C). Similarly, the N-terminal DBD containing first Zn finger ('N' Zn DBD) and the C-terminal DBD containing second Zn finger ('C' Zn DBD) were found to be uniformly distributed between nucleus and cytoplasm (N=C). The DBD containing both Zn finger domains was predominantly localized in nucleus with inclusion of the receptor in the nucleolus. The GFP-LBD comprising ligand-binding domain was also distributed between nucleus and cytoplasm (N=C).

When the 'N' Zn DBD and the 'C' Zn DBD and the DBD containing both Zn finger domains were examined individually, only the DBD containing both zinc fingers was observed to be associated with the mitotic chromatin (Fig. 1d). So, it was concluded that both Zn fingers are essential for mitotic chromatin association, while all other constructs that were devoid of DBD failed to bind the mitotic chromatin.

At least 100 transfected cells were counted in each case for subcellular localization of constructs and graphically represented for the average values of three independent experiments (Fig. 1e) and the percentage association of constructs to the mitotic chromatin (15–20 number of transfected mitotic cells counted in each experiment) is represented in Fig. 1f.

Mitotic binding of DBD of PXR fails to retain the transactivation ability of wild-type PXR

After figuring out the domain responsible for mitotic chromatin association, we attempted to explore whether these truncated constructs could be transcriptionally activated by PXR ligand or not. To accomplish this, promoter-reporter-based luciferase assays were performed in HepG2 cells. Cells were transiently co-transfected with deletion constructs and XREM-Luc promoter-reporter gene. Following transfection, cells were incubated with its standard ligand rifampicin or vehicle (DMSO). As a result, rifampicin significantly enhanced wild-type PXR (full-length) transcriptional activity while all its deletion constructs exhibited subdued or loss of transcriptional activity as shown in Fig. 2. All constructs showed reduced transcriptional activity and the GFP-DBD construct which was able to associate with the mitotic chromatin also failed to show the transcriptional activity.

Receptor association with mitotic chromatin: role of nuclear localization signal region

PXR is constitutively associated with the mitotic chromatin in a ligand-independent manner (Saradhi et al. 2005) suggesting its role in gene bookmarking and transmission of 'transcription memory' from progenitor to progeny cells (Kumar et al. 2012; Festuccia et al. 2016). In the previous section of the study, we have shown that DBD of PXR is responsible for this association. So, the next question arises whether full-length DBD is responsible or only a part of it is competent for this interaction. Kumar and Tyagi 2012 reported that NLS region of AR is mostly responsible for the mitotic chromatin association of AR in a ligand-dependent manner. So, taking this as guiding information, we hypothesized that NLS of PXR may also be responsible for this association. Like AR, NLS of PXR is also bipartite in nature and occupies a region of 27 amino acids starting from amino acid residues 66 to 92 as shown in the figure. This falls in the region of two zinc fingers (Kawana et al. 2003) (Fig. 3).

In silico analysis of NLS region of PXR for association with DNA

The complete 3D structure of PXR protein is not available in PDB and hence we modelled PXR-DBD with the help of I-Tasser. The MD-simulated structure showed stable conformation of PXR. FoldX (a protein design algorithm) results showed that the mutations at **R66A/R67A**, **R91A/R92A**, and **R66A/R67A/R91A/R92A (NLS mutant)** have less stability in comparison to the wild-type (Fig. 4a). Mutant **R91A/R92A** showed least stability which may be due to its presence in the loop of

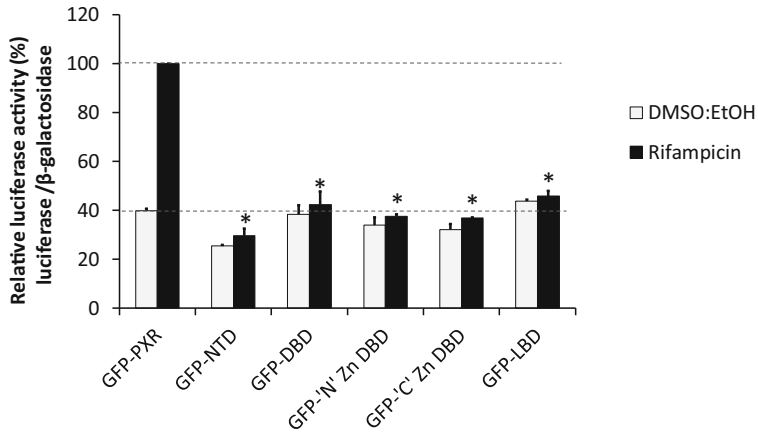

Fig. 2 Modulation of CYP3A4 promoter (XREM-Luc) by PXR deletion constructs. HepG2 cells were co-transfected with XREM-Luc, with GFP-PXR, or with different PXR deletion constructs. After transfection, cells were treated with rifampicin for 36 h and then harvested for luciferase assay. Luciferase values were normalized with respect to transfection efficiency using the corresponding β-galactosidase values and the relative activity was calculated in comparison to rifampicin-induced wild-type PXR activity, which was taken as 100%. Bars represent the mean ± SD of three independent experiments. Asterisks (*) signify luciferase values that differed significantly from the rifampicin-induced wild-type PXR ($p < 0.05$ in Student's t test)

second zinc finger **(**Fig. 3**)**. To understand the interaction between the DNA and PXR, we have generated the 3D structure of DNA (ER6—GATCAATATGAACT CAAAGGAGGTCAGTGAG) using 'Discovery Studio' and docked against full-length NLS of PXR as well as against a small fragment of NLS consisting of 11 amino acid residues, i.e. from 66 to 76 amino acid residues. The HADDOCK docking results showed wild-type PXR has high affinity with the DNA as compared to mutant PXR. The mutant (**R66A/R67A**) protein showed decreased affinity compared to wild-type and other mutants. The docking scores of **R66A/R67A**, **R91A/R92A**, and double mutant (NLS mutant) were − 167.8, − 189.8, and − 179.1 which suggests less affinity as compared to wild-type PXR (− 205.3) **(**Fig. 4b**)**. The docking analysis of wild-type and mutants suggests that amino acid residues at position 66-67 are crucial for DNA binding of PXR.

A number of H bonds between wild-type PXR-NLS region and ER6 DNA were reduced upon mutation in the NLS region. The number of H bonds in interaction of full-length NLS of PXR with DNA was found to be 20 in case of wild-type which was reduced to 16, 17, and 14 in case of mutations R66A/R67A, R91A/R92A, and R66A/R67A/R91A/R92A (NLS mutant) respectively (Supplementary Figure 1a–d). Also, when R66-76R region of PXR-NLS was docked with ER6 DNA, we observed similar kind of results (Supplementary Figure 2a–

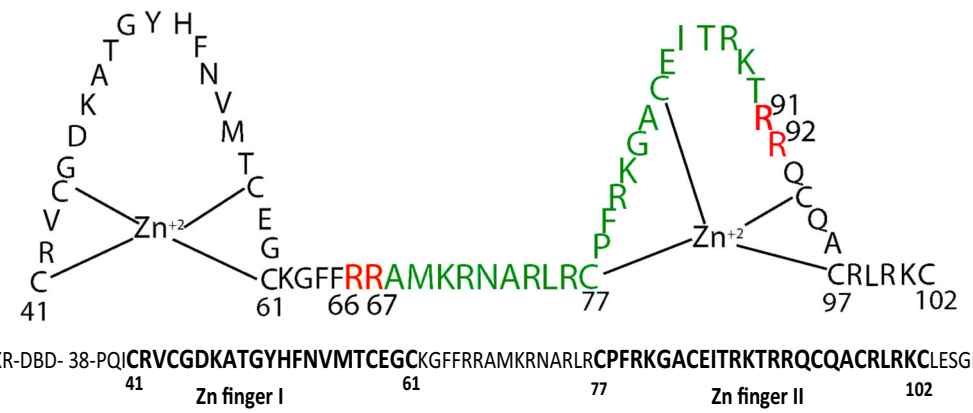

Fig. 3 Details of amino acid composition of two zinc fingers and bipartite NLS (R66-92R) of PXR-DBD. Amino acid residues marked with red and green combinedly represent the complete NLS region of PXR in which the amino acid residues marked in red were predicted to be important as per the previous studies (Kawana et al. 2003)

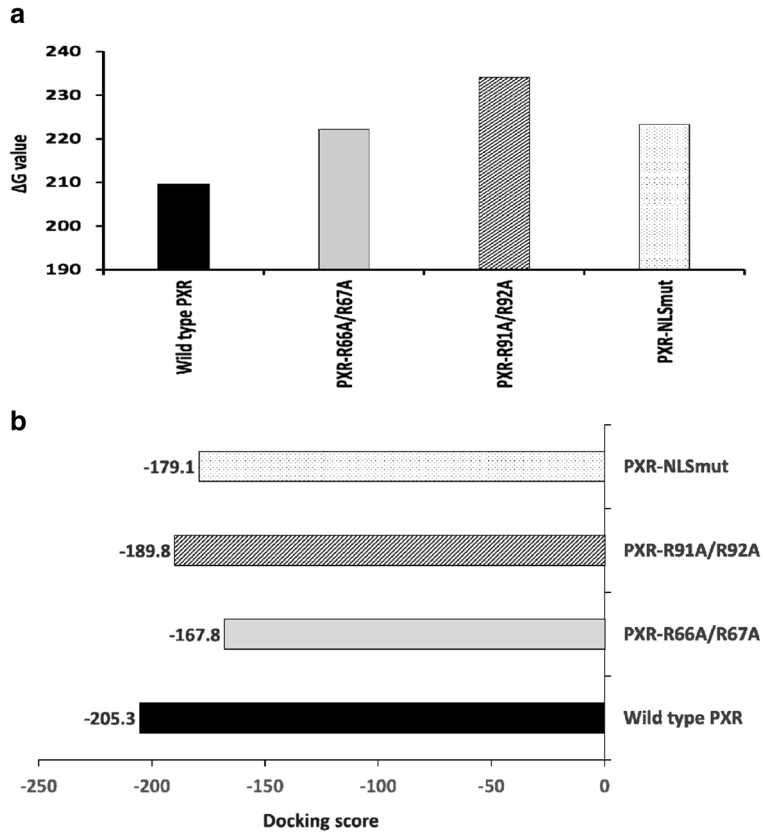

Fig. 4 In silico analysis of NLS region of PXR for association with DNA. **a** Representation of ΔG values of wild-type and different NLS mutants of PXR. PXR-R91A/R92A is found to be least stable which may be due to the position of these two amino acid residues (R91 and R92) in the loop of second zinc finger. Each zinc finger of PXR contains a group of four Cys residues which coordinates a single zinc atom and also helps in proper folding of the protein required for the normal function of PXR. Thus, any mutation in this region can lead to instability of the protein structure. **b** Docking score of suggested mutations in NLS of PXR with ER6 DNA. Docking scores suggest that the two arginines present at amino acid positions 66 and 67 are most critical for interaction of PXR with ER6 region of its promoter

d) confirming that R66 and R67 amino acids are critical for interaction with DNA.

Analysis of subcellular localization and interaction of different regions of zinc finger and NLS of PXR with the mitotic chromatin by live cell imaging

Based on in silico predictions and previous experimental data from our laboratory (Kumar and Tyagi 2012), we can hypothesize that NLS region of PXR may act as a mitotic chromatin binding-determining region (MCBR). In order to further dissect the role of zinc finger and NLS in their interaction with mitotic chromatin, we have generated different RFP-tagged NLS mutants [PXR-R66A/R67A, PXR-R91A/R92A, PXR-R66A/R67A/R91A/R92A (NLSmut), PXR-R66-R76-rev] and different plasmid constructs containing different regions of NLS and zinc finger. Western blot analysis suggested that different RFP-tagged NLS mutants of PXR expressed receptor protein efficiently and in near equal levels (Fig. 5b). Subsequently, studies were conducted for their expression, subcellular localization, and interaction with mitotic chromatin. We also generated RFP-tagged constructs having two classical NLSs, i.e. NLS of SV40 large T antigen (monopartite) and NLS of nucleoplasmin (bipartite) for dissecting the role of NLS in interaction with the mitotic chromatin (LaCasse and Lefebvre 1995).

As expected, different mutations in the NLS region of PXR protein showed cytoplasmic-shifted localization. PXR-**R66A/R67A** was found to be more cytoplasmic than PXR-**R91A/R92A** and the mutant having all these four mutations, i.e. PXR-NLSmut was completely cytoplasmic in localization (Kawana et al. 2003; Dash et al. 2017b). PXR-**R66-76R-rev** showed marginal cytoplasmic-shifted localization as compared to wild-type PXR which is suggestive of the importance of sequence orientation of this region (R66-76R) for nuclear localization of PXR (Fig. 5).

Initial observations in the present study suggested that some specific mutations in the NLS region of PXR cause cytoplasmic shift of the receptor, and the

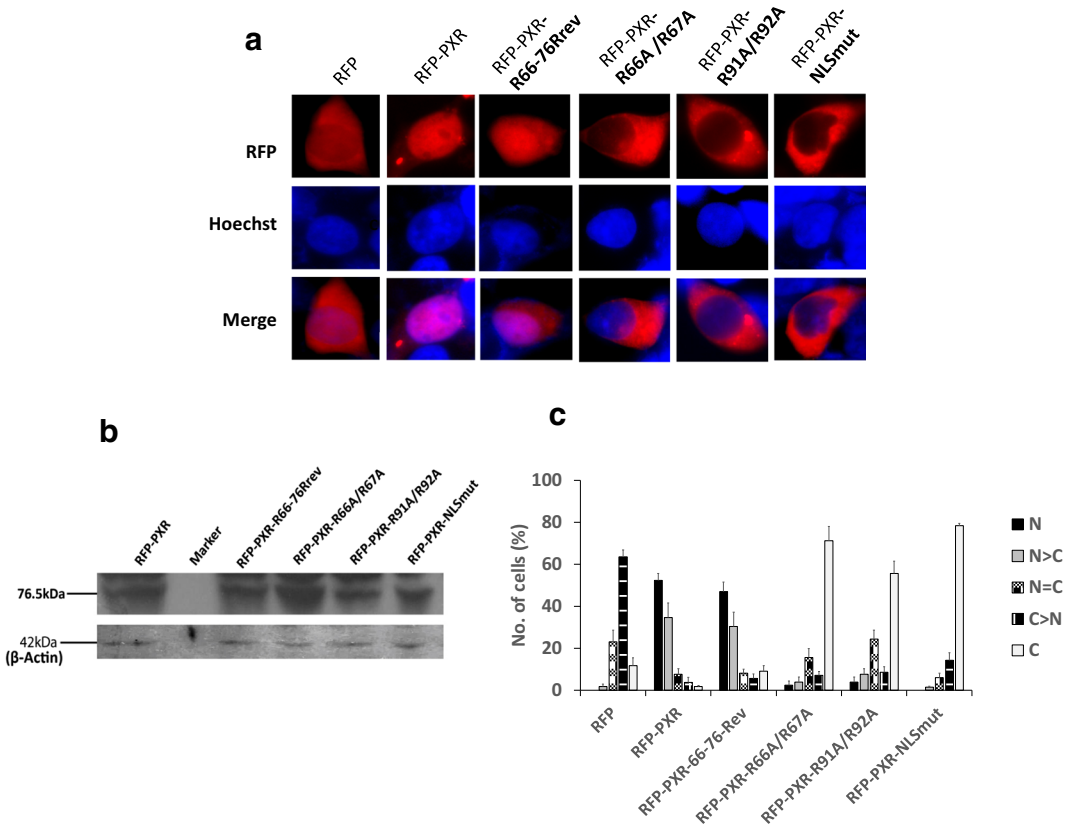

Fig. 5 Mutation in the NLS region shifts localization of PXR to the cytoplasmic compartment. HEK-293T cells were transiently transfected individually with RFP-tagged receptor plasmid constructs as shown in the figure. After 24 h of expression, fluorescent cells were scored for localization and imaged. Reversal of R66-76R results in only minor shift towards cytoplasmic localization. To facilitate visualization of the nucleus, Hoechst dye was added to the live cells at least 2 h prior to imaging. **a** Subcellular localization of different receptor constructs. **b** Western blot analysis of different NLS mutants of PXR with receptor polyclonal antibody showing the protein size and relative expression profile. **c** Quantitative data for receptor subcellular localization were assessed and results represented as the mean ± SD of three independent experiments for all except RFP-PXR-R66A/R67A and RFP-PXR-R91A/R92A shown with six independent experiments. Rev, reverse sequence

amino acid residues R66/67R appear to be the most critical for nuclear localization of PXR. So, in order to re-confirm the specific residues most critical for nuclear translocation, we tagged full-length and different parts of PXR-NLS with RFP as mentioned in Fig. 6 and Table 2. All the RFP-tagged constructs expressed the protein of desired size. Some RFP constructs not only appeared to express higher levels of protein but also exhibited better transfection efficiency showing thicker bands in the blot (Fig. 6c). As expected, RFP-PXR-NLS only (R66-92R) showed complete nuclear localization. Also, the region between the two zinc fingers (K62-76R) showed complete nuclear localization. When we divided K62-76R region into two parts, i.e. K62-67R and A68-76R, none was able to localize to the nucleus. But, interestingly, the construct R66-76R was able to efficiently enter the nucleus like the full-length NLS region. This implied that inclusion

Fig. 6 The amino acid sequence R66-76R is most critical for nuclear localization of PXR. HEK-293T cells were transiently transfected individually with plasmids having RFP-tagged sequences as shown in the figure. After 24 h of expression, fluorescent cells were scored for localization and imaged. To facilitate visualization of the nucleus, Hoechst dye was added to the live cells at least 2 h prior to imaging. **a**, **b** Subcellular localization of different NLS constructs. **c** Western blot analysis showing expression pattern of different RFP-tagged constructs probed with polyclonal RFP antibody (1:1000 dilution). Arrows indicate the regions for specific protein bands whereas asterisk shows the non-specific bands. **d** Quantitative data for subcellular localization of different constructs were assessed and results represented as the mean ± SD of three independent experiments

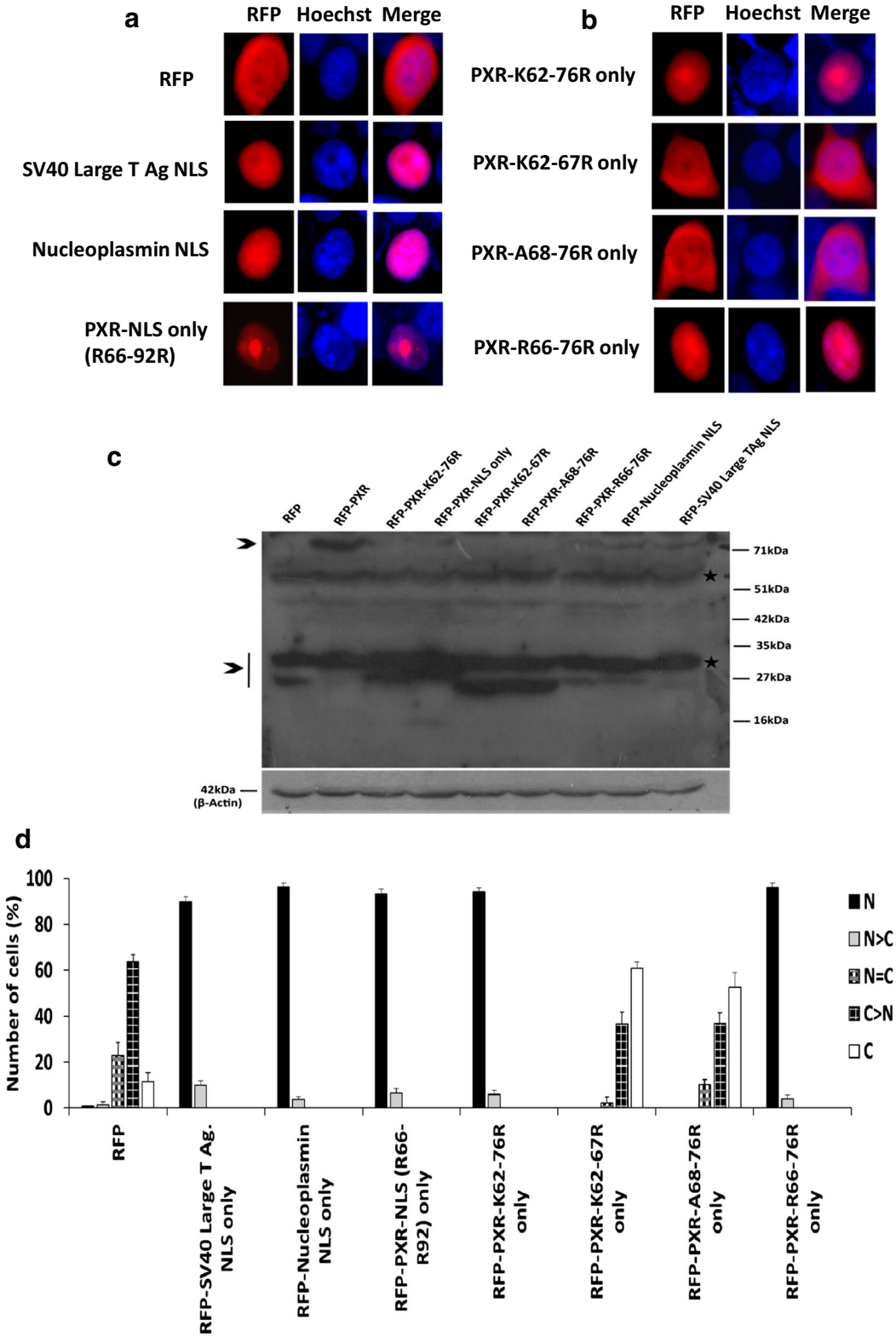
a
RFP
Hoechst
Merge
RFP
SV40 Large T Ag NLS
Nucleoplasmin NLS
PXR-NLS only
(R66-92R)
b
RFP
Hoechst
Merge
PXR-K62-76R only
PXR-K62-67R only
PXR-A68-76R only
PXR-R66-76R only
c
RFP
RFP-PXR
RFP-PXR-K62-76R
RFP-PXR-NLS only
RFP-PXR-K62-67R
RFP-PXR-A68-76R
RFP-PXR-R66-76R
RFP-Nucleoplasmin NLS
RFP-SV40 Large TAg NLS
71kDa
51kDa
42kDa
35kDa
27kDa
16kDa
42kDa
(β-Actin)
d
Number of cells (%)
100
80
60
40
20
0
N
N>C
N=C
C>N
C
RFP
RFP-SV40 Large T Ag. NLS only
RFP-Nucleoplasmin NLS only
RFP-PXR-NLS (R66-R92) only
RFP-PXR-K62-76R only
RFP-PXR-K62-67R only
RFP-PXR-A68-76R only
RFP-PXR-R66-76R only

Table 2 A comprehensive analyses of NLS and zinc finger region of PXR

Region	Subcellular localization*	Mitotic chromatin interaction	Transcriptional activity
SV40 large T Ag. NLS only (classical monopartite NLS)	Nuclear	No	Not applicable
Nucleoplasmin NLS only (classical bipartite NLS)	Nuclear	Yes	Not applicable
Wild-type PXR	Nuclear	Yes	Yes
PXR-**R66A/R67A** (mutation in complete protein)	Predominantly cytoplasmic	No	No
PXR-**R91A/R92A** (mutation in complete protein)	Predominantly cytoplasmic	No	No
PXR-**R66A/R67A/R91A/R92A (NLS mutant)** (double mutation in complete protein)	Cytoplasmic	No	No
R66-92R (NLS) only	Nuclear	Yes	Not applicable
K62-76R only	Nuclear	Yes	Not applicable
K62-67R only	Cytoplasmic	No	Not applicable
A68-76R only	Cytoplasmic	No	Not applicable
R66-76R only **(MCBR)**	Nuclear	Yes	Not applicable
'N' Zn DBD—**C41-72N**	Cytoplasmic	No	No
'C' Zn DBD—**A73-106G**	Cytoplasmic	No	No
PXR-**R66-76R reverse** (reversed in complete protein)	Nuclear	No	No

*Method of subcellular classification is detailed in the 'Materials and methods' section

of R66/67R in conjunction with A68-76R acted as the minimal region (R66-76R) for efficient nuclear translocation. Previously, Fig. 1 showed that C-terminal zinc finger region (A73-106G) which contains A73-92R part of NLS region is also not able to localize to the nucleus. Overall, this suggests that the R66-76R is the minimal region for nuclear localization of PXR and the R66/67R residues are the most critical for nuclear localization of PXR.

In relation to the interaction of PXR with mitotic chromatin, we have observed that all the NLS mutants generated herein lost their constitutive association completely (Fig. 7). To our surprise, we observed that complete NLS region (R66-92R) of the PXR associated strongly with the mitotic chromatin. Therefore, we wished to explore as to which part of the complete NLS region is responsible for association with mitotic chromatin. To address this, we cloned different subregions of this NLS and also the central part of the sequence lying between the two zinc fingers into RFP vectors. Interestingly, the region in between two zinc fingers (K62-76R) associated with the mitotic chromatin whereas the region K62-67R, region R68-76R, N-terminal zinc finger region (C41-72N), and C-terminal zinc finger region (A73-106G) alone failed to associate with the mitotic chromatin. Again, the region R66-R76 strongly associated with the mitotic chromatin which suggested that R66 and R67 amino acid residues in conjunction with full-length NLS region, as well as, C-terminal zinc finger region, are critical for association with mitotic chromatin. Subsequent to expression, we observed that in particular the region R66-76R is sufficient for constitutive association of the receptor with the mitotic chromatin (Fig. 8, Table 2). Since NLS is primarily composed of basic amino acid residues and has an overall positive charge and the DNA has a negative charge on its surface, it may be plausible that these two charges may be responsible for this interaction. So, to ascertain this, we expressed RFP-tagged classical NLS, i.e. NLS of SV40 large T antigen or NLS of nucleoplasmin. Interestingly, we observed that the classical monopartite NLS of SV40 large T antigen does not associate with the mitotic chromatin whereas classical bipartite NLS of nucleoplasmin constitutively associated with the mitotic chromatin. Since PXR-NLS is also a bipartite signal in nature like nucleoplasmin, the mitotic association may be attributed to the bipartite NLS and may not be exclusively to the charge. To further confirm the hypothesis if charge has some role in mitotic chromatin association, we reversed the sequence R66-76R in whole PXR protein and observed that the constitutive

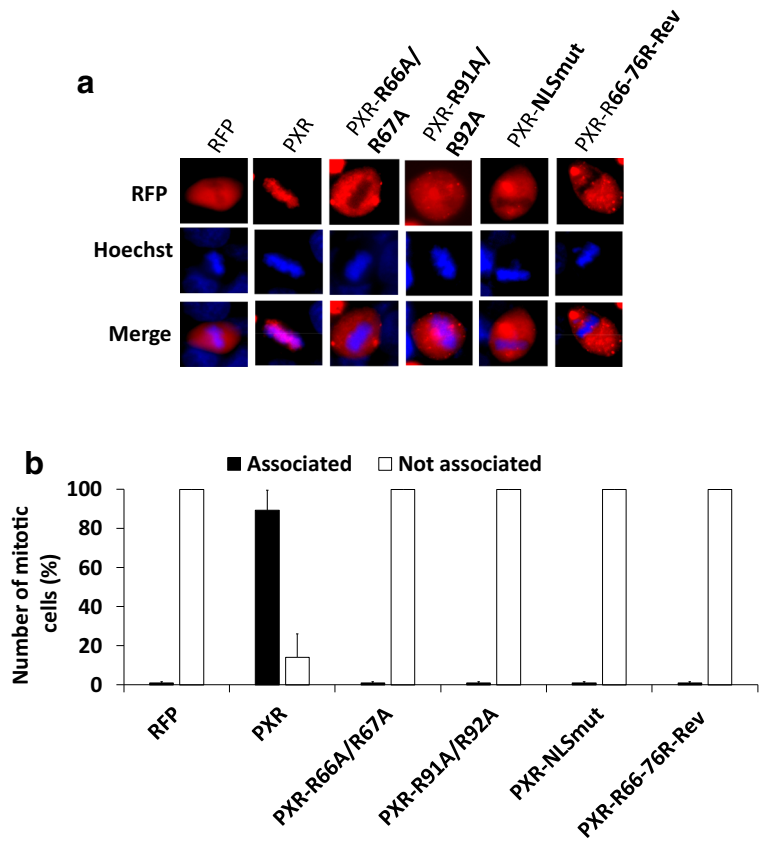

Fig. 7 Mutations in the NLS region of full-length PXR abolished mitotic chromatin interactions. HEK-293T cells were transiently transfected individually with RFP-tagged receptor plasmid constructs as depicted in the figure. Mutations were introduced in the full-length PXR protein. After 24 h of expression, mitotic cells were searched and at least 15–20 mitotic cells were counted in each case. Each experiment was repeated for at least three times. To facilitate visualization of the nucleus, Hoechst dye was added to the live cells at least 2 h prior to imaging. **a** Images of the representative cells showing interaction with the mitotic chromatin. **b** Quantitative data for percentage interaction of the receptor with the mitotic chromatin was assessed and results represent the mean ± SD of three independent experiments. Rev, reverse sequence

association of PXR was completely abolished confirming that orientation of amino acid residues in R66-76R is important but not the positive charge within the sequence (Fig. 7). From in silico predictions, gene expression, and live cell imaging experiments, R66-76R NLS region of human PXR can be termed as the mitotic chromatin binding-determining region (MCBR). The function of this region appears to be dependent on proper orientation of its amino acid sequence but independent of charge of amino acid residues.

In silico analysis showed that mutations in the isolated NLS region of PXR disrupt its association with the ER6 region present in the CYP3A4 promoter which is a known target of PXR. Thus, to validate this in vitro hypothesis, we examined the transcriptional activity of different NLS mutants of PXR on XREM-luc. As expected, all the NLS mutants lost their ability to activate XREM-luc (Dash et al. 2017b). Interestingly, the PXR-R66-76R-rev also lost the ability to transactivate XREM-Luc which indicates that properly oriented R66-76R region of PXR-NLS is the critical region for association with the ER6 region in the CYP3A4 promoter for transactivation of the target genes (Fig. 9). For convenience, a summary of analysis done with the NLS and zinc finger region of PXR is presented in Table 2.

PXR remains bound to its target sequence sites during mitosis

It is now known that PXR is a nuclear protein and remains constitutively bound to the mitotic chromatin during cell division. But this information does not specify if the PXR is associated at the unique or specific sequences during mitosis. Whether PXR associates with its target gene promoters during mitosis and if these specific sequences are accessible to it during cell division? Therefore, to answer these questions, we analyzed well-characterized PXR-binding site for occupancy during mitosis by chromatin immunoprecipitation (ChIP) using PXR antibody against the cross-linked ER-6 region of

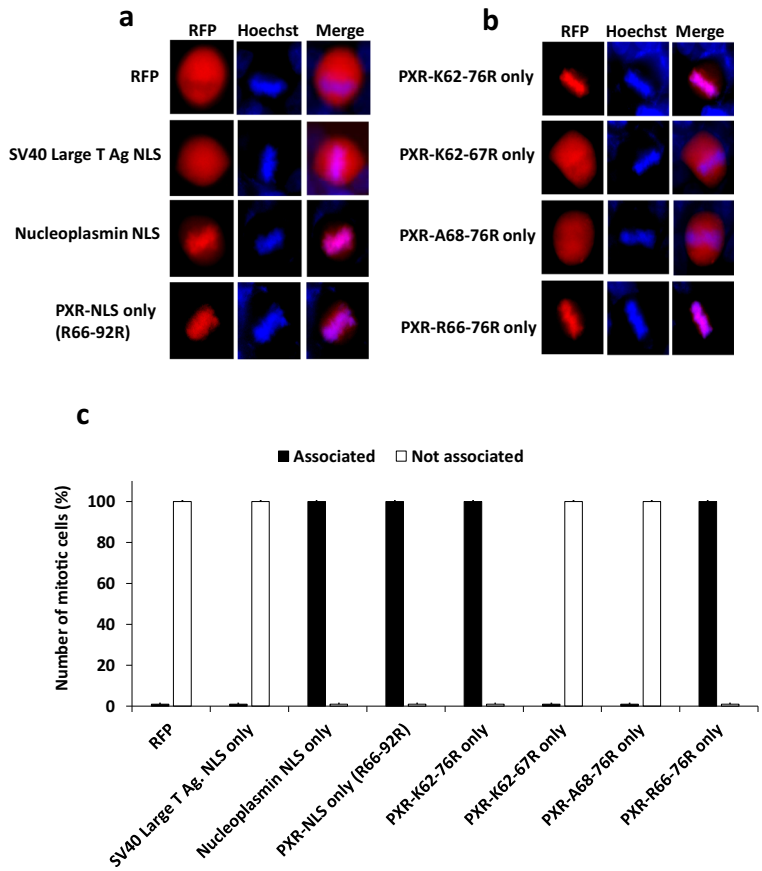

Fig. 8 Amino acid sequence R66-76R (MCBR) in NLS of PXR is important for constitutive association with the mitotic chromatin. HEK-293T cells were transiently transfected individually with RFP-tagged receptor plasmid constructs as depicted in the figure. After 24 h of expression period, mitotic cells were observed and at least 15–20 mitotic cells were counted in each case. Each experiment was repeated for at least three times. To facilitate visualization of the nucleus, Hoechst dye was added to the live cells at least 2 h prior to imaging. **a**, **b** Representative cells showing status of the particular amino acid residue sequence in relation to the interaction with the mitotic chromatin. **c** Quantitative data in percentage for association or not association of the particular amino acid residue sequence with the mitotic chromatin was assessed and results represent the mean ± SD of three independent experiments

CYP3A4 promoter. This promoter is a well-established response element of PXR (Blumberg et al. 1998). Chromatin was prepared from asynchronous HepG2 cells containing interphase chromatin as well as from nocodazole-synchronized and FACS-sorted mitotic cells (Fig. 10b, c). These two sets of cells were used for ChIP analysis.

For mitotic ChIP analysis, HepG2 cells were treated with nocodazole (100 ng/ml) for 16 h and sorted by FACs with propidium iodide staining. This yielded more than 90% pure mitotic cells. The mitotic cells were then cross-linked with formaldehyde and DNA-protein complex was immunoprecipitated with rabbit IgG (pre-immune) and human PXR antibody. Immunoprecipitated DNA was processed and analyzed by PCR. The promoter of human CYP3A4 containing ER6 element in the distal xenobiotic-responsive enhancer module (XREM) was used as the primer sequence for PCR analysis. ChIP assays showed specific PXR binding in asynchronous chromatin as well as in the synchronized mitotic cells. The pre-immune serum served as the negative control (Fig. 10). Overall, the ChIP results indicated the existence of binding of PXR to the ER6 (CYP3A4 promoter) region during the interphase as well as during the mitosis.

Discussion

During cell division, the transition of interphase chromatin to mitotic chromatin is a highly dramatic event which is characterized by chromatin condensation up to 10,000-folds and reduction of the occupied volume as revealed by live cell imaging experiments and FLIM-FRET analyses (Li et al. 1998; Lleres et al. 2009; Woodcock and Ghosh 2010). Condensed chromatin is believed to be characterized by silencing of nuclear transcription. Several mechanisms are suggested to lead to this phenomenon. First, transcriptional repression has been credited to phosphorylation of RNA Pol I and Pol II and of the general transcription factors TFIIH and TFIID (Akoulitchev and Reinberg 1998; Egli et al. 2008). Second, inactivation of transcription factors by

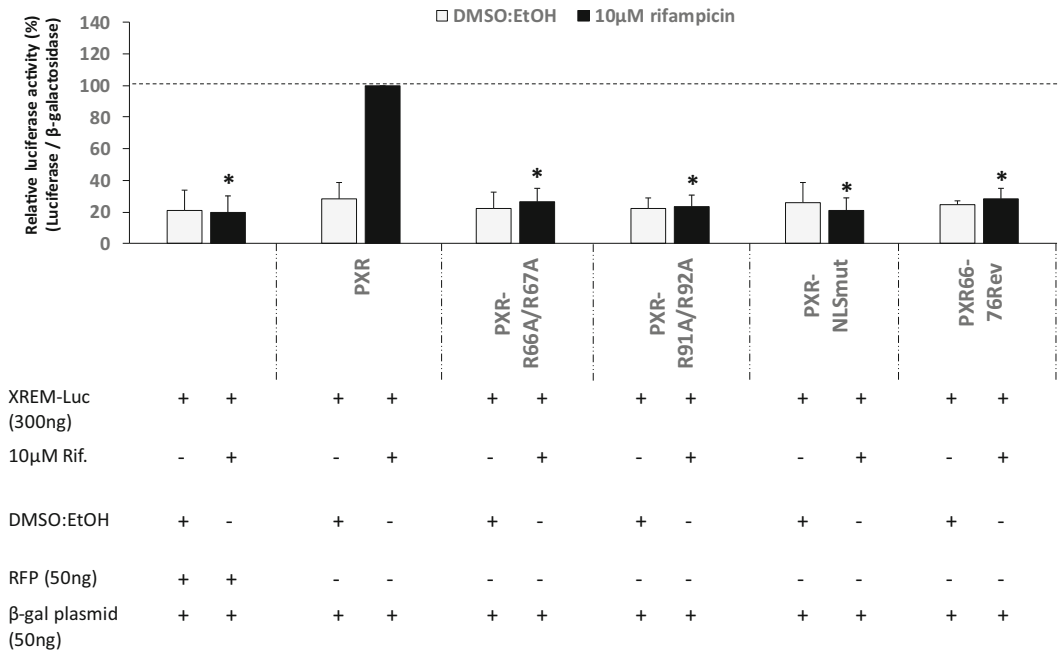

Fig. 9 Effect of mutations in NLS region on transcriptional activity of PXR. HepG2 cells were individually transfected with RFP-PXR, RFP-PXR-R66A/R67A, RFP-PXR-R91A/R92A, RFP-PXR-NLSmut, and RFP-PXR-R66-76R-Rev together with promoter-reporter plasmid XREM-Luc according to the scheme as indicated on the figure. After 14 h of transfection period, cells were treated with 0.1% DMSO:EtOH or 10 μM rifampicin as per scheme shown on the figure. Cells were allowed to express the protein under steroid- and antibiotic-free conditions for 24 h and promoter-reporter luciferase assay was done. Luciferase values were normalized to β-galactosidase and relative luciferase activity was calculated in comparison to rifampicin-induced RFP-PXR activity, which was considered as 100%. Average values of three independent experiments with ± SD are plotted. Asterisks (*) signify luciferase values that differed significantly from the rifampicin-induced RFP-PXR transfected cells ($p \leq 0.05$). RFP-PXR showed almost threefold increase in transcriptional activity while RFP-PXR-R66A/R67A, RFP-PXR-R91A/R92A, RFP-PXR-NLSmut, and RFP-PXR-R66-76R-Rev did not show any significant induction with rifampicin (10 μM). Rev, reverse sequence

Cdks (Heix et al. 1998; Sirri et al. 1999). Third, histone modifications (phosphorylation, methylation, acetylation, etc) or changes in chromatin proteins could contribute to transcriptional silencing (Sif et al. 1998). Finally, reduced accessibility of binding sites may lead to disengagement of transcription factors from their specific target sites.

However, in recent years, a puzzling query is emerging as to why the condensed mitotic chromatin retains only some of the transcription factors while most others are displaced from mitotic chromatin (Saradhi et al. 2005; Kumar et al. 2012; Zaret 2014; Festuccia et al. 2016; Raccaud and Suter 2018). To explain this phenomenon, different justifications have been provided in the literature. A few of these hypotheses suggest that as the cells move out of mitosis and the chromatin opens, the marked sites of these proteins in chromatin serve as a target site for the rapid reassociation and reactivation of transcriptional apparatus. This is suggestive of a phenomenon that is justly termed as 'epigenetic bookmarking'. Another proposed role of bookmarking during cell cycle is to provide specific genes that must be activated in the early G1 phase of the cell cycle, such as hsp70i gene (Xing et al. 2005). The hsp70i gene codes for a heat shock protein critical for cell survival from cellular stress. HSF2, a transcription factor, mediates the hsp70i bookmarking by binding to its promoter sites during mitosis and preventing compaction at the binding site during mitosis. Thereby, the situation provides the emerging daughter cells the ability to quickly recreate the transcription ability on their promoters when the general mitotic repression of transcription is withdrawn (Chen et al. 2005; Kumar et al. 2012; Palozola et al. 2017). Another explanation suggests that the association of transcription factors to the condensed mitotic chromatins might be essential to maintain chromatin architecture of target genes in the interphase nuclei, as revealed in the case of Runx protein and some

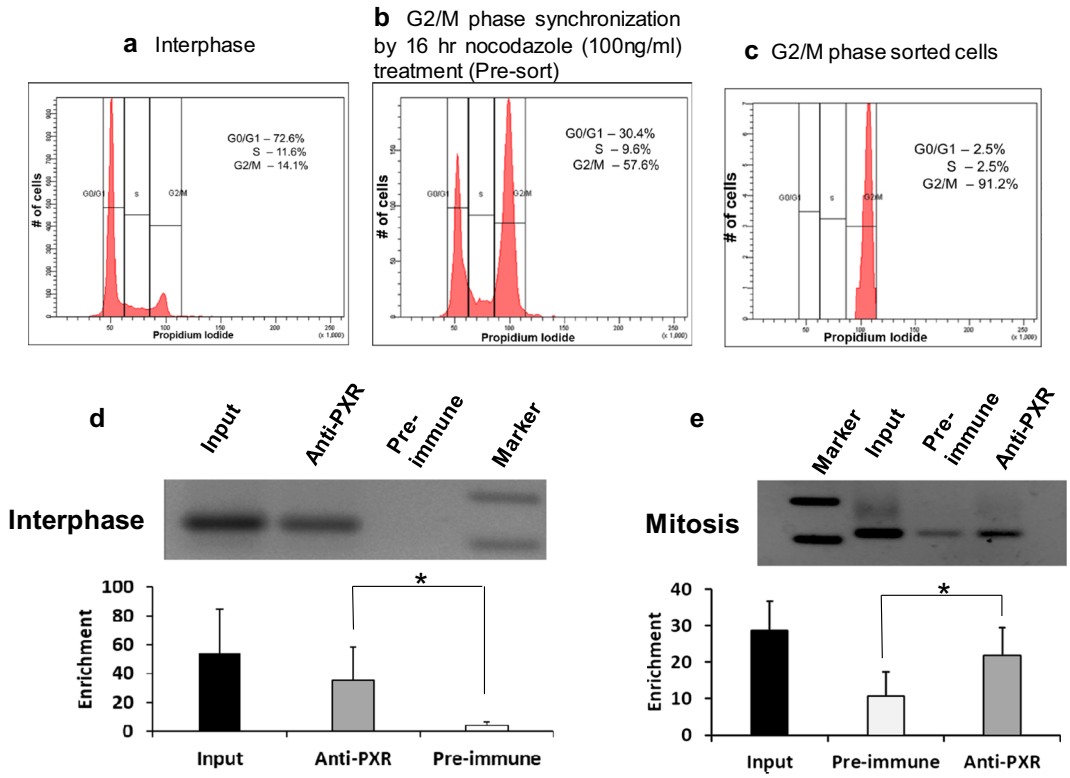

Fig. 10 Human PXR remains associated at its specific target sites both during interphase and during mitosis. Chromatin prepared from **a** interphase (asynchronous) and **b**, **c** mitotic HepG2 cells (synchronized with nocodazole for 16 h and sorted out with about 91% purity by the help of cell sorter) was fixed with 1% formaldehyde for the ChIP assay and precipitated with antibody against PXR or with pre-immune serum (control). One-tenth aliquot of the total lysate (input DNA) was used as a positive control. **d**, **e** DNA was then isolated from the samples and PCR analysis performed with the primers specific to ER6 region of CYP3A4 promoter that yielded an expected product size of 215 bp. Quantitation data represents mean ± SD of three independent experiments. Asterisks (*) signify enrichment values that differed significantly between pre-immune and anti-PXR pulled samples ($p \leq 0.05$)

other transcription factors (Young et al. 2007; Kumar et al 2012; Raccaud and Suter 2018). The significance of this association with mitotic chromatin has been speculated but mechanistic details and physiological implications of this phenomenon remain to be extensively investigated. Kumar et al. (2012) speculated that such a phenomenon plays a crucial role in the maintenance of cell-specific proteome by gene bookmarking during cell cycle. The phenomenon has also been explained through a BIOPIT (Bimolecular Imprints Offered to Progeny for Inheritance of Traits) model to divulge retention and transmission of cellular 'transcription memory' (Kumar et al. 2012). One of the most important observations from the previous reports is that the transcription factors which remain associated to the mitotic chromatin not only may have roles in cell fate decision but also may act as 'pioneer factors' (Kumar et al. 2012; Drouin 2014; Raccaud and Suter 2018). The associating transcription factors include some of the nuclear receptors; however, the physiological significance of their association with the mitotic chromatin is still ambiguous.

In the present study, we have suggested that PXR may function as an 'epigenetic bookmark' during cell cycle by remaining bound to its target regions even during mitosis. In our attempt to identify the specific domain(s) of PXR responsible for mitotic chromatin association, we found that amongst all the PXR domains, it was the DBD of the receptor containing both the Zn fingers, which executed this association. However, as expected, DBD alone failed to transcriptionally activate PXR as was reflected in our promoter-reporter assays.

PXR, the master regulator of the xenobiotic metabolism, due to its promiscuous ligand binding nature, can be activated by a large category of endobiotics and xenobiotics which includes many of the clinical drugs.

In addition to this, PXR plays crucial roles in multiple physiological processes in human body which ranges from bile acid metabolism to glucose homeostasis (Pondugula et al. 2009). Kawana et al. 2003 reported a bipartite NLS in human PXR which comprises of 27 amino acid residues and ranges from R66 to R92 part of which is in the second zinc finger region and remaining in between the two zinc fingers. By in silico analysis, we have observed that the amino acid residues R66/R67 and R91/R92 are the critical residues in the DBD of PXR, that are essential for the interaction with target DNA sequence (e.g. ER6 region in CYP3A4 promoter) or chromatin. This interaction was confirmed by receptor function assays via promoter-reporter analysis. Also, in silico analysis revealed that NLS region of PXR is favourable for interaction with DNA (ER6 region). In this context, all the NLS mutants of PXR showed loss of association with the mitotic chromatin. So, in search for the region/amino acid residues in PXR-DBD, and the role of zinc fingers in interaction with the mitotic chromatin, we dissected the entire zinc finger region into smaller parts. We observed that each of the two GFP-tagged zinc fingers independently and predominantly localized in the cytoplasmic compartment of the cell and was unable to associate with the mitotic chromatin. It is known that the first zinc finger covers the region C41-C61 and second zinc finger C77-102C with NLS region spread over R66-R92. Therefore, this behaviour may be attributed to the absence of complete NLS region in either of the separated zinc fingers. This appears to imply that the zinc fingers may not be critically involved in this initial interaction but for subsequent stabilization and specificity of the receptor-DNA association (Claessens and Gewirth 2004; Cutress et al. 2008; Chang et al. 2010). To gain further insights into the role of NLS region of PXR, we cloned this region with RFPtag and observed the subcellular localization and interaction with the mitotic chromatin. As per expectation, RFP-NLS localized predominantly in the nuclear compartment and showed a strong interaction with the mitotic chromatin, suggesting that NLS region is responsible for the interactions with the mitotic chromatin. Since NLS region is composed of mostly basic amino acid residues and DNA contains the negative charge, it rationally raises the possibility that this interaction may be a consequence of interaction between two oppositely charged molecules. To verify this possibility, we made RFP-tagged constructs of two classical NLS, i.e. monopartite NLS of SV40 large T and bipartite NLS of nucleoplasmin protein (LaCasse and Lefebvre 1995). Interestingly, we observed that RFP-tagged NLS of SV40 large T antigen does not associate with the mitotic chromatin. On the contrary, the bipartite NLS of nucleoplasmin protein associates with the mitotic chromatin. This implied that the positive charged amino acid residues may not play an exclusive role in this phenomenon whereas bipartite nature of NLS may have a role. To further establish the role of PXR-NLS region in mitosis, we also cloned the central portion lying between the two zinc fingers (R62-76R) into RFP vector. To our surprise, we found this to be completely nuclear and it also associated constitutively with the mitotic chromatin. Subsequently, we dissected this region, i.e. R62-76R, into two halves, i.e. K62-67R and A68-76R, and cloned these two regions into RFP vectors. Interestingly, both the constructs when expressed showed predominantly cytoplasmic localization and did not show any interaction with the mitotic chromatin. When R66-76R region was expressed as a RFP chimera, complete nuclear localization was observed. This established that R66 and R67 amino acid residues are critical and the region R66-76R of human PXR is the minimal region determinant of mitotic chromatin association. This was also supported by our in silico analysis as mutation in R66/R67 to A66/A67 resulted in loss of more hydrogen bonds between PXR and DNA (ER6 region) in comparison to R91A/R92A and R66A/R67A/R91A/R92A (NLS mutant). In our functional assays, PXR with these specific mutations revealed no transcriptional activity implying the absence of interaction with ER6 region (used in this promoter assays). To further confirm if this minimal region (R66-76R) acts as the mitotic chromatin binding-determining region (MCBR) and also to examine whether the sequence or charge of this region is important, we reversed the amino acid sequence of this region, i.e. converted R66-R76 to R76-R66, in the full-length PXR protein. Interestingly, it was observed that mitotic chromatin association was completely abolished. This suggested that the sequence rather than charge of this region is essential in this interaction. This observation was further corroborated where a reverse sequence of this specific region failed to bind and activate ER6 in the promoter-reporter assays. The overall observations imply that receptor interaction with chromatin and transcription function are intrinsic features which require (i) specific orientation of MCBR, (ii) zinc finger(s), and (iii) competent receptor conformation. In case any of these features are compromised, the full-length receptor may exhibit only isolated action of mitotic association

without transcription output. Similarly, compromising with MCBR sequence may also abrogate chromatin binding and receptor function. In conclusion, we term this R66-76R region as the 'mitotic chromatin binding-determining region' (MCBR) of human PXR which is involved in constitutive association with the mitotic chromatin. Also, from the receptor function assays, it is suggested that this MCBR region may be responsible for the association with the ER6 region in CYP3A4 promoter. However, how interplay amongst the multiple receptor features along with diverse interacting components of gene transcription operate in cellular context warrants further studies.

In the literature, there are a few reports of some proteins that associate with mitotic chromatin and occupy target gene promoters which are maintained in an open chromatin configuration even during mitosis (e.g. HMGN1, CTCF, FoxI1, Cyclin B1, Esrrb) (Sciortino et al. 2001; Pallier et al. 2003; Harrer et al. 2004; Burke et al. 2005; Yan et al. 2006; Festuccia et al. 2016). In this context, we also show here for the first time that a nuclear receptor interacts with DNA within mitotic chromatin at its target promoter site(s). It is observed that PXR which associates with ER6 region (CYP3A4 promoter) during interphase remains associated at this specific site even during the mitosis as evident from our ChIP assays. So, as per the emerging concept, it is concluded that nuclear receptor PXR can function as a 'gene bookmark' during cell cycle. It implies that transcription of PXR target genes after mitotic exit resumes the progenitor's transcription programmes maintaining cell identity.

Human nuclear receptors constitute a superfamily of 48 transcription factors which govern many important functions of body ranging from metabolism to reproduction. The findings that nuclear receptor bind to mitotic chromatin warrant detailed studies of interactions with their target promoter/enhancer sites and their role in gene transcription reactivations upon mitotic exit. With specific reference to PXR, which is the 'master regulator' of xenobiotic metabolism, it is important to examine if such molecular interactions between receptor and mitotic chromatin have functional physiological significance in some of the aberrant clinical management conditions (Chen et al. 2007; Gupta and Lee 2008; Takeyama et al. 2010). A detailed study of molecular basis of mitotic chromatin binding and their functional significance in 'mitotic bookmarking' not only will enable us to resolve molecular basis of metabolic disorders but also may pave the way for treating several heritable diseases.

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Authors' contributions RKT conceived, designed, and supervised the project. MR and AKD designed and performed most of the experiments. RKT, MR, and AKD analyzed the data and wrote the paper. KP did the in silico experiments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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