Studies on atypical nuclear receptor SHP (NR0B2): its influence on transcription regulation of its interacting partners

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CERTIFICATE

The research work embodied in this doctoral thesis entitled **"Studies on atypical nuclear receptor SHP (NR0B2): its influence on transcription regulation of its interacting partners"** has been carried out at the Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi-110067, India. The work presented here is original and has not been submitted in part or full for any degree or diploma of any University/Institution elsewhere.

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Date: 20/12/2019

Prof. Suman Kumar Dhar
(Chairperson)

Dedicated

to

my

Family

Friends

&

Teachers

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Acknowledgement

PhD journey is the hardest time but most beautiful part of life always!!!

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ABBREVIATIONS

aa	Amino Acid
AF-1	Activation Factor 1
AF-2	Activation Factor 2
APS	Ammonium Persulphate
AR	Androgen Receptor
ATCC	American Type Cell Culture
ATP	Adenosine Triphosphate
bp	Base Pair
BIOPIT	Biomolecular Imprints Offered to Progeny for Inheritance of Traits
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
CAR	Constitutive Androstane Receptor
Cat. No.	Catalogue Number
cDNA	Complementary DNA
CO_2	Carbon Dioxide
Ct	Cycle threshold
CYPs	Cytochrome p-450 Enzymes
DAB	3,3-Diaminobenzidine
DBD	DNA Binding Domain
D-Box	Distal (Dimerization) Box
DDW	Double Deionised Water
DEPC	Diethyl pyrocarbonat
DME	Drug Metabolizing Enzyme
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide Triphosphate
DR-1	Direct Repeat 1
DTT	Dithiothreitol
ECL	Enhance Chemi-Luminescence
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra acetate
et al.,	et alia (and others)
ERα	Estrogen Receptor-α
FBS	Fetal Bovine Serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
FXR	Farnesoid X Receptor
g	Gram
GFP	Green Fluorescent Protein
GR	Glucocorticoid Receptor
HABP1	Hyaluronan Binding Protein 1

HADC	Histone deacetylases
HAT	Histone acetyltransferase
H&E	Hematoxylin and Eosin
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HCC	Hepatocellular Carcinoma
HMG	High mobility-group protein (HMGB1, HMGB2, HMGA1a)
HNF4α	Hepatocyte Nuclear factor 4α
Hr	Hour
HRP	Horse Radish Peroxidase
H_2O	Water
H_2O_2	hydrogen peroxide
HSP	Heat shock protein
HCl	hydrochloric acid
HREs	Hormone Response Elements
ICC	Immunocytochemistry
IgG	Immunoglubin G
IHC	Immunohistochemistry
IPA	Isopropanol
IPTG	Iso-Propyl Thio-D-galacto pyranoside
IR	Inverted repeats
Kb	kilo base
KCl	Potassium Chloride
kDa	kilo Dalton
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2SO_4	Sodium di-hydrogen phosphate
NCBI	National Centre for Biotechnology Information
NP-40	Nonidet P-40
KV	Kilo Volt
LB	Luria Bertani
LBD	Ligand binding domain
Luc	Luciferase
LXR	Liver X Receptor
M	Molar
MCS	Multiple Cloning Site
Mg	Milligram
Mg^{2+}	Magnesium Cation
$MgCl_2$	Magnesium Chloride
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
µg	Microgram
µl	Microliter
µM	Micromolar
min	Minutes
mM	Millimolar
MR	Mineralocorticoid Receptor

MW	Molecular Weight
NaCl	Sodium Chloride
NaOH	sodium hydroxide
NES	Nuclear Export Signal
NR	Nuclear Receptors
NFκB	Nuclear Factor
NLS	Nuclear localizing signal
Nm	Nano meter
NTD	N-Terminal Domain
O_2	Oxygen
OD	Optical Density
Ori	Origin of Replication
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline + Tween 20
PFA	Paraformaldehyde
PPAR	Peroxisome Proliferated- Activated Receptor
PCR	Polymerase Chain Reaction
PMSF	Phenyl Methyl Sulphonyl Fluoride
pH	Power of Hydrogen
Pi	Inorganic Phosphate
PR	Progesterone Receptor
PTM	Post-translational modification
PXR	Pregnane and Xenobiotic Receptor
PVDF	Poly-Vinyl-Di-Fluoride
RA	Retinoic acid
RAR	Retinoic Acid Receptor
RFP	Red Fluorescent Protein (pDsRedExpress)
RIPA	Radioimmunoprecipitation Buffer
RNA	Ribonucleic Acid
Rpm	Revolutions Per Minute
RT	Room Temperature
Runx2	Runt-related transcription factor 2
RXR	Retinoid X Receptor
s	Second
SD	Standard Devision
SDS	sodium dodecyl sulphate
SHP	Small Heterodimer Partner
SMRT	Silencing Mediator for Retinoid and Thyroid-hormone receptors
SNP	Single Nucleotide Polymorphism
snRNP	Small nuclear ribonucleoproteins
SRC	Steroid Receptor Coactivator
STAT3	Signal transducer and activator of transcription 3

TAE	Tris-Acetate-EDTA
TAF	TBP-associated factors
TBE	Tris - borate – EDTA
TBS	Tris Buffer Saline
TBST	Tris-Buffer saline (with 0.1% tween 20)
TEMED	N,N,N',N'- tetramethylethylenediamine tetraacetic acid
TFIIB	Transcription factor IIB
TFIID	Transcription factor IID
TFs	Transcription factors
TR	Thyroid Receptor
TRIS	Tris (Hydroxymethyl) aminomethane
Tris.HCl	Tris hydrochloride
TSS	Transcription start sites
U	Unit
UBF	Upstream binding factor
UBTF	Upstream binding transcription factor (RNA polymerase 1)
VDR	Vitamin D Receptor
WHO	World Health Organization
WT	wild type
XREM	Xenobiotic response element
%	Percentage
+	Positive
-	Negative
α	Alpha
β	Beta
γ	Gamma
Δ	Deletion
~	Approximately
℃	Degree Celsius
≥	Greater than equal to
≤	Lesser than equal to

Introduction

The intricate life cycle starting with embryogenesis and involving coordination of all vital functions within an organism is primarily an outcome of cell-cell signaling. This signaling is assured by messenger molecules, which either bind to membrane receptors or, if they are lipophilic and small enough to cross the membrane, they often interact with the members of the nuclear receptors superfamily [1–3]. Nuclear Receptors (NRs) are ligand-modulated transcription factors that regulate the expression of hundreds of their target genes. NRs play pivotal roles in reproduction, development, metabolism and overall body's homeostasis. Their dysfunction can exert a wide range of proliferative, reproductive and metabolic diseases including obesity, diabetes, inflammation, cancer etc. [1,4–6].

Typically, NRs have characteristic structural and functional features. These features include i) N-terminal ligand-independent transactivation domain-containing activation function 1 (AF-1), ii) a highly conserved DNA binding domain (DBD) containing two zinc fingers, iii) a hinge domain, and iv) a C-terminal ligand-binding domain (LBD) which contains ligand-dependent AF-2 [7]. Uniquely, out of all the 48 human NRs, SHP (NR0B2; Small Heterodimer partner) and DAX-1 (NR0B1; Dosage-sensitive sex reversal, Adrenal hypoplasia critical region, on chromosome X, gene 1) are structurally and functionally different from typical NRs. They contain all the putative domains but lack the most conserved DBD which makes them exceptional [8,9].

SHP was discovered in 1996 on the basis of its interaction with several conventional and orphan receptors of the superfamily [9]. In humans, SHP's genomic architecture consists of two exons that are interrupted by a single intron spanning approximately 1.8 kb and it is located on chromosome 1 at 1p36.1 [10]. SHP protein consists of 257 amino acid residues with a molecular mass of 28 kDa. In humans, its mRNA is predominately expressed in the liver and gallbladder but also in other organs albeit at lower levels [10–12].

SHP is suggested to function as a transcriptional coregulator of gene expression through interaction with several NRs [13,14]. However, the repression mechanisms of SHP are still ambiguous and warrant further investigation at the varied platforms. In one of the suggested mechanisms, SHP binds to AF2 NR helixes through two LXXLL motifs located on the N-terminal helix 1 and C-terminal helix 5 culminating in direct competition with NR-binding coactivators [15,16]. Interestingly, competition between the NR coactivator(s) and the SHP is suggested to influence some NRs function. Apart

from NRs, several new SHP interacting partners have also been identified recently. These include, for example, helix-loop-helix transcription factor BETA2/NeuroD [17] and interaction with Foxo1 [18]. Furthermore, SHP has also been reported to mediate inhibition of transforming growth factor-beta (TGF-β)-induced gene expression by explicit repression of Smad3 transactivation [19]. In addition, it is proposed that SHP may act as a direct transcription repressor by recruiting corepressors, mSin3A/HDAC, category III histone deacetylase SIRT1, G9a methyltransferase and the Brm-containing Swi/Snf remodeling complex [20,21]. SHP is also shown to inhibit transcription function of some of the NRs (e.g. RAR-RXR, RAR-PXR heterodimers and ERα or HNF4α homodimers) using one or more of approaches like promoter-reporter, EMSA and chromatin-immunoprecipitation assays [9,18,22]. In a similar context, SHP is suggested to act as the regulator of several other transcription factors [13,23]. Though not conclusively, is suggested that SHP can function both as a transcriptional repressor or coactivator (e.g. PPARs and NF-kappa B) [12,24].

In addition to the above, various important biological roles of SHP has been reported. For example, SHP i) is shown to act as a negative regulator of NLRP3 inflammasome [25], ii) plays an antitumor role in liver cancer [14], iii) is associated in homocysteine homeostasis with FOXA-1 [19], iv) is involved in hepatic gluconeogenesis through STAT5 pathway [26], v) acts as a novel antihypertrophic regulator by interfering with GATA6 signalling [27], vi) regulates liver lipid metabolism [28] and vii) is also involved in apoptosis signalling (both inhibitory and stimulatory) [16]. These reports are suggestive of SHP as an important member of the NR superfamily.

Several studies were conducted to identify the ligand(s) for SHP, but till date, no direct ligand has been reported [29]. Nonetheless, some reports suggest that FXR ligands GW4064 [30,31], androsterone, bile acids (BA) and chenodeoxycholic acid (CDCA) [12,32] are effective inducers of SHP gene expression. In recent years, it has been shown that steatotic drugs in advanced non-alcoholic fatty acid diseases exhibit repression of SHP [33]. Additionally, post-translational modifications are reported to regulate transcriptional regulatory functions of SHP [34].

The unique structural and functional property of SHP distinguishes it from other conventional NRs. The functional cross-talk of SHP with other interacting partners is interesting and important for normal physiological controls as well as in the states of dysregulation. This warrants for an extensive investigation on the regulatory

mechanisms of SHP and relationship between NRs or other transcription factors. Such studies may not only initiate new avenues to understand the SHP-influenced disease mechanisms may also provide novel therapeutic interventions. From the above discussion, it is apparent that SHP is an emerging atypical nuclear receptor that awaits extensive investigation to assess its subcellular functional dynamics and transcriptional influence on its heterodimeric partners.

Hypothesis of the study

During cell division, termination of transcription is associated with chromatin condensation. It has recently been observed that several transcription factors, including some nuclear receptors, are associated with mitotic chromatin during cell division. The mitotic chromatin binding sites of different nuclear receptors are suggested to be receptor-specific [35]. Till date, different modes of mitotic chromatin associations of NRs have been observed: i) Constitutive association (example: PXR, CAR, FXR and VDR) ii) Ligand-mediated association (example: AR and ERα) and iii) Partner-induced association (example: RXRα).

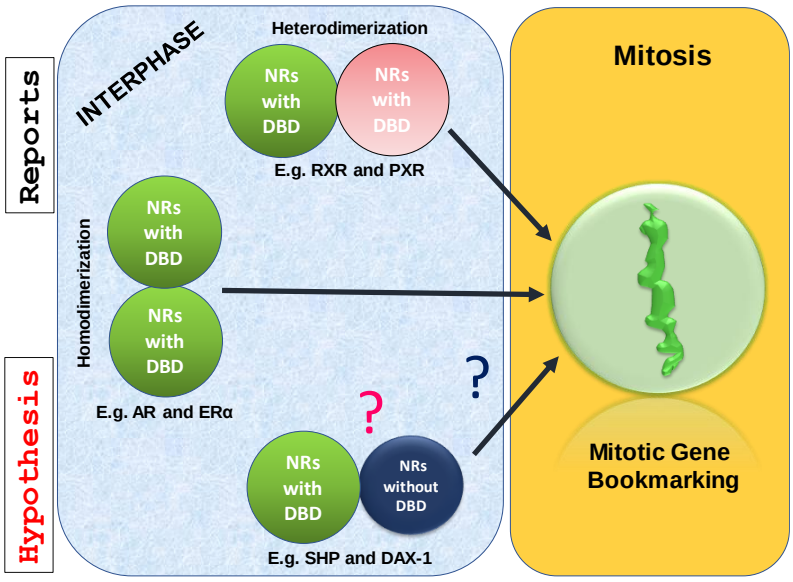


Figure 1. Schematic representation of the hypothesis. *Hypothetical model depicting the docking, retention and transmission of transcription factor blueprint from progenitor to progeny cells. Nuclear receptors (NRs) are intracellular, ligand-modulated transcription factors several of which have reported to associate with the mitotic chromatin (example: PXR, CAR, AR and ER). All these factors contain the DNA binding domain that helps in a mitotic chromatin binding. On contrary, SHP and DAX-1 do not harbour DBD. Whether and how it will be associated with mitotic chromatin is a subject of speculation. If yes, what mode of interaction will be used to achieve such interactions?*

A detailed study from our laboratory reported that for mitotic chromatin association of a nuclear receptor, DBD is essential [36]. Recent reports have suggested that the nuclear localization signal (NLS) region plays a major role in this interaction with mitotic chromatin [36,37]. In this context, interesting questions that arise are: i) Does SHP interact with mitotic chromatin in the absence of its DBD? ii) In case it does interact what would be the mode of its interaction? iii) Do these interactions have physiological relevance? iv) Can these findings explain as to how SHP influences the transcription function of its interacting partners? A schematic presentation on the current understanding of the intermolecular interplay is in context to the proposed hypothesis is shown in **Fig. 1 and 2**.

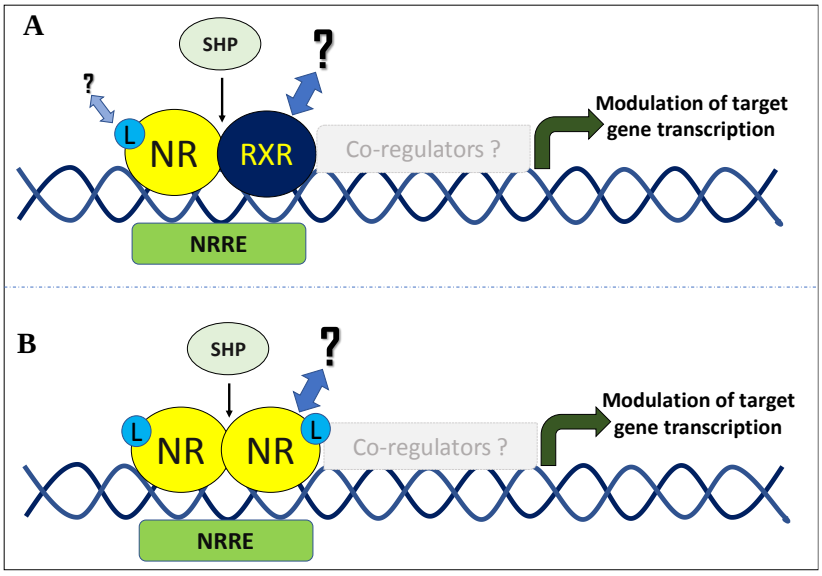


Figure 2. Schematic representation of the hypothesis to depict how (A) SHP would modulate the transcriptional role of heterodimer partners and (B) homodimer partners?

In the view of the recent literature and the hypothesis presented herein, the following three objectives are framed as follows:

Aims and objectives

1. To study the subcellular dynamics of SHP by immunological approaches and live cell imaging.
2. To investigate the inter- and intra-molecular determinants of SHP influencing the transcription function of its interacting nuclear receptors.
3. To examine the influence of SHP on the expression of its interacting nuclear receptors.

Review of Literature

Review of Literature

The Nuclear Receptor Superfamily is a class of ligand-modulated transcription factors which consists of 48 known members in the human genome. Nuclear Receptors (NRs) play pivotal roles in all major events of life including reproduction, development, metabolism and immunity, making them one of the main target areas of advanced therapy and research [38–40]. Their dysfunction can wield a wide range of metabolic diseases, which includes inflammation, diabetes, obesity, endocrine cancers (breast/prostate/endometrial cancers) [41–47]. NRs cover steroid/thyroid endocrine receptors and orphan/adopted receptors. They function as intracellular transcription factors (TF) to control the expression of plenty of genes in response to their cognate ligands.

Interestingly, NRs conjointly can also function as "epigenetic marks" for the retention and transmission of cellular "transcriptional memory". These receptors primarily operate, either as homodimers or as heterodimers with RXR (Retinoid X Receptor). Sometimes they may also function as monomers to induce or repress the transcription function via orphan receptors such as SHP and DAX-1. Markedly, about 15% of the clinically approved drugs target NRs. Being 'drug-responsive', these receptors promise an enormous scope for exploring new molecules and chances for improving upon the existing ones [28,48,49]. However, plenty of the recently discovered members of the NR family remain partially understood in terms of their physiological roles and activating ligands. In brief, NRs possess considerable potential for drug discovery and their underlying mechanisms of action [6,50].

An evolving story of Nuclear Receptor Superfamily

NRs belong to a superfamily of major transcription factors that bind to lipophilic ligands selectively and transmits signals which can alter gene program [51]. Other than human NRs, there are about 900 NRs in the whole animal kingdom, considering their occurrence from simplest to the most complex [50,52]. The human NR superfamily is categorized into six evolutionary conserved groups supported by sequence alignment and phylogenetic tree construction.

Estrogen Receptor (ER) was the first NR of the family to be identified biochemically or "estrophilin" as Jensen called it [53]. He measured ER as a means to

predict hormone responsiveness in human breast cancer by developing monoclonal antibodies against the receptor [53].

In the mid of 1970s, Gustafsson and his co-workers found out that the Glucocorticoid Receptor (GR) consists of three distinct domains, the DBD, the LBD and the immunoreactive domain. The immunoreactive domain was discovered using the monoclonal and polyclonal anti-GR antibodies and is also called as N-terminal domain (NTD) [54]. It was also observed that the NTD is more susceptible to proteolytic digestion than the remaining two domains. A portion of GR cDNA was cloned and northern blots with GR mRNA was performed from lymphoma cell lines to study their peculiar glucocorticoid signaling [55]. However, the three-domain structure of the NR was confirmed only after full-length GR cDNA was available [56]. Next, the cDNA of ER [57] and Mineralocorticoid Receptor (MR) [58] were elucidated. The steroid hormone receptors were grouped as a subfamily under NR superfamily of transcription factors. Later, a small group of non-steroidal receptors was also added to the family, namely the thyroid hormone receptor (THR) [59], and the retinoic acid receptor (RAR) [60].

In 1988, Evan's group reported about the LBDs in NRs whose cognate ligands were not known; hence these NRs were named as orphan receptors. They reported the ER (alpha, beta, and gamma) as orphan receptors [61]. Later, the other orphan receptors such as TRs (TR alpha and beta) [62,63], all-trans RAR (α, β and γ) [60,64], and RXRβ were also cloned. Subsequently, in 1992, the RXR was found to be a heterodimeric partner of several NRs, including the orphan receptors [65,66]. Later, another orphan receptor named as Liver X receptor (LXR) was discovered and it was shown to have two isoforms LXRα and LXRβ. At first, the LXRs were classified as orphan receptors. Later on, it was found that endogenous oxygenated derivatives of cholesterol or oxysterols act as ligands of LXRs[67,68]. In addition to this, it was discovered that the LXR with the obligate partner RXR undergoes heterodimerization to form LXR/RXR heterodimer complex which can be activated by the agonist of both LXR and RXR. The activation of LXR/RXR heterodimer complex prevents bacteria-induced macrophage apoptosis, which suggested the possibility of using the LXR/RXR agonists in enhancing the innate immunity [69]. Also, in mammalian system the enactment of LXR/RXR signaling can hinder cell proliferation and activates apoptosis in pancreatic β-cells [70].

In 1996, Gustafsson and his co-workers discovered the second isoform of ER during the cloning of nuclear receptors from rat prostate and ovary and named it as ERβ [71]. The group reported the opposite expression pattern of ERβ to that of ERα in cancer diseases. In addition to cancer, ERβ was also found to influence CNS related disorders, therefore making ERβ a novel target to cure several physiological disorders [72]. Simultaneously in the same year i.e. 1996, an unusual receptor DAX-1 and atypical nuclear receptor SHP were discovered [9,73]. These receptors were devoid of the most conserved DBD region in their NR modular structure.

The pregnane X receptor (PXR) and constitutive androstane receptor (CAR) is the xenobiotic receptors. They are capable of transcriptionally modulating the expression of transporters, enzymes of phase I and phase II. David Moore's laboratory cloned CAR, a human nuclear receptor in the year 1994, and the mouse PXR and human PXR were cloned independently in Kliewer laboratory and Evans laboratory in the year 1998, respectively [74,75]. The name PXR is given due to its activation by the pregnanes (21-carbon steroids) by Kliewer's lab. Majorly, the PXR and CAR are involved in drug metabolism, drug-drug interactions and drug toxicity [76].

In the meantime, one more receptor was discovered during the 1990s in Xenopus frog, which induced proliferation of peroxisomes in cells and thus called Peroxisome proliferator-activated receptors (PPARs) [77]. PPARα was identified in rodent liver tissue as a peroxisome proliferator [78]. PPARδ was discovered in humans in 1992 [79]. At present, three types of PPARs have been identified namely PPARα, PPARβ/δ, and PPARγ. All PPARs bind with RXR to form heterodimers, which further bind to a gene called peroxisome proliferator hormone response elements (PPREs) at a specific region on the DNA. The natural ligands of PPARs include free fatty acids and eicosanoids. Till date, the researchers have found potential roles of PPARs in different pathological conditions like atherosclerosis, inflammation, cancer, infertility, and demyelination treatment of several chronic diseases [80].

Evans group had also identified one more nuclear receptor that formed a heterodimer with RXR which binds to ligand farnesol, thus, named as Farnesoid X Receptor (FXR). Primarily FXR functions as a bile acids sensor in liver and intestine as well as plays a vital role in the biotransformation of different metabolites [81]. Summary of landmark discoveries in the field of nuclear receptor research is mentioned in **(Fig. 3)**.

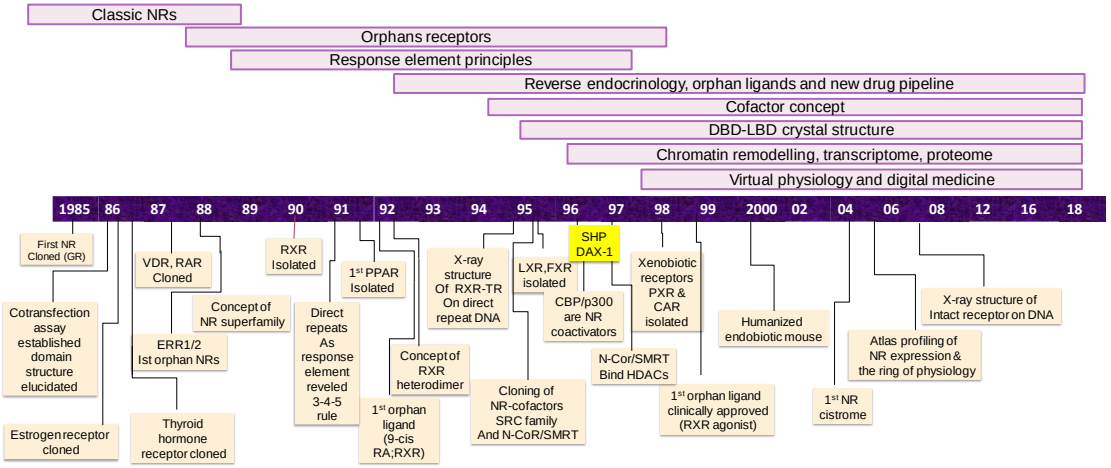


Figure 3. A timeline for the discovery of Nuclear Receptors and associated interacting partners. *A pictorial representation of milestone in the field of NRs. The entries start from the cloning of the first Glucocorticoid Receptor (GR) cDNA to more recent ''omics'' findings. SHP was shown to discovered in 1996. (Adapted and modified from Evans and Mangelsdorf 2014* [7] *)*

Generalized classification of Nuclear Receptors

Researchers working in the field of nuclear receptors classified different receptors, arranged them for a comprehensive study and determined their future perspective**.** In this case, they were classified according to a comparison of their sequences. The most similar sequences were grouped in the same set. This similarity between sequences also indicates an evolutionary history [82].

Homology classification

The homology classification system is an evolution-based system of the well-conserved domains of NRs, i.e. the DNA-binding C domain (most highly conserved domain in NR structure). This classification was recommended by the Nuclear Receptors Nomenclature Committee in 1999 [82]. It offers a practical and significant framework to which subsequent genes can be added in the Nuclear Receptors Nomenclature Committee [41]. A detailed summary of nuclear receptors is mentioned in **(Table 1)**.

Table 1: A summary of human nuclear receptors symbol, accession no, ligands and associated diseases

Subfamily and Group	Name of nuclear receptor / Abbreviation	Accession ID	NRNC symbol	Ligand	Diseases
0B	Dosage-sensitive sex reversal, adrenal hyperplasia critical region, on chromosome X, gene 1/ DAX1	P51843	NR0B1	*Orphan*	*Bone, Breast, Prostate*
	Small heterodimer partner/ SHP	Q15466	NR0B2	*Orphan*	
1A	Thyroid hormone receptor-α/ TRα	P10827	NR1A1	*Thyroid Hormone*	*Liver, Kidney, Pituitary, Breast, Thyroid*
	Thyroid hormone receptor-β/ TRβ	P10828	NR1A2	*Thyroid Hormone*	
1B	Retinoic acid receptor- α / RARα	P10276	NR1B1	*Retinoid acid*	*Leukemia, Breast, Head &Neck, Lung, Oral tissue, Cervix Ovary, Esophageal, Prostate*
	Retinoic acid receptor- β / RARβ	P10826	NR1B2	*Retinoid acid*	
	Retinoic acid receptor- γ / RARγ	P13631	NR1B3	*Retinoid acid*	
1C	Peroxisome proliferator-activated receptor- α / PPARα	Q07869	NR1C1	*Fatty acids*	*Brain, Lung, Stomach & Colon, Liver, Pancreas, Bladder, Breast, Testis, Bone*
	Peroxisome proliferator-activated receptor- β/δ / PPARβ/δ	Q03181	NR1C2	*Fatty acids*	
	Peroxisome proliferator-activated receptor- γ / PPARγ	P37231	NR1C3	*Fatty Acids*	
1D	Rev-erbAα / Rev-erbAα	P20393	NR1D1	*Heme*	*Neurological*
	Rev-erbAβ / Rev-erbAβ	Q14995	NR1D2	*Heme*	
1F	RAR-related orphan receptor-α / RORα	P35398	NR1F1	*Sterols*	*Lymphoma, Breast, Prostate*

	RAR-related orphan receptor-β / RORβ	Q92753	NR1F2	*Sterols*	
	RAR- related orphan receptor-γ /RORγ	P51449	NR1F3	*Sterols*	
1H	Liver X receptor-α / LXRα	Q13133	NR1H3	*Oxysterols*	*Prostate, Breast, Colon, Pancreas, Esophageal, Liver*
	Liver X receptor-β / LXRβ	P55055	NR1H2	*Oxysterols*	
	Farnesoid X receptor / FXR	Q96RI1	NR1H4	*Bile acid*	
1I	Vitamin D receptor / VDR	P11473	NR1I1	*1a,25-dihydroyvitaminD3 /Lithocholic acid*	*Colon, Prostate, Breast Colon, Breast, Ovary, Prostate, Esophagus, Endometrial Liver*
	Pregnane X receptor / PXR	O75469	NR1I2	*Endobiotic and Xenobiotics*	
	Constitutive androstane receptor / CAR	Q14994	NR1I3	*Xenobiotics*	
2A	Hepatocyte nuclear factor-4-α / HNF4α	P41235	NR2A1	*Fatty acids*	*Liver, Colon, Breast, Prostate*
	Hepatocyte nuclear factor-4-β / HNF4β	Q14541	NR2A2	*Fatty acids*	
2B	Retinoid X receptor-α/ RXRα	P19793	NR2B1	*9-Cis-RA/ docosahexaenoic acid*	*Breast, Colon*
	Retinoid X receptor-β/ RXRβ	P28702	NR2B2	*9-Cis-RA/ docosahexaenoic acid*	
	Retinoid X receptor-γ / RXRγ	P48443	NR2B3	*9-Cis-RA/ docosahexaenoic acid*	
2C	Testicular receptor 2/ TR2	P13056	NR2C1	*NA*	*Unkown*
	Testicular receptor 4/ TR4	P49116	NR2C2	*NA*	
2E	Homologue of Drosophila tailless gene / TLX	Q9Y466	NR2E1	*NA*	*pineal gland development, reproduction, circadian clock function*
	Photoreceptor cell-specific nuclear receptor / PNR	Q9Y5X4	NR2E3	*NA*	
2F	Chicken ovalbumin upstream promoter	P10589	NR2F1	*Orphan*	*Prostate, Lung, Breast*

	transcription factor 1/ COUP-TF1				
	Chicken ovalbumin upstream promoter transcription factor 2/ COUP-TF2	P24468	NR2F2	*Orphan*	
	V-erbA-related/ EAR-2	P10588	NR2F6	*Orphan*	
3A	Estrogen receptor-α / ERα	P03372	NR3A1	*Estrogens*	*Breast, Cervix, Colon, Liver, Lung, Pancreas, Prostate, Thyroid, Esophageal, Ovary, Adrenocortical*
	Estrogen receptor-β / ERβ	Q92731	NR3A2	*Estrogens*	
3B	Estrogen-related receptor-α/ ERRα	P11474	NR3B1	*Orphan*	*Prostate, Breast, Defenses, Bone metabolism*
	Estrogen-related receptor-β/ ERRβ	O95718	NR3B2	*Orphan*	
	Estrogen-related receptor-γ/ ERRγ	P62508	NR3B3	*Orphan*	
3C	Glucocorticoid receptor / GR	P04150	NR3C1	*Glucocorticoids*	*Prostate, Breast, Lung, Pancreas Ovary, Endometrial, Gallbladder, Breast Prostate, Breast*
	Mineralocorticoid receptor / MR	P08235	NR3C2	*Mineralocorticoids/ Glucocorticoids*	
	Progesterone receptor / PR	P06401	NR3C3	*Progesterone*	
	Androgen receptor / AR	P10275	NR3C4	*Androgens*	
4A	Nerve Growth Factor 1B/ Growth factor-inducible immediate early gene nur 77/ NGFIB/ Nur77	P22736	NR4A1	*Orphan*	*Breast, Colon, Prostate*
	Nuclear receptor related 1/ NURR1	P43354	NR4A2	*Orphan*	
	Neuron-derived orphan receptor 1/ NOR 1	Q92570	NR4A3	*Orphan*	
5A	Steroidogenic factor 1/ SF1	Q13285	NR5A1	*Phospholipids*	*Prostate, Adrenocortical Tumors Breast, Colon*
	Liver receptor homolog- 1/ LRH-1	O00482	NR5A2	*Phospholipids*	
6A	Germ cell nuclear factor/ GCNF	Q15406	NR6A1	*Orphan*	*NA*

[Adapted and modified from Roshan-Moniri et al. 2014; Dash and Tyagi 2016 [6,46]]

An overview of the Nuclear Receptor modular structure

Nuclear receptor superfamily shares structurally and functionally conserved proteins are having the molecular masses ranging from 25,000 to 100,000 Daltons (Da). They are comprised of a series of a distinct stretch of sequences, referred to as domains A to F originally defined by Krust et al., [83]. The 'NTD' is structurally and functionally the most variable domain while the 'DBD' is the most conserved region, with the latter being responsible for binding to the response element. The domain for ligand binding is denoted as 'LBD' **(Fig. 4)**.

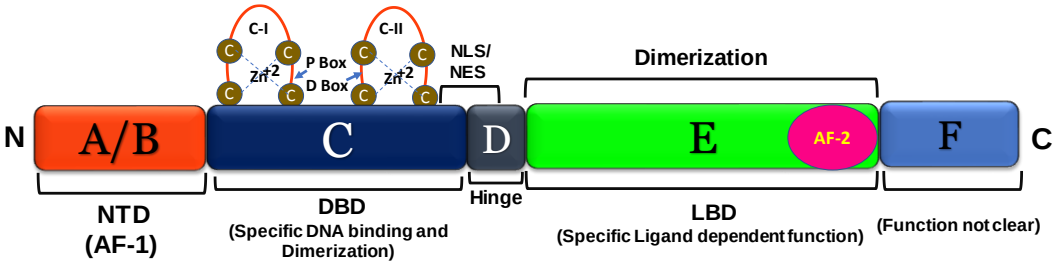


Figure 4: A general domain structures of nuclear receptors. *Different letters starting from A (N-terminal) and ending with F (C-terminal) signify different structural and functional domains whose details are explained in the text. A/B refers to N-terminal domain (NTD), C refers to DNA binding domain (DBD), D refers to Hinge region, E refers to Ligand binding domain (LBD) and F shown at the C-terminus is absent in most of the receptors. [Adopted and modified from (Dash and Tyagi 2016* [6]*].*

N-Terminal Domain (NTD): Structurally, most diverse and poorly understood A/B domain of NRs have a high rate of sequence diversity among NRs. The regions A and B cannot be separated from each other. The tertiary structure of this region has not been fully explained so far, which may be due to its high mobility. NTD of many NRs shows isoform-specific differences and these variations in sequence may induce different feedback to response elements and the process of transcriptional regulation [84]. This region incorporates one or more autonomous transcriptional activation function (AF-1) domain, which works in a ligand-independent manner and activates the basal transcription machinery when coupled to a heterologous DBD, [85] with an exception in case of steroid hormone receptors. It is suggested that in an unliganded state, steroid hormone receptors may have a silent AF-1. The length of NTD varies significantly from 23 amino acid residues (VDR) to 550 amino acid residues (AR, MR and GR). In order to produce a strong modulation of the target gene expression, AF-1 synergistically acts along with AF-2 which is present in the LBD of the NRs [6].

DNA-binding domain (DBD): DBD is highly conserved in different families and subfamilies of NRs and is accountable for sequence-specific DNA recognition. Due to its unique response element recognition and dimerization properties, this domain has been a significant focus of an investigation by many researchers. Several studies related to DBD of NRs yielded numerous X-ray, nuclear magnetic resonance (NMR) and protein crystallization data in their DNA complexed and un-complexed forms. The DBD is comprised of two zinc-finger motifs, the N-terminal motif Cys-X2-Cys-X13-CysX2-Cys (C-I) and the C-terminal motif Cys-X5-Cys-X9-Cys-X2-Cys (C-II). Each zinc-finger has four cysteine residues that chelate with one Zn^{2+} ion. In addition, the DBD encompasses many sequence elements (termed P-, D-, T- and A-boxes) that have now been characterized, and define (i) specificity of the response element (ii) an interface for dimerization and (iii) interaction with the DNA and (iv) DNA core recognition sequence. The NRs, are synthesized on ribosomes outside the nucleus. The nuclear localization signal (NLS), which is placed near the edge of the C and D domains imports the NR into the nucleus. DBD in most of the NRs contains the nuclear localization signal (NLS) and also the nuclear export signal (NES). These observations have also been made in the case of GR, ER, AR, LXR, RXR, PR, RAR, RevErb, TR and VDR [86–88]. However, xenobiotic receptors PXR and CAR make an exception as they are reported to have a leucine-rich NES in their LBD [6,89,90].

Hinge Region: Unlike region C and E, the Hinge region is less conserved. This domain primarily functions as a 'hinge' between the C and E domains and hence is termed as ‘hinge region’. It appears to execute cellular compartmentalization functions by helping DBD and LBD to overcome steric hindrance and in adopting different conformations. To be precise, this region confers conformational flexibility to the receptor. Thus, it indirectly helps region C and E in contributing to dimerization interfaces by promoting some receptors to lodge their specific heterodimeric partners and response elements for transactivation of the target gene. Region D further contains NLS or NES which contribute to the nucleo-cytoplasmic shuttling of the receptors [86,91,92]. The visible intracellular localization of NRs, therefore, could be a consequence of a dynamic balance between the viable strengths of these localization signals [6,87].

Ligand Binding Domain (LBD): NRs lipophilic ligand is sensed by high-affinity C-terminal LBD, which is characteristic of many nuclear receptors. The

LBD/region E can be regarded as the hallmark of an NR as it is very ordered and translates a means of diverse physiological functions, mostly operated in a ligand-dependent manner. Among the various NRs, this domain is highly conserved in its structure and moderately conserved in sequence. It can be regarded as the second most conserved region after DBD. The LBD also serves as a major binding site for different coactivators and corepressors. AF2 that acts a significant dimerization interface and sometimes features in a repression function forms a part of LBD [93–96]. Crystal structures of the LBD alone or in certain state with agonists, antagonists and coregulator peptides helped to review the detailed mode of action of NRs. Among all the domains of NRs, the LBD contributes most for the receptor dimerization process [6,84].

F-domain: The F domain is found at the C-terminus of LBD in only some of the NRs and exhibits least evolutionary conservation. The length varies from smaller to longer as in instances of estrogen and retinoic acid receptors. The function of this C-terminal sequence is still ambiguous. Some studies advocate that this region plays a part in recruiting the coactivator to the LBD and in determining its specificity [82,97]. This domain inherits symbolic structural options, however, it fine-tunes the transcription related events associated with the transcriptional properties executed *via* the LBD, or the whole receptor [98].

NR Signaling Mechanisms

NRs are multifunctional protein complexes that transmit their signal by binding to the cognate ligands with high affinity and thus controlling target gene expression through many distinct mechanisms, which includes activation and repression [39]. Following ligand binding, NRs undergo conformational changes and interact with a specific group of associated proteins within the nucleus, known as co-activators and corepressors [99]. Inside the nucleus, they bind to the specific DNA sequences called "hormone-responsive elements (HREs)" with a single copy of themselves or with another NR (e.g. RXR). Binding to HREs is managed through the NR's DBD's P-box. Such HREs are the bipartisan elements consisting of two half-site motifs of the hexameric core. These consensus HRE sequences are systematized either as direct (DR), inverted (IR) or everted (ER) repeats which are separated by the variable length of nucleotide spacers [6,38,39]. Ligand-bound NR's interaction with HRE signals the basal transcription machinery to either increase or decrease the target gene's

transcription. While the entire NR superfamily uses similar basic mechanisms for controlling the signaling, the specificity is dealt with by other mechanisms that are also essential to the abundance of biological effects of many receptor-related hormones and ligands. A widespread and pictorial mechanism of action by NRs is shown in **(Fig. 5)**.

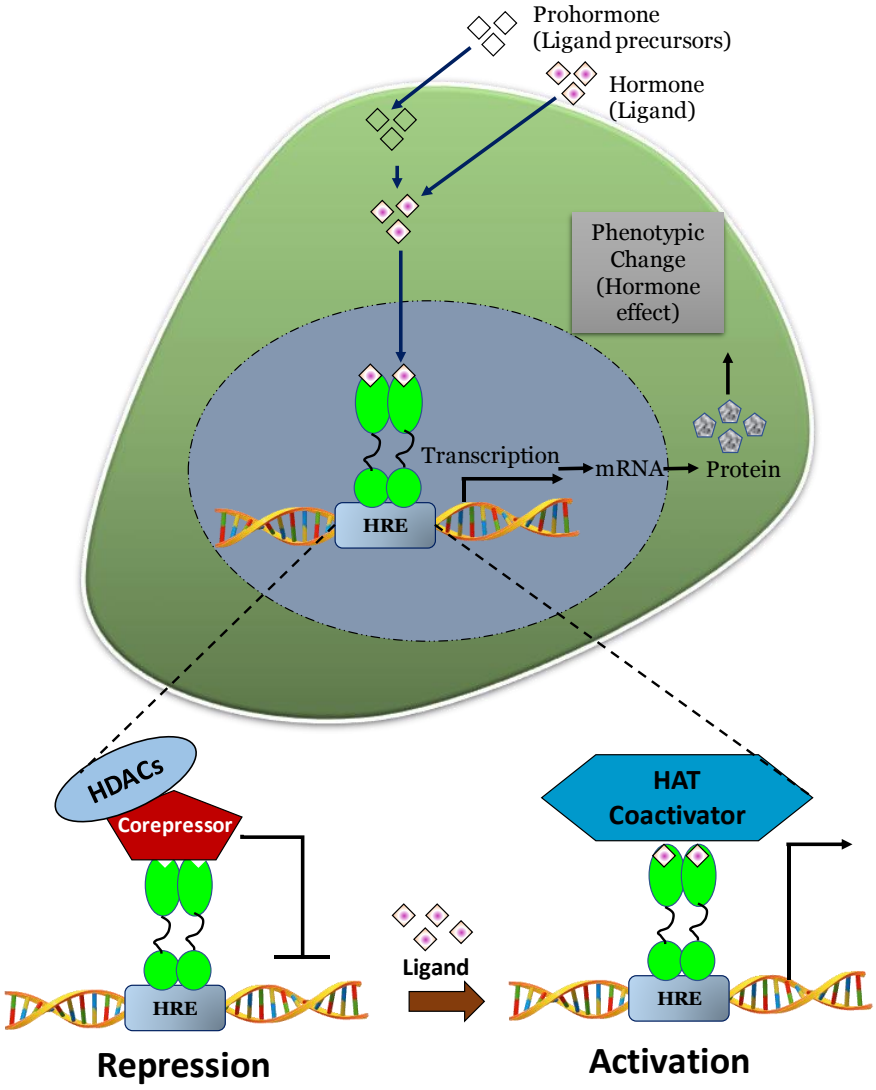


Figure 5. A simplified signaling model for the nuclear receptor. *Hormone and prohormone signal transduction that works by nuclear receptors is shown in the figure. In the absence of ligand, NRs together with corepressors such as SMRT and NCoR form a repressive complex with HDACs. Ligand binding induces the dissociation of corepressors and NR binds in the upstream promoter sequences of the specified NR target genes as a homodimer or heterodimer to specific DNA elements known as HRE. Subsequently, associates and coactivators act as a communication bridge between the receptor and several components of the general transcription machinery, thereby triggering the expression of NR-regulated genes. HRE, hormone-responsive element; SMRT, silencing mediator of retinoid and thyroid receptors; NCoR, nuclear receptor corepressor [adapted and modified from Sonoda et al. 2008; Santos et al. 2011*[39,99]*]*

Sub-types of NR Superfamily

Depending on their sequence homology, dimerization properties, activation mechanism, and ligand specificity, the NR family can be divided into separate groups **(Fig. 6)**. The endocrine receptors are the first and most widely characterized class of NRs, which are also called classical receptors. This group contains, for example, receptors for steroid hormones. AR, ER, GR, MR, and PR that respond to steroid hormones and are critical for the maintenance of homeostasis, cell growth, and differentiation [100]. The steroid receptors, in principle, bind as homodimers to their response element. While with the other NR like VDR, TR, RAR, PXR, and CAR form heterodimers with RXR [7,101,102]. They display high affinity for their ligands [39]. The orphan receptors are a subfamily of nuclear receptors that are proteins with all of a nuclear receptor's structural characteristics but have not yet been identified as physiological ligands. However, once their natural ligands are identified, they are not characterized as orphan receptors anymore. Some of these nuclear receptors may also act as monomers (e.g. RORα), but most of them act as dimers, either on their own (homodimer) or with the RXR (RXR heterodimer) nuclear receptor [7].

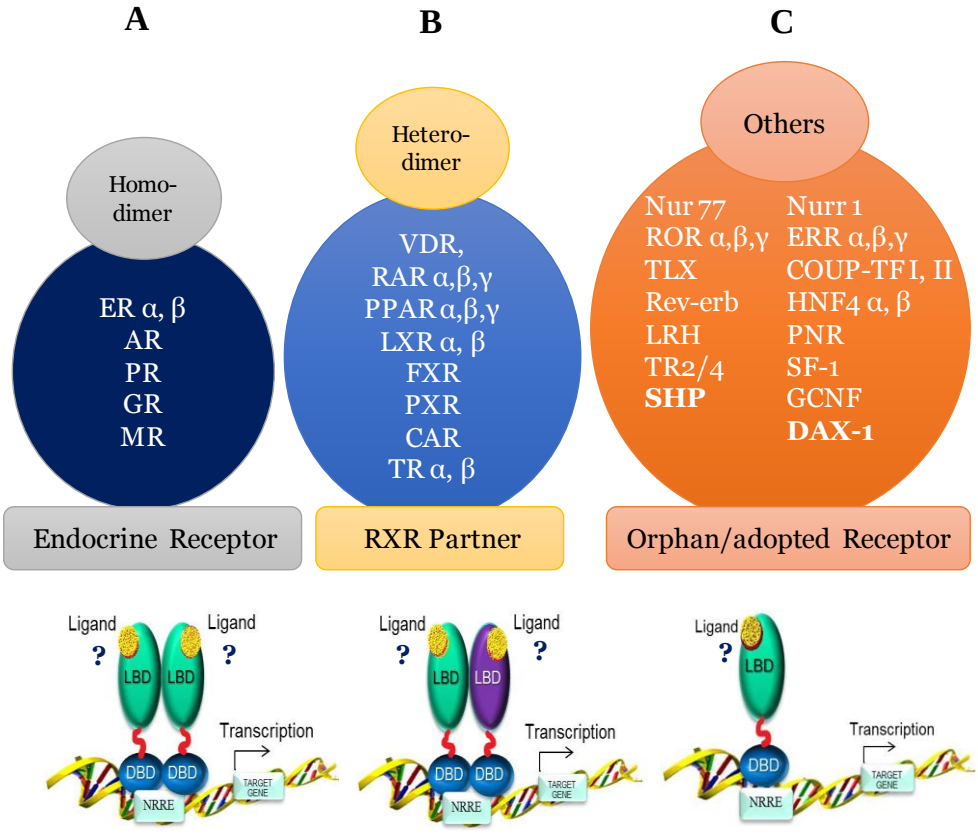


Figure 6: Nuclear receptor classification based on their different dimerization modes. *The classical steroid receptors are known as NRs for which specific ligands*

have been identified. ***(A)*** *This group's NRs bind as homodimers to specific DNA elements.* ***(B)*** *The others use RXR to form a heterodimer. This group is classified as an orphan/ adopted NR that does not identify endogenous ligands. Orphan NRs bind as monomers or homo-or hetero-dimers to specific elements of DNA.* ***(C)*** *The figure shows the adoption of orphan receptors. Members of this class are RXR heterodimers. Examples are listed in their respective panels of each class. [Adapted & modified from Gronemeyer et al. 2004; Sonoda et al. 2008; Imai et al. 2013* [39,103,104]*]*

An overview of Nuclear Receptor modulators: Coactivators and corepressors as two hands of NR action

During gene transcription, a large complex of factors, including basal transcription factors, regulates the activity of RNA polymerase and is broadly referred to as general transcription factors (GTFs) [105]. Coregulators are the two hands of NRs that regulate the gene expression mechanism [106]. GTFs are stimulated to recruit positive acting cofactors, called coactivators, by ligand-bound nuclear receptors. These coactivators are molecules which improve the expression of NR-mediated genes [107,108]. Many coactivators have a conserved motif with the sequence LxxLL or FxxLF (where x is any amino acid) so-called 'NR box' required for direct recruitment to nuclear receptors [109]. They dramatically change the position of nuclear receptor protein Helix 12 (H12) along with H3, H4, and H5 that form a hydrophobic cleft. These are multi-component protein complexes that contribute to many enzyme-specific processes such as DNA unwinding; histone acetylation and displacement that open the chromatin structure, polymerases are recruited, and NR is then released from the promoter region to increase gene transcription rates [110].

On the contrary, corepressors are negatively acting factors that interact primarily with unliganded NRs and repress the expression of the target gene [106,108]. NR Corepressor (N-CoR) and Silencing Mediator for Retinoid and Thyroid Receptors (SMRT) are the two major negative acting factors (corepressors). N-CoR and SMRT's transcriptional functions are the ligand-dependent activation mirror image. In corepressors, a similar sequence of the NR box (LxxH/IIxxxI/L) was identified and called the CoRNR box [111,112]. They recruit multiple histone deacetylases (HDAC) that result in a condensed chromatin state by reversing the role of histone acetylation. To date, in the literature that works as a multi-protein complex, more than 300 coactivators are identified [108]. Therefore, in summary, we should think of

coregulators as molecules that can control NR target protein expression by organizing and controlling transcription, splicing, and mRNA translation.

Chromatin remodeling by NRs: an interplay with coactivators and corepressors

Mitosis and transcription factor

A typical eukaryotic cell cycle is comprised of four distinct phases. The S-phase where DNA synthesis occurs and M-phase where mitosis, i.e. somatic cell division occurs. In between these two phases, the G1 and G2 phase occurs. The mitotic phase consists of five sub-phases, namely prophase, prometaphase, metaphase, anaphase and lastly telophase, which is concluded with cytokinesis. After cytokinesis, the two daughter cells enter an early G1 phase. However, sometimes, instead of entering early G1 as per the requirement, the daughter cells enter into the quiescent state or G0 leading to cell cycle arrest. A typical mammalian cell cycle takes about 24 hours to complete. G1phase takes approximately 12 hours and S- and G2-phase takes about six hours each. The smallest phase, i.e. M-phase take only about 30 minutes. To maintain the fidelity of the cell cycle, the cell has three checkpoints, i.e. at G1/S, G2/M and the spindle checkpoint during M-phase.

Association of NRs with mitotic chromatin and the concept of gene bookmarking

The metazoan cells convey major dynamic changes in their nuclear structure and profile of gene expression during mitosis [113]. The nuclear envelope is disassembled temporarily, resulting in the massive release into the cytoplasm of soluble nuclear constituents [114]. Simultaneously, chromosomes condense and move towards prophase [115], leading to transient transcription silencing. Many transcription factors dissociate themselves from DNA [116]. During the transmission and reactivation of genetic programmes, rod-shaped mitotic chromosomes are formed [117–121]. Some nuclear receptors have been found to remain associated with chromatin during all stages of the cell cycle (including mitosis) under normal or specific physiological conditions [122]. It implies that during division, the cells inherit a biomolecular blueprint via transcription factors from progenitor to progeny to express and sustain their unique proteome and cellular identity **(Fig. 7)**.

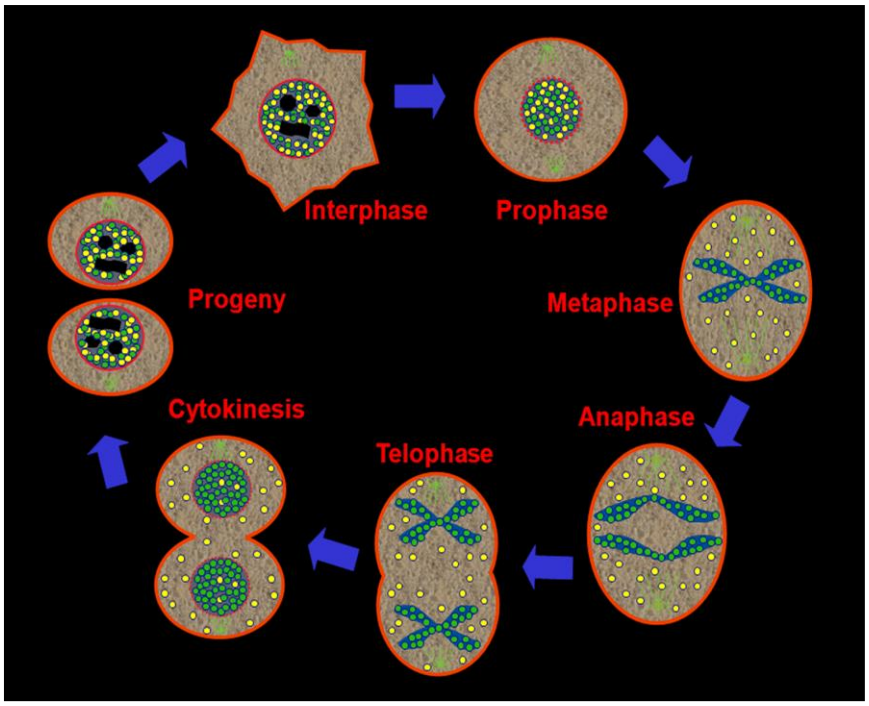


Figure 7. Maintenance of cell proteome and traits by mitotic gene bookmarking by NRs. *During mitosis, most of the DNA is occupied by condensed chromatin with transcription factor (TF) dislodged. The above portrayal elucidates nuclear foci retention and transmission as a transcription memory formed by ligand-activated transcription factors. Agonist-activated NRs (green dots) interacting with co-activators (yellow dots) in the interphase nucleus give rise to transcriptionally competent nuclear foci. With the onset of mitosis, the interaction of NRs with specific nuclear coactivators starts to decrease with coactivators leaving the transcription complex, while the NR remains associated with the condensing chromatin from prophase to cytokinesis. Post-mitosis, with the advent of daughter cells, the chromatin-pre-occupying NRs will promptly enlist the dislodged components of transcription machinery again and revert to the inherited effective gene transcription state. NRs interaction with compressed mitotic chromatin serves as 'gene-bookmarks' for conducting transcription memory retention and transmission. [Adopted from Kumar et al. 2012* [122]*].*

Many reported findings indicate that during mitosis several transcriptional regulators such as transcription factor, nuclear receptors, [122,123] coactivators [124] and chromatin modifiers [125] as well as sequence-specific transcription factors such as Runx2 [124], Gata1 [125], Hnf1 [126], and FoxA1 [127] associate with mitotic chromatin and remain bound to their targets [128]. Therefore, mitotic bookmarking is coupled with an essential regulator of expression of genes, and it is propagated to maintain the transcriptional identity of the cells [129]. In recent years, it is being recognized that the impairment of the function of specific bookmarking factors may hinder the expression of target genes after a cell cycle [125,130].

Every individual cell in the body has its own unique identity. In order to keep this identity intact, the maintenance of 'epigenetic marks' carried by the specific transcription factors is very important. However, this may be challenged during the process of cell division. A cell cycle-dependent phosphorylation cascade is responsible for halting of cellular transcriptional machinery during mitosis [131]. With the progression of mitosis, the chromatin undergoes condensation, nuclear envelope gets disassembled, and most of the transcription factors (TFs) are detached from the mitotic chromosomes [132,133] giving rise to the hypothesis that TFs may remain inaccessible to the mitotic chromatin. To maintain the normal cellular architecture, these inactive transcription factors must regain their property as soon as the cell exits from the mitosis. Recently, some processes have been described which play a crucial role in regaining of the transcriptional machinery after mitotic exit [122,134–136]. These include preserving patterns of DNA methylation (causing heritable silencing) and modifications of the histone. However, some reports claim that this alone is not enough. In fact, it was observed that throughout the mitosis, DNase I hypersensitive sites on the human hsp70 locus remain anchored to the mitotic chromatin [114]. The operation of these sites serves as a bookmarker to mark the area available for the digestion of nuclease. The transcription starts sites (TSSs) of certain genes that are expected to be activated (after mitosis exit was observed) to retain their sensitivity to mitosis permanganate oxidation in mitosis implies that TSS of these genes has a structural conformation advantage [119,137]. Reports often highlight that throughout the mitosis, some TFs bound to mitotic chromatin. Xing et al. reported in 2005 that during mitosis, HSF2 binds at the hsp70 locus [128]. Several TFs have been recorded to be correlated with mitotic chromosomes with the development in live-cell microscopy [120–123,130,134,138], which marks the revelation of mitotic bookmarking era by transcription factors. The human transcription factors associated with the mitotic chromatin throughout the mitosis are listed in **Table 2** [139]. Teves and group reported that contrary to the ongoing published literature, most of the TFs remain attached to the mitotic chromatin throughout the mitosis and the exclusion of which was observed in previous experiments was primarily due to a formaldehyde-based cross-linking and other experimental artefacts. This was reported based on a combined approach comprising *in vitro* biochemical assays, genome editing and fixed versus live cell imaging experiments [139]. To justify they also gave an example of Sox2, excluded from the mitotic chromatin as observed after the chemical fixation. On the contrary, it

was found to be associated with the mitotic chromatin with high affinity when observed in live-cell imaging. They also hypothesized that the competence of nuclear imports and the binding ability of the respective TFs were necessary for the interaction of TFs with mitotic chromatin. They explained the halt in transcription during mitosis by high mobile/dynamic binding of TFs (e.g. Sox2) to mitotic chromatin. This explanation could give an idea of the absence of transcriptional activity during mitotic stages rather than the global inaccessibility of DNA. [139]. These studies suggested that for DNA binding, NLS region and nuclear import are prerequisites for the association of Sox2 with the mitotic chromatin. The mutations in the DNA binding domain abolished the association of Sox2 with the mitotic chromatin. On mutating both the NLS regions (monopartite and bi-partite) of Sox2 simultaneously, they observed the complete exclusion of Sox2 from the mitotic chromatin. This phenomenon was also observed and reported from our laboratory in 2012 [122]. The NLS region of AR acts as 'mitotic chromatin binding-determining region (MCBR)' [122]. In 2016, Lerner et al. reported nuclear import-dependent enrichment of HNF1B mutants on mitotic chromatin (after the cold shock) [140]. Teves et al., 2016 also suggested that both sequence and nuclear import of transcription factors are necessary for association with mitotic chromatin, as they observed that SV40 Large T Antigen NLS was stably associated with mitotic chromatin when fused with Halo Tag and expressed in embryonic stem cells (ES) of the mouse [139]. Whereas under similar conditions, a plant NLS fused with Halo-Tag could not show enrichment on mitotic chromatin. They also reported that HMG domain of Sox2 which interacts with the DNA is stabilized by the TAD domain, which is required for transcription initiation and interaction with other transcription factors. Since, during mitosis, transcription is halted, they proposed that Sox2 interacts with mitotic chromatin through HMG domain during mitosis whereas TAD domain remains inactive. Caudron et al., hypothesized that NLS containing proteins accumulates on chromosomes and blocks the nuclear import process resulting in a TF mutant being dissociated from the mitotic chromosome [141,142]. Teves et al., subsequently suggested in 2016 that the NLS-dependent enrichment of TFs on mitotic chromatins would be through the Ran-GTP gradient involved in the nuclear import process [139]. This Ran-GTP gradient allows importins to transport NLS-containing proteins onto the mitotic chromatin. This phenomenon of mitotic bookmarking has many important consequences as the bookmarking of important transcription factors on the mitotic chromatin, and their safe release from chromatin following mitotic exit determines

several important physiological processes. In 2012, Caravaca et al., reported that many transcription factors responsible for liver regeneration remain bound to the mitotic chromatin [127]. Out of these transcription factors, FoxA1 was found to be most enriched in the mitotic chromosome [127]. Previously, our laboratory has reported two types of association between nuclear receptors and the mitotic chromatin. One is constitutive and other is ligand-mediated **(Fig. 7)** [123,138]. PXR was reported to be associated with the mitotic chromatin constitutively throughout the mitosis [123]. PXR is the major xenobiotic receptor involved in drug metabolism and elimination. Thus, this association of transcription factors with the mitotic chromatin bookmarks the active gene profile which is crucial for transmission of the transcription/epigenetic memory to progeny cells [122]. There were also records of ligand-dependent interaction of two important sex steroid hormone receptors AR and ER with mitotic chromatin [138]. Nevertheless, to date, in the context of NRs action, the perception and significance of 'mitotic or genomic bookmarking' phenomenon is in its infancy.

Table 2. List of TFs/ coactivators associated with mitotic chromatin

Serial No.	Transcription factors/ Coactivators	Function	References
1	Topoisomerase II	Maintains structural component of mitotic chromosome scaffolds	Earnshaw et al. 1985 [143]
2	p67 SRF (Serum Response Factor)	Modulation of genes required throughout the G1 period	Gauthier-Rouviere et al. 1991 [144]
3	TFAP2A (AP-2)	Helps in the displacement of TFs from mitotic chromatin	Martinez-Balbas et al. 1995 [114]
4	C/EBPb (CCAAT/enhancer binding proteins b)	Role in adipocyte differentiation and mitotic clonal expansion expressed during early differentiation program	Tang and Lane 1999 [145]
5	Topo IIa (Topoisomerase IIa)	An essential nuclear enzyme, role in DNA metabolism, chromosomes origination and bound to mitotic chromosomes	Mo and Beck 1999 [146]
6	MCAP (mitotic chromosome-associated protein)	Role in G2/M transition and associates with mitotic chromatin	Dey et al. 2000 [147]

7	NC2 (Negative cofactor 2)	Act as a ubiquitous mitotic transcription repressor	Christova and Oelgeschläger 2002 [148]
8	Transcription factor IID (TFIID)	TFIID and TFIIB remains associated with active gene promoters during mitosis	Christova and Oelgeschläger 2002 [148]
10	Transcription factor IIB (TFIIB)		
11	TBP-associated factors (TAFs)	Constitutively associated with condensed mitotic chromosomes	Chen et al. 2002 [149]
12	Upstream binding factor (UBF)		
13	Double bromodomain protein (Brd4)	Recognizes acetylated histone codes and passes to progeny	Dey et al. 2000, 2003; Behera et al. 2019 [120,147,150]
14	High mobility group protein 1 (HMGB1)	Interaction with mitotic chromosomes at HMG-box A and B	Pallier et al. 2003 [151]
15	(HMGB2		
16	TFIIIB	Disrupts during mitosis by hyper-phosphorylation, and support transcription	Fairley et al. 2003 [152]
17	High mobility group protein-a (HMGA1a)	Involved in changes in chromatin structure	Harrer et al. 2004 [153]
18	CTCF (CCCTC binding factor)	Bound to mitotic chromatin	Burke et al. 2005 [154]
19	Pregnane and Xenobiotic Receptor (PXR)	Constitutively associates with mitotic chromatin	Saradhi et al. 2005 [123]
20	Heat shock transcription factor-2 (HSF2)	Associated with gene-bookmarking and regulate hsp70i induction and survival of stressed cells in the G1 phase	Xing et al. 2005 [128]
21	Calreticulin (CRT)	Regulation of chromatin dynamics on the surface of mitotic chromosomes	Kobayashi et al. 2006 [155]
22	Forkhead transcription factor (FOXI1)	Stably associates and remodel mitotic chromatin	Yan et al. 2006 [156]
23	Transcriptional coactivator α-PC4	Role in association and organize chromatin	Das et al. 2006 [157]
24	Co-repressor BS69	Role in gene repression and chromatin remodeling	Velasco et al. 2006 [158]

25	Interleukin-33 (IL-33)	Colocalizes with mitotic chromatin and has transcriptional repressor property	Carriere et al. 2007 [159]
26	Erythroid-specific activator NFE2 (p45)	Associated with its binding sites on the globin gene loci	Xin et al. 2007 [160]
27	Runt-related transcription factor 2 (Runx2)	Controls lineage commitment, cell proliferation and associates with mitotic chromatin	Young et al. 2007a [161]
28	Upstream Binding Factor 1 (UBF-1)		
29	Androgen Receptor (AR)	Associates with mitotic chromatin in a ligand-dependent manner	Kumar et al. 2008 [138]
30	Estrogen Receptor (ER)		
31	HMGN	Weakly associates with mitotic chromatin	Cherukuri et al. 2008 [162]
32	MLL (Mixed lineage leukemia)	Maintains the gene activity through preserving chromatin structure and associates mitotic chromatin.	Blobel et al. 2009 [125]
33	MEN1 (Menin)		
34	Rbp5 (Retinoblastoma binding protein 5)		
35	HNF1B (Hepatocyte Nuclear Factor 1-homeobox B)	Association with the mitotically condensed chromosomal barrels	Verdeguer et al. 2010 [126]
36	TLE1 (Transducin-like enhancer protein 1)	Associates with rRNA genes during mitosis and interphase through interaction with Runx2	Ali et al. 2010 [163]
37	GATA1 (GATA-binding factor 1)	Role in mitotic bookmarking and maintains of cellular maturation, lineage fidelity	Kadauke et al. 2012 [164]
38	PcG (Polycomb Group)	Associate with mitotic chromatin, mediate stable inheritance of gene expression patterns through mitotic divisions	Follmer et al. 2012 [165]
39	FOXA1 (Forkhead box protein A1)	Implicated in early steps of liver development, also bookmarks targets genes during mitosis.	Caravaca et al. 2013a [127]
40	GATA4 (GATA binding protein 4)	A regulatory and early development actor in the	Caravaca et al. 2013a [127]

		liver partially bound to mitotic chromatin	
41	SOX2 [SRY (sex-determining region Y)-box 2]	Dynamically associates with mitotic chromatin	Teves et al. 2016 [139]
42	ESSRβ (Estrogen Related Receptor Beta)	A major pluripotency TF remains bound during mitosis to key regulatory regions.	Festuccia et al. 2016 [129]
43	OCT4	TF promoting faithful and efficient propagation of stemness after cell division and bookmarking by histone modifications.	Liu et al. 2017 [137]

All about NR0B subfamily members, SHP and DAX-1

DAX-1 and SHP are atypical receptors with common characteristics of both the structure and function of conventional NRs [13]. The NR0 subfamily of NRs comprises of the two divergent receptors DAX-1 and SHP, encoded by NR0B1 and NR0B2 respectively. Both DAX-1 and SHP are devoid of the central DBD with two zinc-finger motifs and AF-1 domain which is common to the NR superfamily [103]. They contain a CTD homologous to the LBD of superfamily members and also contain the AF-2 transactivation domain. However, in case of NTD DAX-1 consists of novel repeats from 65- to 70-amino acid motif, while on other hand SHP contains an extremely short 57-residue NTD which is DAX-1 NTD homologous **(Fig. 8)**.

DAX-1 and SHP are atypical receptors which are devoid of classical receptor function; instead, they have been suggested to involve in the regulation of several NRs' functions primarily through heterodimeric interactions [9,166]. The general function of any NR is in the modulation of gene expression by binding to the promoter region on their target gene, followed by the recruitment of coregulator protein (Burris et al. 2001). SHP and DAX-1 are an exception to the classical activation function of NRs. Instead, they are proposed in the repression of the transcriptional function of some NRs and interacting partners. To facilitate the transcriptional modulation, SHP and DAX-1 have LXXLL motif also known as 'NR box' which bind to the AF-2 domain of NRs. SHP and DAX-1 contain two and three LXXLL motifs respectively and known as

corepressors. Both SHP and DAX-1 repress via a different mode of action by forming a ternary complex with dimeric complex on the DNA [167].

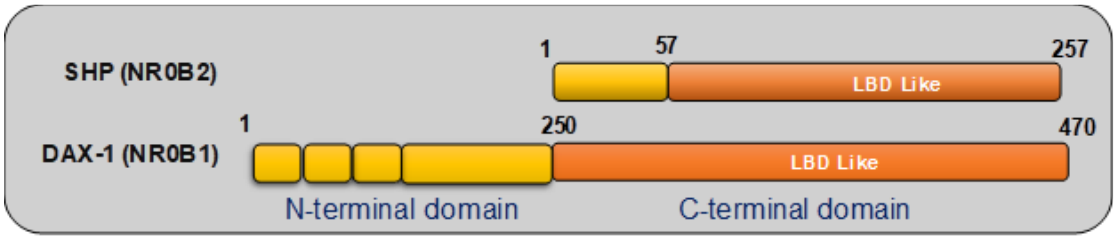


Figure 8: Schematic representation of the structural and functional domain of SHP and DAX-1.

Small Heterodimer Partner (SHP; NR0B2): basic facts and insights

The SHP gene contains two exons, which are interrupted by approximately 1.8 kb intron. By analysis of a somatic cell, a hybrid mapping panel mapped the NR0B2 gene to chromosome 1 [13], cytogenetic localization is at 1p36.1position [168]. Other details are summarized in **(Table 3)**.

Table 3: Basic information of SHP and DAX-1

Details of human SHP	**ID: SHP**	**ID: DAX-1**
Approved symbol	NR0B2	NR0B1
Cytogenetic location	1p36.11	Xp21.2
Genomic coordinates (GRCh38)	26911489-26913966	X:30,304,205-30,309,389
NCBI Accession	NP_068804	NP_000466.2
NCB Reference Sequence	NM_021969	NM_000475.5
Uniport	Q15466	P51843
HUGO Gene Nomenclature Committee	7961	7960
Entrez Gene	8431	190
Ensembl	ENSG0000013190	ENSG00000169297
OMIM	604630	300473
Exons	2	2
Introns	1	1
Length (amino acid)	257	470
Molecular Weight	28058 Da	51718 Da
PDB 3D structure	1YUC, 2Q3Y, 2Z4J, 4DOR, 4ONI, 5UFS	3F5C, 4RWV

Expression of SHP

SHP mRNA is expressed predominantly in the liver and varying levels in a wide range of tissues ranging from the duodenum, adipose, pancreas and heart [167,169]. Transcript of NR0B2 was identified by Northern blot analysis of human tissues and detection of human NR0B2 genomic sequence in the adult intestine, spleen, fetal liver and adrenal gland [170].

Mechanism and mode of action of SHP

SHP appears to interact with many key NRs, including conventional and orphan receptors. The list of interacting partners starts from retinoid receptors RAR, RXR, THR and CAR [9] and includes half of the nuclear receptor and many transcription factors. The known function of SHP is primarily negative regulation of NR members with whom it is projected to interact [9,13,29].

Most ligand-activated NRs are well studied on their molecular mechanisms. Liganded NRs recruit the AF-2 helix in canonical LBD with coactivators (Containing LXXLL motifs) [171]. Conversely, antagonist destabilize the AF-2 helix in LBD and opens a widespread grove in NR corepressors for interaction with LXXXLXX motifs [172].

SHP was discovered, based on its distinct mode of inhibitory action with different NR and transcription factors (TFs). The first mechanism of SHP indicates that it directly associate to the AF-2 helix and interfere with the LXXLL-relate motif of coactivators to play a repressive role[13,173]. This mode of SHP mediates repression in the case of HNF4, LRH-1, ERs, ERRs, AR, GRs, LXRs, Nur77, RXR induced transcription functions [14]. Interestingly, the second mode of repression appeared with ERs, LRH and HNF4, in which SHP directly recruits the corepressors and inhibit the transcription activity [20,21,174,175]. Apart from the discussed mechanism, SHP is also suggested to directly inhibit the DNA binding of interacting NRs/TFs [13]. In this mechanism, SHP-NRs complex inhibits the binding with the promoters and thus, hindering the transcriptional activity. This mode of inhibition was observed with the RAR-RXR heterodimers, RAR-PXR heterodimers and HNF4 homodimerization [9,18,22].

Interestingly, the discussed mechanism of SHP-mediated repression is not exclusive to the NRs but many other transcription factors are also inhibited via different

modes **(Fig. 9)**. In conclusion, the repressive mechanism is tissue and cell-specific. The detailed list of SHP interaction with its partners is presented in (**Table 4)**.

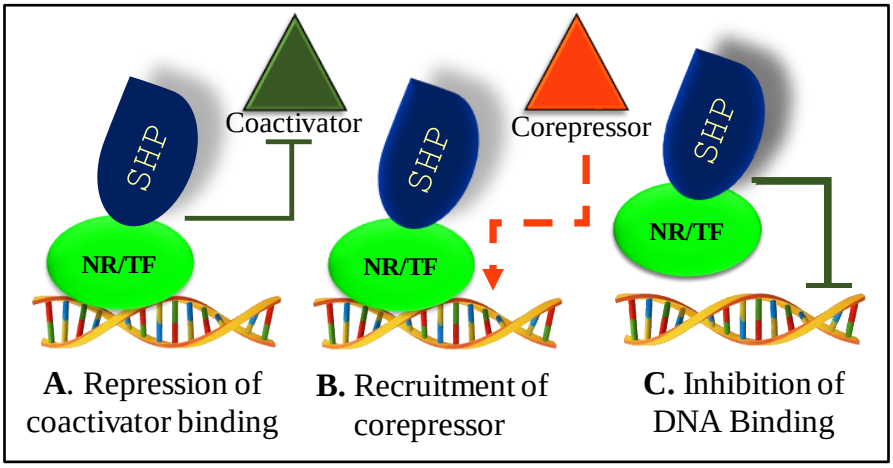


Figure 9: SHP's distinct mode of transcription repression. *SHP inhibits nuclear receptor (NR) or transcription factor (TF) mediated transactivation through* ***(A)*** *NR-binding coactivator competition,* ***(B)*** *SHP-associated corepressor recruitment and* ***(C)*** *DNA binding inhibition. Through these three inhibitory steps, SHP can act in a cell type and target gene-specific manner alternatively or sequentially. [Fig. adapted and modified from Zhang et al. 2011a; Zou et al. 2015* [13,14]*].*

SHP and its interacting partners

SHP interacts with more than half of the NRs and many other transcription factors. SHP can widely interact and subsequently modulate their biological role and their target genes. Recently, many new interacting partners have been identified [14]. The crosstalk of SHP with its interacting partners maintains the homeostasis in diverse biological processes. Summary of the interacting partners is presented in **Table 4.**

Table 4: Summary of SHP mediated influence on functions of different transcription factors

NRs	SHP Function	Reference
AR	Inhibition of ligand-dependent transactivation and competition with AR coactivator.	Gobinet et al. 2001 [176]
ERα	Interaction with ERα and inhibition of ERα transcriptional activity.	Risinger 2002[177]
ERβ	Interaction with AF2 domain of ERβ and inhibition DNA binding.	Johansson et al. 2000a [15]

ERRα, β, γ	Physical interaction dependent ERRγ transactivation.	Sanyal et al. 2002 [11]
CAR	Inhibition of CAR dependent transactivation function.	Seol et al. 1997 [178]
PXR	Repression of PXR transactivation function in the presence of chenodeoxycholic acid, cholic acid-dependent SHP upregulation.	Ourlin et al. 2003 [22]
RXR	Transcriptional repression of RXRα.	Lee et al. 2000 [179]
FXR	Downregulation of the PCK1 gene.	Lu et al. 2000 [30]
GR	Inhibition of GR transcription function via LXXLL motif.	Borgius et al. 2002 [180]
HNF4	Inhibition of DNA Binding of HNF4 via interaction with AF-2 domain and N-terminal domain of HNF4.	Lee et al. 2000 [179]
LRH-1	Interacts with AF-2 domain and directly compete with the other coactivators and repress the transcriptional activity.	Lee and Moore 2002 [181]
Nur77	Inhibition of the transcription function of Nur77.	Lee and Moore 2002 [182]
PPARα	Upregulation the PPARα mediated transcriptional activity.	Kassam et al. 2001 [183]
PPARγ	SHP has induced activation through the PPARγ through a direct binding to the PPARγ DBD / hinge region and inhibiting the NCoR repressor function.	Kassam et al. 2001 [12]
DAX-1	Heterodimerization with DAX-1 with the involvement of AF-2 domain of DAX-1.	Iyer et al. 2006b, 2007 [167,184]
LXRα, β	Repression the transcription activation of LXR.	Brendel et al. 2002 [185]
RARα	Inhibit the DNA binding of RARα with its partners.	Seol et al. 1996, 1997 [9,178]
TRβ	Inhibition of DNA binding of TRβ.	Seol et al. 1996, 1997 [9,178]
SF-1	Has a cooperative mechanism with SF-1.	Lu et al. 2000 [30]
Non-NR Transcription Factor		

ARNT	Direct binding to the AHR/ARNT and repression of the binding with xenobiotic response element and repression of CYP1A1 and UGT1A6 activity.	Kress and Greenlee 1997; Klinge et al. 2001 [186,187]
BET2/ NeuroD	Inhibitor role in BET2/NeuroD mediated transcription function *via* physically interfering with p300 coactivator.	Kim et al. 2004a [188]
C/EBPα	Repression of the C/EBPα-driven PEPCK gene transcription.	Park et al. 2007 [189]
Foxo-1	Repression of the FOXO-1 mediated G6Pase transcription.	Yamagata et al. 2004 [190]
HNF3	Inhibition of the transcriptional activity of HNF3.	Kim et al. 2004b [191]
Jun D	Directly bind with Jun D and represses the DNA binding of its adaptor protein (AP)-1 induced by thrombin.	Fiorucci et al. 2004 [192]
NF-κB	Positive regulation the NF-κB in macrophage cell line RAW-264.7.	Kim et al. 2001 [24]
Smad	Repression the Smad-induced transactivation by competing with coactivator p300.	Kim et al. 2004a; Ji et al. 2006 [188,193]
TRAF-6, p65	Negative regulation the TLR signaling and represses the p65.	Yuk et al. 2011a [194]
Bcl2	Interaction with Bcl2 with a function in cell-dependent cell proliferation.	Zhang et al. 2010 [195]
Gli	The decrease in Gli target gene expression by suppressing Gli1's transcriptional activity.	Kim et al. 2010 [196]
P53	SHP, p53, and Mdm2 act in concert to determine susceptibility to carcinogenesis.	Yang et al. 2012 [197]
Transcriptional coregulators		
Coactivator		
CBP	Bins CBP and contends with Nur77 and ultimately repress the transcriptional activity of Nur77.	Yeo et al. 2005 [182]
SRC-1		Kim et al. 2001 [24]
Corepressor		

EID-1	Interaction with EID1 to provide an inhibitory function.	Båvner et al. 2002, 2005 [20,198]
GPS2	Downregulation of the GPS2.	Sanyal et al. 2007 [199]
G9a, HDAC	Provides a repressive mechanism in G9a and HDAC mediated target genes.	Fang et al. 2007 [174]
SIRT1	Recruitment of the SIRT1 and repression of LRH1-mediated transcriptional function and, also inhibit the target gene of LRH1	Chanda et al. 2010 [200]
SMRT/ N-CoR	Involved in CAR-mediated transactivation of CYP2B6 genes and work in concert with HDAC3-N-CoR-SMRT complex.	Bae et al. 2004 [201]
BRM, BAF155, BAF47, mSin3A, Swi/Snf	At the level of chromatin, direct interaction with the corepressor complex and mediation of the mSin3A-Swi / Snf-Brm chromatin complex to the promoter CYP7A1, SHP interacts with the Swi/Snf complex protein (BAF155, NAF47) and leads to hepatic bile acid synthesis inhibition.	Kemper et al. 2004 [21]
Others		
RNA Pol II	Interaction with RNA Pol II and inhibition of both basal and induced transactivation.	Brendel et al. 2002 [185]

SHP agonist

The SHP domain structure contains LBD and is conserved on the basis of protein homology-based similarity with many prominent NRs [29]. So far, endogenous SHP ligands have not been identified and remain in the ‘Orphan Nuclear Receptor' category. Several artificial retinoid-like compounds (AHPN/CD437) and 4-[3-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid (3Cl-AHPC) [202] and many more altered isoxazole derivatives all-trans retinoic acid (ATRA) were prepared with different side chains close to that of the well-documented SHP agonist [203]. The AHPN and 3Cl-AHPC ligand binds to the SHP protein and encourages the interaction of SHP with peptide-containing LXXLL [204].

Role of SHP in different cellular pathways

Several reports have identified that many proteins of biological pathways are directly involved in SHP and directly regulate their functions. In line with this, SHP is a pleiotropic regulator that directly or indirectly affects multiple target genes that participate in different biological processes. It also includes the regulation of metabolic pathways, apoptosis, autophagy, cell proliferation, drug detoxification, cell cycle control stress and inflammatory response [13,205–207].

- **Signaling pathways that modulate the expression of SHP**

Stress signaling mechanisms such as mitogen-activated kinase (MAPK), extracellular signal-regulated mechanisms (Erk1/2) and AMP-activated protein kinase (AMPK) stimulate SHP [208–211]. Activation of PI3K or MEK1/2 pathways has been shown in a recent study which results in repression of SHP in non-alcoholic fatty liver diseases [33].

Numerous transcription factors including nuclear receptors induce the expression of SHP are LRH-1, SF-1, FXR, HNF4α, ERRγ, LXRα, ERα, PXR, PPARγ, adaptor protein (AP1) c-jun, E2A gene product, SREBP-1c, CLOCK-BMAL1 [14]. In the advanced condition of NAFLD, FOXA1 and C/EBPα repress the expression of SHP [33].

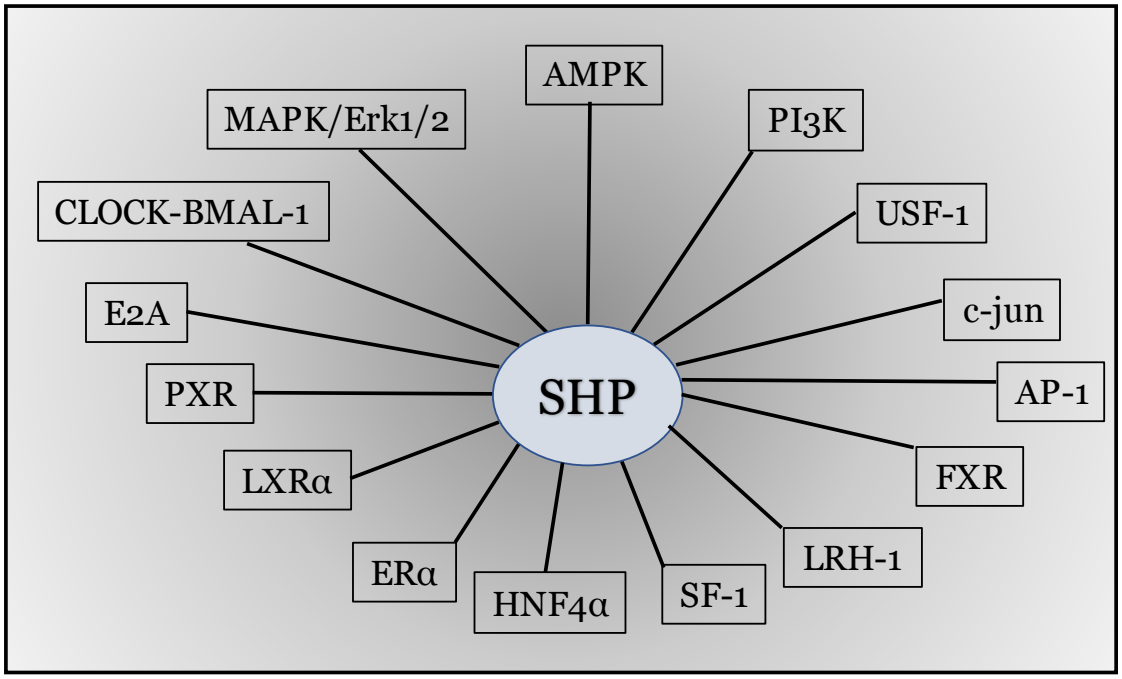


Figure 10: Signaling pathways and transcriptional factors that affect the cellular expression of SHP. *[Figure adapted and modified from Zou et al. 2015* [14]*]*

- **SHP in cell proliferation**

Cell proliferation is the mechanisms of maintaining and replacing a damaged cell of the body during injury or shading. It is a tightly controlled mechanism of multiple pathways, which however, upon any deviation from its principle model pathway (for e.g. cell cycle protein dysregulation) leads to cancer. SHP, therefore, inhibits cyclin D1 expression, providing a molecular foundation for the tumor suppression function of SHP during HCC **(Fig. 11)** [212,213]. SHP knockout in mice resulted in enhanced malignant transformation and proliferation of hepatocytes leading to HCC [214]. The balance between the expression of FXR and SHP is critical in HCC [215]. Some studies showed that induction of SHP serves as an essential mechanism of FXR in suppressing gene expression, thus establishing a link between SHP and FXR [212]. In another study, FXR, which is an activator of SHP, showed beneficiary effect in nude mice [216].

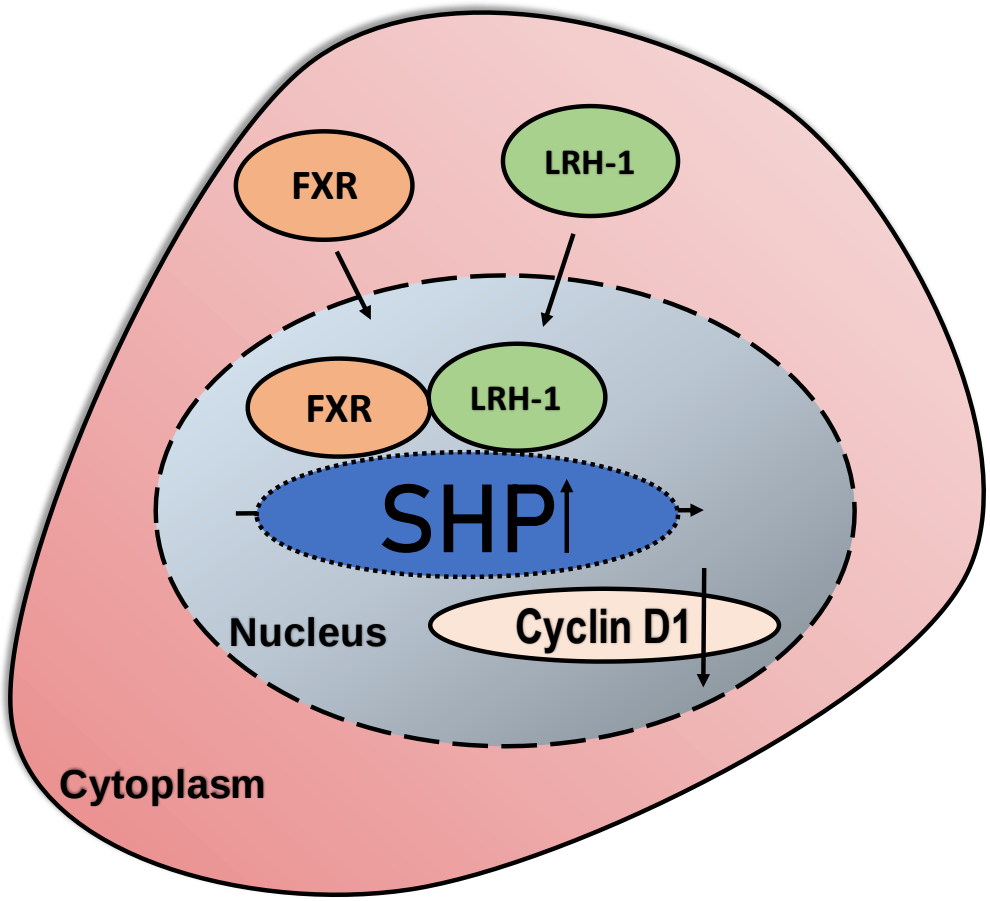


Figure 11: Role SHP in cell proliferation. *FXR and LRH are inducers of SHP expression. The SHP directly regulate the cell cycle protein CDK1 and thus regulate the cell proliferation. [Figure adapted and modified from Zhang et al. 2008* [212]*]*

- **SHP in cell apoptosis**

Apoptosis is vital for maintaining homeostasis of tissue and proliferation of cells. The cells that bypass the mechanism enter the stage of cell growth leading to cancer. Animal studies involving the knock out models have established the role of SHP in cell-specific apoptosis. The SHP knockout mouse reduces apoptosis, leading to Fas-mediated apoptosis [195]. It shows that SHP is a critical contributor to the signals of apoptosis. AHPN and 3Cl-AHPC synthetic ligands are inductors of apoptosis. Such ligands associate with the SHP and facilitate the build-up of the N-CoR, Sin3A, histone deacetylase 4 and heat shock protein 90 (HSP90) corepressor complex [204]. In contrast, SHP also showed anti-apoptotic activity in Nur77-mediated apoptosis. However, a detailed summary of the SHP mediated apoptotic cell signaling is cited for contrast observations **(Fig. 12)** [14,182].

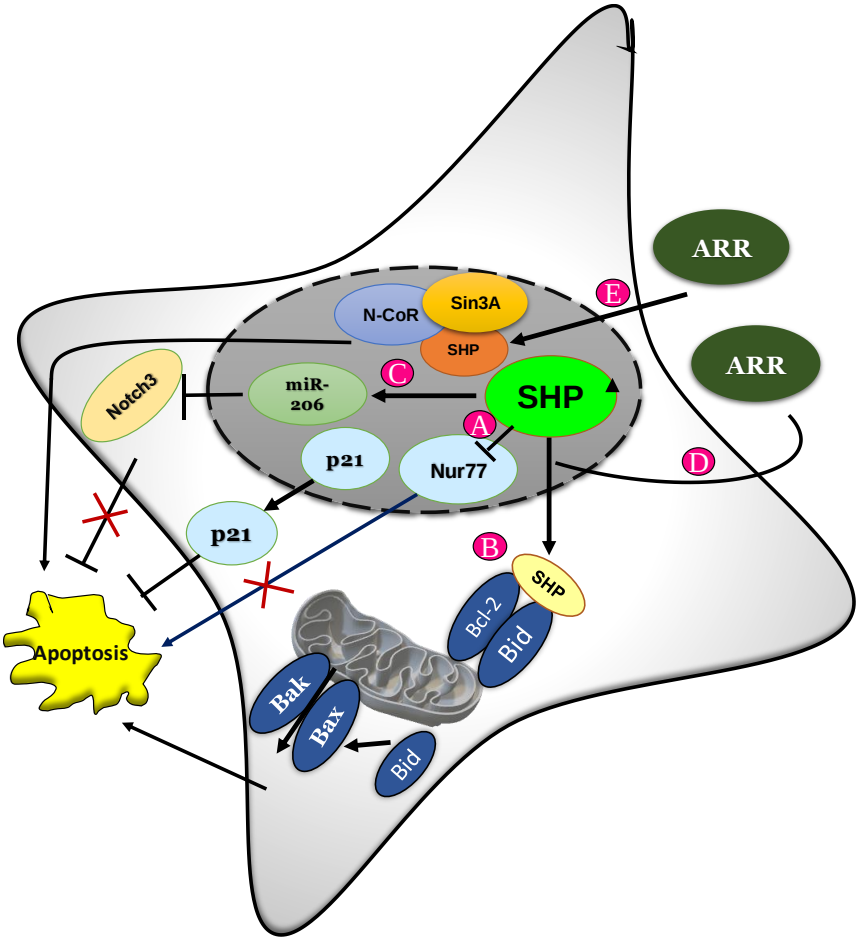


Figure 12: A proposed model showing an SHP-mediated apoptotic signaling pathway. ***(a)*** *SHP, which inhibits Nur77 transcription and represses apoptosis through cytoplasmic p21WAF1 sequestration.* ***(b)*** *SHP translocate to mitochondria, binds to anti-apoptotic Bcl-2 protein and disrupts interactions between Bcl-2 and Bid causing the release of cytochrome c and resulting in apoptosis suppression.* ***(c)*** *by controlling the miR-206 expression, SHP can trigger apoptosis and may prevent the antiapoptotic*

behavior of Notch3. ***(d)*** *The AHPN and 3-Cl-AHPC compounds of adamantyl-substituted retinoid-related (ARR) also enhance the stabilization of the SHP protein by reducing the disruption and increasing mitochondrial targeting of SHPs.* ***(e)*** *SHP is binding on ARRs directly. This promotes the formation of the Sin3A and Nuclear Receptor Co-Repressor (N-CoR) corepressor complex for the activation of apoptosis. [Adapted and modified from Zhang et al. 2011a* [13]*].*

- **SHP in inflammation**

The beneficial host response against extracellular challenge or injury restores the tissue structure and function [217]. If prolonged, it becomes detrimental to the host and leads to a diseased state. The nuclear receptor SHP has a repressive mechanism in the response of inflammation via protein-protein interactions [218]. The inflammatory role of SHP inhibits the development of iNOS and COX-2 in vascular smooth muscle cells when it is activated via FXR ligand GW4064 [219]. Inflammatory pathways are also associated with SHP through interaction with the cytoplasm p65 subunit and inhibition of its nuclear translocation process [220]. This further helps in interaction with TLR4 pathway, NF-κβ p65 and TRAF6 and inhibits the inflammatory response of innate immune cells [221]. SHP also maintain the NLRP3 mediated inflammasome by directly binding to NLRP3 [217,222]. SHP negative regulation role extends to inflammatory reactions from the other non-TLR pathogenic receptors (Nod2, Mda5 and Rig-I) and TLR (TLR2-TLR6, TLR1-TLR2, and TLR3) [223]. To conclude, SHP regulates inflammation negatively and its loss may lead to more cytokine and chemokine production, leading to the development of disease-related conditions.

- **SHP in autophagy**

SHP plays a large role in many biological processes, molecular and cell components [13,203]. The previous reports suggest that many autophagic genes are influenced by SHP, and some are common to both. Autophagy is the most commonly preserved homeostatic process that recycles cytoplasmic cell components [224]. The SHP targets (Human Autophagy Database http:/autophagy.llu/) are autophagic components, effectors and regulator genes. During nutrient deprivation, autophagy plays an essential part in mobilizing energy supplies [224,225]. An organism's health stems from cell life and death, cell fatality assessment, genome integrity maintenance, immune responses, and metabolic circuitry. The autophagy mechanism plays a crucial role in critical processes through its various functions. Such as cell death, stem cell survival, tumor invasion, durability and metabolic protection [14,226].

SHP in metabolic pathways

Apart from cellular pathway regulation, SHP regulates numerous metabolic pathways, such as BA, lipid, cholesterol, fatty acid metabolism, reproductive biology, glycemic homeostasis, and their target genes [213].

- **SHP in bile acid and lipid homeostasis**

SHP maintains homeostasis of cholesterol and BA by inhibiting the conversion of cholesterol to BAs [13]. The BA is synthesized in the liver and processed in the gall bladder. They are physiological detergents that mediate lipophilic molecule absorption, transportation and dissemination [227,228]. The concentration of BAs is strictly regulated in serum, liver and intestine to prevent enterohepatic tissue damage [229]. In humans, CYP7A1 is the main enzyme that limits bile acid synthesis [230]. In the coordination of BAs biosynthesis, NRs also play a key role. The LRH-1, HNF4α, FXR and SHP provides a feedback mechanism for firmly regulating BAs through the CYP7A1 and CYP8B1 enzymes [231].

FXR is abundantly expressed in the liver and intestine which are the primary site of synthesis of BAs. In turn, FXR binds the cytoplasm to BAs and translocate it in the nucleus to the promoter of its target genes with its heterodimeric partner RXR [232]. The binding of bile acids represses CYP7A1 with FXR, leading to SHP transcription [231,232]. Elevated SHP Protein then inactivates LRH-1 by forming a heterodimer complex resulting in promoter-specific CYP7A1 and SHP repression **(Fig. 13)**. Likewise, LXRα attaches to oxysterols and mediates the initiation of feed-forward induction by BA receptor, FXR; LRH-1 promoter-specific activator; and SHP promoter-specific repressor [30,31,229]. This auto-regulatory cascade is regulated by nuclear receptors to promote the catabolism of hepatic cholesterol [31].

Animal studies have also indicated towards BA as a potent ligand of FXR which stimulates fibroblastic growth factor 15 (FGF15) expression [206,231]. FGF15 is a target gene of FXR and leads to the limitation of BA biosynthesis in the liver and intestine [233], in coordination with the SHP repression mechanism of CYP7A1 enzyme. Eventually, FGF15 activation retains the presence of intestinal FXR in the hepatic BAs [206].

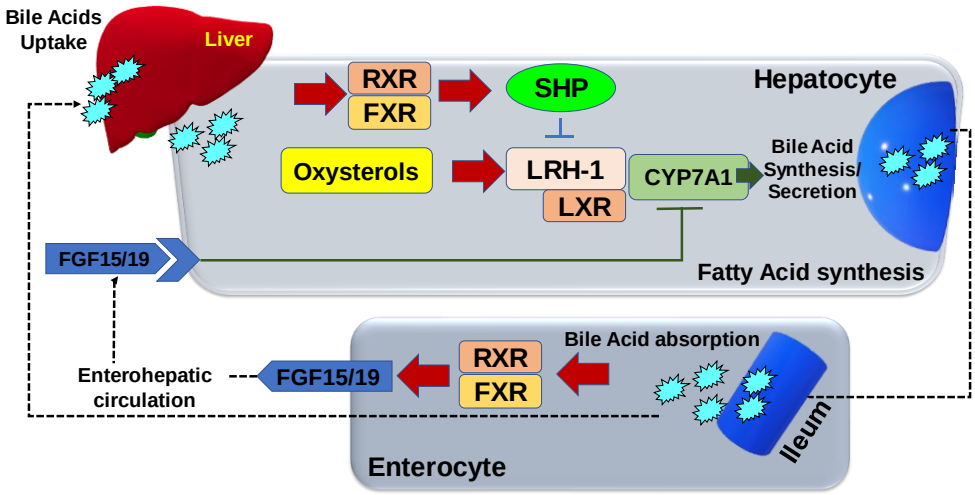


Figure 13. Model depicting the crosstalk in liver and intestine regulated cascades for the control of bile acid homeostasis with the participation of NRs LXR and FXR-SHP-LRH-1. *FXR binds to bile acids and heterodimerizes to RXR resulting in increased SHP production. SHP, in effect, is associated with LRH-1 and forms an inhibitory complex. This step prevents the activation of bile acid and fatty acid synthesis target genes. Intestine frequently modulates the production of bile acid and stimulates FXR, resulting in the secretion of FGF15 (growth factor of fibroblast). FGF15 circulates in the intestine and liver, resulting in the synergy with SHP repression of CYP7A1. In this way, bile acids effectively down-regulate their own synthesis. [Image Adapted and modified from Goodwin et al. 2000; Lu et al. 2000; Ory 2004; Inagaki et al. 2005; Garruti et al. 2012* [30,31,213,233,234]*].*

- **SHP in glucose metabolism and energy homeostasis**

Glucose homeostasis in the body is a well-coordinated integration of several physiological systems, primarily the liver and peripheral tissues that balance the production of glucose. This mechanism is usually controlled by coordinated insulin secretion and action on one side and epinephrine, glucagon, cortisol, and growth hormone on the other side [235]. The transcription of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), which recruit a rate-determining enzyme in hepatic gluconeogenesis is known to regulate by a variety of transcription factors and cofactors such as PGC-1α [236]. SHP is expressed predominantly in the liver and also interacts with the NR involved in gluconeogenesis. GR is a critical mammalian blood glucose regulator. It has been observed that GR signalling is hindered by the SHP [180]. In fact, GR's inhibition mechanism is related to its PGC-1α coactivator, which is antagonised by SHP leading to PEPCK expression inhibition. SHP PCG1α and GR cascading imply a physiologically relevant role for SHP in modulating the action of hepatic glucocorticoids [13].

- **SHP in xenobiotic metabolism**

Bile acids induce SHP expression, initiating SHP interaction with ligand-dependent PXR and causing PXR-mediated CYP3A induction to repression in the liver [22]. The liver is the body's main pharmaceutical processing site where the cytochrome P450 enzyme converts proactive drug or metabolites into active drug or inactive metabolites [11]. PXR and CAR are xeno-sensors of broad specificity that identify multiple ranges of synthetic drugs and endogenous compounds such as BAs, steroids and their precursors [237]. PXR induces CYP3A upon activation and inhibits CYP7A, suggesting that PXR can act on and eliminate BA synthesis. Indeed, CYP7A and CYP3A are involved in biochemical pathways leading to the conversion of cholesterol into primary BAs and to the detoxification of secondary toxic BA derivatives [238]. Contrary to the view that SHP would be a negative regulator of xenobiotic metabolism, recent studies have shown that SHP positively modulates xenobiotic-detoxifying CYP enzymes including Cyp1a2, Cyp2a5, Cyp2b10, and Cyp3a11 [239].

Disease association and clinical significance of SHP

- **SHP in cancer**

NRs were involved in the progression and initiation of various types of cancers. SHP's function as an effective tumor suppressor and has been demonstrated in this direction [240]. Loss of SHP function has shown to result in liver, breast, renal and prostate-related cancers [13,14,29,241,242]. In hepatocellular carcinoma, SHP prevents tumor *via* cellular growth and activation of apoptosis [16,195,212,243].

HCC is a well-coordinated progression through various cellular pathways and epigenetic mechanisms [14]. SHP deficiency a cell proliferation via cyclinD1 [212], reduce apoptosis through Bcl2 [244], and results in genome-wide epigenetic changes by Dnmts [245,246], including DNA hypomethylation and tumor suppressor gene hypermethylation ([16].

Worldwide, Breast cancer is the most prevalent form of cancer among women globally and comes after lung cancer in terms of mortality among its patients [247]. Estrogen is one of the common factors of breast cancer and exercises its biological function by ERα and ERβ in a ligand-dependent manner [248]. Anti-estrogen therapy plays an essential role in the diagnosis of ER-positive type breast cancer. SHP is related to ER signaling pathways as it tends to interact directly with ER [249] by the following

pathways involving the inhibition of ER-mediated transcriptional activation by SHP [250] and SHP's ER-dependent transcriptional regulation in a feedback loop [251]. In the menopausal women, Estrogen-dependent cancer is locally facilitated by the surrounding adipose tissue, along with aromatase like CYP19 in the critical role[181,252]. In that connection, SHP enhances the expression of PPARγ via binding to its promoter.

Further, the ligands of PPARγ are an effective inhibitor of the aromatase in adipose tissue [253]. Reports suggest the SHP regulate the estrogen signaling at multiple levels [13]. Also, the ligand of FXR GW4064 was found to upregulate the expression of SHP, by inhibiting the expression of aromatase and induce apoptosis [254]. Thus, it suggests that the axis of FXR-SHP-LRH-1 may provide a therapeutic platform to repress proliferation and induce apoptosis in breast cancer treatment.

However, in renal carcinoma patient tissue, SHP was observed to be downregulated as compared to healthy tissue [29]. In this regard, SHP also has an impact on renal cell carcinoma development and progression [29].

- **SHP in obesity**

SHP is reported to play a crucial role in obesity [255,256]. Obesity is a polygenic, multifactorial disorder proposed to be governed by the abnormal molecular mechanisms. Obesity is an imbalance between nutrient energy gain and energy dissipated as heat and increase of body fat mass[257]. In obesity, a major function is played by the contrast of brown adipose tissue (BAT) and white adipose tissue (WAT). SHP knockout mice do not appear to be susceptible to high-fat-diet-induced obesity from the reports [255]. SHP also acts as a negative regulator of energy production through the modulation of PCGα1 expression in the BAT [258]. BAT-specific overexpression of SHP led to increased adiposity and body weight. However, SHP deletion could not overcome leptin-deficient obesity [213].

In addition to changes in the molecular level, genetic polymorphism also affects the early onset of obesity. Several studies have suggested that SHP gene mutations are not only a prevalence's of severe obesity, but genetic variation can also influence birth weight and body size [259]. However, similar results were not obtained from Caucasian cohorts [213]. Whereas, the reports on SHP in various populations were oriented separately towards the occurrence of SHP variants [258,260].

- **Single nucleotide polymorphisms (SNPs) of SHP associated metabolic disorders**

SNPs are the well-established form of genetic variations representing more than 80% of all human genome variations [261]. Several studies have attempted to explore the role and functional variations of SNPs in genes following the completion of the human genome project. 'SNP is a DNA point variation at a single base pair position with a frequency of more than 1% in a population'.

Although several SNPs in SHP have been reported, still no comprehensive insight into the critical parameters of receptor functioning and SNPs have been conducted so far **(Table 5)**.

Table 5: Summary of SNPs present in the exon region of SHP and its association with diseases.

SNP position (S)	Protein Stability	Disease Association	Reference
R38C	Normal	type 2 diabetes	Enya et al. 2008 [262]
R38H	Normal	early-onset obesity CADASIL (Cerebral Autosomal Dominant Arteriopathy With Subcortical Infarcts and Leukoencephalopathy)	Zhou et al. 2010 [263]
R45P	Normal	type 2 diabetes	Enya et al. 2008 [262]
R54C	Reduced		
R57W	Reduced	early-onset obesity	Kanamaluru et al. 2011 [264]
G93D, V105G	Reduced	type 2 diabetes	Enya et al. 2008 [262]
P139H	Normal		
K170N	Reduced	CADASIL	Zhou et al. 2010 [263]
G171A	Normal	CADASIL	Nishigori et al. 2001; Zhou et al. 2010 [169,263]
D178N	Normal	type 2 diabetes	Enya et al. 2008 [262]
G189E	Reduced		
A195S, R213C	Reduced	early-onset obesity	Nishigori et al. 2001 [169]
R216H	Normal		

In summary, SHP's SNPs are involved in various metabolic diseases such as type 2 diabetes, early-onset obesity, and CADASIL etc. with different residues of amino acids changing. Such specific variants in the SHP gene may be associated with increased body weight in the case of obesity, increased insulin resistance in the case of diabetes and two SHP mutations (R38H, K170N) in both healthy and CADASIL-like patients and finding that the K170 residue regulates SHP ubiquitin and acetylation correlated with SHP's protein stability and repressive activity [263].

- **SHP in miRNA and noncoding RNA regulation**

SHP performs an indispensable role in controlling different cellular and physiological processes and diseases by modulating the RNA-level expression of disease-specific genes. The transcribed RNA transcript, such as microRNAs (miRNAs), small non-coding RNA and long non-coding RNAs (lncRNAs), is not translated into active proteins but controls gene expression and therefore modulates cellular pathways **(Table 6)** [265]. SHP's current understanding of miRNA control has been supported by some recent reports [265]. SHP also functions as an essential regulator of the expression and function of lncRNA in addition to its regulation of miRNAs **(Table 6)**.

Table 6. SHP regulated miRNA and long non-coding RNA and their functional role

miRNA	Function	References
miR-433	Inhibits liver cancer migration by targeting cAMP response element-binding protein (CREB).	Song and Wang 2008a; Yang et al. 2013a [266,267]
miR-127	Inhibits HCC cell migration by targeting transforming growth factor-β (TGF-β)-mediated MMP and also represses high-mobility-group protein 2 (HMGB2) to modulate pluripotency of mouse embryonic stem cells and liver tumor-initiating cells.	Song and Wang 2008b; Yang et al. 2013b; Song et al. 2017; Zhao et al. 2017 [265,268–270]
miR-206	Turns on target Notch3 to activate apoptosis.	Song et al. 2009 [271]

miR-200c	It has a role in tumorogenesis, cell migration, oxidative stress. It is regulated *via* feedback mechanism of PPARα and LRH-1.	Zhang et al. 2011b [272]
miR-34a	Regulates the gene expression of SIRT1 gene and have a role in metabolic diseases like obesity and tumorogenesis.	Chang et al. 2007 [273]
lncRNA		
H19	Roles in hepatic fibrosis, hepatocellular carcinoma, bladder cancer	Matouk et al. 2007 [274]
MEG3	Inhibition of MEG3 gene transcription function, which has a role in tumor suppression.	Zhou et al. 2012; Zhang et al. 2017 [275,276]

In conclusions, SHP is devoid of DBD which makes it atypical NR. SHP sequences have LBD (although endogenous ligand is yet not identified), perturbs the transcription function of it's interacting partners and influences the several cellular and metabolic signaling pathways by directly associating with diseases.

Materials & Methods

Materials

Bacterial and mammalian cell culture media, supplements, antibiotics, ligands and inhibitors

Product Name	Company	Cat. No.
Agar	Himedia, INDIA	RM301
Luria Broth Powder	Himedia, India	M575
Ampicillin	Himedia, INDIA	RM645
Kanamycin	Himedia, INDIA	RM210
Geneticin (G418)	G-Biosciences	RC169
Charcoal Stripped FBS	PAN Biotech, GmbH, Germany	P30-2301
CITCO	Sigma-Aldrich, St. Louis, MO, USA	C6240
Phenytoin	Sigma-Aldrich, St. Louis, MO, USA	PHR1139
Rifampicin	Sigma-Aldrich, St. Louis, MO, USA	R-8883
Estradiol	Tokyo Chemicals (India) Pvt. Ltd.	E0025
9-Cis-Retinoic acid	Sigma-Aldrich, St. Louis, MO, USA	R4643
DHT	Sigma-Aldrich, St. Louis, MO, USA	A8380
CD437	Tocris	1549
DMSO	Sigma-Aldrich, St. Louis, MO, USA	D2650
Escort III	Sigma-Aldrich, St. Louis, MO, USA	L3037
Escort IV	Sigma-Aldrich, St. Louis, MO, USA	L3287
Lipofectamine 2000	Invitrogen, Life Tech, CA, USA	11668019
Lipofectamine 3000	Invitrogen, Life Tech, CA, USA	L3000015
DMEM (high glucose)	Sigma-Aldrich, St. Louis, MO, USA	D7777
Sodium bicarbonate	Sigma-Aldrich, St. Louis, MO, USA	S5761
PSA	Himedia, India	A002A
FBS	PAN Biotech, GmbH, Germany	3302
PBS	Sigma-Aldrich, St. Louis, MO, USA	D-5652
Trypsin-EDTA	Sigma-Aldrich, St. Louis, MO, USA	T3924

General laboratory chemicals

Product name	Company	Cat. No.
3-Amino Phthalhydrazide (Luminol)	Biochemika Fluka	73660
Acetic Acid	Merck, INDIA	60006325001730
Acrylamide	Sigma-Aldrich, St. Louis, MO, USA	A3553
Agarose	Sigma-Aldrich, St. Louis, MO, USA	A9539
Ammonium persulphate	Sigma-Aldrich, St. Louis, MO, USA	A3678
β-Mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA	M7522
Bromophenol Blue	Himedia, INDIA	RM117
BSA	Himedia, INDIA	RM105
Calcium Chloride	Sigma-Aldrich, St. Louis, MO, USA	22231-3
Chloroform GR	Merck, INDIA	S13SF53306
Coomasie Briliant Blue R-250	Himedia, INDIA	RM344
Diethyl pyrocarbonate	Sigma-Aldrich, St. Louis, MO, USA	D5758
Di-Sodium Hydrogen Phosphate	Himedia, INDIA	RM1416
DTT	Sigma-Aldrich, St. Louis, MO, USA	D9163
EDTA disodium salt	Sigma-Aldrich, St. Louis, MO, USA	E5513
Ethanol	Merck, Germany	1009830511
Ethidium bromide	Himedia, INDIA	RM813
Formaldehyde	Ranbaxy, INDIA	F0070
Formamide	Qualigens Fine Chemicals, India	24015
Freund’s adjuvant complete	Sigma-Aldrich, St. Louis, MO, USA	F-5881
Freund’s adjuvant incomplete	Sigma-Aldrich, St. Louis, MO, USA	F5506
Glutathione-Agarose beads	Sigma-Aldrich, St. Louis, MO, USA	G4510
Glycerol	Qualigens Fine Chemicals, India	15455
Glycine	Sigma-Aldrich, St. Louis, MO, USA	G8898
Hoechst 33258	Sigma-Aldrich, St. Louis, MO, USA	86140-5
Hydrochloric Acid	Fisher Scientific, USA	29505

Hydrogen Peroxide	Rankem, INDIA	H0120
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich, St. Louis, MO, USA	I6758
Isopropanol	Merck, India	P0790
Sodium Lauryl Sulfate (SDS)	Sigma-Aldrich, St. Louis, MO, USA	L3771
L-Glutathione reduced	Sigma-Aldrich, St. Louis, MO, USA	G4251
Methanol	Merck, India	CAS 67-56-1
4-Morpholinepropanesulfonic acid (MOPS)	Sigma-Aldrich, St. Louis, MO, USA	M1254
N, N'-Methylene-Bis-Acrylamide	Sigma-Aldrich, St. Louis, MO, USA	M7279
N-Lauroyl Sarcosine sodium salt	Sigma-Aldrich, St. Louis, MO, USA	L9150
Nonyl phenoxypolyethoxylethanol (NP40)	Himedia, INDIA	RM 2352
Orthophosphoric acid	Qualigens Fine Chemicals, India	29905
p-Coumaric Acid	Sigma-Aldrich, St. Louis, MO, USA	C9008
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, St. Louis, MO, USA	P7626
Potassium chloride	Rankem, INDIA	P0240
Potassium dihydrogen orthophosphate	Rankem, INDIA	P0320
Protease inhibitor cocktail	Sigma-Aldrich, St. Louis, MO, USA	P8340
Skim milk powder	Titan Biotech Ltd., INDIA	651
Sodium azide	Sigma-Aldrich, St. Louis, MO, USA	S2002
Sodium chloride	Sigma-Aldrich, St. Louis, MO, USA	S5886
Sodium hydroxide	Rankem, INDIA	S0270
TEMED	Sigma-Aldrich, St. Louis, MO, USA	T9281
TRI reagent	Sigma-Aldrich, St. Louis, MO, USA	T9424
Triton X-100	Sigma-Aldrich, St. Louis, MO, USA	T8787
Trizma base	Sigma-Aldrich, St. Louis, MO, USA	T6066
Tween-20	Sigma-Aldrich, St. Louis, MO, USA	P5927

Primary and secondary antibodies

Product name	Company	Cat. No.
Anti-rabbit IgG HRP conjugated	Sigma-Aldrich, St. Louis, MO, USA	A0545
Anti-rabbit IgG-cy3	Sigma-Aldrich, St. Louis, MO, USA	C2306
Anit-mouse-FITC	Sigma-Aldrich, St. Louis, MO, USA	F1262
Rabbit anti-Glutathione-S-transferase	Sigma-Aldrich, St. Louis, MO, USA	G7781
Rabbit anti-SHP	This study	--
Rabbit anti-human PXR	Raised in our laboratory	--
Rabbit anti-β-actin	Raised in our laboratory	--
Rabbit anti-LC3	Sigma-Aldrich, St. Louis, MO, USA	L8918
Mouse anti-NR0B2	SANTA CRUZ Biotechnology, USA	Sc-271511

Enzymes

Product name	Company	Cat. No.
BamHI HF	NEB, England	R3136S
Calf Intestinal Phosphatase	NEB, England	M0290S
dNTP	Fermentas Int. Inc., Canada	R0181
DpnI	Fermentas Int. Inc., Canada	ER1701
EcoRI HF	NEB, England	R3101S
GoTaq Flexi DNA polymerase	Promega, Madison, WI, USA	M8295
Lysozyme	Sigma, St. Louis, MO, USA	L-6876
Phusion High Fidelity DNA polymerase	NEB, England	M0530S
RNasin ribonuclease inhibitor	Promega, Madison, WI, USA	N21111
T4 DNA Ligase	Fermentas Int. Inc., Canada	EL0015
Taq DNA Polymerase	NEB, England	M0273L
KpnI	NEB, England	R3142S

Protein and DNA standard size markers for gel electrophoresis

Product name	Company	Cat. No.
Prestained Protein Marker	Fermentas Int. Inc., Canada	SM0671
Unstained Protein Marker	Sigma-Aldrich, St. Louis, MO, USA	SDS7
1Kb DNA Size Standard	Fermentas Int. Inc., Canada	SM0311
100bp DNA Size Standard	Fermentas Int. Inc., Canada	SM0241
50 bp DNA Size Standard	Fermentas Int. Inc., Canada	SM0371

Kits

Product name	Company	Cat. No.
HiYeldTM Gel/PCR DNA Mini kit	RBC, TAIWAN	YDF100
Luciferase assay kit	Promega, Madison, WI, USA	E1501
Plasmid DNA extraction mini prep	MDI, Ambala, INDIA	MIPK50

Plasticware

Product name	Company	Cat. No.
Microtips (0.2 - 10 µl)	Tarson, Kolkata, INDIA	521000
Microtips (2 - 200 µl)	Tarson, Kolkata, INDIA	521010
Microtips (200 - 1000 µl)	Tarson, Kolkata, INDIA	521020
0.5 ml microcentrifuge tubes	Tarson, Kolkata, INDIA	500000
1.5 ml microcentrifuge tubes	Tarson, Kolkata, INDIA	500010
2 ml microcentrifuge tubes	Tarson, Kolkata, INDIA	500020
PCR 0.2 ml tubes	Tarson, Kolkata, INDIA	B79001
15 ml falcons	Tarson, Kolkata, INDIA	546020
50 ml falcons	Tarson, Kolkata, INDIA	546040
Cell culture plates 12 well	Corning, NY, USA	CLS3513
Cell culture plates 24 well	Corning, NY, USA	CLS3526
Tissue-culture treated culture dishes 35 mm	Corning, NY, USA	CLS3430165
Tissue-culture treated culture dishes 60 mm	Corning, NY, USA	CLS3430166
Tissue-culture treated culture dishes 100 mm	Corning, NY, USA	CLS3430167
Bacterial Petridishes 35 mm	Tarson, Kolkata, INDIA	460035
Bacterial Petridishes 100 mm	Tarson, Kolkata, INDIA	460095
Cell scrapper	Corning, NY, USA	CLS3020

Miscellaneous materials

Product name	Company	Cat. No.
Developer	Kodak, INDIA	4908216
Disposable filter paper (0.22μ)	MDI Ambala, INDIA	CN
Fixer	Kodak, INDIA	4908232
PVDF Membrane	MDI Ambala, India	SVF
Salmon sperm DNA	Agilent Tech., USA	201190
Whatman Filter Paper 3 MM	Whatman, England	3030917
Whatman Filter Paper No.1	Whatman, England	100125
X-Ray Film	Kodak, INDIA	4910022

Plasmids used in the present study

Plasmid Name	Nature of Plasmid	Source
pDs Red-Express-C1 vector	BD Biosciences, USA	8331-1632430
pCMX-SHP	Mammalian expression vector pCMX encoding full-length human SHP	A gift from Prof. Steven A. Kliewer (University of Texas, USA)
RFP-SHP	Mammalian expression vector encoding full-length human SHP cloned at KpnI & BamHI site of pDS-Red-Express C1 vector	Amit Kumar Dash PhD Thesis
GFP-SHP	Mammalian expression vector encoding full-length human SHP cloned at KpnI & BamHI site of GFP-C1 vector	This Study
GFP-ERα	Mammalian expression vector encoding full-length human ERα cloned at KpnI & BamHI site of GFP-C1 vectory	Dr. M. A. Mancini (Baylor College of Medicine, Houston, USA)
pSG5-PXR	Mammalian expression vector encoding human PXR gene sequences cloned in pSG5 Vector	Dr. S.A. Kliewer (University of Texas, Southwestern Medical Center, Dallas, USA) and
XREM-luc	Promoter-reporter expression Plasmid encompassing a distal xenobiotic responsive enhancer module from CYP3A4 gene	Dr. C. Liddle (University of Sydney at Westmead Hospital, Australia)
pCMV-β galactosidase	Mammalian expression vector encoding β Galactosidase	(Kumari et al. 2015)[277]
GFP-PXR	Mammalian expression vector encoding full-length human PXR cloned at EcoRI & BamHI site of GFP-C2 vector	(Saradhi et al. 2005b)[123]

GFP-PXR-NTD	Mammalian expression vector encoding NTD of PXR cloned at EcoRI & BamHI site of GFP-C2 Vector	(Rana et al. 2018)[36]
GFP-PXR-'N' Zn DBD	Mammalian expression vector encoding 'N' terminal Zn finger of PXR cloned at EcoRI & BamHI site of GFP-C2 vector	(Rana et al. 2018)[36]
GFP-PXR-'C' Zn DBD	Mammalian expression vector encoding 'C' terminal Zn finger of PXR cloned at EcoRI & BamHI site of GFP-C2 vector	(Rana et al. 2018)[36]
GFP-PXR-DBD	Mammalian expression vector encoding both Zn fingers of PXR cloned at EcoRI & BamHI site of GFP-C2 vector	(Rana et al. 2018)[36]
GFP-PXR-LBD	Mammalian expression vector encoding hinge and LBD domains of PXR cloned at EcoRI & BamHI site of GFP-C2 vector	(Rana et al. 2018)[36]
GFP-PXR-NTD-DBD	Mammalian expression vector encoding NTD and DBD domains of PXR cloned at EcoRI & BamHI site of GFP-C2 vector	(Rana et al. 2018)[36]
GFP-PXR-DBD-LBD	Mammalian expression vector encoding DBD and CTD domains of PXR cloned at EcoRI & BamHI site of GFP-C2 vector	(Rana et al. 2018)[36]
GFP-ERα-NTD	Mammalian expression vector encoding NTD of ERα cloned at KpnI & BamHI site of GFP-C1 Vector	This study
GFP-ERα-DBD	Mammalian expression vector encoding DBD of ERα cloned at KpnI & BamHI site of GFP-C1 Vector	This study
GFP-ERα-LBD	Mammalian expression vector encoding LBD of ERα cloned at KpnI & BamHI site of GFP-C1Vector	This study
GFP-ERα-ΔNTD	Mammalian expression vector encoding ΔNTD of ERα cloned at KpnI & BamHI site of GFP-C1 Vector	This study
GFP-ERα-DBD-LBD	Mammalian expression vector encoding DBD of ERα cloned at KpnI & BamHI site of GFP-C1 Vector	This study
GFP-ERα-ΔLBD	Mammalian expression vector encoding ΔLBD of ERα cloned at KpnI & BamHI site of GFP-C1 Vector	This study
RFP-SHP R216E	Mammalian expression vector encoding full-length Human SHP cloned in RFP-C1 vector Arginine replaced with Glutamic acid at amino acid 216	This study
RFP-SHP R216A	Mammalian expression vector encoding full-length human SHP cloned in RFP-C1 vector	This study

	Arginine replaced with Alanine at amino acid 216	
RFP-SHP W206H	Mammalian expression vector encoding full-length human SHP cloned in RFP-C1 vector Tryptophan replaced with Histidine at amino acid 206	This study
RFP-SHP W206A	Mammalian expression vector encoding full-length human SHP cloned in RFP-C1 vector Tryptophan replaced with Alanine at amino acid 206	This study
RFP-SHP E204R	Mammalian expression vector encoding full-length human SHP cloned in RFP-C1 vector Glutamic acid replaced with Arginine at amino acid 204	This study
RFP-SHP E204A	Mammalian expression vector encoding full-length human SHP cloned in RFP-C1 vector Glutamic acid replaced with Alanine at amino acid 204	This study
RFP-SHP E154R	Mammalian expression vector encoding full-length human SHP cloned in RFP-C1 vector Glutamic acid replaced with Arginine at amino acid 154	This study
RFP-SHP E154A	Mammalian expression vector encoding full-length human SHP cloned in RFP-C1 vector Glutamic acid replaced with Alanine at amino acid 154	This study
RFP-SHP N176D	Mammalian expression vector encoding full-length human SHP cloned in RFP-C1 vector Asparagine replaced with Aspartic acid at amino acid 176	This study
RFP-SHP N176A	Mammalian expression vector encoding full-length human SHP cloned in RFP-C1 vector Asparagine replaced with Alanine at amino acid 176	This study
RFP-SHP K225A	Mammalian expression vector encoding full-length human SHP cloned in RFP-C1 vector Lysine replaced with Alanine at amino acid 225	This study

Oligo nucleotides used for site-directed mutagenesis of RFP-SHP in this study

Oligo Name	Sequence (5'----------3')	PCR Profile Temperature
RFP-SHP R216E	**FP: CGC CTG ACC GAA GTC CTC CTC ACG GCC** **RP: GAG GAG GAC TTC GGT CAG GCG GCC TTG**	66℃
RFP-SHP R216A	**FP: CGT GAG GAG GAC AGC GGT CAG GCG GCC T** **RP: AGG CCG CCT GAC CGC TGT CCT CCT CAC G**	66℃

RFP-SHP W206H	**FP: CTG GAA CCC CAC TGC CCA GCA GCC CAA** **RP: TGC TGG GCA GTG GGG TTC CAG GAC TTC**	66℃
RFP-SHP W206A	**FP: GTC CTG GAA CCC GCG TGC CCA GCA GCC** **RP: GCT GCT GGG CAC GCG GGT TCC AGG ACT**	66℃
RFP-SHP E204R	**FP: GAA GTC CTG CGA CCC TGG TGC CCA GCA** **RP: GCA CCA GGG TCG CAG GAC TTC ACA CAG**	66℃
RFP-SHP E204A	**FP: TGT GAA GTC CTG GCA CCC TGG TGC CCA G** **RP: TGG GCA CCA GGG TGC CAG GAC TTC ACA C**	66℃
RFP-SHP E154R	**FP: TGC TGT CTG CGG TCC TTC TGG AGC CTG** **RP: CCA GAA GGA CCG CAG ACA GCA TTG AAG**	66℃
RFP-SHP E154A	**FP: CTT CAA TGC TGT CTG GCG TCC TTC TGG AGC CTG** **RP: CAG GCT CCA GAA GGA CGC CAG ACA GCA TTG AAG**	66℃
RFP-SHP N176D	**FP: ATC CTC TTC GAC CCC GAT GTG CCA GGC** **RP: CAC ATC GGG GTC GAA GAG GAT GGT CCC**	66℃
RFP-SHP N176A	**FP: GGG ACC ATC CTC TTC GCC CCC GAT GTG CCA GG** **RP: CCT GGC ACA TCG GGG GCG AAG AGG ATG GTC CC**	66℃
RFP-SHP K225A	**FP: TGG TCG GAA TGG ACG CGA GGG TGG AGG CCG** **RP: CGG CCT CCA CCC TCG CGT CCA TTC CGA CCA**	66℃

Primers used for RT-PCR

NRNC Symbol	NR Common Name	ACCESSION No.	Primer Sequence
NR0B1	DAX-1	NM_000475	FP: GCCATCAAGTGCTTTCTTTCC
			RP: GCACGTCCGGGTTAAAGAG
NR0B2	SHP	NM_021969	FP: GCCTGAAAGGGACCATCCTC
			RP: CCAGGGTTCCAGGACTTCAC
NR1I2	PXR	NM_003889	FP: CGGCATGAAGAAGGAGATGAT
			RP: GTCCCTGTCCGTTCACTTT
NR1I3	CAR	NM_001077481	FP: CTGAGGAACTGTGTGGTATGTG
			RP: TTGCTGACTGTTCTCCTGAAG
NR2A1	HNF4α	NM_000457	FP: GTGTCCATACGCATCCTTGA
			RP: GGCATCTGGGTCAAAGAAGAT
NR2B1	RXRα	NM_002957	FP: CTTCTCCGTACGATTGTCTCTG
			RP: CCTCTCACACTTCTCCCTTTG
NR3A1	ERα	NM_001122741	FP: TAGAATGTGCCTGGCTAGAGA
			RP: CCTGTCCAAGAGCAAGTTAGG
NR3C4	AR	NM_000044	FP: CGCTGAAGGGAAACAGAAGTA
			RP: CGAAGACGACAAGATGGACAA

Methods

Bacterial strains and growth conditions

For cloning and amplification of plasmid DNA, *Escherichia coli (E. coli)* strain DH10β was used. For recombinant protein expression, *E. coli* strain BL21 (DE3) was used. Both the bacterial strains were cultured in Luria Bertani (LB) medium with an appropriate antibiotic (50 µg/ml kanamycin or 100 µg/ml ampicillin) overnight at 37℃ with constant vigorous shaking (250 rpm).

Preparation of bacterial competent cells and transformation

E. coli strains (DH10β and DE3) competent for transformation were prepared by Calcium chloride ($CaCl_2$) method according to the protocol described in Sambrook et al. 1989. Briefly, a primary culture was set up by inoculating a single colony of the appropriate *E. coli* strain in 5 ml LB medium and grown overnight at 37℃ with constant shaking at 250 rpm. The following day, 0.1% of this overnight grown, saturated primary culture was used to inoculate 50 ml LB medium and again incubated at 37℃ with constant shaking at 250 rpm. This secondary culture was allowed to grow until its OD_{600} was between 0.4 - 0.6, which indicated the log-phase of bacterial growth. After attaining the desired OD, the culture was chilled on ice for about 15 minutes and pelleted by centrifugation at 6000 rpm for 10 minutes at 4℃. The supernatant was discarded, and the cell pellet was resuspended in 15 ml of sterile, pre-chilled 0.1M $CaCl_2$ by gentle swirling and incubated on ice for 10 minutes. This cell suspension was again centrifuged at 6000 rpm for 10 minutes at 4℃. The resulting cell pellet was resuspended in 1.5 ml ($1/10^{th}$ of the starting volume of $CaCl_2$) of ice-cold 0.1M $CaCl_2$ and kept on ice for 2 hours. Once the incubation period was over, sterile, pre-chilled glycerol was added dropwise with gentle mixing to the cell suspension to a final concentration of 15%. The competent cells thus obtained were stored as 100 µl aliquots at -80℃ until further use.

For transformation, the competent *E. coli* cells were thawed on ice and 100 ng of the plasmid DNA of ligated DNA product was added per 100 µl aliquot. The cells were incubated on ice for about 30 minutes with intermittent mixing. After the incubation period, the cells were given a heat shock at 42℃ for 90 seconds and immediately followed by chilling on ice for 5 minutes. LB medium (1 ml) was added to these cells and allowed to grow for 1 hour at 37℃ with vigorous shaking. The cells

were then plated on LB agar plates containing appropriate antibiotic and incubated at 37℃ for 16 hours. The following day, the plates were observed for bacterial colonies.

Mini-scale plasmid DNA isolation by alkaline lysis method

Plasmid DNA isolation at mini-scale was performed by alkaline lysis method as described by Sambrook et al. 1989, with minor modifications. Transformed *E. coli* cells harbouring the plasmid of interest were grown in 5 ml LB medium containing appropriate antibiotic for 12-16 hours with vigorous shaking at 37℃. The cells were centrifuged at 4,000 rpm for 1 min and the supernatant was discarded. The pellet was resuspended in 100 µl of resuspension buffer (25 mM Tris-Cl pH 8.0, 50 mM glucose, 10 mM EDTA pH 8.0 and 20 µg/ml RNase A) by mixing thoroughly. Cells were lysed by adding 200 µl of lysis solution (0.2 N NaOH and 1% SDS) and mixing by gently inverting the tube (8-10 times) followed by incubation at room temperature for 2-5 minutes. The suspension was neutralized by adding 350 µl of the neutralizing solution (3 M potassium acetate and 2 M glacial acetic acid) and gentle mixing by inversion. Cells were centrifuged at 4,000 rpm for 15 minutes to clear the cell debris. The clear supernatant was extracted with 1:1 mixture of phenol and chloroform followed by centrifugation at 4,000 rpm for 5 minutes. The upper aqueous phase was carefully removed and re-extracted with chloroform by mixing and centrifugation at 4,000 rpm for 5 minutes. The upper aqueous phase was separated into a fresh microcentrifuge tube and DNA was precipitated by adding two volumes of absolute ethanol and incubating at -80℃ for 1 hour. The precipitated plasmid DNA was separated by centrifugation at 12000 rpm for 10 minutes and washed with 70% ethanol. The DNA pellet was allowed to air dry and resuspended in 100 µl of TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0) and stored at -20℃ until use.

Quantification of plasmid DNA

The isolated plasmid DNA was quantified by measuring its absorbance at 260 nm. A ratio of the ODs at 260 nm and 280 nm was used to estimate the purity of eluted DNA. To quantify, OD_{260} of the appropriately diluted plasmid DNA sample was measured on a spectrophotometer keeping TE buffer as sample blank. DNA concentration was estimated by utilizing the following equation:

DNA concentration (ng/µl) = OD at 260 nm X 50 X dilution factor

Agarose gel electrophoresis

Agarose gel electrophoresis of DNA samples was performed as described by Sambrook et el. 1989. In brief, 1% agarose was dissolved in TAE buffer (40 mM Tris-Acetate, 1.0 mM EDTA, pH 8.0) by heating followed by cooling of the solution to about 37℃ before adding ethidium bromide (0.5 µg/ml). The gel was immediately cast on a gel casting tray and allowed to solidify. DNA samples were mixed with DNA gel loading buffer and loaded onto the gel. The samples were electrophoresed at 5 V/cm in TAE buffer and the resolved DNA was visualized on a UV transilluminator.

Prokaryotic expression and purification of human SHP

SHP was subcloned into the prokaryotic expression vector pET-30b(+) and transformed into *E. coli* BL21 (DE3) cells. This vector expressed SHP with an N-terminal His (6X-Histidine) tag. Expression of His-SHP was induced by adding 0.2 mM IPTG to the medium containing transformed *E.* coli cells growing in log phase (OD_{600} between 0.4 - 0.6). The induction was given for 18 hours at 25℃ with constant vigorous shaking at 180 rpm. The cells were centrifuged at 5,000 rpm for 10 minutes at 4^0C and lysed with lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 1 mM PMSF, 1 mM DTT, 100 µg/m lysozyme and 1 µg/ml each of pepstatin, leupeptin and aprotinin) followed by an incubation at 4℃ for 1 hour. The lysates were sonicated and centrifuged and the protein was found to be in both the fractions, i.e. soluble as well as inclusion bodies. The soluble fraction from lysate was bound with Ni-NTA-agarose beads for 16 hours at 4℃. The beads were then washed three times with wash buffer (100 mM Tris-Cl pH 8.0, 1 mM PMSF, 1 mM DTT and protease inhibitor cocktail). His-SHP bound to the beads was eluted by incubating with elution buffer (100 mM Tris-Cl pH 8.0, 1 mM PMSF, 1 mM DTT and protease inhibitor cocktail) on a rotor at 4℃ for 8 hours. The purity and amount of His-SHP were checked by running the samples on SDS-PAGE.

Polyclonal antibody generation

Ethical clearance for the use of rabbit to generate polyclonal antibody against human SHP was obtained from Institutional Animal Ethical Committee Jawaharlal Nehru University, New Delhi, India (IAEC code 06/2016). To generate the polyclonal antibody against SHP, about 250 µg of His-SHP protein were electrophoresed on SDS-

PAGE and specific band at ~34kDa was cut. The excised band was chopped into fine pieces and thoroughly mixed with equal volumes of PBS and Freund's complete adjuvant. The resulting antigen-adjuvant mixture was injected subcutaneously at 3 sites in a 3 months old female New Zealand white rabbit. Booster doses of 150 µg purified His-SHP protein processed similarly was given every 21 days for the next 90 days (4 boosters). Test serum was collected 7 days post every booster according to the standard antibody generation protocol [278]. The generation and specificity of an anti-SHP antibody were examined by testing the anti-serum through western blotting and immunocytochemistry.

Mammalian cell lines, growth conditions and cryopreservation

Cell lines used in this study were HepG2 (human hepatocellular carcinoma cell line), COS-1 (African green monkey kidney cell line) and HEK293T (human embryonic kidney cell line). These ATCC hallmarked cell lines were procured from National Cell Repository, National Centre for Cell Science (NCCS), Pune, India. The cell lines were routinely cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum containing 100 µg/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin. The cells were maintained at 37℃ in a humidified incubator in 5% CO_2 and 95% air atmosphere.

Throughout regular culturing, the cells were allowed to grow till 90-95% confluency was attained and then subcultured by detaching the cells from plates using trypsin-EDTA. During trypsinization, the culture medium was removed, and cells were washed with sterile PBS. Then 1 ml trypsin-EDTA per 100 mm plate was added for a few minutes and subsequently removed. The plate was then incubated in a CO_2 incubator for a few minutes and observed under a phase contrast microscope for rounding up and detachment of cells from the plate surface. Once detached, the cells were resuspended in fresh culture medium and reseeded in fresh culture dishes.

For cryopreservation, the cells were detached from the plates by trypsinization, resuspended in fresh culture medium and then centrifuged at 1000 rpm for 3-4 minutes. The medium was discarded and the cell pellet was gently resuspended in complete DMEM containing 5 or 10% DMSO depending on the cell line as recommended by ATCC. This cell suspension was transferred to 1 ml cryovials and slowly frozen at a cooling rate of -1℃ per minute which is achieved by using cryo-cooler (Tarsons, India)

placed at -80℃. After 24 hours, the vials were transferred to liquid nitrogen (-196℃) container for long term storage.

Liposome-mediated transient transfections

Transient transfections were performed in 35mm, 12-well or 24-well tissue culture plates as per the experimental requirement. Cells were seeded in DMEM complete medium (with FBS and antibiotics) so as to achieve about 60% confluency after 24 hours of incubation at 37℃ in a CO_2 incubator. Following day, the medium was removed and the cells were washed with 1 ml of OPTI-MEM I medium to remove traces of serum and antibiotics. Then, fresh OPTI-MEM I was added to the wells (800 µl for 35mm plate, 500 µl/well for 12-well plate and 250 µl/well for 24-well plate) and incubated for 45 minutes in the incubator. Meanwhile, DNA-lipid complexes were prepared by mixing appropriate amounts of plasmid DNA (500-800 ng for 35 mm plate, 600 ng/well for 12-well plate and 300 ng/well for 24-well plate) and transfection reagent (5 µl Lipofectamine 2000/Escort IV for 35 mm plate, or 2.5 µl/well or 1.25 µl/well Escort III for 12-well or 24-well plate, respectively) to a final volume of 50 µl, 100 µl and 200 µl for 24-well, 12-well and 35 mm plate, respectively, in OPTI-MEM I and incubated for 40 minutes at room temperature. Once the incubation period was over, the DNA-lipid complexes were added to the plates dropwise and the plates were again incubated at 37℃ in CO_2 incubator for 10-12 hours. Following the transfection period, the complexes were removed by aspirating the medium and the cells were supplemented with 5% charcoal-stripped serum containing DMEM without antibiotics and allowed to grow for a further 24-36 hours. These transiently transfected cells were then processed according to the experimental requirement.

MTT Assay

About $0.2x10^5$ cells were seeded in each well of a 24 well culture cluster. The samples were collected at the required time point in triplicates. 10 µl of MTT (1mg/ml) dye was added to each well and incubated at 37℃ for 4 hours in a humidified CO_2 incubator. The precipitate formed was solubilized in 150 µl of the DMSO after discarding the media and the absorbance was recorded when all samples were collected. The colored formazan product is stable at 4℃ for several days. The absorbance was recorded at 570 nm.

Preparation of whole cell lysates from cultured cells

For preparing whole cell lysates, cultured cells were washed twice with ice-cold PBS to remove the culture media and then mechanically scraped from the dish surface using an ice-cold cell scrapper while still in PBS. The cells were transferred to cold microcentrifuge tubes and centrifuged at 1,500 rpm for 5 minutes. PBS was removed, the cell pellet resuspended in ice-cold lysis buffer (20 mM Tris pH 7.6, 150 mM NaCl, 0.5 mM DTT, 1 mM EDTA, 1 mM PMSF, 0.1% NP-40 and protease inhibitor cocktail) and incubated on ice for 30 minutes with intermittent tapping. After the incubation period, samples were centrifuged at 12,000 rpm for 15 minutes at 4℃ and the clear supernatant representing whole cell lysate was transferred to fresh, ice-cold microcentrifuge tubes. The lysates were stored at -80℃ until use.

Electrophoresis of proteins on Sodium Dodecyl Sulphate-Polyacrylamide Gel (SDS-PAGE)

Polyacrylamide gel electrophoresis of proteins was performed under denaturing conditions (in the presence of 0.1% SDS) according to Laemmli's method [279]. The proteins were stacked in a gel containing 5% acrylamide, 0.106% N, N'-methylene bisacrylamide, 0.125 M Tris-Cl pH 6.8, 0.01% TEMED and 0.1% ammonium persulfate. The proteins were resolved at pH 8.8 in a resolving gel that consisted of 10% acrylamide, 0.33% N, N'-methylene bisacrylamide, 0.375 M Tris-HCl pH 8.8, 0.01% TEMED and 0.1% ammonium persulfate. SDS-PAGE sample buffer consisting of 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and 5% β-mercaptoethano [279] was used to prepare protein samples for electrophoresis. The samples were boiled at 95℃ for 5 minutes, cooled and loaded onto the gel after a brief spin. The samples were electrophoresed in a running buffer composed of 0.025 M Tris-base, 0.192 M glycine pH 8.3 and 0.1% SDS. Standard molecular weight markers were also run alongside the protein samples to estimate the molecular size of the resolved proteins.

Western Blotting

Proteins resolved on 10% SDS-PAGE gel were transferred onto polyvinyl difluoride (PVDF) membrane (freshly charged with methanol) using a wet transfer system (Invitrogen, USA). The size-fractionated proteins were then transferred onto a PVDF membrane, which was blocked with 5% non-fat milk in PBS for 1 hours. Primary antibody dilutions in PBS (pH 7.2), against the respective protein, was incubated with

the membrane overnight at 4℃. After washing three times with PBS containing 0.05% Tween-20, the membrane was incubated with horseradish peroxidase (HRP) or alkaline phosphatase (AP) conjugated secondary antibody at room temperature for 1 hour. The membrane was again washed three times, and immune complexes were detected by enhanced chemiluminescence (ECL substrate) or AP substrate staining (66 µl NBT and 34 µl BCIP in 10 ml Alkaline Phosphatase buffer).

Enhanced Chemiluminescence (ECL)

Blots were treated with 10 ml of 100 mM Tris-Cl, pH-8.5, containing 22 µl p-coumaric Acid (90 mM), 50 µl luminol (250 mM), 3µl H_2O_2, after throwing away PBST and monitored until bands fluoresce. S-ray cassette was wiped with wet tissue and the blot was kept in it, covered with saran wrap and on top of that, a film of appropriate size was placed. The film was removed after exposure and put in Developer Solution, washed in water and then fixed in Fixer solution until film becomes transparent. The film was then kept in the water.

Fluorescence microscopy

a) Live cell imaging: Cells cultured in 35 mm plates were transfected with Lipofectamine 2000 or Escort IV transfection reagent as described previously [280]. After completion of the transfection period, the cells were supplemented with 5% charcoal-stripped serum containing DMEM without antibiotics and incubated for a further 24-36 hours to allow for protein expression. Two hours prior to imaging, DNA staining dye Hoechst 33258 at 0.5 µg/ml was added to the plates to facilitate visualization of the nucleus. The cells were observed under a Nikon fluorescent microscope fixed with a water immersion objective lens and subcellular localization or chromatin association was recorded depending on the experimental requirement. Subcellular localization was categorized into 5 sub-types depending on the distribution of fluorescence between cytoplasm and nucleus. When fluorescence was restricted to the cytoplasm, the localization was counted as cytoplasmic (C). When the majority of the fluorescence was observed in one of the compartments and lower in the other, the localization was accordingly considered as either predominantly cytoplasmic (C>N) or predominantly nuclear (N>C). Completely nuclear fluorescence with none in the cytoplasm was scored as nuclear (N) localization. When the fluorescence was more or

less equal in both the compartments, the receptor localization was considered to be uniform in cytoplasm and nucleus (N=C).

b) Immunocytochemistry: For indirect immunodetection, the cells were cultured on sterile glass coverslips and transfected with appropriate plasmids. Following transfection, the medium was decanted and the cells were washed twice with PBS to remove traces of the medium. The cells were then fixed with chilled methanol and kept on ice for 20 minutes. After the incubation period, the coverslips were washed thrice with PBS and blocked with 3% BSA-PBS (BSA prepared in PBS) for 30 minutes at room temperature. Subsequently, the coverslips were probed with the primary antibody, appropriately diluted in 2% BSA-PBS and incubated in a humid chamber at 4℃ overnight. Following day, the cells were washed thrice with PBS to remove unbound primary antibody and incubated with appropriate Cy-3 conjugated secondary antibody combined with Hoechst 33258 (0.5 μg/ml) in a humid chamber for 1 hour at room temperature. The cells were again washed thrice with PBS and mounted on glass slides with 40% glycerol. The edges and corners of coverslips were sealed with transparent nail polish, allowed to air dry and observed under the fluorescence microscope.

Hematoxylin-Eosin Staining

Hematoxylin-Eosin staining is the most commonly used method for morphological analysis of cells under a light microscope. Hematoxylin stains the basophilic components, whereas Eosin stains the acidophilic components of a cell. Cells fixed in chilled methanol were processed for Hematoxylin-Eosin staining. At first, the methanol was aspirated out followed by 2 changes of 99% ethanol for 2 min each. Then 2 changes of 95% ethanol for 2 mins each were given. After washing with distilled water for 1 min Hematoxylin was put on the coverslips and kept for 1 min. After thorough washing in running water for 3 min, Eosin was put and kept for 30 sec only. Rinsed in running tap water for 30 sec. Dehydration steps were followed by giving two changes of 95% ethanol for 2 minutes each and again two changes of 99% ethanol for 2 minutes each. At last, the coverslips with stained cells were mounted with 50% glycerol sealed with nail enamel and observed under light microscope.

Luciferase reporter assay for gene expression

The luciferase promoter-reporter assay is the most widely used assay for analysis of gene expression. This assay is based on the principle that when a transcription factor occupies the promoter region, it induces the expression of the downstream gene. We utilized different promoter cloned upstream of the *Luciferase* gene which encodes luciferase enzyme. Luciferase acts on the substrate luciferin to produce luciferin with the emission of light **(Fig. 14).** Thus, detection of this light gives a measure of *luciferase* gene expression and in turn, promoter occupancy by the protein of interest, in our case SHP with respective genes.

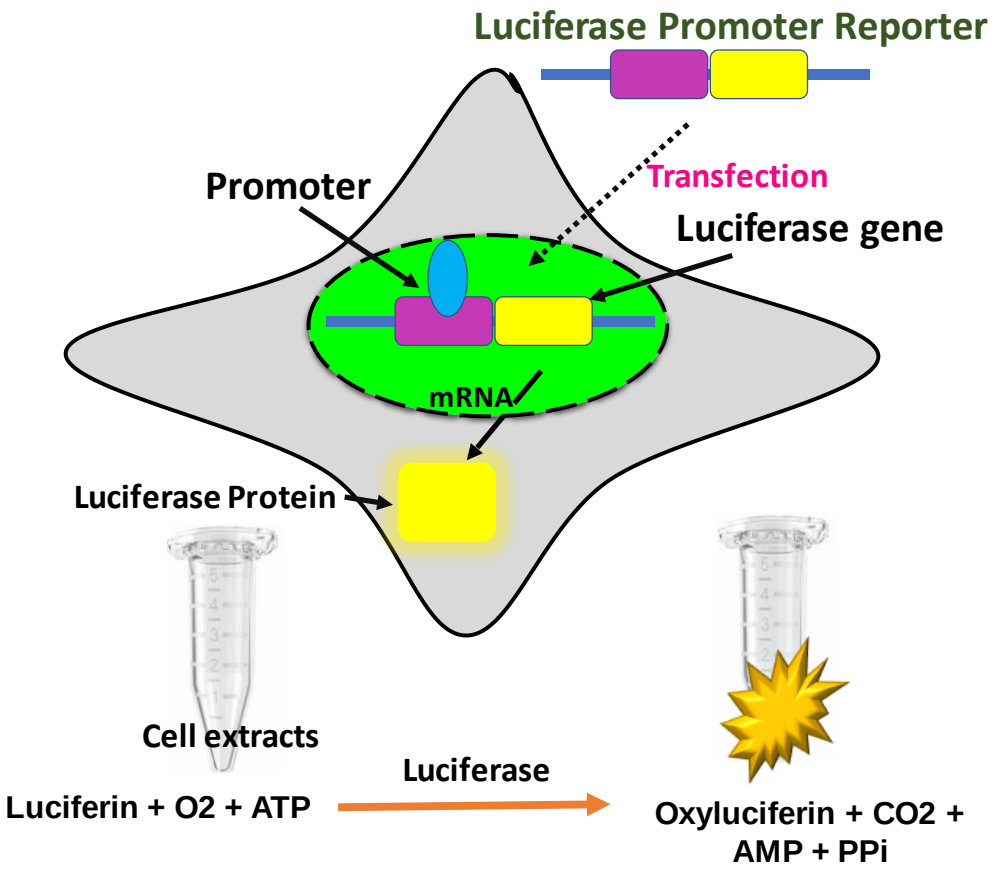


Figure 14: Schematic presentation of principle of a simple luciferase reporter assay. *The reporter plasmid vector consists of the target promoter sequence and a luciferase gene sequence. After transfection of the plasmid into target cells, the promoter region regulates the expression of luciferase gene in living cells. The expressed luciferase protein catalyzes a reaction with luciferin to produce light. In the transient transfection luciferase assay, luciferase-expressing cells are lysed for an appropriate period. The amount of expressed luciferase protein can be estimated from the light intensity, which indicates the promoter activity in living cells. In this case, the promoter activity is normalized by cell numbers or cellular enzymatic activity. [Adopted and modified from* [281]*]*

To perform this assay, HepG2 cells were seeded in either 12-well or 24-well plates and transiently transfected with Escort III transfection reagent as described previously. After 12-14 hours of transfection period, the medium was changed to 5% charcoal-stripped serum containing DMEM, and appropriate treatments were given. The cells were incubated in a humidified CO_2 incubator at 37℃ for 24 hours. Following day, the cells were washed twice with PBS and harvested with reporter lysis buffer (125 mM Tris-phosphate pH 7.8, 10 mM DTT, 10 mM 1, 2-diaminocyclohexane-N, N, N´, N´-tetraacetic acid, 50% glycerol and 5% Triton X-100). The cell lysates were centrifuged at 12,000 rpm for 10 minutes and luminescence was measured on a TD20/20 DLReady™ luminometer after adding luciferin substrate (Promega, Madison, WI, USA) at a 5:1 dilution.

For normalization, β-galactosidase expressing plasmid DNA was cotransfected as an internal transfection control. To perform the normalization, 50 µl of cell lysate was mixed with 50 µl of β-galactosidase assay buffer (200 mM sodium phosphate buffer pH 7.4, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol and 1.33 mg/ml o-Nitrophenyl-β-galactopyranoside). The mixture was incubated at 37ºC until a faint yellow color developed. The absorbance of the samples was measured at 415 nm using a microplate reader (Infinite 2000, Tecan Austria GmbH).

Site-directed mutagenesis

Specific amino acid substitutions in SHP protein were performed by site-directed mutagenesis. Inverse PCR utilizing high fidelity thermostable DNA polymerase was used to incorporate the desired mutations. Mutagenic primers were designed such that the desired mutation was at the center of the complementary primer pair. 50 ng template DNA (RFP-tagged SHP) was amplified using Phusion high fidelity DNA polymerase (NEB, England) for 18-25 cycles. Following PCR, the products were treated for 1 hour with 1 µl of DpnI restriction endonuclease that specifically recognizes and digests methylated and hemi-methylated DNA sequences. 10 µl of the digested product was checked on 1% agarose gel for amplicon size and presence of undigested template DNA. *E. coli* DH10β competent cells were transformed with the remaining digestion product and positive clones were confirmed by DNA sequencing.

Analysis of RNA

Glassware used for RNA isolation and analysis were baked at 200℃ for 16-18 hours. Plastic wares were treated with 3.0% (w/v) H_2O_2 for 2 hours and later autoclaved at 15 lb/sq. in for 30 minutes. All the solutions (except Tris buffer) were prepared in DEPC treated water. DEPC was dissolved (0.1%, 86 v/v) in Milli-Q water with vigorous stirring for 2 hours followed by overnight incubation at 37℃ and then autoclaved.

RNA extraction and Real-Time quantitative PCR

The primers used in this study were designed using PrimerQuest tool of Integrated DNA Technologies (IDT) and verified by NCBI primer blast tool according to the mRNA sequences of the target gene and internal control GAPDH and β-actin and synthesized by IDT for human specific primers. Total RNA was extracted with TRI Reagent according to the manufacturer protocol (Sigma-Aldrich, USA). Total 1 µg RNA was reversed transcribed using iScript cDNA Synthesis Kit for first-strand cDNA synthesis following manufacturer protocol priming for 5 minutes at 20℃, reverse transcription for 20 minutes at 46℃ and RT inactivation for 1 minute at 95℃ (Catalog Number 1708890, Bio-Rad, California, USA), and first-strand cDNA samples were diluted in 1:6 ratio and subjected to quantitative PCR by using SYBER Green (PowerUp™ SYBR™ Green Master Mix Applied Biosciences). Real-time quantitative PCR was performed using Step One System (Applied Biosystems). In the PCR reaction, DNA template was pre-incubated for 2 minutes at 50℃ and the denatured for 5 minutes at 95℃ followed by amplification steps cycles of 30 seconds denaturation at 95℃, 1-minute annealing at 60℃. The amplification steps in the PCR reaction were for 15 seconds only. RT-qPCR was carried out by using the specific primer mentioned in **Table**, and the relative amplification of the target genes were calculated using the $2-\Delta\Delta Ct$ method, where $\Delta\Delta Ct$ is $\Delta Ct_{(Control)}$-$\Delta Ct_{(Experimenat)}$, ΔCt is $Ct_{(Target\ gene)}$ - $Ct_{(House\ keeping\ gene)}$ and Ct is the cycle, at which threshold crossed. PCR product quality was monitored using a post-PCR melt curve analysis.

In silico modelling of SHP-PXR and SHP-ERα complexes and its interactions

The complex structure of small heterodimer partner (SHP) and pregnane X receptor (PXR) was modelled to understand the binding mode and nature of the mechanism of its interaction. As the X-ray structure of human SHP was not available, so, first we built the 3D structure of human SHP using MODELLER [282]. Crystal structure of *E. coli* expressed mouse SHP was taken as the template (PDB ID: 4NUF) with 81% sequence identity. The stereochemical quality of the modelled structure was evaluated using the Ramachandran Plot [283]. Crystal structure of pregnane X receptor (PXR) (PDB code: PX0R) and the crystal structure of ERα (PDB code: 4DMA) are available. The structures of PXR, SHP and ERα, were energy-minimized using GROMOS96 forcefield [284] in order to remove steric clashes. To model the structure of SHP with the complexes of SHP-PXR and SHP-ERα, protein-protein docking [285,286] was performed using Cluspro software [287]. The potential interactions of the respective protein-protein complexes were further analyzed using pyMOL (PyMOL) and Dimplot [288].

Molecular Dynamics (MD) Simulations

To validate the stability of the docked complexes, the MD simulations were done using GROMACS 5.1.2 package with the GROMOS 96 force field. The structures were solvated in a solvation box with 10Å distance. For creating electro-neutrality conditions, NaCl counter ions were included. By utilizing the steepest descent method, the energy was minimized. Parrinello-Rahman pressure coupling and Berendsen temperature coupling were employed to maintain a stable system environment (300 k, 1 bar). The coupling constants for temperature and pressure were set to 0.1ps and 2.0 ps, respectively. The partial mesh Ewald (PME) algorithm was implemented for the measurement of electrostatic and Van der Waals interactions. For the small-range VdW (rvdw) cut-off distance was fixed to 1.4 nm. Coulomb cut-off (r coulomb) and neighbor list (rlist) were set to 0.9 nm. To measure all the constrained bond lengths, the LINCS algorithm was used, and the time step was set to 0.002 ps. The complexes in a medium were equilibrated for 100 ps in NPT and NVT ensembles, respectively. For both native and mutant complexes of SHP-PXR and SHP-ERα complex, a 25 ns molecular dynamics simulation was carried out [283,288]. All trajectories obtained were stored every 2ps for further analysis.

Structural Analysis of Molecular Dynamics (MD) Simulations

The trajectory files obtained from MD simulations were used for calculating the structural properties of the SHP-PXR and SHP-ERα, and its mutant complexes with the built-in modules of GROMACS 5.1.2 [286,289]. Through the use of g_rmsd and g_gyrate, root-mean-square deviation (RMSD) and radius of gyration were analysed respectively with the built-in functions of GROMACS. To calculate, the number of hydrogen bonds present within the protein during simulation, the g_bond utility was used. DIMPLOT was used to determine the formation of H-bonds. For, DIMPLOT the donor-acceptor distance was smaller than 3.6 Å and of donor-hydrogen-acceptor angle larger than 90°. In order to create plots for three-dimensional backbone RMSD, of carbon-alpha, gyration of backbone (Rg) Graphing Advanced Computation and Exploration (GRACE) program were used (http://plasma-gate.weizmann.ac.il/Grace/) for plotting graphs.

Statistical analysis

Most of the experiments were performed in triplicates and the results represent mean ± SD of at least three independent experiments. Statistical analysis was done on GraphPad Prism (version 8.0.1) software. Student's t-test and ANOVA was used to calculate the significance of results that differed significantly from the control experiments. Asterisks (*) represent p values less than 0.05.

Chapter-I

Investigation into the subcellular localization & dynamics of SHP

Background

The NR superfamily consists of ligand-activated transcription factors which play diverse roles in cell differentiation, development, proliferation and metabolism [51,290]. They are associated with numerous pathologies such as cancers, cardiovascular diseases, inflammation, and reproductive abnormalities [291]. Members of this family have a modular structure containing an NTD, a central highly conserved zinc-finger harboring DBD and a CTD. Ligand binding to a cognate nuclear receptor results in the transactivation of specific genes within its target tissue [38,100].

Uniquely, out of all the 48 human NRs, SHP and DAX-1 are structurally and functionally differ from typical NRs [8,178]. They harbour all the putative domains but lack the most conserved DNA binding domain, which makes them an exceptional entity within the superfamily. In humans, SHP is predominately expressed in the liver but its presence is also reported in heart, pancreas, kidney, spleen, small intestine, adrenal gland and stomach [292]. The expression of SHP gene in many tissues potentially has essential biological functions and reportedly can be affected by genetic variations such as insertions, deletions, repetitive elements, single nucleotide polymorphisms (SNPs) and large chromosomal rearrangements which in turn might lead to onset of diseases [263,293].

The physiological relevance of SHP appears to lie in its ability to interact with some of the NRs and other interacting proteins as a repressor of transcription function and gene expression [13,29]. The proposed interaction mechanism(s) of SHP is similar to corepressor/activator-like proteins [23,294]. SHP has two functional LXXLL related motifs which bind to the AF-2 domain of NRs i.e. (the C-terminal transcription activation domain located within the LBD of ligand-regulated and constitutively active NRs) [13].

The ability to localize and translocate proteins to specific compartments is fundamental to the organization and functioning of all living cells. The regulation of gene expression by steroid/nuclear receptors occurs mainly through the subcellular compartmentalization of liganded and unliganded receptors [87]. When the cellular localization and dynamics of critical proteins are compromised, it has severe impact on normal cellular functions thereby leading to diseases [295]. Therefore, a detailed understanding of these processes may provide insight for molecular diagnosis and disease management.

Several groups have shown that nuclear receptors continuously shuttle between the cytoplasm and the nucleus. The steady-state localization of a nucleocytoplasmic shuttling protein is a consequence of a fine balance between the operational strengths of nuclear localization signal (NLS) and nuclear export signal (NES) [86,87,296]. Although, GFP-tagged SHP has been reported to localize predominantly in the nucleus [297], confirmed by coimmunoprecipitation experiments [168], previous studies on subcellular localization and functioning of SHP are inconclusive or controversial.

In the present study, we initially generated tools to study the subcellular localization of SHP in fixed and living cells. The discovery of fluorescent proteins like GFP (Green Fluorescent Protein) and RFP (Red Fluorescent Protein) have revolutionized the area of cell biology. Hence, GFP and RFP-tagged SHP constructs have been created, and subsequently utilized for visual localization of the receptor in the living cells. Further, we also attempted to explore nuclear import and export signal through which many nuclear receptors translocate between the nuclear and cytoplasmic compartments. To study the cellular changes at transcript and protein levels, SHP overexpressing stable cell line was generated and characterized. Normally, in cells transcription factors are not expressed at high levels. Therefore, to make sensitive immunodetections we successfully raised polyclonal antibodies against full-length human SHP.

Results

Generation of SHP polyclonal antibody against full-length receptor protein

Detection of endogenous level of the NR (transcription factors) *via* immuno-techniques is generally challenging because the abundance of their expression in the cells and tissues is low as compared to the other proteins. So, the polyclonal antibody raised against the full-length receptor provides a better alternative over others as polyclonal antibody is expected to recognize the multiple epitope sites on the proteins without compromising with specificity.

A. Purification of full-length recombinant SHP protein from prokaryotic expression system and production of polyclonal antiserum against SHP

To detect the expression of SHP and to study the functional and behavioral aspects of the receptor in details, we required a highly specific, sensitive and well-characterized antibody. Therefore, polyclonal antibody against full-length SHP was

generated in rabbit. For this purpose, full-length SHP was subcloned into pET30b(+) expression vector tagged with 6X-His **(Fig. 14A)**. For prokaryotic expression, *E. coli* BL21 (DE3) cells were transformed with pET30b (+)-SHP and protein expression were induced with 0.2 mM IPTG for 18 hrs at 25℃. The IPTG induced cultures were lysed in lysis buffer and sonicated. The majority of SHP protein was found in the inclusion bodies **(Fig. 14B, lane 3)**. SHP protein containing fractions of inclusion bodies were subsequently extracted with urea extraction buffer and sarcosine extraction buffer. Finally, sarcosine solubilized His-tagged-SHP protein was purified by using Ni^{2+} NTA beads. On SDS-PAGE gel, the purified protein yielded a band corresponding to the expected molecular weight of 34 kDa **(Fig. 14B, lane 4)**.

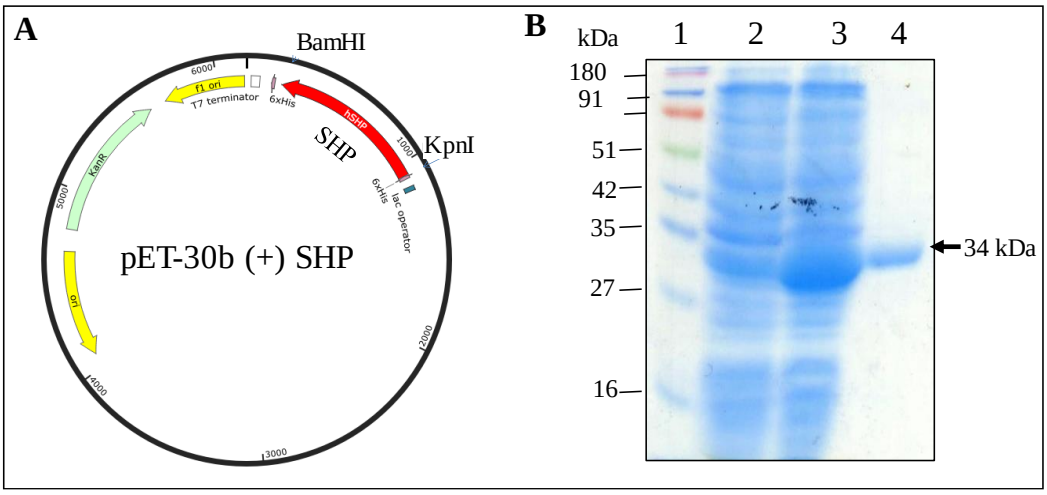


Figure 14: Cloning of SHP into pET-30b(+) prokaryotic expression vector and its purification. *(A) Schematic representation of SHP encoding sequence cloned into pET-30b(+). SHP ORF was amplified by polymerase chain reaction with specific primers and using pCMX-SHP plasmid as a template. Amplified product and pET-30(b) vector were digested using KpnI and BamHI restriction enzymes to generate appropriate sticky ends. Digested SHP ORF and pET-30b(+) plasmid were ligated together by ligase enzyme at 16°C for 16 hrs generating pET-30(b)-SHP construct. The vector map shown here was generated using 'SnapGene' software. (B) Bacterial expression and purification of His-tagged SHP protein resolved on 12.5% SDS-PAGE. BL21(DE3) cells were transformed with pET-30(b)-SHP clone and induced with 0.2 mM IPTG for protein expression at 25°C for 18 hrs. Bacterial cells were harvested and lysed for protein purification. The expressed SHP protein was found to be present in the inclusion bodies. The pellet was solubilized using 8M urea and purified using Ni-NTA affinity chromatography. Arrow indicates the position of purified and solubilized SHP band. Lane 1 indicates protein molecular weight marker in kDa; Lane 2 represents lysate of uninduced culture expressing the recombinant protein, while; Lane 3 shows induced culture lysate of recombinant protein from E. coli BL21 (DE3) strain and in Lane 4, partially purified SHP protein is indicated.*

B. Characterization of polyclonal antibody raised in rabbit against full-length SHP

The antibody raised against the full-length protein has some advantages over a sequence specific (peptide) or a partial length immunogen. The antibody generated against the full-length protein has increased sensitivity as it can bind on multiple epitopes of the antigen/receptor. In addition, it may work on isolated receptor domains since epitopes are spread over the entire protein length. So, to ascertain the purity of antigen, the apparently purified receptor was further resolved by SDS-PAGE, and the receptor protein band was cut out. This process helps in eliminating any other contaminating immunogenic bacterial proteins that may be co-purified with receptor antigen. Gel retrieved purified antigen was used to immunize the rabbit for the generation of polyclonal antibodies using protocol mentioned in 'Materials and Methods'. After priming of the animal with antigen, 3-4 booster doses were given as per standard protocol, and test bleeds were collected from the immunized animal after two weeks of every booster dose. Antibody containing sera was separated from the whole blood after coagulation reaction. Standard immunological methods as described in 'Materials & Methods' were employed to determine the titer of SHP polyclonal antibodies along with the specificity, selectivity and reproducibility. Since exploring the function of SHP in mammalian systems was the main emphasis of the study, we assessed whether the generated polyclonal antisera could specifically recognize SHP in cultured mammalian cells. For this purpose, COS-1 cells were transiently transfected with SHP expression plasmid i.e. RFP-SHP, GFP-SHP and pCMX-SHP. Cell extracts were prepared from transfected and untransfected cells. Western blot analysis was performed with the generated SHP antibody after resolving equal amounts of protein from the experimental extracts. Specific protein band in transiently expressed receptor samples for tagged-SHP was detected at the expected size of ~56 kDa contributed by GFP/RFP tag and SHP (28 kDa). The wild type untagged SHP was detected at 28kDa while untransfected sample did not exhibit band for SHP **(Fig. 15A, blot 2)** implying that endogenous SHP was low.

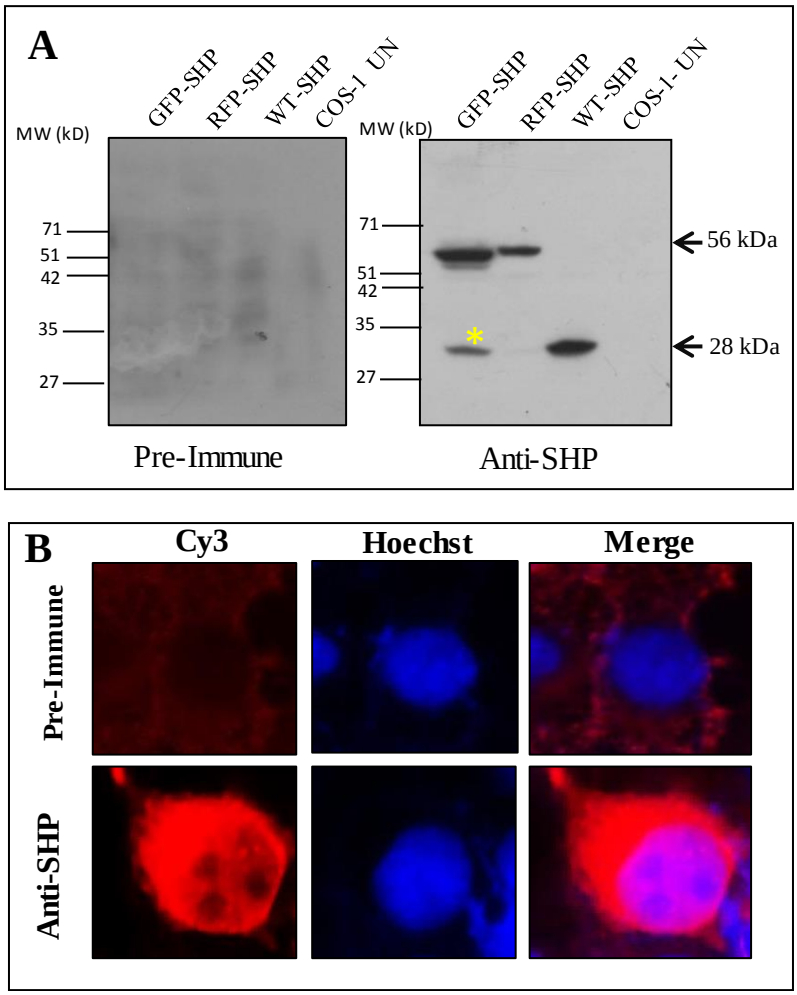


Figure 15: Characterization of SHP polyclonal antibody. ***(A)*** *Immunoblot analysis to detect the expression of SHP in transiently transfected cultured cells. COS-1 cells were independently transfected with GFP-SHP, RFP-SHP, WT-SHP constructs and harvested after 24 hrs of expression period and lysed in lysis buffer. An equal amount of protein was electrophoresed and resolved onto 12.5% SDS-PAGE and subsequently, western blot analysis was performed using anti-SHP antibody (1:2500). Anti-SHP antibody detected a band at the expected size for SHP while no band was detected in the untransfected extracts.* ***(B)*** *Indirect immunofluorescence staining of COS-1 cells transiently transfected with SHP. Cells were fixed and processed for immunodetection by anti-SHP antibody at a dilution of 1:300 as described under 'Materials and Methods'. The left panel shows the distribution patterns of receptor in immunodetected cells. The middle panel shows the Hoechst staining for visualizing the nuclei/DNA and the right panel shows the merged images for both Hoechst and SHP. [In Fig. 2 UN= untransfected, and (*) showing possibly degraded receptor].*

Further, the specificity of the generated polyclonal antibody against SHP was examined *via* immunofluorescence assay. To perform this experiment, wild type SHP (pCMX-SHP) plasmid was transfected in COS-1 cells. Fixed cells were

immunodetected with SHP antibody followed by incubation with cy3-conjugated anti-rabbit secondary antibody. Subsequent fluorescence microscopic analysis revealed SHP protein to be localized primarily in the cytosol, and to partial extent in the nuclear compartment. Immunodetection with pre-immune sera did not show any specific immunostaining for SHP **(Fig. 15B)**.

Generation and characterization of a cell line stably expressing SHP

A major advantage provided by stably transfected cells over transiently transfected cells is the consistent, stable expression of protein of interest over indefinite or extended periods of time. Transiently transfected cells can only be harvested up to 24-96 hrs after the transfection, depending on the cell type and expression of the gene. The process of generating stably expressing cells starts with transient transfection. In a small population of transfected cells, the foreign gene is integrated into the cell's genome. The transfected foreign gene gets integrated into the genome and becomes part of the host cell genome and thus can be replicated. A suitable antibiotic is used to selects the stably integrated cell. The subsequent propagation of these transfected cells, therefore, will also express the integrated gene, resulting in a stably transfected cell line. Stable cell lines are utilized in multiple research applications ranging from cell biology to protein production, antibody generation, drug discovery and many functional studies.

HepG2 cells were transfected in a ratio of 10:1 with wild-type SHP expressing plasmid and the vector that contained the neomycin-resistance gene (pcDNA3.1) which is the selectable marker. The detailed protocol is mentioned in 'Materials & Methods'. Wild-type SHP and pCDNA3.1 empty vector was co-transfected for 16 hours. Fresh complete media was added after additional 24-30 hours the cells were serially diluted in three plates and selected with G418 (geneticin) containing DMEM complete media. Only the stably transfected cells with antibiotic resistance survived in long-term cultures, allowing for the selection and expansion of the desired clonal cells. Over three weeks after the selection period, single colonies were propagated and several clones were selected. These clones were subsequently confirmed by western blotting and immunofluorescence using anti-SHP antibody. The final clone selected after these preliminary observations was designated as HepSHP and stored as multiple frozen aliquots for future needs.

Characterization of HepSHP stable cell line at receptor transcript and protein level

Integration of SHP in HepG2 cells was confirmed at the mRNA transcript level by RT-PCR. From five selected clones, total RNA was isolated using exponentially growing cells. Quality of RNA was analyzed and quantification was done from absorbance values at 260nm and 280nm, using a nanodrop based approach. The cDNA was generated using an equal concentration of RNA (1µg) by following the protocol mentioned in 'Materials and Methods'. PCR amplification was performed using SHP and GAPDH specific primers. RT-PCR showed an equal quantity of the amplified product for GAPDH (136 bp) in HepG2 and HepSHP clone. A clear band of 116 bp was observed for the RT-PCR product from HepSHP clone in comparison to a very faint band in the control HepG2 cell line confirming stable overexpression of SHP in HepG2 cells **(Fig. 16A)**. Since the amplified product of SHP was found to be higher in quantity in HepSHP clone, this clone (HepSHP) was used for all further studies. RT-PCR was carried out by using the specific primer mentioned in **Table 1** under 'Material and Methods'. The relative mRNA expression was observed to be ~14 folds higher in HepSHP as compared to that of HepG2. The increase in the abundance of SHP transcript proved stable integration of SHP in the HepG2 genome.

Further, SHP protein expression in the stable HepSHP was examined by western blotting using polyclonal anti-SHP antibody. Whole cell lysates of HepG2 and HepSHP were prepared with RIPA buffer **(Fig. 16B)**. Equal amounts of protein were loaded on to a 12.5% SDS-PAGE, transferred onto PVDF membrane and probed with anti-SHP and anti-GAPDH antibody. The blots were developed through ECL as described under 'Material and Methods' and bands were densitometrically quantified by ImageJ software. After normalization with GAPDH expression, HepSHP stable cell line showed 8.5-fold increase in expression of SHP as compared to the parent cell line HepG2 **(Fig. 16B)**.

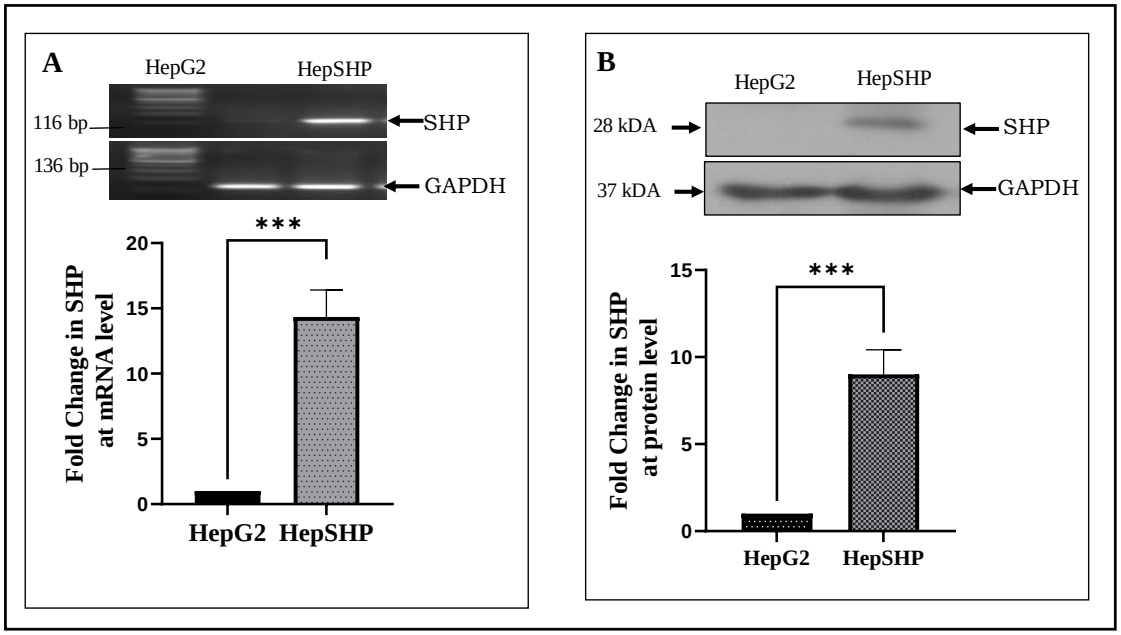


Figure 16: Quantitation of SHP mRNA expression in parental HepG2 and stable HepSHP cell lines. ***(A)*** *Total RNA was extracted from exponentially dividing cultures of HepG2 and HepSHP cells. SHP transcript levels were quantitated by RT-PCR. Enhanced SHP mRNA expression (~14 folds) could be observed in HepSHP cells.* ***(B)*** ***T****o confirm the stable integration and expression of full-length SHP in HepSHP immunodetection with anti-SHP antibody was performed. Equal amounts of whole cell lysates of HepG2 and HepSHP prepared in RIPA buffer were electrophoresed and immunodetected using anti-SHP antibodies along with anti-GAPDH antibody as a loading control. Intensity of bands was analyzed densitometrically using ImageJ software. An 8.5-fold increase in SHP level was observed in HepSHP cells as compared to the HepG2 cells.*

Characterization of HepSHP cell line by immunocytochemistry

Indirect immunofluorescence assay was performed on HepG2 and HepSHP cells cultured on sterile glass coverslips for 24 hrs and fixed for immunodetection. Subsequently, the polyclonal SHP antibody was used to probe the receptor **(Fig. 17)**. Upon microscopic analysis using Olympus inverted fluorescence microscope (model IX71), significantly higher expression of SHP was evident in the SHP overexpressing cell line, HepSHP as compared to the parent cell line, HepG2.

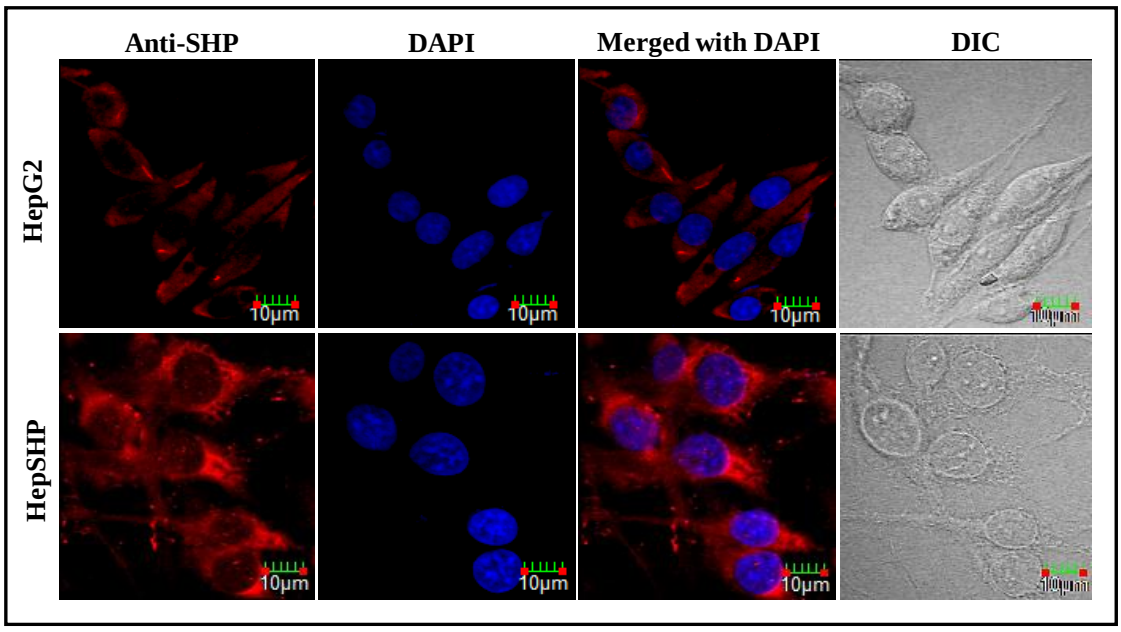


Figure 17: Enhanced cytoplasmic expression of SHP in HepSHP cells. *Immunofluorescence assay performed on fixed HepG2 and HepSHP cells utilizing the SHP polyclonal antibody, indicates elevated cytoplasmic expression of SHP in the stably overexpressing cell line HepSHP as compared to the parental, HepG2. HepSHP cells also showed enhanced cell size. DAPI was used to visualize the nucleus. The DIC images on the right panel reveal the morphology of the two different cell lines.*

Morphology and growth pattern analysis of experimental cell lines

For morphological analysis of HepSHP cell line, Hematoxylin and Eosin (H&E) staining was performed by standard protocol for HepSHP along with the parental HepG2 and HABP1 (Hyaluronan-binding protein 1) overexpressing HepG2 (HepR21) cell lines. HepR21 stable cell line has been previously reported to be highly proliferative with increased tumorigenic properties [298]. H&E stained cells were visualized under the brightfield channel and images were captured using a Nikon upright microscope (model 80i). The morphology of HepSHP cells remained the same with the progression of time. It is interesting to note that the HepSHP cells appeared to be enlarged as compared to the HepG2 cells **(Fig. 18A)**. Cellular proliferation assays were performed and the growth pattern of different cell lines at various time points are graphically plotted. The growth curve indicated an increased growth rate of HepSHP as compared to HepG2 cells, but the proliferative rate of HepR21 cells was much higher than that of both the HepG2 and HepSHP cells **(Fig. 18B)**.

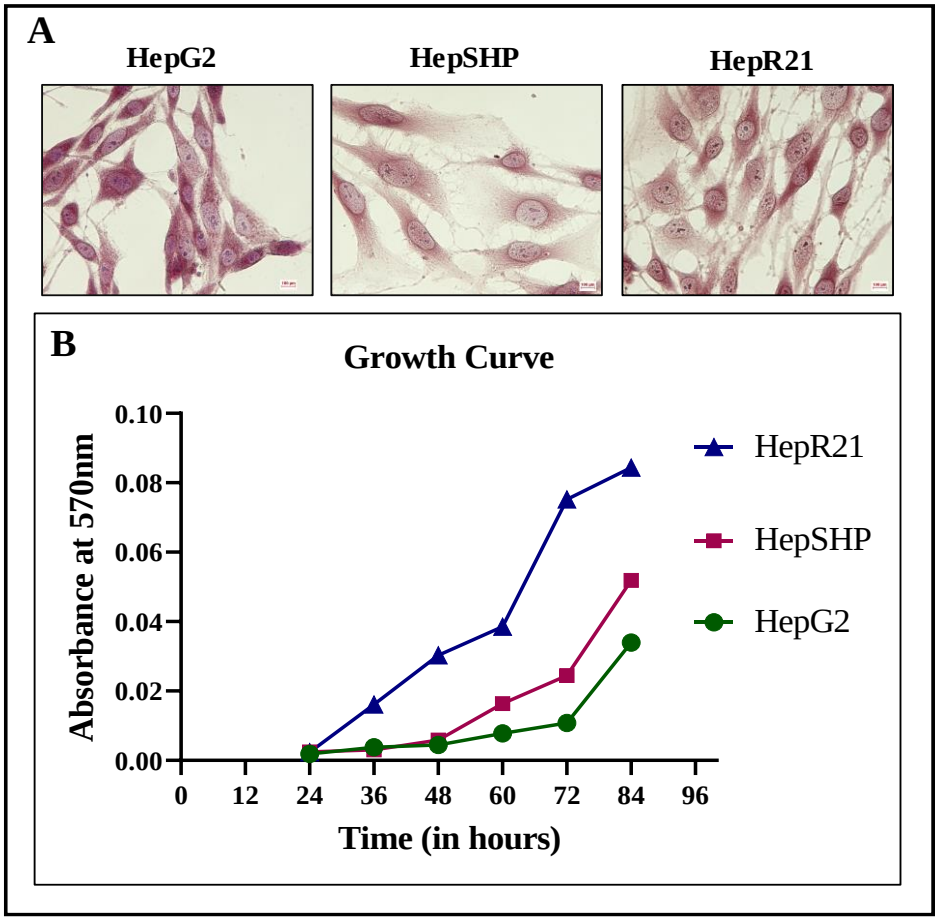


Figure 18: Morphological and growth pattern changes in the HepSHP cell line. **(A)** *Hematoxylin and Eosin staining of HepG2 cells and the stable clones show a considerable difference in their morphology. HepSHP cell size was much larger than that of HepG2 cells.* ***(B)*** *HepG2, HepSHP and HepR21 cells were grown in complete media and MTT assay for cell proliferation was done at different time points from 0 to 84 hours. The media was not changed both for experimental and control sets at any point during the course of the study. HepR21 and HepSHP cells were shown to have better survival rates over an extended period of time as compared to HepG2 cells.*

Status of SHP during cell division by immunofluorescence of HepSHP cells

Nuclear receptors are a class of transcription factors which in general can associate with the interphase chromatin, and some (PXR, AR, ERα, CAR etc.) also with mitotic chromatin [135]. This class of transcription factors have the most conserved DNA binding domains, which helps binding with chromatin by different mechanisms during cell division [36]. However, SHP is an atypical NR which lacks the DBD. To gain more insight into the receptor biology, it was crucial to study the chromatin association and functioning of SHP in the HepSHP cell line. HepSHP cell line was fixed with chilled methanol (-20℃) and probed with anti-SHP antibody and Cy3-conjugated

secondary antibody. Different stages of mitosis were observed but no binding of SHP with mitotic chromatin was detected at any stage of cell division **(Fig. 19)**. This failure to associate with mitotic chromatin may be attributed to the absence of DBD in the receptor.

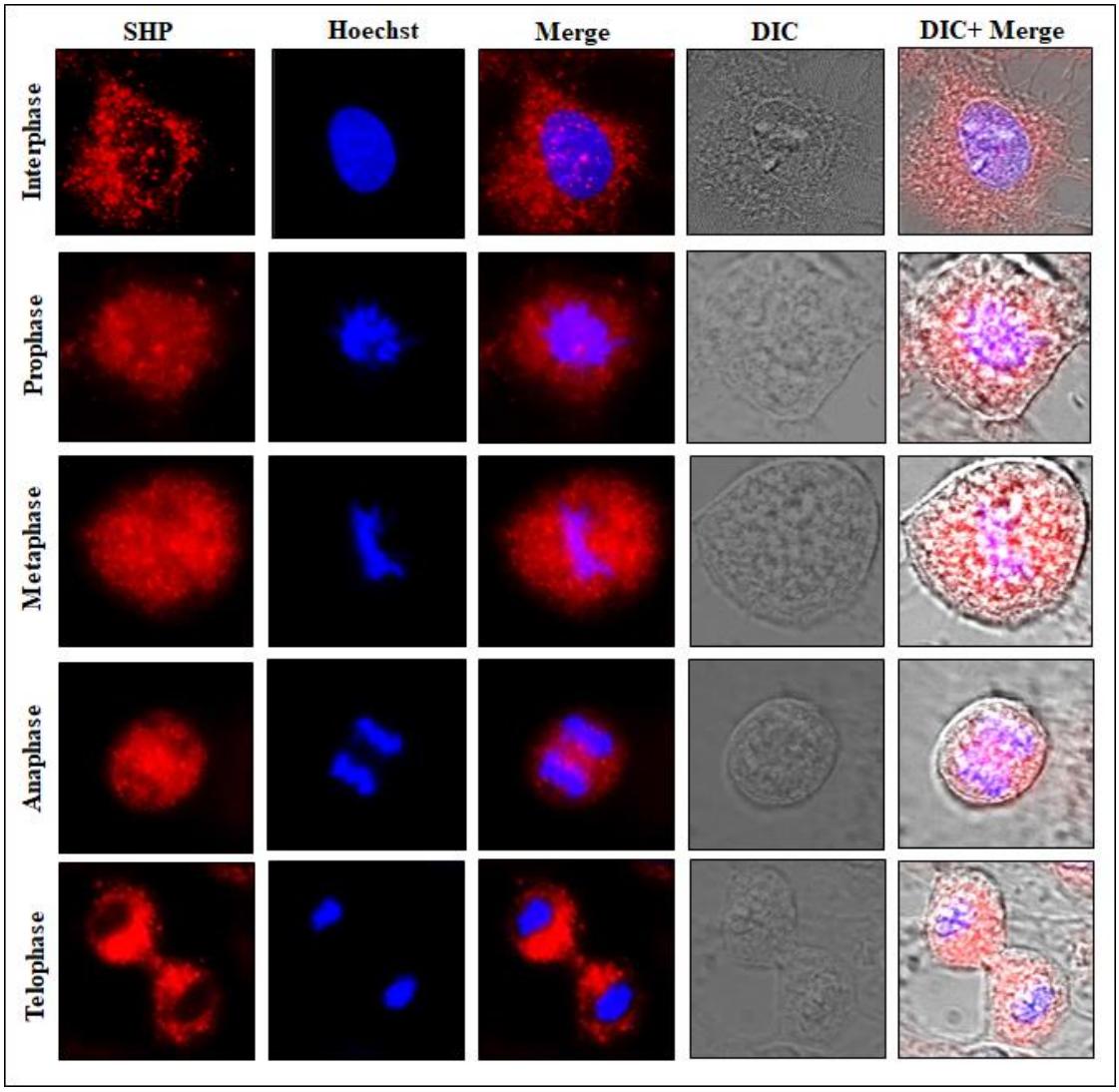


Figure 19: Absence of association of SHP during mitosis in HepSHP stable cell line. *Indirect immunofluorescence staining of SHP in HepSHP stable cell line. Cells, after the fixation, were processed for immunostaining following the protocol mentioned in 'Material and Methods'. Rabbit polyclonal anti-SHP antibody was used as the primary antibody. Cy3-conjugated secondary antibody was used along with nuclear stain DAPI. The first panel shows the distribution patterns of receptor in immunodetected cells at different stages of mitosis. The second panel shows DAPI staining for visualizing the chromatin/DNA and the third panel shows the merged images for the two fluorescence channels. The fourth panel shows the DIC images while the extreme right panel shows the merged images for all the channels.*

Subcellular localization of fluorescent protein-tagged SHP in living cells

Discovery of fluorescent proteins has opened new avenues in cell biology studies. We constructed GFP and RFP chimeras of SHP by sub-cloning SHP into pEGFP-C1 and the pDS-Red express-C1 vectors between restriction sites KpnI and BamHI. In order to study dynamics of SHP in living cells, we expressed both GFP- and RFP-tagged SHP plasmid in HEK293T, COS-1 and HepG2 cell lines and monitored the subcellular localization post-transfection. After 24 hours, the expression of SHP-tagged with green and red fluorescent proteins was visualized under the fluorescent microscope **(Fig. 20)**.

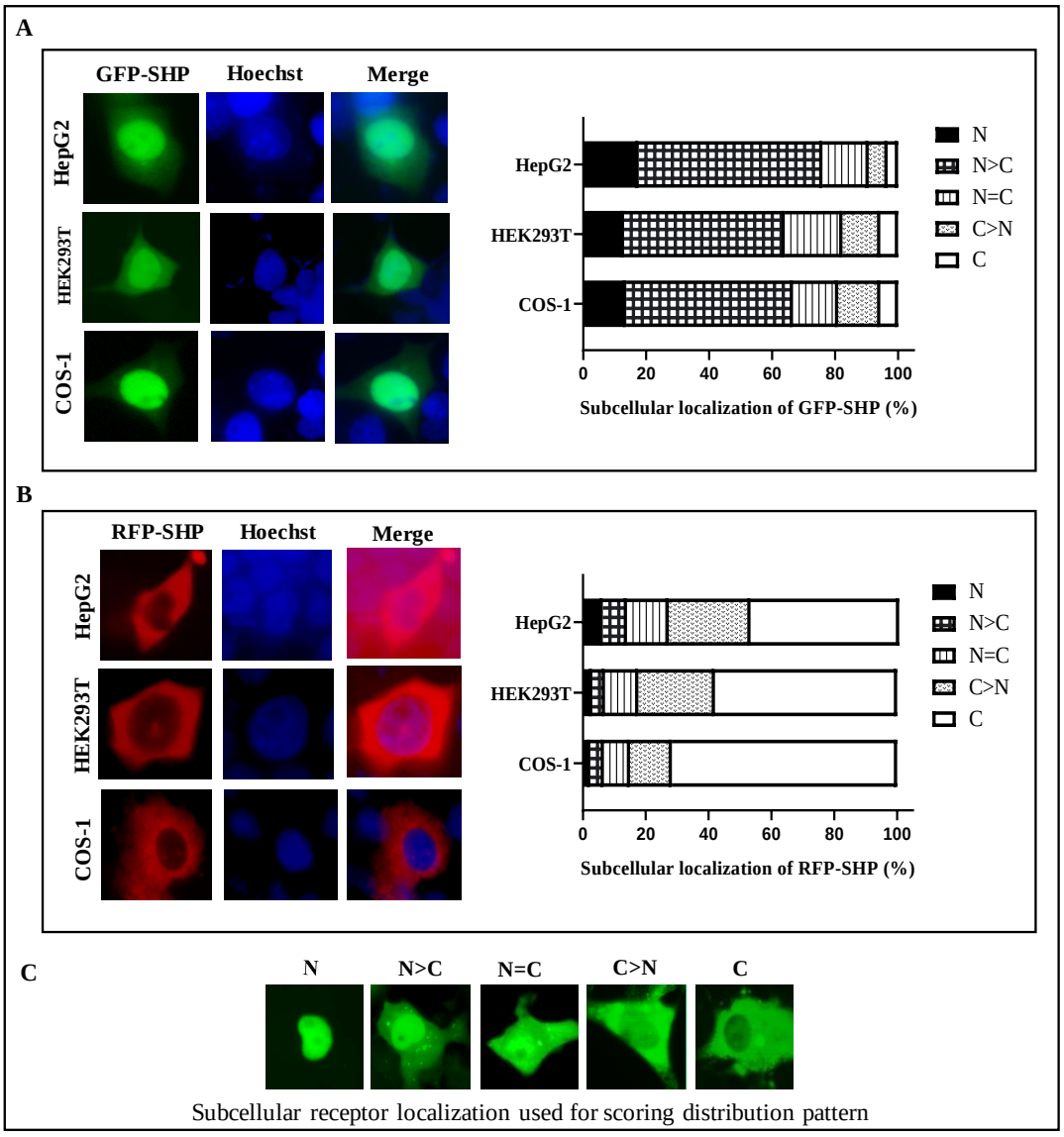


Figure 20: Subcellular localization pattern of SHP in different cell lines with GFP or RFP tag. ***(A)*** *GFP-SHP and* ***(B)*** *RFP-SHP were transfected in different cell lines as indicated and live cell imaging was performed to establish the localization of both GFP-*

and RFP-tagged SHP in these cell types. HepG2, HEK293T and COS-1 cells were transfected with GFP-SHP or RFP-SHP and localization of the tagged receptor was monitored by live cell imaging after 24 hrs of expression period. In each case, localization of SHP was recorded in at least 100 transfected cells and the average values of three independent experiments were plotted. Hoechst was used to stain the nuclei. ***(C)*** *Image classification used for subcellular distribution of fluorescent-tagged protein in live cell imaging is shown. Fluorescence was considered nuclear (N) when it was exclusively in the nucleus or (C) when present exclusively in the cytoplasm. When the protein was present primarily in the nucleus or cytoplasm, it was considered N>C and C>N, respectively. When the protein was uniformly distributed in the nucleus and the cytoplasm, it was scored as N=C. The graphical representation of subcellular localization of RFP-SHP was cytoplasmic and GFP-SHP was mostly nuclear.*

We observed that GFP-tagged SHP was mostly in the nuclear compartment **(Fig. 20A)**. Interestingly, expression of RFP-tagged SHP appeared mostly in the cytoplasmic compartment, and this result was similar in HEK293T, COS-1 and HepG2 cell lines **(Fig. 20B)**. Cytoplasmic retention of SHP could be due to the weak multimerization property of RFP [299]. The shift of SHP localization towards the cytoplasmic compartment provides us with the opportunity to study not only the receptor interaction with heterodimeric partners but also the nuclear translocation events in the living cells.

SHP lacks NLS and NES like sequences

The importins are known to mediate the nuclear import of proteins from cytoplasm to nucleus *via* the nuclear pore complex [300]. Nuclear export on the other hand is mediated by proteins called exportins [301]. These events are mediated with the involvement of the NLS or NES present on the cargo proteins. NLS of simian virus 40 (SV40) large T-antigen (PKKKRKV) and nucleoplasmin (KRPAATKKAGQAKKKK) have been most studied [302]. The NLS may harbour sequences that are classical monopartite and/or bipartite NLS motifs [303]. Other non-classical NLS motifs have also been defined, but many more still remain uncharacterized. NES motifs have remained difficult to predict, with only the prototypical chromosome region maintenance 1 (CRM1)-dependent leucine-rich NES sequence being well identified [303]. Hitherto, a large number of different families of proteins have been searched for the NLS and NES sequences. Nuclear receptors are ligand-modulated transcription factors which have significant physiological impact owing to their continuous shuttling between the cytoplasm and nucleus [86,296]. In

principle, all steroid/nuclear receptor in the ligand-bound state localize into the nucleus [91]. Hormone or ligand binding to the receptors lead to the conformational changes resulting in their dissociation from the cytoplasmic chaperone or corepressor proteins, leading to translocation of the receptor into the nucleus and binding to the receptor-responsive genes as homo- or hetero-dimer. However, in the absence of ligand many nuclear receptor exhibit varied subcellular localization ranging from exclusive nuclear to exclusive cytoplasmic [87,304].

In recent years, the importance of regulated nucleo-cytoplasmic shuttling of transcription factors in target gene regulation has become apparent leading to keen interest in identifying novel NLS and NES motifs in these factors [301]. The nuclear export mechanisms of these nuclear receptors are still uncharted and represent an active area of investigation. Nuclear export activity has been mapped in the DBD region of multiple nuclear receptors, but there is also evidence for NES in LBD as reported in the case of AR [305]. Notably, sequence analysis has suggested that at least one of the 48 nuclear receptors (NGIF-B), has a hydrophobic NES recognized by CRM1 [306].

Leptomycin B (LMB), which is a specific inhibitor of CRM1-dependent nuclear export pathway, has been widely used in studying general nucleo-cytoplasmic shuttling of proteins. In the present study, p65-GFP (RelA-GFP) was cotransfected with super-repressor IκB and the localization of p65-GFP was observed in the cytoplasm **(Fig. 21A row 1)**. Alongside, in another set of experiments with similar cotransfection the cells were treated with LMB (20 ng/ml). p65-GFP was observed in the nuclear compartment as it rapidly shuttles using a leucine-rich NES, and LMB renders it non-operational (**Fig. 21A row 1 & 2)** [307,308]. The observations were similar to the previous studies [307]. p65-GFP protein trapped into the nuclear compartment in the LMB-treated cells indicated the inhibition of exportin-1 mediated nuclear export pathway [308].

Next, we computationally predicted NES signal sequences in SHP protein using Net-NES 1.1 server **(Fig. 22)** [309]. This prediction is based on HMM and the Artificial Neural Network (ANN), which are based on Leucine-rich NES [310]. The calculated NES score of SHP protein sequences was below the threshold score indicating that SHP does not harbor leucine-rich NES **(Fig. 22)**.

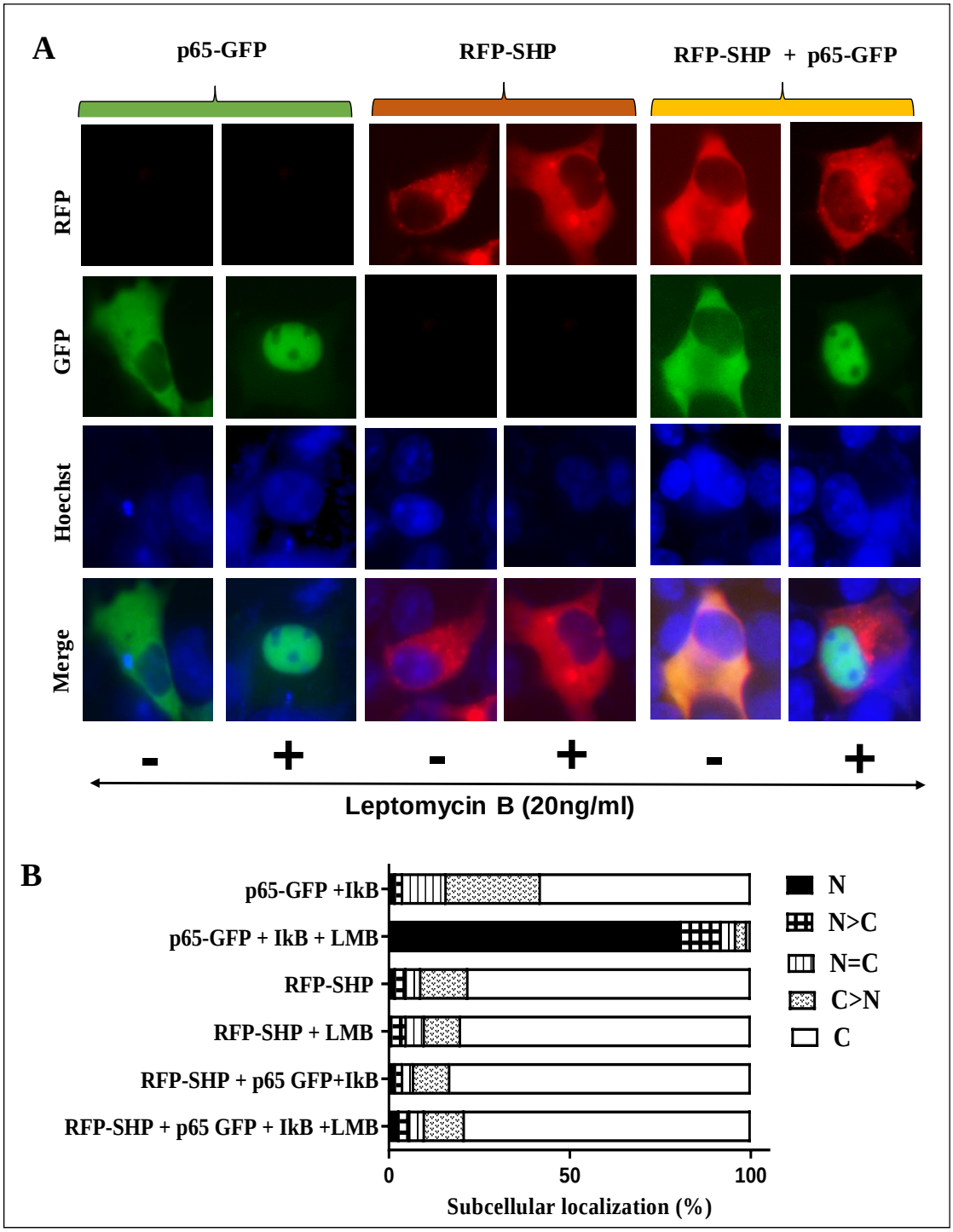


Figure 21: Leptomycin B does not alter the localization of RFP-SHP. ***(A)*** *COS-1 cells were transiently transfected individually with 500 ng of each plasmid (p65-GFP, RFPSHP, and pCMV-IκB) as described under 'Materials and Methods'. Following the transfection period, the cells were treated with vehicle (DMSO: ethanol,1:1) or LMB (20 ng/ml) for 4 hours. p65-GFP along with IκB localized in the cytoplasm of the cell treated with vehicle alone but in LMB treated, it localized in the nuclear compartment implying that its nuclear export was blocked. Similarly, RFP-SHP transfected cells were treated with either vehicle or LMB for 4 hours. RFP-SHP subcellular dynamics*

was unaltered in both the treatments. Further, to examine inter-molecular interactions, RFP-SHP was expressed along with p65-GFP and IκB when subcellular localization of RFP-SHP was cytoplasmic. Treatment with LMB did not affect the nuclear accumulation of p65-GFP indicating that SHP and p65 do not interact with each other. ***(B)*** *Quantitation of subcellular localization of p65-GFP+IκB, RFP-SHP and RFP-SHP+p65-GFP+IκB with vehicle and in LMB treated cells were observed and classified (N, N>C, N=C, C>N and C) by fluorescence microscopy. In each case, localization of SHP was recorded in at least 100 transfected cells, and the mean values of three independent experiments are plotted.*

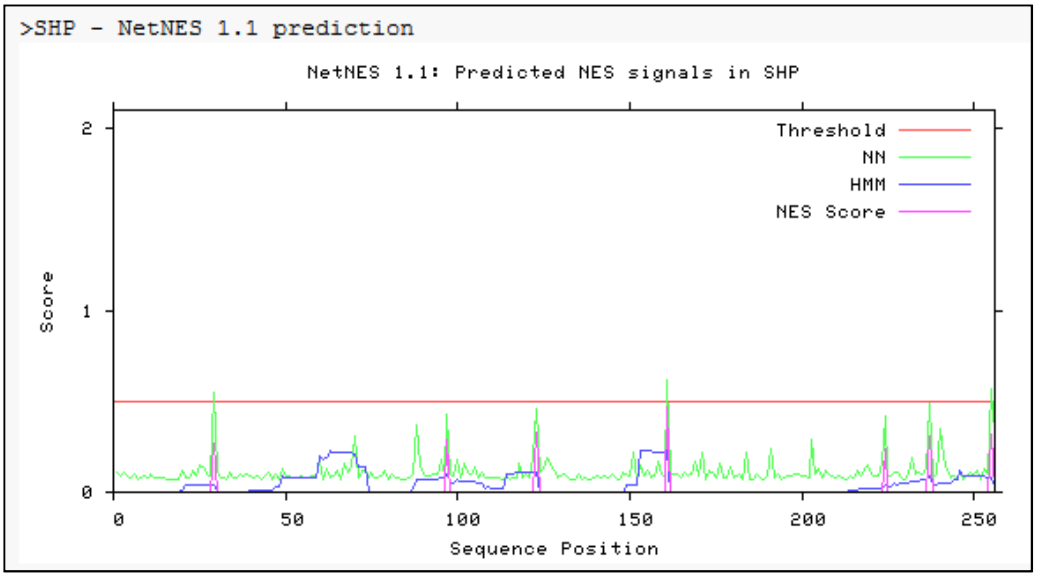


Figure 22: Identification of putative NES signal in SHP protein sequence using Net-NES 1.1 server. *An in silico prediction for NES using the Net-NES 1.1 software from CBS (Center for Biological Sequence Analysis, Technical University of Denmark), which identify LxxxLxxLxL (L = Leucine or hydrophobic amino acid and X= any amino acid) motif. No putative leucine rich NES was found in SHP supporting the observation made with LMB.*

Discussion

Nuclear receptors play diverse essential roles in many physiological events to maintain cellular homeostasis. Atypical nuclear receptor SHP, along with other receptor DAX-1, is a distinct receptor when compared with other members of the NR superfamily. Due to its distinctiveness in the absence of DBD it is exciting to study the role of SHP, which appears to play a central regulatory role with several other NRs [14,213].

NRs are transcription factors with relatively low cellular expression levels; hence, may be challenging to detect. Therefore, in order to detect the nuclear receptor,

polyclonal antibodies raised against full-length antigen have proved to be highly useful [278]. In the current study, a polyclonal antibody against full-length SHP protein was raised and characterised by the immunoblotting and immunofluorescence techniques. SHP polyclonal antibody could successfully and specifically detect SHP in all the immunological assays tested. Immunocytological assay with transiently expressed wild type SHP showed it to be distributed in both, the cytoplasmic and the nuclear compartments of the cell.

Stable cell line expressing SHP was generated using established methods described in 'Materials and Methods' and was designated as HepSHP. The cell line was characterized at the transcript and protein level. At the transcript level, we observed SHP expression was about fifteen-fold higher as compared to the parental cell line HepG2. The receptor protein level nearly undetectable in HepG2 cells but clearly visible in HepSHP stable cell line. Simultaneously, immunocytology was performed to study the subcellular expression of SHP in fixed cells. Higher expression of SHP in HepSHP cell line was observed by immunofluorescence assay. Some of the nuclear receptors (PXR, ERα and AR) associate with condensed chromatin during mitosis [36,135]. However, SHP did not associate with mitotic chromatin as inferred from immunofluorescence studies performed in fixed, as well as, in live cells expressing SHP. Such associations was not expected since SHP is an atypical nuclear receptor lacking DBD required for chromatin/DNA interactions [9].

Interestingly, while they were routinely cultured, morphological changes were also observed in stable cell line HepSHP under the phase-contrast microscope. When culturing the cells, it was observed that HepSHP cell growth was slower as compared to HepG2 cells. Thus, hematoxylin and eosin staining were conducted to assess and compare the cellular morphology of HepG2 and HepSHP cells. Differential interface contrast (DIC) microscopy was performed on both the cell populations. Interestingly, we observed that HepSHP cells had a bulky/bloated appearance when compared with HepG2 cells. The nuclear and the cytoplasmic volumes were substantially more as shown in **Fig. 18A** and **B**. Subsequently, upon further examination with MTT assay, HepSHP cells were found to be more proliferative than the HepG2.

Depending on functional status the NR localization may be cytoplasmic or nuclear. When cytoplasmic its nuclear translocation is a pre-requisite step to transactivates or repress the target genes. Previous studies have suggested that SHP subcellular localization is either predominantly nuclear or equally distributed between

the two compartments of the cell, irrespective of the cell line expressing it. RFP-tagged SHP showed a cytoplasmic shift across different cell lines which may be due to weak multimerization property of RFP as reported recently [299]. This cytoplasmic retention of RFP-SHP allows the investigator an option to study the effect of different ligands as well as interactions with other nuclear receptors.

Nucleocytoplasmic shuttling and dynamic movement of NRs are critical for accomplishing their transcriptional regulatory functions [36,311]. The balance between nuclear import and export of NRs appears to be an important mechanism for the regulation of transactivation or repression of target genes. Nuclear import of steroid/nuclear receptors involves recognition of NLS by adapter molecules like importin-α and importin-β [296]. These import factors continuously shuttle between the nucleus and cytoplasm to carry out the subsequent cycles of translocation [300]. Although the mechanism of nuclear import of steroid/hormone receptors is now relatively well documented, their export to the cytoplasm is only partially explored [311]. The best characterized nuclear export pathway uses exportin-1/CRM-1, which recognizes specific leucine-rich NES present on most cargo proteins. Discovery of potent exportin-1/CRM-1 inhibitor LMB has facilitated the identification of many transcription factors which utilize this pathway for their export [307,312,313]. As steroid receptors and importins do not contain such leucine-rich NES, it was reasonable to hypothesize that these proteins may not follow the classical CRM-1/exportin-1 pathway for their export [87,300]. In the present study, we also confirmed that there was no effect of LMB on the subcellular localization of SHP. However, GFP-tagged-p65 which has classical leucine-rich NES was able to accumulate in the nucleus with LMB treatment unlike SHP whose localization remained unaffected (**Fig. 21A)**. Further, *in silico* predictions have also confirmed that SHP lacks classical NES-like sequences **(Fig. 22)**. Overall, the study implied that subcellular localization of SHP is operated independent of classical NLS/NES sequences.

Chapter-II

Functional characterization of SHP with key interacting partner(s)

Background

Maintenance of cell identity, proliferation and growth properties during clonal expansion must be maintained during genome replication and cell division [36,137]. Every cell accurately replicates its genome and transmits the information to its progeny [124]. It is a well-organized sequence of events for distribution of molecular belongings, ultimately poised for substantial dynamic changes in nuclear organisation and gene expression of the cell [113]. The mitotic phase of the cell cycle is indispensable event in the life of a cell, and it has attracted immense attention in recent years. During mitosis, the nuclear envelope is temporarily disassembled, which leads to massive release and mixing of soluble nuclear constituents with the cytoplasm [114]. At the same time, interphase chromatin begins to condense and progress towards the beginning of prophase [115]. It further leads to transient silencing of gene transcription, and dissociation of most but not all transcription factors from chromatin [116,122,314,315].

Recent observations suggest that during mitosis, some transcriptional regulators associate with mitotic chromatin and remain bound to their targets promoters during the entire process [35,128]. A similar phenomenon mitotic binding has also been reported for some nuclear receptors [36,122,138,316] coactivators [124] and chromatin modifiers [125] and for sequence-specific transcription factors such as RUNX2 [124], GATA1 [125], HNF1 [126] and FOXA1 [127]. This phenomenon appears to be appropriately referred to as 'genomic- or gene-bookmarking'. It was proposed that mitotic gene-bookmarking is coupled with early onset of essential regulatory genes, that help in maintenance of specific cell proteome, identity and survival [122,129]. Recently, it has been postulated that functional impairment of specific bookmarking by transcription factors can result into delayed or impaired re-expression of target genes after a cell exists mitosis [130,164].

The observation that a nuclear receptor PXR associates with mitotic chromatin was a novel and unexpected finding, and also a persuasive directive for further advances [123]. In that direction, our laboratory investigated the association of androgen receptor (AR) and estrogen receptor-α (ERα) with the mitotic chromatin [122,135]. However, unlike PXR mitotic chromatin association of AR and ERα was agonist-mediated. Further, a detailed study of AR conferred that pure antagonists did not provoke such an association, even though these ligands could bind and translocate AR from the cytoplasm into the nucleus [317]. The functional significance and molecular basis of

such an association remains to be comprehended completely and await more insights. However, as of current understanding it has been contemplated that such an association may have a role in gene-bookmarking, which is a biological phenomenon believed to function as an epigenetic mechanism for transmitting the interphase pattern of gene expression from progenitor to progeny cells *via* mitosis [116,123,129,130,318].

The nuclear receptor PXR act as a master xenobiotic-regulated transcription factor and forms heterodimeric complex with retinoid X receptor (RXR) [280]. Investigations suggested that PXR constitutively associates with mitotic chromatin [278]. Further, detailed studies suggested that a specific region encompassing the NLS (Nuclear localizing Signal) residing in DBD (DNA binding domain) of PXR is essential for mitotic chromatin binding [36,122]. Whereas, estrogen receptor which functions as homodimer associates with chromatin similar to AR in ligand-dependent manner [37,122,319].

In view of the recent finding that PXR-DBD is central to the gene-bookmarking through interaction with chromatin [36]. We decided to validate these findings further with atypical nuclear receptor SHP. Though SHP is devoid of DBD it is reported to interact with a few nuclear receptors and modulates their transcription function. This makes it distinct from other conventional NRs. The functional cross-talk of SHP with other interacting partners is compelling and has importance in normal physiological controls and the state of dysregulation. This warrants for extensive investigation not only on the unique regulatory mechanisms of SHP but also its relationship with other NRs and transcription factors. This may initiate ways to understand the SHP-mediated disease mechanisms (diabetes, cancer etc.) as well as its potential in therapeutic interventions [13]. From the simplistic point, it is apparent that SHP is an emerging atypical nuclear receptor and awaits an extensive investigation to assess its subcellular functional dynamics and molecular basis of transcriptional influence on its heterodimeric interacting partners. Also, a detailed study is warranted to unravel the emerging regulatory implications of gene-bookmarking during mitosis which may have implications on the onset and progression of a disease.

Using live cell fluorescence microscopy, we document that unlike some known nuclear receptors, SHP does not associate with mitotic chromatin by similar mechanisms. However, it plays a key role in influencing the mititic chroamtin association of its interacting partners in mutually responsive manner to influence gene-bookmarking events. Finally, we report that the SHP interacts with some of the key

nuclear receptors (like PXR and ERα) implying its role in their cell regulatory functions. Altogether, we propose that SHP may act in a *hog-tie* manner to serve as an indirect physiological regulator.

Results

In silico analysis of SHP interactome

Interactome3D is a web service for the structural annotation of protein-protein interaction networks. This server (https://interactome3d.irbbarcelona.org/) gives the interactions from all the available structural data for both, the individual interactors and the interactions themselves [320]. Additionally, we can also visualize and download structural information for interactions involving a set of proteins or interactomes for one of the precalculated organisms. Interactome3D includes binary interactions identified between chains of the same PDB structure. Interactome of SHP uniport ID Q15466 was predicted against *Homo sapiens* genes. A total of 75 interactions and 31 proteins were predicted **(Fig. 23)**. The different color coding is marked in the legend, which shows the interaction based on the experimental structure of the protein or model or without the structure of the protein. Further, we conducted our detailed study with some selected nuclear receptors.

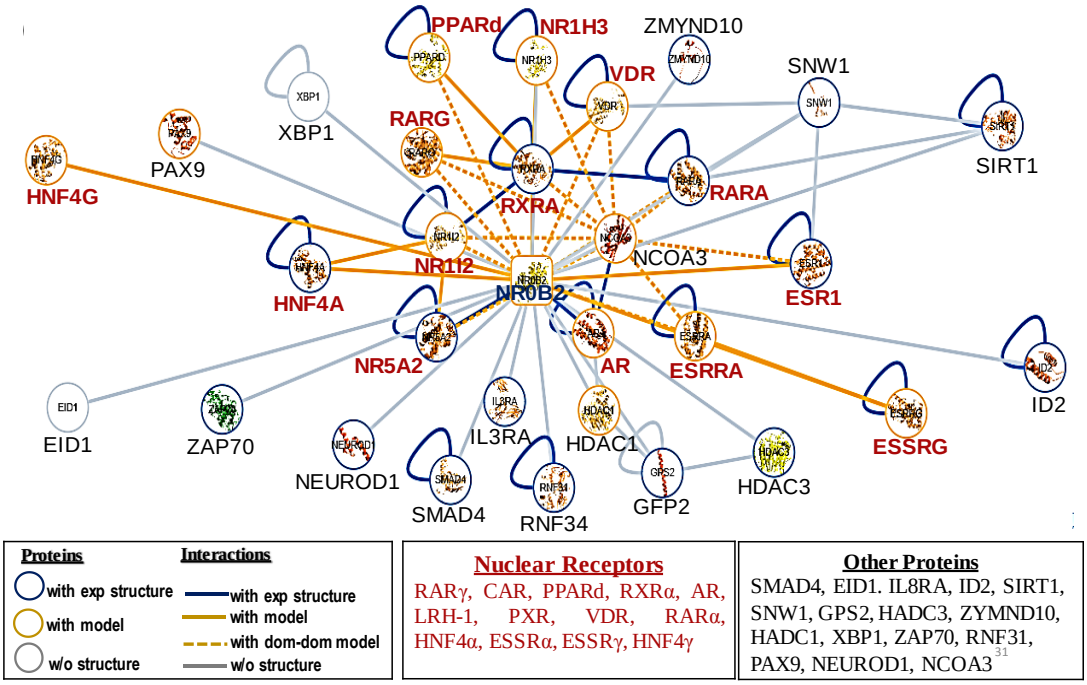


Figure 23: Prediction of SHP interactomes. *Uniport ID of SHP (NR0B2) Q15466 was set in the query box and interactome was predicted against Homo sapiens. Total*

75 interactions and 31 proteins were predicted. The color coding is marked in the box, showing the interaction based on the experimental structure in the blue color. Saffron color shows the interaction with modelled structure and the grey color shows interaction without any structure. The protein interactome predicted interactions with some of the key nuclear receptors which are involved in major metabolic diseases.

Functional interaction of SHP with selected NRs: an experimental approach

SHP is a key nuclear receptor which interacts with several nuclear receptors in particular as well as other proteins **(Fig. 23)**. SHP regulates the transcription function of interacting partners *via* different modes of interactions [13]. Since nuclear translocation is a prerequisite step for transactivation by NRs we queried whether SHP could also influence the subcellular localization of its interacting partners. For this purpose, subcellular dynamics of SHP with DAX-1, RXRα, PXR, CAR, HNF4α1, AR and ERα were examined. To visualize the subcellular localization of SHP, it was tagged with a red fluorescent protein (RFP). The RFP-SHP plasmid was co-transfected with different GFP-tagged NRs in COS-1 cells. After 24 hours of expression period, subcellular localization of different GFP-tagged proteins alone and coexpressed in combination with RFP-SHP were observed in live cells. It was observed that RFP-SHP shifted predominantly towards the nucleus (>70% cells) in the presence of GFP-tagged PXR, ERα and HNF4α1. Whereas GFP-CAR showed mild nuclear localization of SHP (~30%). However, SHP showed no translocation and remained in cytoplasm (~80 % cells) when coexpressed with GFP-DAX-1, GFP-RXRα and GFP-AR (**Fig. 24A)**. This implied existence of intermolecular interactions between SHP and some of the NRs, resulting in alternation of subcellular localization. The interacting NR constructs identified here were subsequently used for the study of receptor-receptor interactions. The study was also extended further with mitotic chromatin interactions and in identification of key receptor domains or amino acid residues involved in such interactions.

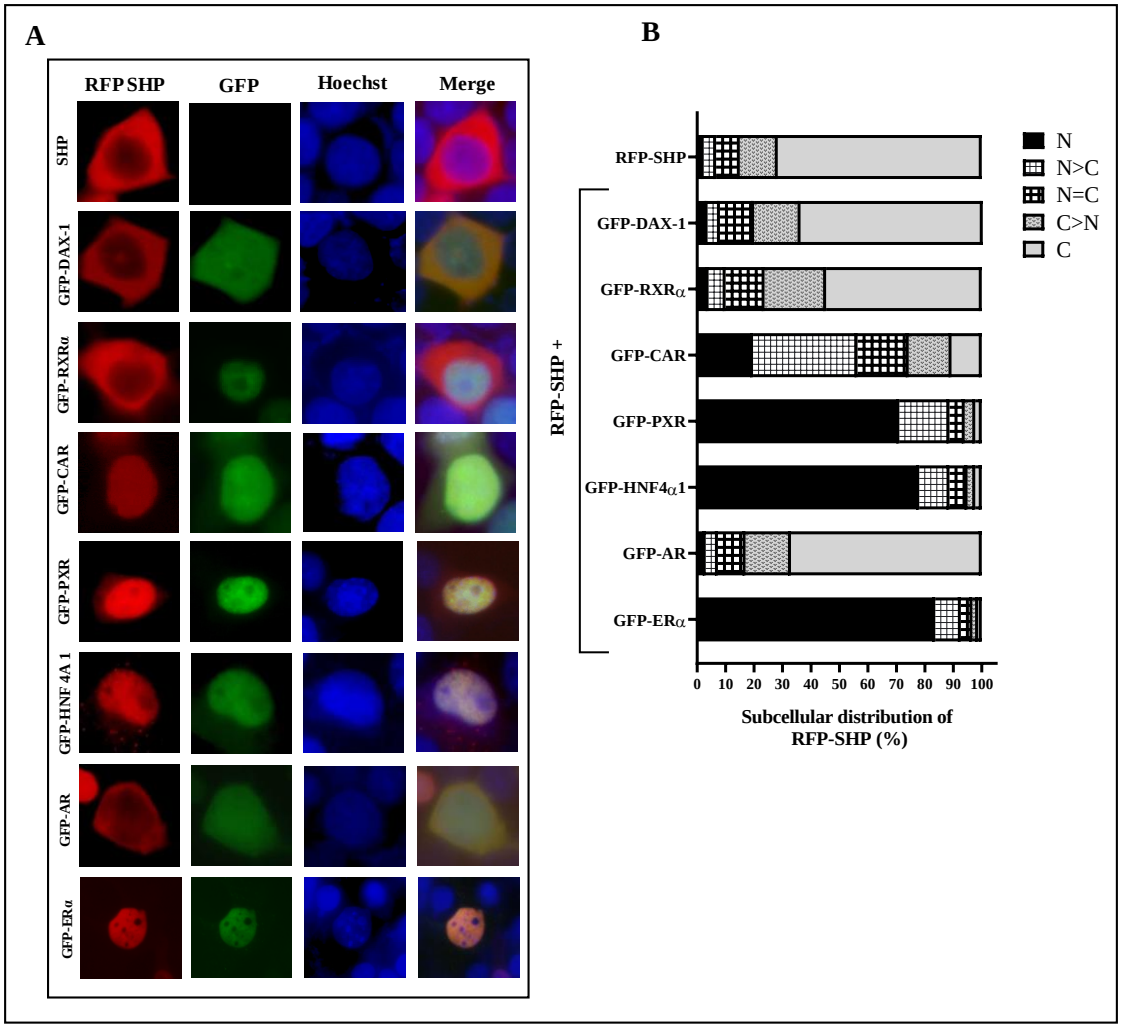


Figure 24: Identification of SHP interaction with selected nuclear receptors. ***(A)*** *The shift in subcellular localization pattern of SHP in the presence of different nuclear receptors (DAX-1, RXRα, CAR, PXR, HNF4α1, AR & ERα).* ***(B)*** *Quantitation of shift in subcellular localization of SHP. COS-1 cells were transiently transfected with RFP-tagged SHP with different GFP-tagged nuclear receptors. Subcellular localization of SHP was observed and classified into groups (N, N>C, N=C, C>N, C) by fluorescence microscopy. In each case, localization of SHP was recorded in at least 100 transfected cells and average values of three independent experiments were plotted. The values are average of at least three separate experiments which exhibit similar patterns with values differing between 5-10%. Unlike a few other, nuclear receptor PXR, ERα and HNF4α1 strongly influenced the subcellular dynamics of SHP. CAR influenced SHP localization only weakly.*

SHP inhibits the transcription function of PXR, CAR, ERα and RXRα in HepSHP stable cell line

It is evident from the above experiments that SHP interacts with PXR, ERα and CAR, and is co-translocated from cytoplasm to the nuclear compartment of the cells.

In order to shed light on the effect of SHP on the transcriptional function of its interacting partners, i.e. PXR, ERα and CAR, the interacting partner were transiently co-transfected with their promoter-reporter constructs. In addition, RXRα, which is also an interacting partner of many other NRs was also included. The promoter-reporter experiments were performed in a cell line stably integrated with SHP. Control experiments were conducted with the parent cell line HepG2. To inquire about the influence of SHP on the transcriptional activity of PXR, HepG2 cells were cotransfected with PXR and XREM-Luc promoter-reporter plasmid as control. Similarly, HepSHP constitutively expressing SHP cells were also cotransfected with above two plasmids. Both the cells were treated with PXR ligand rifampicin or vehicle (DMSO: EtOH). After the expression period of 24 hrs, the cells were harvested for luciferase assay following the protocol as under 'Materials and Methods'. The promoter activity of rifampicin activated PXR was ~3 fold higher as compared to vehicle treated. Interestingly, the promoter activity of rifampicin treated HepSHP cells was ~1.5 fold, which was lower in the comparison of HepG2 cells **(Fig. 25A)**. These results demonstrate that presence of SHP repressed transcriptional activation of PXR target promoter. Similarly, expression plasmid ERα was transiently cotransfected with ERE-Luc promoter-reporter and treated with 17β-estradiol. The promoter activity of ligand-activated ERα was ~1.5 fold compared to solvent only and it was reduced to ~0.8 in HepSHP expressing cells **(Fig. 25B)**. Furthermore, CAR was transiently cotransfected with its CYP2B6-Luc promoter-reporter and treated with its ligand CITCO. The ligand-activated promoter activity of CAR was ~2 fold as compared to the control solvent treated HepG2 cells. However, the promoter activity of CAR was reduced by 60% in HepSHP cells **(Fig. 25C)**. Expression plasmid of RXRα was cotransfected with its promoter-reporter RXRE-Luc. The ligand (9-cis-retinoic acid) induced promoter activity of RXRα up to ~45 fold in HepG2 cells and it drastically reduced to ~10 fold in HepSHP cells **(Fig. 25D)**. Therefore, overall, we observed that transcriptional activity of PXR, ERα, CAR and RXRα were repressed in HepSHP cell line which constitutively overexpress SHP. The repression by the SHP, in HepSHP cells was ~50% when compared to the response obtained in HepG2 **(Fig. 25)**. Interestingly, our results showed that the repression of the target promoter activity of PXR, ERα, CAR and RXRα were SHP-mediated. These repressions of transcription function of PXR, ER α and CAR can be explained by their physical interaction with SHP as observed in

Fig. 24. However, since RXRα did not exhibit physical interaction its transcription repression may be due to indirect modes or its broad interactions with diverse other NRs.

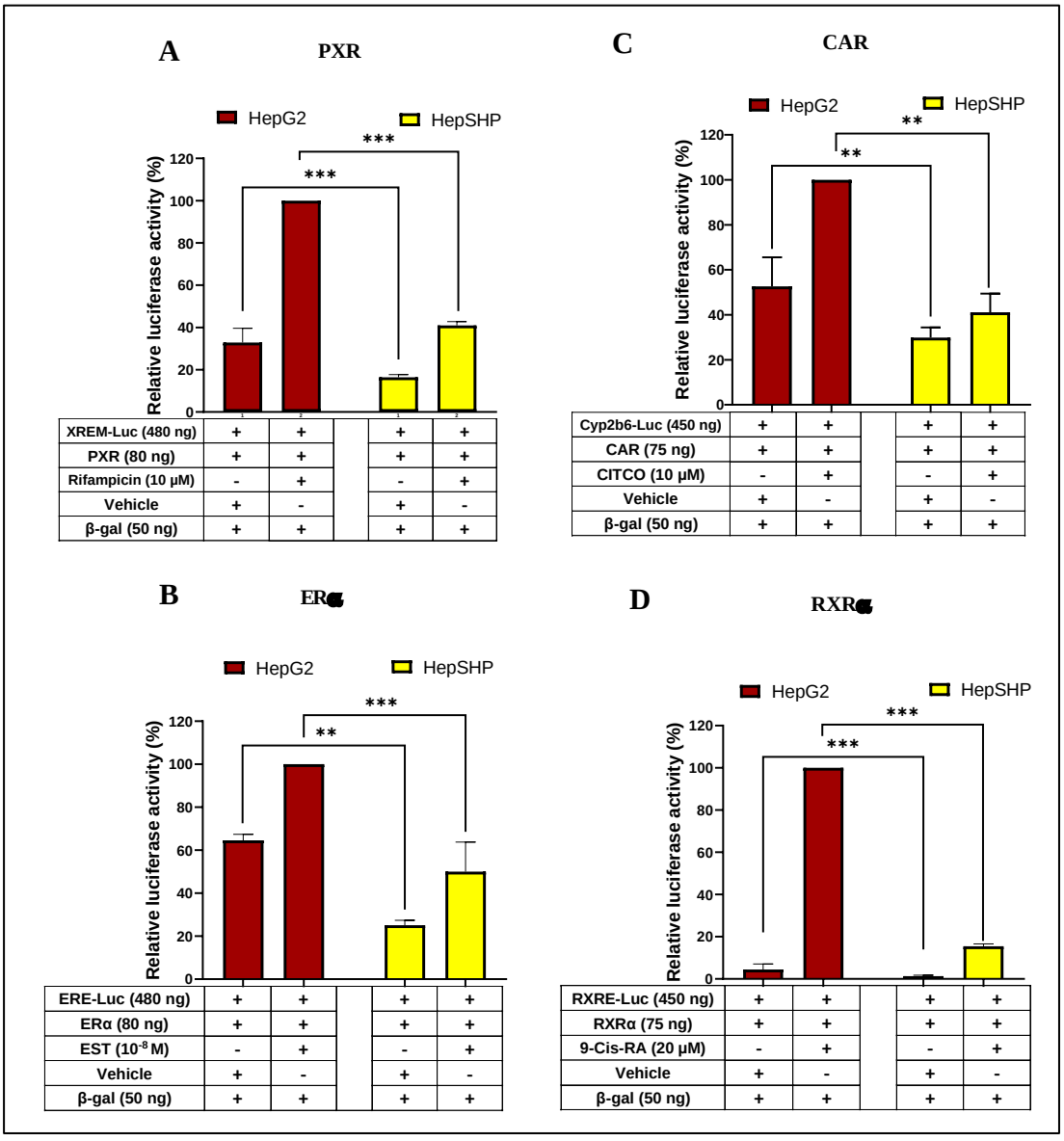


Figure 25: SHP represses the transcription function of PXR, ERα, CAR and RXRα in HepSHP stable cell line. *In all the set of experiments mentioned here, HepG2 and HepSHP cells were cotransfected with:* ***(A)*** *wild-type PXR and XREM-Luc promoter reporter and plasmid. After transfection, the cells were treated with vehicle and rifampicin for 24 hours. Relative luciferase activity was calculated in comparison to rifampicin-induced wild-type PXR activity.* ***(B)*** *wild-type ERα and ERE-Luc promoter plasmid. After transfection, the cells were treated with vehicle and 17β-estradiol for 24 hours. Relative luciferase activity was calculated in comparison to 17β-estradiol-induced wild-type ERα activity.* ***(C)*** *wild-type CAR and CYP2B6-Luc plasmid. After transfection, the cells were treated with vehicle and CITCO for 24 hours. Relative*

luciferase activity was calculated in comparison to CITCO-induced wild-type CAR activity. ***(D)*** *wild-type RXRα and RXRE-Luc promoter plasmid. After transfection, the cells were treated with vehicle and 9-cis-retinoic acid. Relative luciferase activity was calculated in comparison to 9-cis-retinoic acid-induced wild-type RXRα activity. In all set of experiment 50 ng of β-galactosidase plasmid was also used as an internal control. After 24 hrs, cells were harvested for luciferase and β-galactosidase assays. Asterisks (*) signify luciferase value that differed significantly (p<0.0001 in two-way ANOVA). Overall, the SHP appears to repress the transcriptional activity of selective NRs, primarily through physical interactions.*

Downregulation of expression of some key nuclear receptor at the transcript level in SHP expressing stable cell line

Expression of different nuclear receptors at transcriptomic and proteomic level in harmonized manner is indispensable for the regulation of metabolism and bodily functions. For the study of influence of SHP on the expression of selected nuclear receptor at the transcript level we generated a stable cell line HepSHP. Stable cell lines have proved to be a consistent tool to generate reliable and reproducible observations. Therefore, we used HepSHP cell line to study the expression of selected nuclear receptor like *SHP, DAX-1, PXR, CAR, HNF4α, RXRα, AR and ERα* at the transcript level. For this purpose, quantitative real-time polymerase chain reaction (qRT-PCR) was performed using syber green chemistry using appropriate protocol and primers as mentioned in 'Materials and Methods'. From the data, we observed a significant down-regulation of mRNA transcript for nuclear receptor genes *DAX-1, PXR, CAR, HNF4α, RXRα, AR and ERα* at the varied level as compared to the HepG2 mRNA. As expected, HepSHP cell line showed high expression levels of SHP **(Fig. 26)**. The results obtained with qRT-PCR were in accordance with the results of our transcription assays implying that SHP functions as a repressor for nuclear receptors.

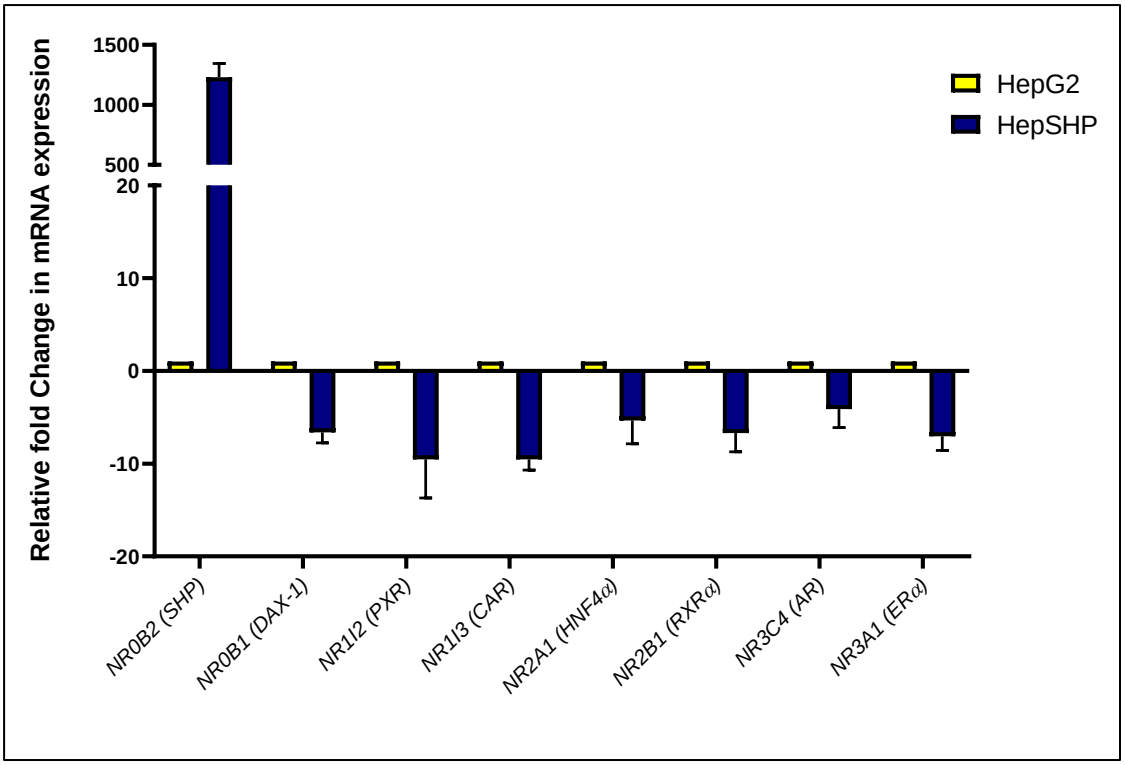


Figure 26. SHP overexpressing HepSHP cell line exhibits downregulated expression of some key nuclear receptors at the transcript level. *Quantitative Real-time mRNA expression profiling of SHP, DAX-1, PXR, CAR, HNF4α, RXRα, AR and ERα nuclear receptor genes. The relative quantification of mRNA expression calculated using* $2^{-\Delta\Delta Ct}$ *method and the fold change was plotted after the normalization with GAPDH as an internal control. Statistical analysis was done by two-way ANOVA. The significance of 'p value' was <0.0001 (***) which was calculated by Graph Pad prism software package 8.0.1.*

Association of SHP with mitotic chromatin during mitosis:

Previously our laboratory has investigated the association of nuclear receptor PXR, AR and ERα with the mitotic chromatin [122,135,278]. The observation made herein on mitotic bookmarking gave insight into further investigations with the other members of nuclear receptor superfamily.

SHP does not associate with mitotic chromatin

To investigate the behavior of SHP during mitosis, HEK293T cells were transfected with 500 ng RFP-SHP plasmid using standard transfection reagents. Subsequent to expression time, live cell imaging was performed under the upright Nikon 80i model fluorescence microscope with water immersion objectives. Mitotic cells were analyzed by receptor fluorescence and Hoechst staining patterns. RFP-SHP was tracked and

recorded in these cells for its localization. We observed that SHP was not associated with the mitotic chromosomes during any phase of mitosis contrary to what has been previously observed with nuclear receptor PXR **(Fig. 27)** and also with AR and ERα. This phenomenon has been observed in naturally dividing living cells, i.e., without imparting any chemical arrest. Conceivably, SHP does not associate with mitotic chromatin owing to architectural lack of its DNA binding domain (DBD).

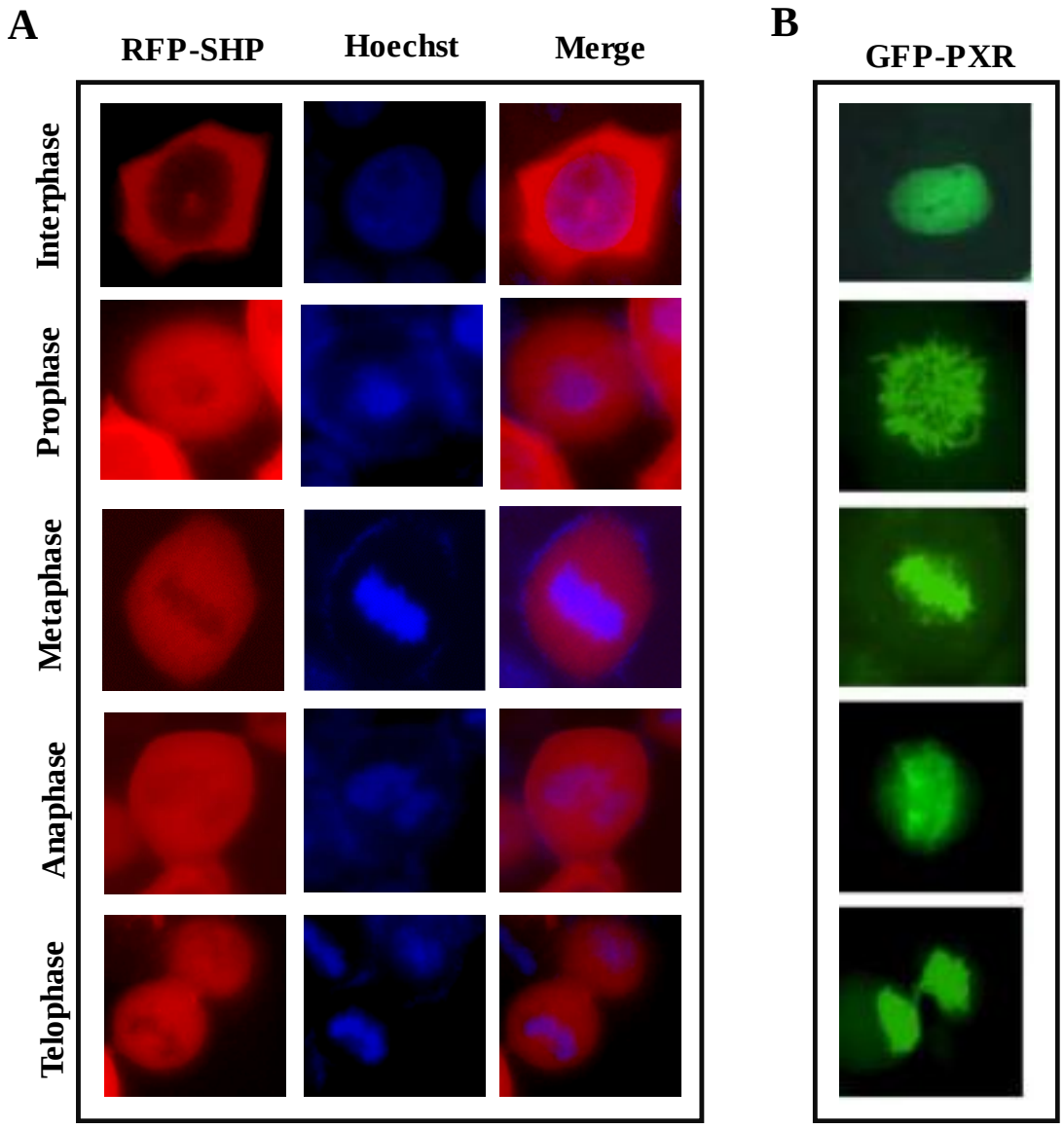


Figure 27. SHP does not associate with mitotic chromatin like several other nuclear receptors. *HEK293T cells were transiently transfected with 500 ng of RFP-SHP and incubated in steroid-free medium for 24 hrs. Images of naturally dividing live cells were recorded using a fluorescence microscope equipped with water immersion objectives. RFP-SHP does not associate with mitotic chromosomes* ***(A)*** *like PXR (extreme right panel)* ***(B)*** *as observed in live cells by fluorescence microscopy. Hoechst was used as a fluorescent dye to visualize corresponding nuclei/condensed*

chromosomes. In the set of images, the left panels show the RFP-tagged SHP; the middle panels show the Hoechst stained DNA of the corresponding cells while the right panel shows the merged images of the two fluorescence. The interphase cells and the mitotic stages of the naturally dividing cells are indicated. The live cell imaging observations showed that nuclear receptors devoid of DBD are not able to bind with mitotic chromatin during cell division.

Influence of SHP on chromatin binding of interacting partner

In the previous section, we have shown that PXR, ERα and HNF4α can facilitate nuclear translocation of SHP through protein-protein interactions (**Fig. 24)**. In the transcription function assays, we observed that SHP represses the transcription activity of its interacting partners **(Fig. 25)**. So, in view of this, we further examined the role of SHP on the mitotic chromatin binding of its interacting partners. For this purpose, RFP-SHP was coexpressed with its interacting partner GFP-PXR and GFP-ERα. Thereafter, we performed live cell imaging experiments of these proteins under defined conditions and observed receptor localization on mitotic chromatin. Several interesting observations were made which are elaborated underneath hereafter.

SHP abrogates the mitotic chromatin binding of PXR and ERα

From our live-cell imaging experiments, it was evident that like PXR, AR and ERα, the RFP tagged SHP (RFP-SHP) does not associate with the mitotic chromatin **(Fig. 28A)**. Based on this observation, we next attempted to determine the chromatin binding behavior of SHP when co-expressed along with its interacting partners during mitotic stages. For this purpose, HEK293T cells were cotransfected with 500 ng of RFP-SHP and GFP-PXR plasmids using Lipofectamine 3000. After protein expression, localization of both the proteins were observed under fluorescence microscope **(Fig. 28A)**. As previously reported, we observed that GFP-PXR alone binds constitutively to the mitotic chromatin **(Fig. 28A)**. The observations were revalidated by cell counting for localization of RFP-SHP in presence and absence of PXR coexpression. Average values exhibiting i) receptor chromatin association (strong + weak binding) or ii) receptor non-association were graphically plotted and are shown in **Fig. 28B**. The results from the live-cell imaging experiments revealed that under coexpressed conditions, the two receptors exhibit varied colocalization on the mitotic chromatin **(Fig. 28A).** The data showed significant abrogation of PXR proteins from the mitotic chromatin by SHP **(Fig. 28A & B)**. In conclusion, under the coexpressed condition of

RFP-SHP and GFP-PXR, mitotic chromatin association of GFP-PXR is not only significantly abrogated but a fraction of RFP-SHP was also observed to be localized on the mitotic chromatin with GFP-PXR. These results are of immense relevance as RFP-SHP alone does not exhibit any significant association with mitotic chromatin. This novel observation could only be attributed to the inter-molecular interaction between the two receptors.

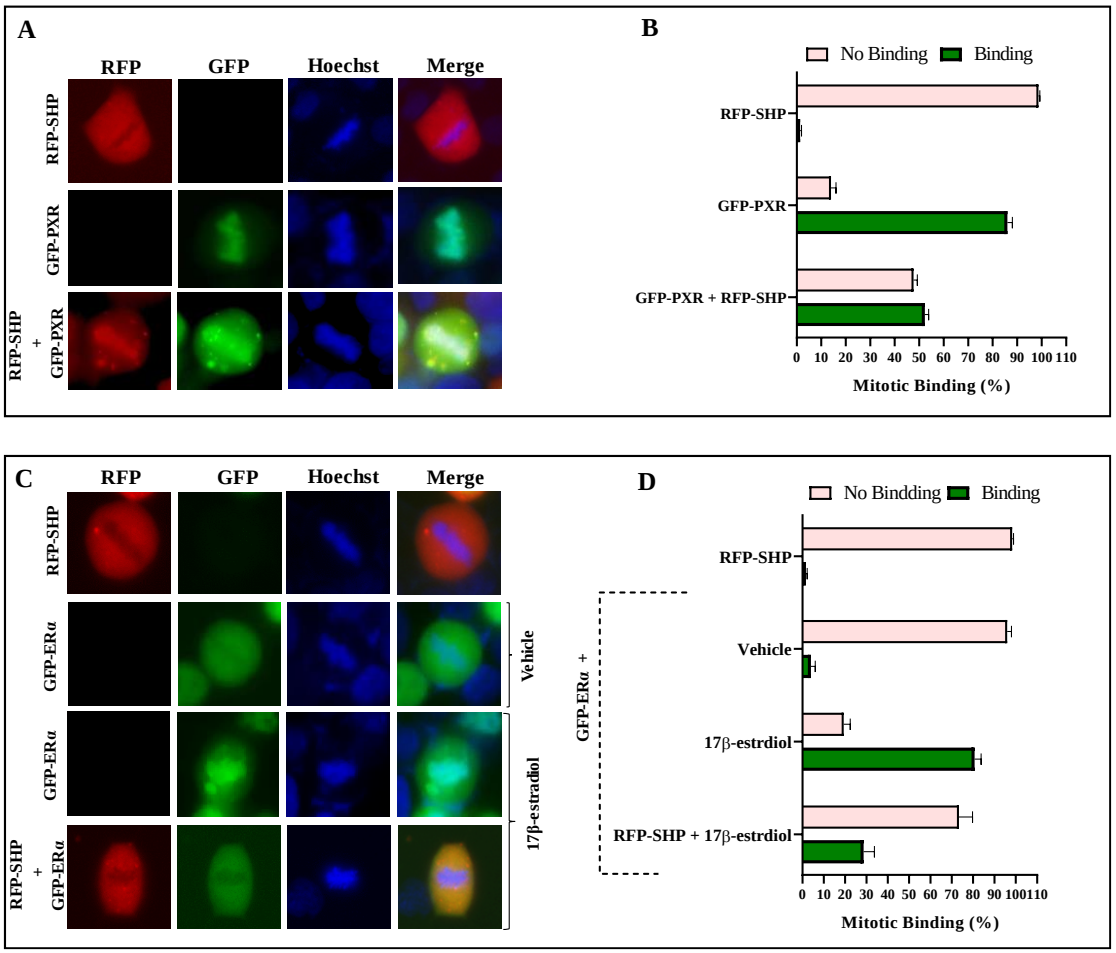


Figure 28: SHP impedes mitotic chromatin binding of its interacting partners while exhibits its own partial mitotic chromatin binding. *HEK293T cells were transiently transfected with 500 ng of GFP-PXR, GFP-ERα and RFP-SHP plasmids either alone or in combination as depicted in the figure and expressed in steroid-free conditions as described under 'Materials and Methods'. Images of naturally dividing live cells were recorded using a fluorescence microscope equipped with water immersion objectives.* ***(A)*** *SHP impeded mitotic binding of PXR while promoted its own partial binding.* ***(B)*** *Quantification of mitotic associated and non-associated cells.* ***(C)*** *SHP abrogates the ligand-dependent mitotic binding of ERα. Following receptor expression, the control cells (unliganded) were treated with the vehicle alone or treated (liganded) with its cognate hormone 17β-estradiol at 10^{-8} M as indicated in the figure.* ***(D)*** *Quantification of mitotic associated ERα and abrogation of its association in the*

presence of SHP. For quantitation, 20–25 cells were scored per sample. The values are average of at least three separate experiments which exhibited similar patterns with values differing between 5-10%. The abortive influence of SHP for mitotic binding of PXR and ERα was evident for both the receptors. Apparently, ERα was aborted more strongly as compared to PXR.

The abrogation of mitotic chromatin association in the presence of SHP was not exclusive to GFP-PXR but also observed with GFP-ERα. In subsequent study, ERα expressing cells were treated with optimal concentration of ligand 17β-estradiol (10^{-8} M), or vehicle alone (DMSO:EtOH 1:1). Mitotic cells coexpressing both RFP-SHP and GFP-ERα were imaged and their colocalization onto the mitotic chromatin was quantitated. We observed that unliganded ERα and SHP, when expressed separately, do not show any significant association with mitotic chromatin **(Fig. 28C)**. However, estradiol bound ERα associated strongly with the mitotic chromatin. Subsequently, we also examined the effect of coexpression of both the receptors under similar conditions. We observed that the ligand-induced ERα associated with mitotic chromatin and was significantly abrogated from the mitotic chromatin in the presence of coexpressed SHP **(Fig. 28C and D)**. The abrogation of PXR and ERα from the mitotic chromatin implies potential role of SHP in regulation of gene-bookmarking event in the life of the cell.

Identification of minimal domain(s) of selected receptor interacting with SHP

The interaction of SHP with the full-length receptors (PXR, ERα, HNF4α1 etc.) prompted us to identify the minimal domain of the receptor required for this action. To identify the minimal domain(s) of the receptor, different domains of the receptor were subcloned either individually or in combination. Subsequently, each was co-transfected with SHP and subcellular localization and shift of RFP-SHP were recorded in live cells.

I: <u>DNA binding domain of PXR is essential for intermolecular interactions with SHP</u>

Our above observations exhibited existence of intermolecular interaction between SHP and PXR. Therefore, to identify the specific domain/region of PXR which interact with SHP we generated different domain plasmid constructs of PXR. For this purpose, COS-1 cells were cotransfected with RFP-SHP and GFP-PXR plasmid and its different domain deleted constructs for receptor interactions and subsequent translocation studies **(Fig. 29A)**. After the expression period of 24 hours, live-cell

imaging was done, images were recorded and quantification was done **(Fig. 29A & B)**. Full-length GFP-PXR protein was able to shift the subcellular localization pattern of RFP-SHP from cytoplasm to the nuclear compartment. The SHP shift was observed to occur in ~80% of the transfected cells. In addition, other than full-length PXR protein, GFP-PXR-DBD and GFP-PXR-DBD-LBD were also able to shift the localization pattern of RFP-SHP. The constructs expressed without DBD did not translocate SHP. Interestingly, only those deletion constructs of PXR containing DBD were able to shift RFP-SHP to the nuclear compartment **(Fig. 29B)**. In conclusion, all the deletion constructs having PXR DBD appeared to interact with SHP and shift it towards the nucleus albeit to different extent.

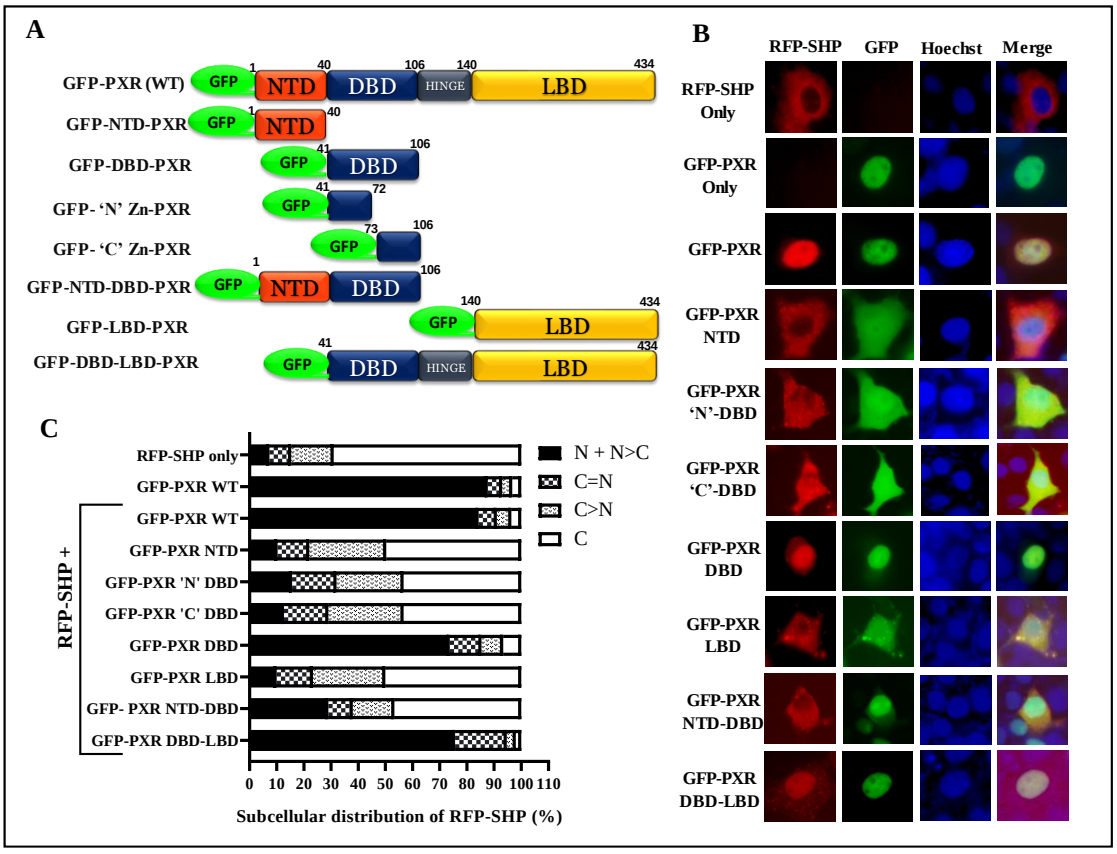


Figure 29: Subcellular localization patterns of RFP-SHP in living cells in the presence of different GFP-tagged PXR deletion constructs. ***(A)*** *Schematic representation of full-length and different deletion constructs of PXR used in the study are shown.* ***(B)*** *COS-1 cells were transiently transfected with 500 ng of RFP-SHP and each of the GFP-tagged wild-type and different deletion construct of PXR. Images were recorded using a fluorescence microscope equipped with water immersion objectives. The RFP-SHP and GFP fluorescence visualize the distribution pattern of receptors. Hoechst stained images are for visualizing the corresponding nuclei while the merged images for visualizing the corresponding nuclei with different deletion variants of PXR.*

***(C)** Quantitation of sub-cellular localization of RFP-SHP. COS-1 cells were transiently transfected with RFP-SHP and GFP-tagged PXR and its deletion constructs. Sub-cellular localization of SHP was observed and classified into groups (N+N>C, N=C, C) by fluorescence microscopy. 'N+N>C' refers to predominantly nuclear receptor localization where a small fraction of receptor may also be present in the cytoplasm, 'N=C' is assigned to the cells where receptor is expressed equally in both compartment and 'C' is assigned predominantly cytoplasmic localization of the receptor. In each case, localization of SHP was recorded in at least 100 transfected cells and average values of three independent experiments were plotted. The values are average of at least three separate experiments which exhibit similar patterns with values differing between 5-10%. The observations suggested that PXR interacts with SHP via its DBD.*

II: Identification of minimal domain of ER*α* required for interaction with SHP

To identify the domain/region in ERα required for interaction with the SHP, GFP-ERα and its different domains deletion constructs were prepared. For this purpose, COS-1 cells were co-transfected with relevant plasmid constructs **(Fig. 30A)**. After the expression period of 24 hours, live-cell imaging was performed and quantification was done **(Fig. 30B & C)**. In these experiments, GFP-ERα full-length protein was able to shift the localization pattern of RFP-SHP from cytoplasm to nuclear compartment. Similarly, other than the full-length ERα protein, only GFP-ERα without NTD (ΔNTD) was also able to shift the localization pattern of RFP-SHP. Surprisingly, DBD containing GFP-ERα was nuclear but did not shift of RFP-SHP to nucleus (**Fig 30A)**. GFP-ERα-ΔNTD which showed significant nuclear localization contains DBD, Hinge and LBD portion of ERα. This prompted us to conclude that LBD of ERα might be involved in interaction with SHP. Further, in our next experiments, we attempted to identify the minimal LBD region which interacts with SHP.

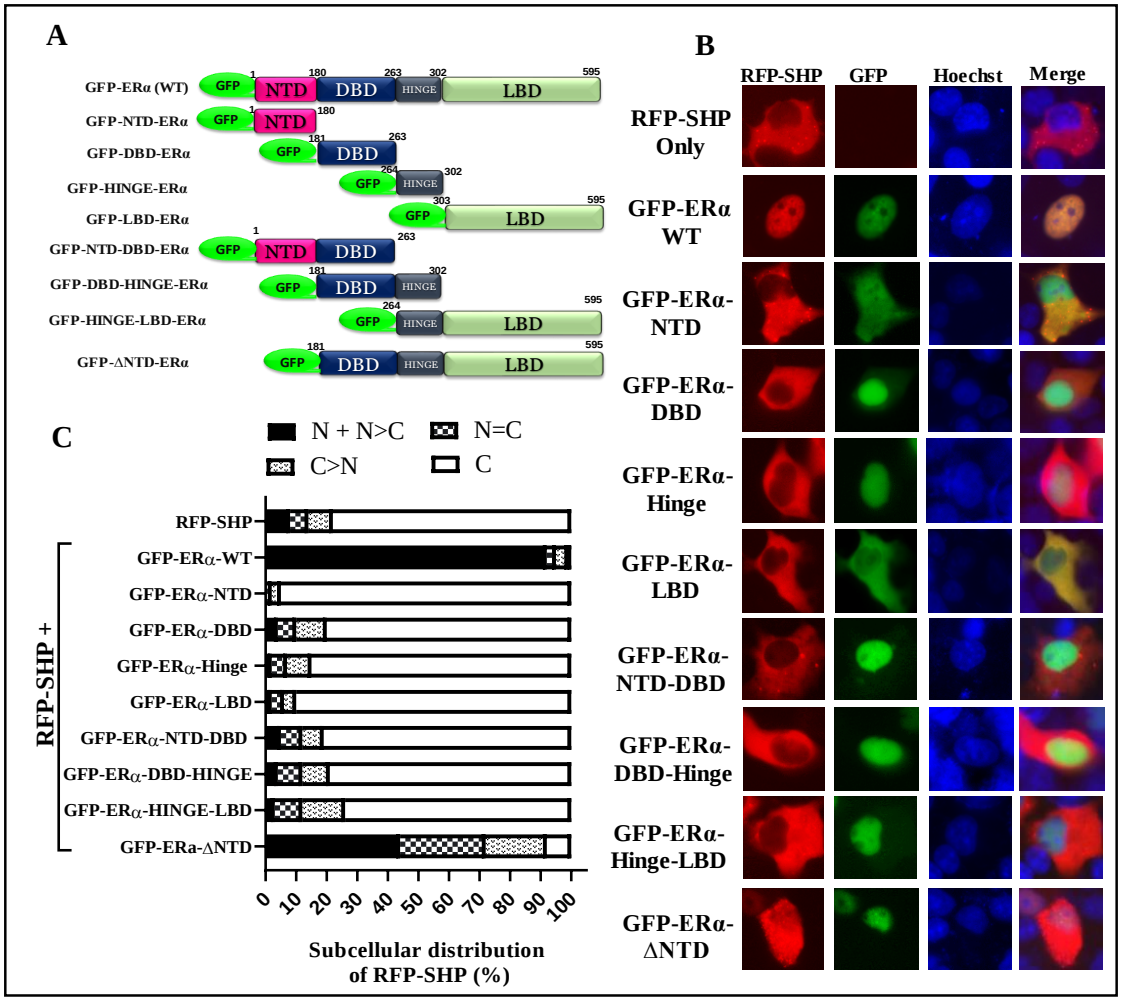
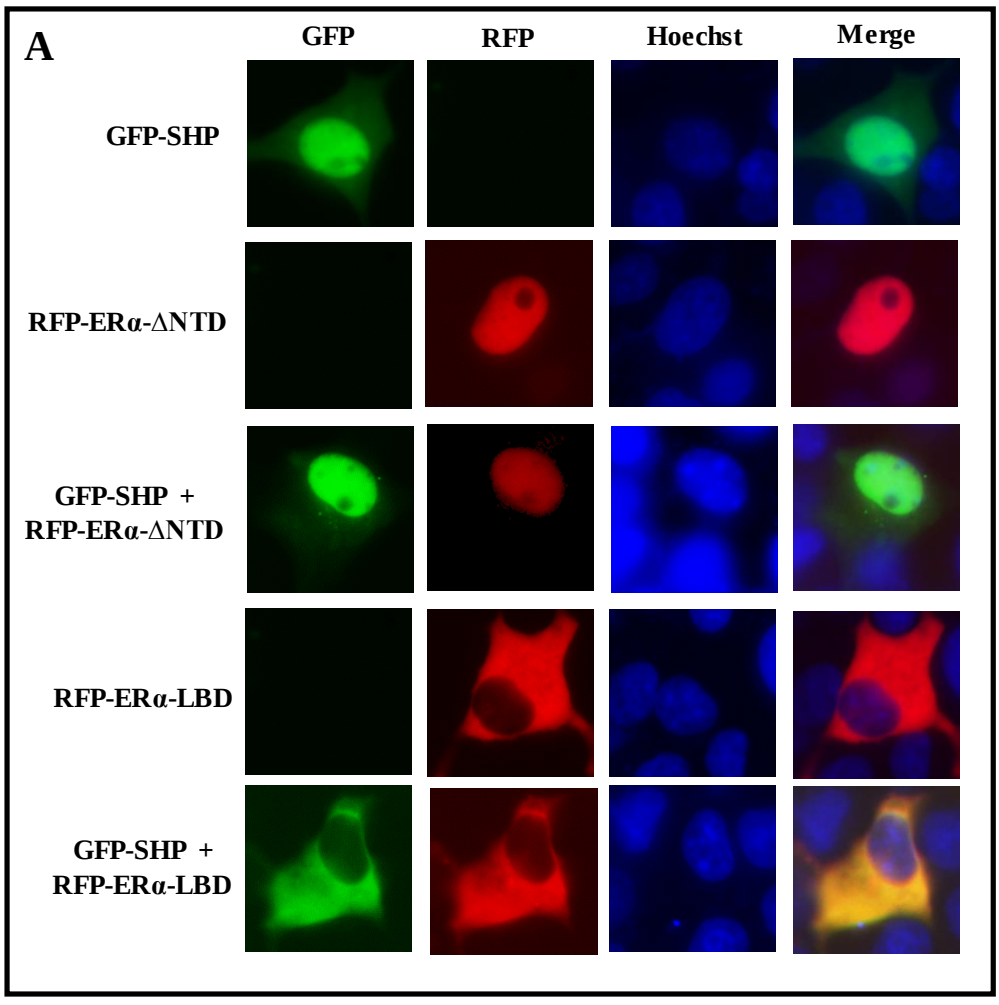


Figure 30: Subcellular localization patterns of RFP-SHP in the presence of different GFP-tagged ERα deletion constructs in living cells. ***(A)*** *Schematic representation of full-length and different deletion constructs of ERα used in the present study.* ***(B)*** *COS-1 cells were transiently transfected with 500 ng of each of RFP-SHP and GFP-ERα or along with one of its different deletion constructs as depicted in the figure. After receptor expression, the images were recorded using a fluorescence microscope equipped with water immersion objectives. The RFP and GFP fluorescence visualize the distribution pattern of specific receptors. Hoechst stained images are for visualizing the corresponding nuclei and the merged images for visualizing the corresponding nuclei with different domains of ERα.* ***(C)*** *Quantitation of subcellular localization of RFP-SHP. COS-1 cells were transiently transfected with RFP-SHP or GFP-ERα or along with one of deletion constructs of the latter. After the expression period subcellular localization of SHP as RFP signal was recorded. In each case, localization of SHP was recorded in at least 100 transfected cells and average values of three independent experiments were plotted. The values are average of at least three separate experiments which exhibit similar patterns with values differing between 5-10%. The GFP-ERα-ΔNTD partially shifted the localization of SHP from the cytoplasmic compartment to nucleus. This suggested that ERα interacted with SHP either via DBD or LBD.*

III: Ligand binding domain (LBD) of ERα is essential for interaction with SHP

The results above provided only initial evidence to show that SHP interacts with the ERα LBD but were not confirmatory. Therefore, to confirm further, RFP constructs with two critical domains of ERα i.e. ΔNTD and LBD domains were generated. These deletion constructs were then individually co-transfected with GFP-SHP plasmid which is mostly nuclear when expressed alone. After the expression period of 24 hours, the two fluorescent proteins were observed and quantified. When the RFP-ERα-ΔNTD (with deleted NTD region) was coexpressed with GFP-SHP, the subcellular localization of both was nuclear, as expected **(Fig. 31A).** RFP-ERα-LBD was observed to be cytoplasmic **(Fig. 31A)** as it does not harbor NLS containing DBD. Interestingly, when GFP-SHP was co-expressed with the RFP-ERα-LBD, GFP-SHP was retained into the cytoplasmic compartment **(Fig. 31A & B)**. This, observation can be attributed to the interaction between the shuttling GFP-SHP and the cytoplasmic retained RFP-ERα-LBD. This result indicated that LBD of ERα interacts with the SHP to shift the localization towards cytoplasm.



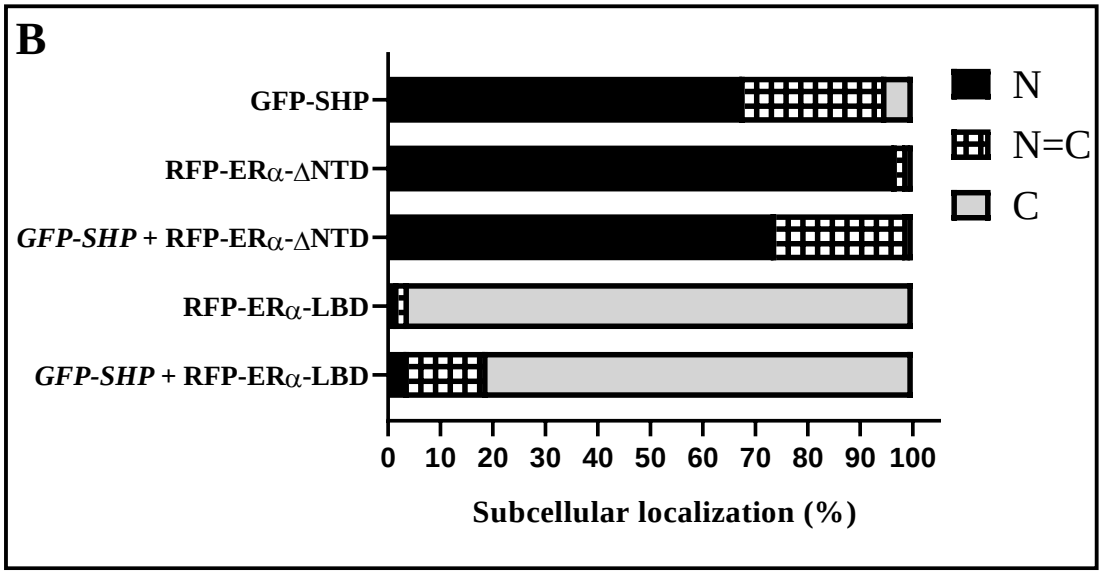


Figure 31: Ligand binding domain (LBD) of ERα is essential for interaction with SHP. (*A*) *COS-1 cells were transiently cotransfected with GFP-SHP and RFP-ERα-LBD or RFP-ERα-ΔNTD. Same constructs were also transfected individually for comparisons purposes. Cell images for subcellular localization during interphase were recorded using a fluorescence microscope. The GFP fluorescence visualizes the distribution pattern of SHP while the RFP fluorescence visualizes the distribution pattern of deletion constructs of RFP-ERα-LBD and RFP-ERα-ΔNTD. Hoechst stained images are for visualizing the corresponding nuclei and the merged images show the colocalization of SHP and deletion constructs of RFP-ERα-LBD and RFP-ERα-ΔNTD.* ***(B)*** *In each case, localization of SHP was recorded in at least 100 transfected cells and average values of three independent experiments were plotted. The values are average of at least three separate experiments which exhibit similar patterns with values differing between 5-10%. It was confirmed that ligand binding domain of ERα interacts with SHP.*

Identification of critical amino acid residues in SHP involved in the interaction with PXR and ERα

In the study above, we characterized the specific domains of SHP that interact with its interacting partners. Initially, the analysis was performed *in silico* to identify the critical amino acid residues that influence the intensity of interaction of SHP with its interacting partner PXR or ERα. For this purpose, we first constructed the *in silico* working model of SHP as shown in **Fig. 32**.

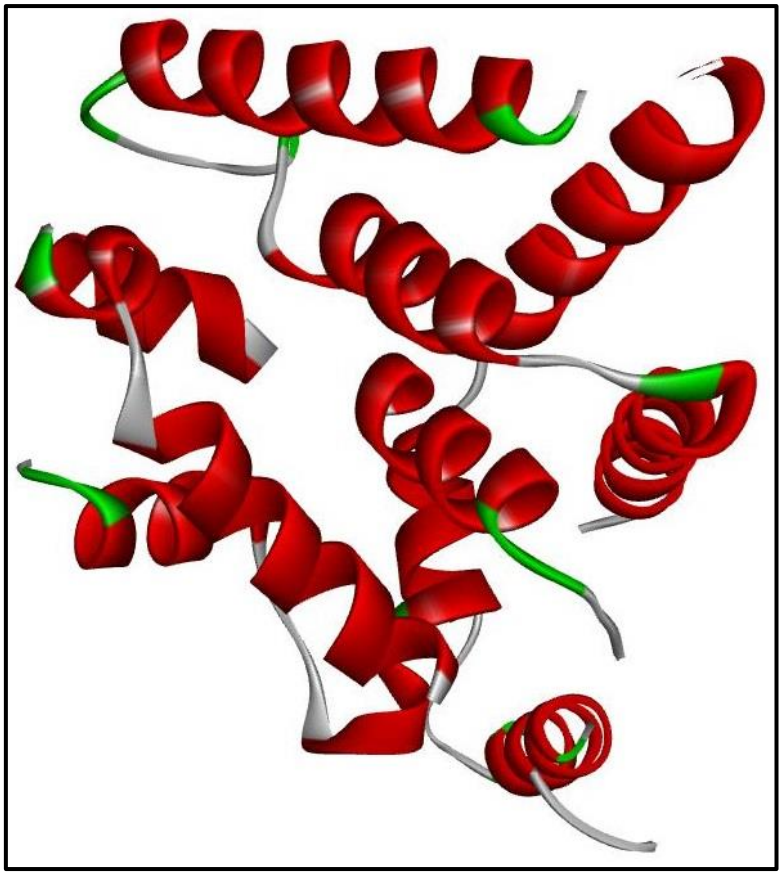


Figure 32: Three-dimensional structure of human SHP constructed using homology modelling approach.

Construction of human SHP model

The crystal structure of human SHP is still not available. Therefore, for modelling the complex with other proteins, BLAST was used to search for the known homologues in Protein Data Bank against SHP [321]. We found a crystal structure of mouse SHP protein that shows 81% sequence identity with human SHP [321]. A three-dimensional model of human SHP as shown in **Fig. 32** was built using Modeller software [322]. The phi-psi angles of the model were evaluated using the Ramachandran plot [283] **(Fig. 33)**. Around 93% of the residues fell under the favorable region, while 7% of residues were observed under additional allowed regions **(Fig. 33)** and no residues were found in the disallowed region **(Fig. 33)**.

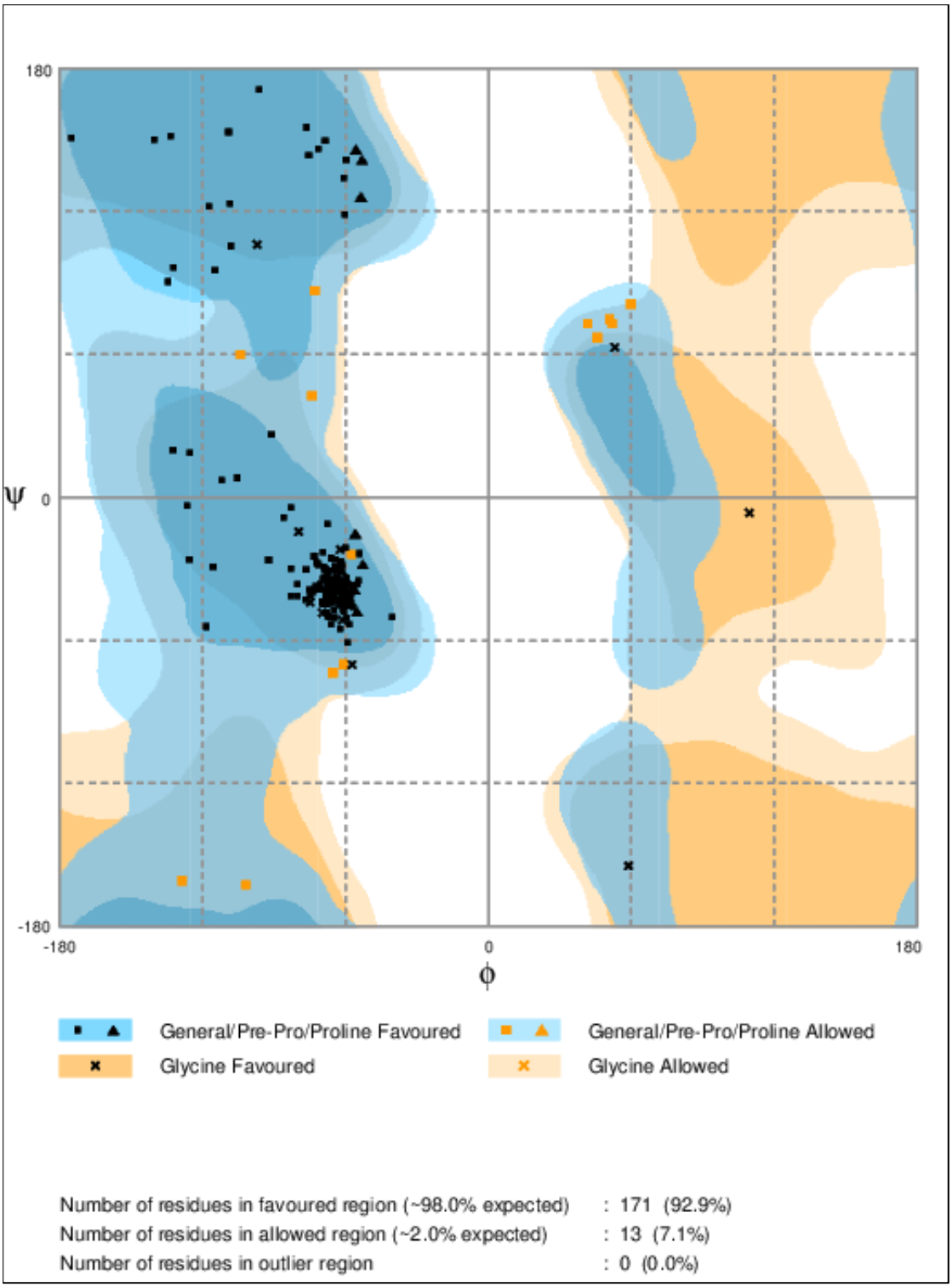


Figure 33: ***The Ramachandran plot evaluated the stereochemical quality structure of human SHP show that there are no residues felling under disallowed regions.***

Protein-protein docking experiments were employed to understand the molecular mechanism and identifying the mode of binding and the conformations having least protein-protein binding energy and its essential interactions [286,323]. Docking of

SHP-PXR and SHP-ERα complexes were carried out by ClusPro server [287]. The binding free energy weighted score for SHP-PXR was -1055.4 whereas for SHP-ERα complexes was -1071.1. Thus, in order to find the crucial amino acid residues that are responsible for interactions at the interface of SHP-PXR and SHP-ERα complexes, we performed *in silico* analysis as mentioned in 'Materials and Methods' section and found that R216A, W206H in SHP-PXR complex and N176A and R216E in ERα were critical. The lowest energies obtained from the docked conformations of native and mutants of SHP-PXR and SHP-ERα complexes were taken for molecular dynamics simulations.

Identification and characterization of amino acid residues in SHP responsible for interaction with PXR and ER*α*

Our previous section of the study suggested that SHP interacts with PXR and ERα. The protein-protein interaction of native proteins and the identified minimal domains lay the basis to investigate the interface residues that form contacts between the two interacting partners. Further, using *in silico* tools we attempted to identify these amino acid residues in such interactions.

***In silico* approach to assessing the protein-protein interaction between SHP and its interacting partners**

In our initial approach, we performed docking of SHP with its interacting nuclear receptors, PXR and ERα based on the binding affinities.

The further mutational scan was performed, on that basis SHP mutants, R216E, W206H, E204A were docked with PXR and showed the binding affinity were reduced compared to native SHP. Among the mutants, R216E showed significantly decreased binding affinity of -860.6. Further R216 and W206 position of amino acid residues were replaced with the simplest amino acid alanine and their binding energy was calculated **(Table 7, Fig. 34)**.

Table 7: Estimation of binding affinities of native SHP and its mutants with PXR by ClusPro server tool. The results show that the binding affinity of SHP mutant decreased compared to the native SHP structure

No.	Receptor-1	Receptor-2 (SHP)	Binding affinity
1	PXR	SHP native	-1071.1
2	PXR	SHP-R216E	-860.6
3	PXR	SHP-W206H	-753.1
4	PXR	SHP-E204A	-822.1
5	PXR	SHP-R216A	-715.3
6	PXR	SHP-E204R	-869.6

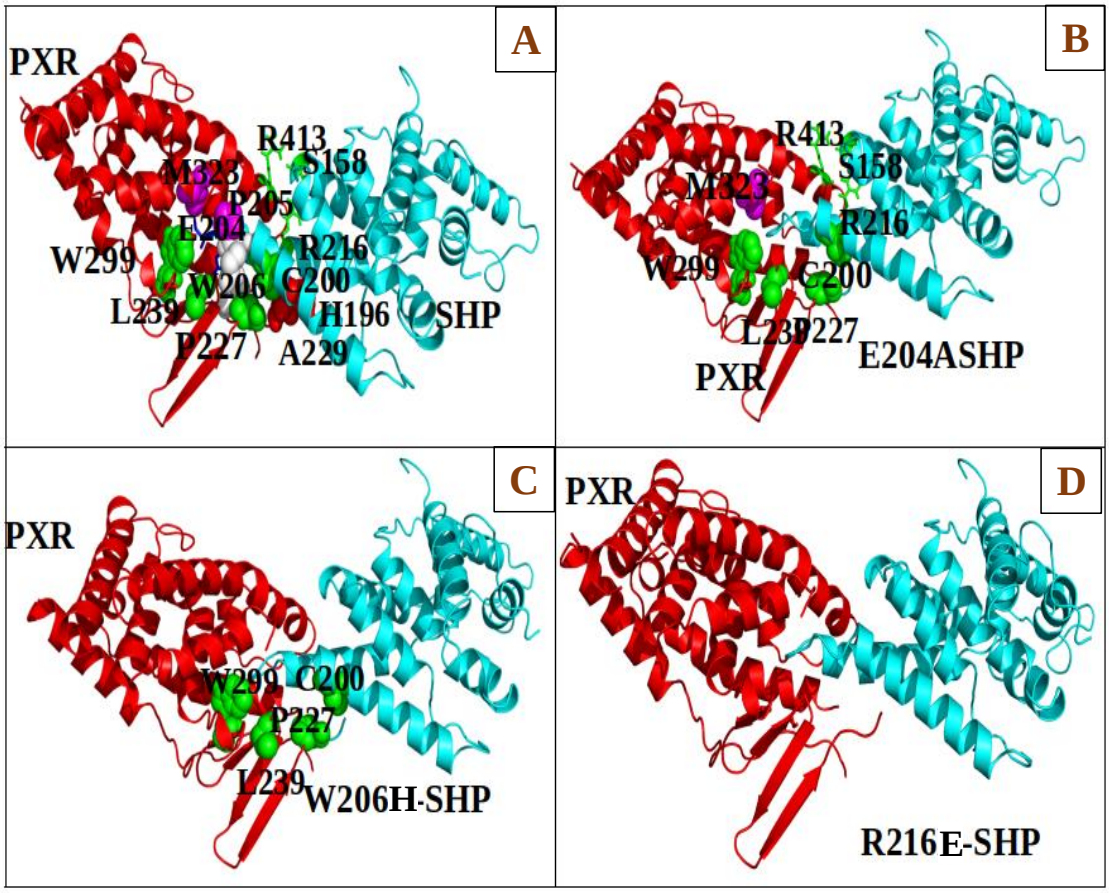


Figure 34: Interactions at the interface of native and mutants SHP forms with PXR. (*A*) *Native SHP and PXR.* ***(B)*** *SHP-E204A and PXR* ***(C)*** *SHP-W206H and PXR* ***(D)*** *SHP-R216E and PXR. In all the interactions cyan color protein ribbon shows the native SHP and mutated SHP while the red color represents PXR residues.*

Molecular dynamic simulation of SHP-PXR and its mutant complexes

Molecular dynamics simulations were carried out over 10 ns for the SHP-PXR complexes, and the stability of the structures was analyzed using Root Mean Square Deviation (RMSD), Radius of Gyration (Rg), solvent accessible surface area (SASA) and the hydrogen bonds interactions **(Fig. 35)**. The overall stability of the SHP-PXR and the mutant complexes of SHP (E204R, E204A, W206H, W206A, R216E and R216A) throughout the molecular dynamics simulations were monitored by the RMSD of Cα, as shown in **Fig. 35A**. Seven independent simulations were carried out for the SHP-PXR (native) and with SHP mutant complexes (E204R, E204A, W206H, W206A, R216E and R216A) for a total of 10 ns simulation time. We found that the mutant complexes R216A / W206D showed significantly distinct trajectories compared to SHP-PXR (native), the native RMSD was observed between 0.2 nm **(Fig. 35A).** After 5 ns, a stable trajectory was observed throughout the simulation till 20 ns. In the case of all the SHP mutants, simulation tend to reach a higher equilibrium when compared to the native structure of SHP. This remained distinguishable throughout the simulation, which resulted in Cα RMSD of 0.2 to 0.4 nm. The radius of gyration has been used to calculate the MD simulations of protein-protein complexes over 10 ns for competence, shape and folding **(Fig. 35B)**. Protein-protein complexes show that the radius of gyration for PXR-SHP (wt) or with SHP mutant complexes (E204R, E204A, W206H, W206A, R216E and R216A) exhibited a Rg score of 2.4 nm which is similar to native. The radius of gyration for E204R remained at Rg value of 2.8 nm, which was slightly higher as compared to that for the native and mutant complexes (E204A, W206H, W206A, R216E and R216A). The radius of gyration for all the complexes was maintained at 2.3 nm and remained stable after 2.5 ns as observed in all the complexes towards the end of the simulation **(Fig. 35B).** This indicates that the radius of gyration for mutant complexes (E204R, E204A, W206H, W206A, R216E and R216A) was stabilized under simulated dynamic condition. The solvent accessible surface area (SASA) was calculated for the SHP-PXR (native) and the SHP mutant complexes (E204R, E204A, W206H, W206A, R216E and R216A). In the case of E204A, W206D and R216A, SASA values (240 nm) were observed similar to native **(Fig. 35C)**. We obtained higher values of SASA (255 nm) with time for E204R mutant **(Fig. 35C).** Hence, the observations are in direct correlation with changes to the SASA which attains lower stability against the binding of SHP-PXR complexes. The SASA results were comparable with the RMSD and radius of gyration plot.

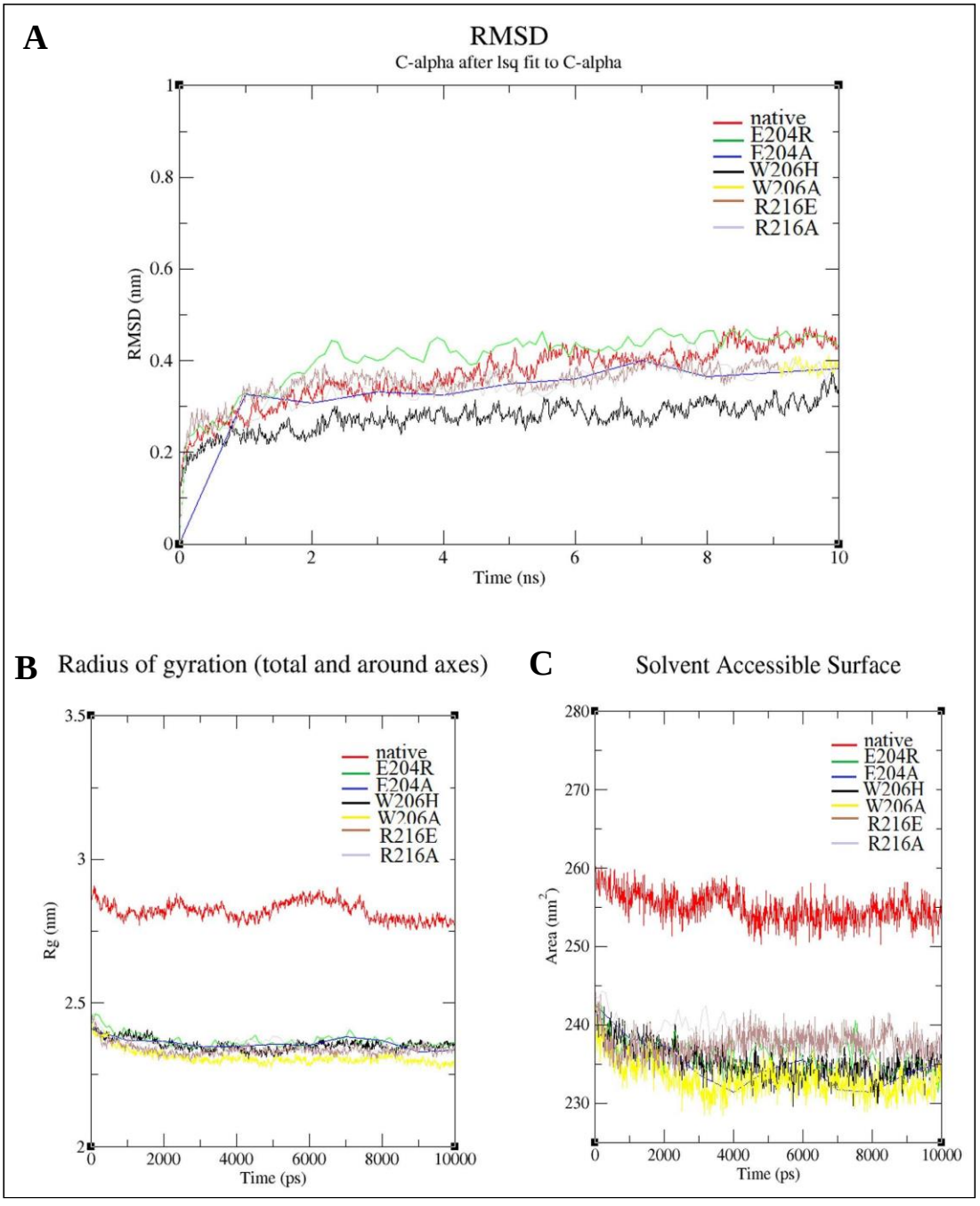


Figure 35: Molecular dynamic simulation of SHP in complex with wild type PXR or its mutant. ***(A)*** *Time evolution of backbone Root Mean Square Deviations (RMSDs) of the native and mutant structures.* ***(B)*** *Rg of the protein backbone over the entire simulation. The ordinate is Rg (nm), and the abscissa is residue.* ***(C)*** *Solvent Accessible Surface Area (SASA) and the hydrogen bonds interactions.*

***In silico* docking of SHP with its interacting partner ERα, and an approach for essential amino acid prediction**

Similarly, was performed with PXR, the SHP mutants, R216E, N176D, K225A, E154A were docked with ERα, which exhibits reduced binding affinities with native SHP protein **(Table 8, Fig. 36)**. However, this phenomenon needs to be further validated by molecular dynamics simulation.

Table 8: Estimation of binding affinities of SHP and its mutants with ERα by ClusPro server tool. The results show that the binding affinity of SHP mutant decreased compared to the native SHP structure

No.	Receptor-1 (ERα)	Receptor-2 (SHP)	Binding affinity
1	ERα	SHP native	-854.8
2	ERα	SHP-R216E	-794.3
3	ERα	SHP-N176D	-832.5
4	ERα	SHP-K225A	-571.2
5	ERα	SHP-E154R	-619.0
6	ERα	SHP-E154A	-672.4
7	ERα	SHP-N176A	-545.7
8	ERα	SHP-R216A	-536.1

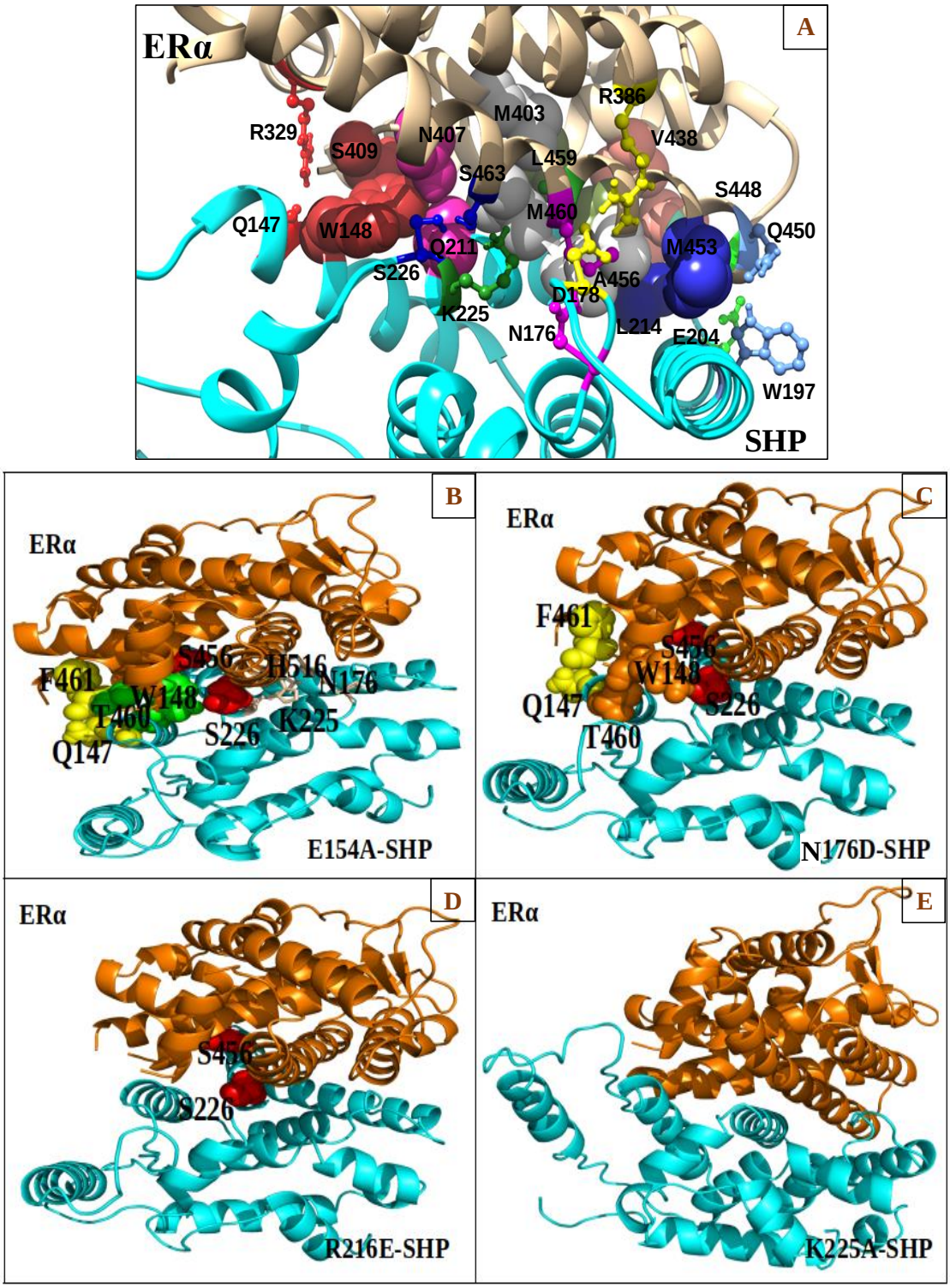


Figure 36: Interactions at the interface of ERα and native SHP and SHP mutants. ***(A)*** *Native SHP and ERα* ***(B)*** *SHP-E154A and ERα* ***(C)*** *SHP-N176D and ERα* ***(D)*** *SHP-R216E and ERα* ***(E)*** *SHP-K225A and ERα. In all the interactions cyan color protein ribbon represents native SHP and mutated SHP and the red color represent PXR residues.*

Molecular dynamic simulation of ERα in complex with wild type SHP or its mutants

We performed molecular dynamics simulations over 10 ns for the SHP-ERα and its mutant SHP-ERα complexes (E154A, E154R, N176D, N176A, R216E, R216A and K225A) [285,289]. The stability of the structures was analyzed using Root Mean Square Deviation RMSD, Radius of gyration (Rg), Solvent Accessible Surface Area (SASA) and the hydrogen bonds interactions. The overall stability of the native SHP-ERα and SHP mutant in complex with ERα throughout the molecular dynamics simulations were examined by the root-mean-square deviation (Cα RMSD) **(Fig. 37A)**. Eight independent simulations were carried out for the Cα for a total of 20 ns simulation time. We observed that the mutant complexes of SHP-ERα have similar trajectories when compared to SHP-ERα (native). The native RMSD was observed between 0.2 nm-0.4 nm **(Fig. 37A)**. After 2 ns, a slight deviation was observed in the trajectory leading to stable equilibrium as throughout the simulation till 10 ns. The E154A, E154R, N176D, N176A, R216E, R216A and K225A simulation tend to reach a lower equilibrium as compared to the native and remained stable throughout the simulation resulting in Cα RMSD of 0.35 nm and 0.4 nm. The radius of gyration has been employed to calculate the MD simulations of protein-protein complexes over 10 ns for competence, shape and folding **(Fig. 37B)**. The radius of gyration for SHP-ERα (native) and its mutant complexes with a Rg score of 2.25 nm obtained lower trajectories in comparison to the native. Whereas for the mutants, N176A, R216E exhibited a higher pattern with a Rg score of 2.2 nm. The radius of gyration remained at Rg value of 2.4 nm which was slightly increased than that for the mutant complexes (N176A, R216E and R216A). In addition, the radius of gyration for all the complexes was maintained stable plateau after 2.5 ns as observed in all the complexes towards the end of the simulation **(Fig. 37C)**. The SASA was calculated for the SHP-ERα (native) and the SHP mutant complexes. We obtained higher values of SASA (257 nm) for SHP-ERα (native) while in the case of mutant complexes (E154A, K225A, N176A and R216E) SASA value was lower at 255 nm. Hence, the observations are in direct correlation with changes in the SASA, which attains lower stability against the binding of SHP-ERα mutant complexes.

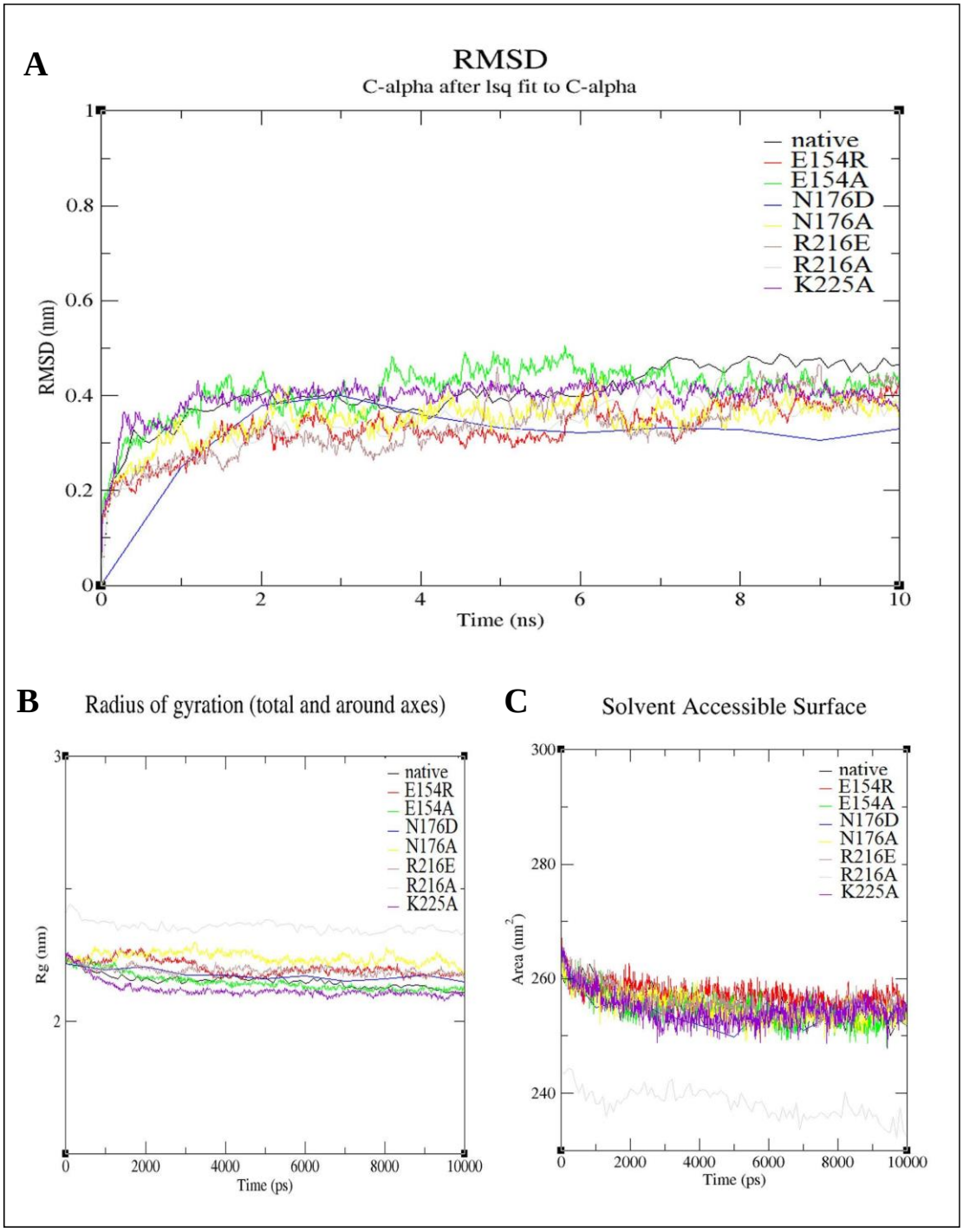


Figure 37: Molecular dynamic simulation of SHP-ERα and SHP mutant complexes ***(A)*** *Time evolution of backbone Root Mean Square Deviations (RMSDs) of the native and mutant's structures.* ***(B)*** *Rg of the protein backbone over the entire simulation. The ordinate is Rg (nm), and the abscissa is residue.* ***(C)*** *Solvent Accessible Surface Area (SASA) and the hydrogen bonds interactions.*

In vivo characterization of *in silico* predicted minimal amino acid residues

We further validated the *in silico* observation of protein-protein interaction at the level of subcellular localization.

- **Assessment of predicted amino acid residues of SHP on interaction with PXR**

The *in silico* studies in previous section showed that the amino acid E204, W206, R216 are critical residues of SHP which can alter the protein-protein interaction of native SHP with PXR **(Table-7)**. Therefore, to validate the *in silico* results the predicted amino acid residues were replaced as E204R, E204A, W206H, W206A, R216E and R216A. The selection of substituted amino acids was based on the simplicity of amino acid charge, score of the mutational scan and protein docking score. Further validation was done by molecular dynamics simulation approach which could influence the binding of mutated SHP with PXR. Additionally, to examine the influence of these amino acids, the residues in wild type RFP-SHP were replaced with E204A, W206H, W206A, R216E and R216A using the site-directed mutagenesis protocol elaborated under 'Materials and Methods'. Firstly, RFP-SHP and its different mutants were generated, confirmed by sequencing and validated by transient expression in the COS-1 cells. Similar to previous experiments, subcellular localization of RFP-SHP and its different mutant forms were visualized under the fluorescence microscope and cells were quantified for subcellular localization. The localization patterns of the native SHP and mutated SHP were cytoplasmic (not shown). Furthermore, GFP-PXR was cotransfected with mutated RFP-SHP plasmid **(Fig. 38B)**. The results were in agreement with the observation predicted by in *silico* approach **(Fig. 36)**.

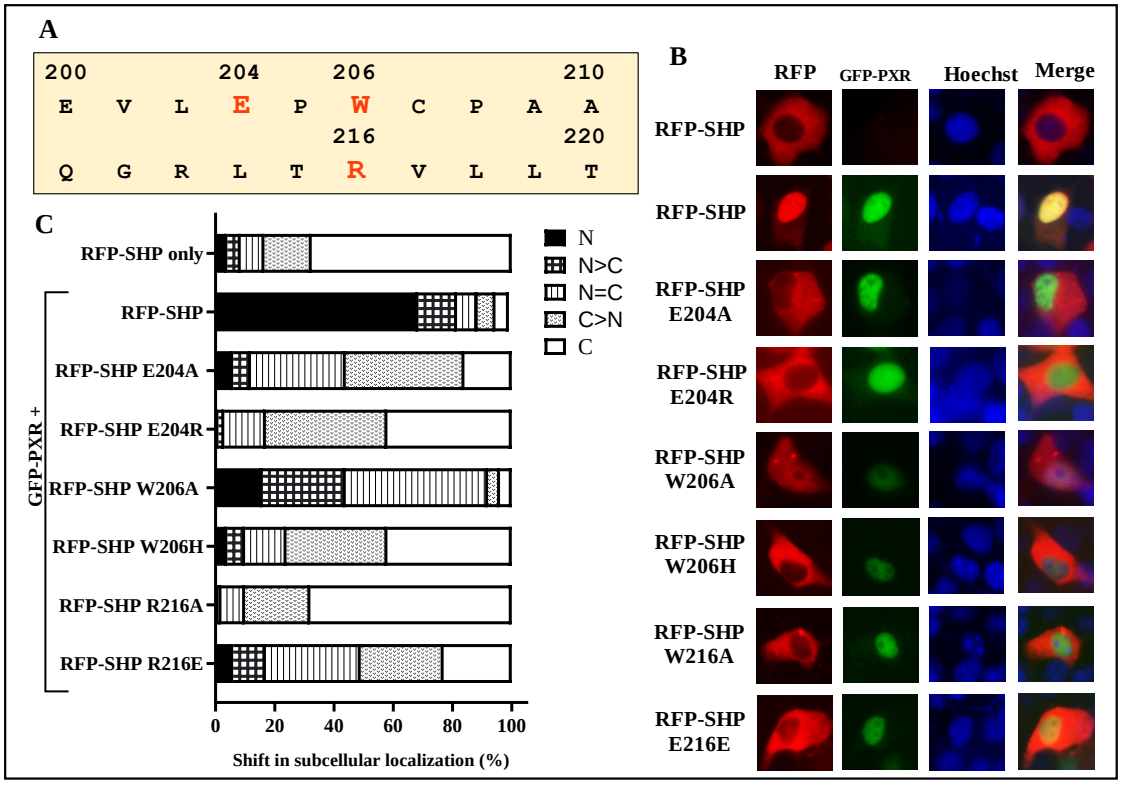


Figure 38: Subcellular localization patterns of RFP-SHP mutants in the absence and presence of GFP-PXR in living cells. *(**A**) In the box, the mutated amino acid residues in SHP are marked in the red color. (**B**) COS-1 cells were transfected with 500 ng of different plasmid constructs alone or in combination with GFP-PXR as indicated in the figure. Live cell images were recorded using a fluorescence microscope equipped with water immersion objectives. The RFP-SHP and GFP fluorescence show the distribution pattern of the two receptors either alone or in combination. Hoechst stained images are for visualizing the corresponding nuclei and the merged images for visualizing the corresponding nuclei with different mutant of SHP with PXR. (**C**) Quantitation of subcellular localization of RFP-SHP and its critical mutants. In each case, localization of SHP was randomly recorded in at least 100 transfected cells. The values are average of at least three separate experiments which exhibit similar patterns with values differing between 5-10%. The protein-protein interaction of mutated SHP at W206 and R216 amino acid residues are critical for interaction with PXR.*

The protein-protein interaction between wild type SHP and PXR shifted the former from cytoplasmic to nuclear compartment. However, the substitution of critical amino acid residues in SHP identified by *in silico* study exhibited diminished interaction with PXR. Of all the mutants, SHP-E204R and SHP-R216A exhibited near complete absence of interaction with PXR while other mutant residues hampered this interaction with PXR at varied levels. Overall, the study identified and concluded that

a few of the amino acid residues in SHP are vital for the protein-protein interaction with PXR.

- **Validation of predicted amino acid residues of SHP on interactions with ERα**

The previous *in silico* observations (**Fig. 37 and Table-8**) showed that amino acid E204, W206, R216 were critical residues in SHP, and change in these residues can alter the protein-protein interaction with ERα **(Table-8)**. To validate, the *in silico* predicted amino acid residues were replaced with E154R, E154A, N176D, N176A, E204R, E204A, R216E, R216A, and K225A. Here too, the selection amino acid residue was done as in the section above. The site-directed mutagenesis approach was applied as mentioned in 'Material and Methods'. The mutant constructs, after the sequence confirmation, were transiently transfected into the COS-1 cells and visualized under the fluorescence microscope. The subcellular localization of SHP mutant proteins was mostly cytoplasmic. Further, to examine the influence of mutated SHP variants (E154R, E154A, N176D, N176A, E204R, E204A, R216E, R216A, and K225A) on ERα, cotransfection experiments were conducted. After the expression period of 24 hours, cells were recorded and quantified **(Fig. 39B & C)**. The interaction of mutant forms of SHP with ERα diminished to varied level as compared to wild type SHP. The mutated amino acid residue N176D and A showed negligible or no interaction with GFP-ERα. Similarly, the other amino acid residue R216 showed no interaction suggesting its importance in the interaction. The results obtained here strongly supported our *in silico* predictions **(Fig. 39)**. In conclusion, the observation imply that protein-protein interactions are dependent on a single amino acid residue (R216) as its replacement caused severe impairment between two interacting receptors.

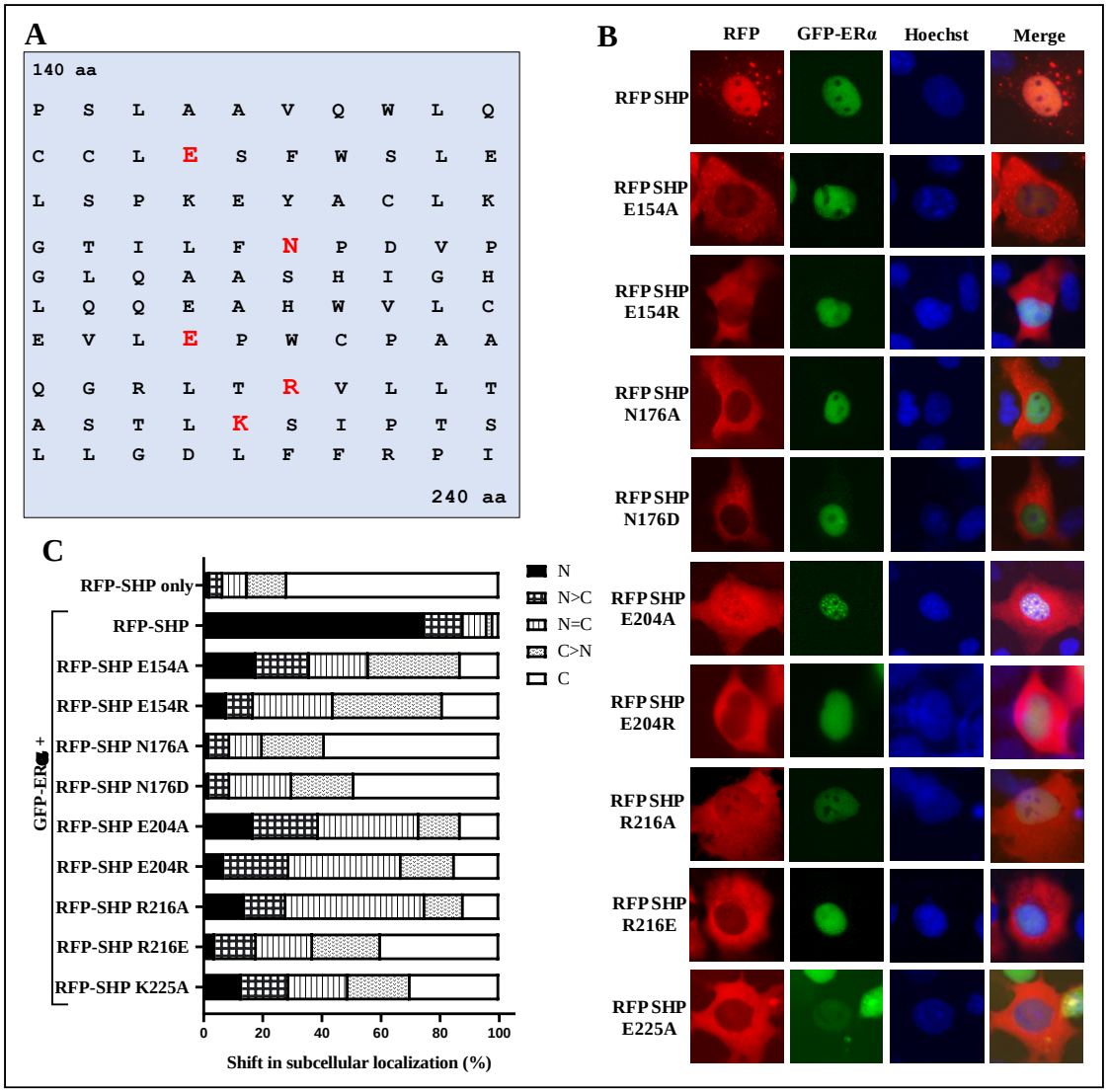


Figure 39: Subcellular localization patterns of RFP-SHP mutants in the presence of GFP-ERα in living cells. *(**A**) In the box, the mutated amino acid residues are marked in red color. (**B**) COS-1 cells were transiently transfected with 500 ng of RFP-SHP or its mutants along with GFP-ERα. Live cell images were recorded using a fluorescence microscope equipped with water immersion objectives. The RFP-SHP and GFP fluorescence visualize the distribution pattern of the two receptors. Hoechst stained images are for visualizing the corresponding nuclei and the merged images for visualizing the corresponding nuclei with different mutants of SHP with ERα. (**C**) Quantitation of subcellular localization of RFP-SHP and its varied mutants. In each case, localization of SHP was recorded in at least 100 transfected cells. The values are average of at least three separate experiments which exhibit similar patterns with values differing between 5-10%. The amino acid residue N176 of SHP is identified as critical for the interaction with ERα.*

Discussion

Homeostasis is a life supporting, cooperative equilibrium among different cells, tissues and organs of the body that relies on a rigorous communication and sensory network as a means to sustain bodily functions in all metazoans [7]. SHP was discovered in 1996 and has been reported to interact with variety of transcription factors, including nuclear and non-nuclear receptors, which in turn are established to regulate the expression of many target genes in a tissue-specific manner. This wide variety of SHP-interacting partners is indicative of higher order of regulatory role that it plays in many cellular and physiological pathways, thereby establishing itself as a critical transcriptional coregulator in cellular metabolic processes [14].

PXR functions as is a broad-specificity metabolic and xeno-sensing transcription factor. It interacts with a wide variety of small molecules of endogenous (bile acids, hormones etc.) and exogenous (e.g. synthetic drugs, herbals) nature to protect the body from harmful chemical insults [31]. Upon activation, PXR induces CYP3A and inhibits CYP7α, suggesting that PXR can act on both bile acid synthesis and their elimination [213]. Indeed, CYP3A and CYP7α are involved in biochemical pathways leading to cholesterol conversion into primary bile acids, whereas CYP3A also participates in the detoxification of xenobiotics and endobiotics. Based on our transcriptional assays, receptor translocation and mitotic chromatin binding observations, along with some indications from the previous report, we suggest that PXR is a target for SHP [22]. All the observations derived from multiple approaches supported this conclusion.

Previously, it has been reported that PXR associates with mitotic chromatin in ligand-independent manner [123], whereas ERα associates with mitotic chromatin in a ligand-dependent manner [138]. On the contrary, SHP when expressed alone did not associate with mitotic chromatin, a property that was attributed to the absence of DBD. The observations made here with live cell imaging indicated that coexpression of either PXR or ERα strongly shifted the cytoplasmic SHP to the nuclear compartment in interphase cells. A few other NRs (like AR, FXR, RXR) did not exhibit such shift for SHP (data not shown) implying the specificity of inter-molecular interactions with PXR and ERα. In accordance with the previous reports, we observed that SHP modulates the transcription function of some of its interacting partners. To further validate the

findings, we explored the involvement of SHP on the mitotic binding property of PXR and ERα.

Interestingly, we attempted to study the association of SHP during mitosis, and we observed that SHP alone does not associate with mitotic chromatin. Recent reports suggests that a 'Mitotic chromatin Binding Region' (MCBR), that is present in NR DBD, is essential for association with mitotic chromatin [36,122]. Thus, SHP which is devoid of MCBR-DBD is unexpected to have this property.

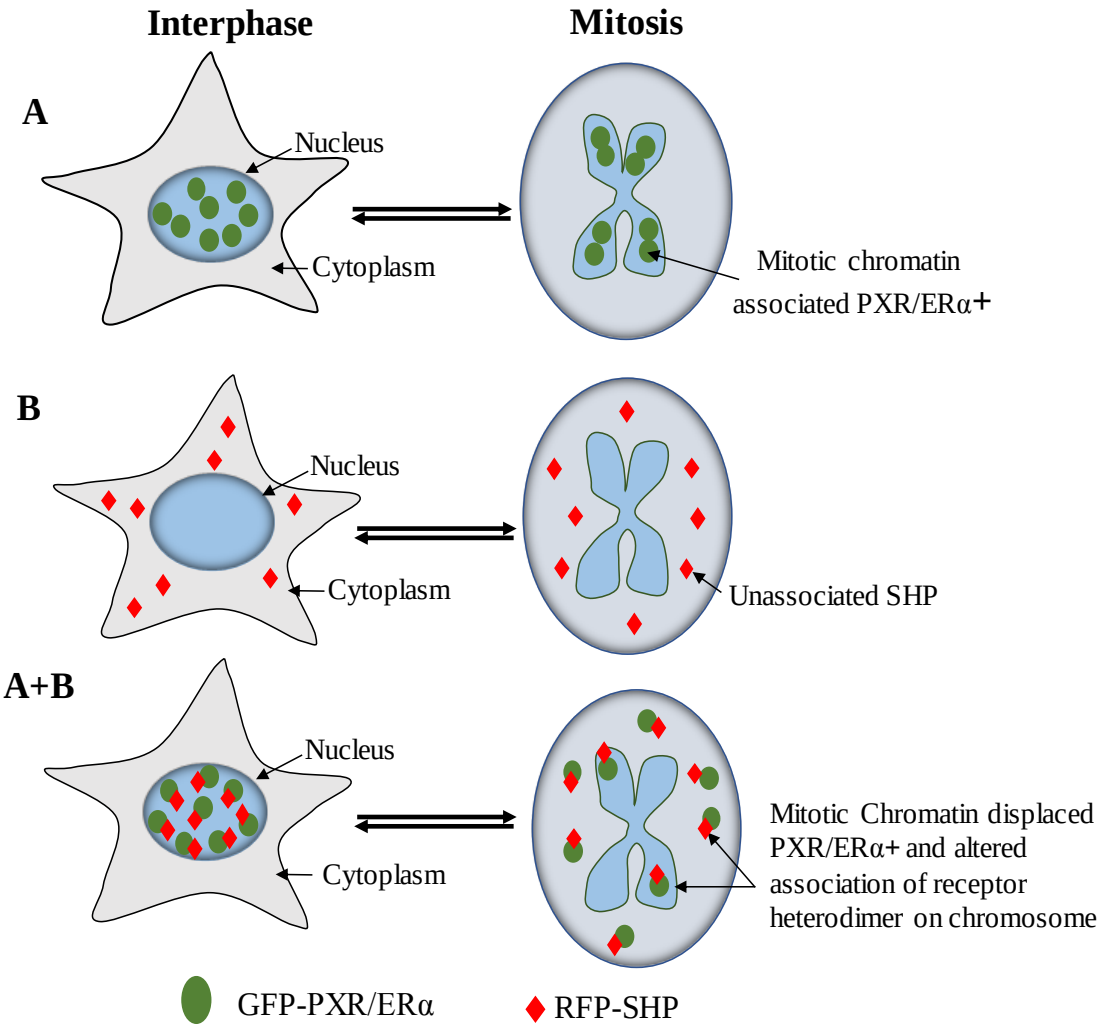


Figure 40: A schematic model to show the mechanism by which SHP abrogates association of its interacting nuclear receptors from mitotic chromatin in a *hog-tie* manner. *The left figure panel shows the receptor localization during interphase and right panel shows receptor association with mitotic chromatin.* ***(A)*** *PXR and ERα, whether in unliganded or liganded* state, *are predominantly localized in the nucleus. PXR associates constitutively with mitotic chromatin while ERα associates in a ligand-dependent manner.* ***(B)*** *Free SHP is localized in cytoplasmic compartment during*

interphase, and it does not associate to mitotic chromatin during cell division due to absence of DBD in its modular structure. ***(A+B)*** *During interphase SHP shifts to the nuclear compartment in the presence of PXR or ERα. This implies existence of inter-molecular interaction between SHP and these two receptors. However, during cell division presence of SHP interferes with PXR and ERα association with mitotic chromatin. This is attributable to inter-molecular interactions of SHP with the chromatin bound receptors. '+' represents liganded ERα.*

However, when SHP was coexpressed along with one of its interacting partners (i.e. PXR or ERα) we observed that presence of SHP impeded or nearly abolished the mitotic chromatin binding property of the partner. Interestingly, a small fraction of SHP was seen associated with the remnant portions of the bound interacting partner **(Fig. 40)**. This is suggestive of occurrence of intermolecular interactions between SHP and its interacting partners. This is reflected not only in cytoplasmic to nuclear shift studies but also in alteration in mitotic chromatin binding behavior of these receptors. This receptor-based interaction is also evident in inhibition of transcription function of the interacting partners.

Further, detailed studies with individual structural domains of PXR indicated that DBD of PXR is essential for the interaction with SHP. This conclusion was derived from the finding that GFP-PXR-DBD was able to translocate cytoplasmic SHP into the nuclear compartment. Similarly, a detailed study with ERα indicated that LBD domain of ERα is minimal domain which is crucial for the interaction with SHP. Further studies are required to gain concrete insights into the mechanistic details of this phenomenon.

In our subsequent study, we attempted to identify the critical amino acid residues in SHP which are required for the protein-protein interactions. Using *in silico* 'Molecular Docking and Molecular Dynamics Simulations' (MD simulations) we identified a few crucial residues in PXR (E204, W206, and R216) that are responsible for intermolecular interactions at the interface of SHP-PXR. In our next approach, *in silico* mutation analysis was performed to investigate the role of individual amino acid residues in SHP-PXR complex and mutants of SHP (mutants E204R, E204A, W206H, W206A, R216E and R216A) in complex with PXR**.** Similarly, we analyzed the SHP-ERα complex and observed that the active site residues E154, N176, R216, and K225 of SHP form crucial interactions at the interface. The mutated SHP residues 154A, 154R, 176D, 176A, 216E, 216A and 225A were observed to break the stable complex formation with ERα implying the importance of native amino acid residues in the

intermolecular interactions. The residues that are involved in the formation of interactions are conserved in the native simulation whereas the hydrogen bond networks were lost during simulations. MD simulation analysis revealed that SHP mutations (E204R, E204A, W206H, W206A, R216E and R216A) and (E154A, E154R, N176D, N176A, R216E, R216A and K225A) may not promote PXR or-ERα interactions respectively with polar contacts resulting in loss of their physical interactions. Our experimental data exhibited that SHP mutants R216A, W206D in presence of PXR, and Q176A and R216E mutants in presence of ERα, do not induce significant nuclear translocation as was observed with wild type SHP. The numbers of hydrogen bonds formed between SHP (native and mutant) with PXR and ERα during the MD simulation were also calculated. From our analysis, it is revealed that native complex of SHP-PXR and SHP-ERα form several NH bonds and a higher number of van der Waals interactions while the mutant complex R216A, W206D in SHP–PXR complex, and Q176A and R216E in SHP-ERα, exhibited no such interactions**.** Thus, the MD simulations and comparative experimental study revealed that the structure and interaction network are influenced by these substituted mutations and cause the loss of stable interactions, and consequently the receptor function.

Further, the critical amino acid residues in SHP identified by *in silico* analysis, which influence the inter-molecular interactions, were confirmed by live cell imaging analysis. Native SHP interacts efficiently with PXR and ERα which was observed in receptor shift from the cytoplasmic to nuclear compartment. However, after mutation of critical amino residues in SHP, its interactions with PXR and ERα were observed to be drastically reduced as compared to the interaction with the wild type SHP.

Finally, the investigations from this part of the study emphasize that atypical nuclear receptor SHP by itself does not associate with mitotic chromatin. However, SHP abolishes the mitotic chromatin of some of the key nuclear receptors while it weakly promotes its own association by hitch-hiking mechanism. SHP is observed to interacts efficiently with some of the key nuclear receptors (like PXR and ERα) implying its role in their regulatory functions during cell cycle. A detailed study of the biological significance and underlying mechanisms of retaining or excluding specific TFs from ‘mitotic chromatin’ warrant further investigation to resolve the molecular and cellular basis of such behavior.

Chapter-III

Overexpression of SHP leads to induction of autophagy

Background

SHP, which is devoid of functional DNA binding domain, functions primarily as an epigenetically regulated transcriptional regulator and is widely associated in many biological processes, cellular components and molecular functions [324]. Although it has a dimerization domain and a putative ligand binding domain (LBD), yet the knowledge about its interactions and endogenous ligands is illusive. SHP has been purported to exert its action primarily through protein-protein interactions with NRs and other transcription factors [13,203]. Dysfunction of NR signalling has been implicated in a wide array of diseases, including reproductive and metabolic diseases like obesity, diabetes, and cancers [14]. Since SHP is epigenetically modulated [243] and is mostly involved in transcriptional repression of several other NRs, it becomes quintessential for us to study its molecular mechanisms modulating different cellular events. Apart from its nuclear function as a transcriptional repressor, SHP has been reported to translocate from the nucleus to mitochondria, inducing cytochrome C release, thereby inducing apoptosis, under the influence of apoptosis inducer 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN or CD437) [195]. SHP activation and apoptosis induction have been observed to inhibit peritoneal pancreatic tumor growth [195]. One report suggests that SHP, which otherwise is predominantly a repressor of gene transcription, upregulates the transcriptional activity of peroxisome proliferator-activated receptor γ (PPARγ) [12,24]. It is important to mention that SHP has been observed to physically interact with PPARγ and also that PPARγ can transcriptionally activate SHP in the liver [325,326]. PPARγ is a ligand-induced NR, considered to be a tumor suppressor by induction of apoptosis upon ligand activation. PPARγ activation has also been observed to induce autophagy in breast cancer cells [327].

Autophagy is an extremely conserved homeostatic process that recycles cytoplasmic components [224]. It plays a key role during nutrient deprivation and mobilizes alternative sources of energy stores [224,225]. The autophagy machinery, via its diverse functions, plays a crucial role in key processes like cell death, preservation of stem cells, tumor suppression, longevity, and defense against metabolic diseases [226]. The ability of autophagy in recycling raw materials might help in the survival of cancer cells under nutrient stress or during hypoxia. Several tumor suppressors like Beclin 1, PTEN and p53 are considered as autophagy inducers, which are often found

to be downregulated in several cancers [328,329]. A decline in tumorigenicity and autophagic cell death has been observed upon overexpression of such modulators in cancerous cells [330].

As already mentioned in the previous section of this study, a stable cell line overexpressing SHP has been generated from HepG2 and termed as HepSHP. We observed numerous conspicuous vacuolated cells in this stable cell line. This observation triggered our curiosity towards the genesis of those vacuolated cells, and we were eager to learn whether the vacuoles were autophagic in nature or not? Previous studies from our group, have revealed that overexpression of autophagic modulator hyaluronan-binding protein 1 (HABP1) in HepG2 cells leads to increased proliferation and tumorigenicity concomitant with decreased proteolysis or autophagy owing to excess generation of polymeric hyaluronan (HA) [298,330]. HABP1, considered to be a biomarker of cancer progression, is a multifunctional, multiligand binding protein having a myriad of subcellular localization under various physiological conditions. While exploring the functions of HABP1, its regulatory involvement in a wide variety of cellular events like cellular growth, cell cycle regulation, host-pathogen interaction, apoptosis induction and autophagy have been revealed [331]. The assortment of HABP1 functions are possible due to its ability to interact with diverse proteins depending on its subcellular localization [331]. It is quite interesting to note that the stable overexpression of HABP1 in normal fibroblasts results in the protein being localized in the mitochondria [331]. These cells show the excess generation of ROS, breakdown of polymeric HA, autophagic induction and flux, which ultimately leads to apoptosis [332,333]. These observations are contrary to what has been observed upon stable overexpression of HABP1 in HepG2. In the stably HABP1 overexpressing HepG2 cells (HepR21), the protein has been observed to be primarily localized on the cell surface leading to increased generation of polymeric HA. This induced a cell proliferative signaling resulting in increased tumorigenicity as compared to its parental cell line HepG2 [298,330].

In ChIP-sequencing experiment, around 85 of the autophagy related ‘gene binding peaks’ detected within 10 kb of transcription start site (TSS) of SHP suggests its interaction with several autophagy regulatory factors [334–336]. Considering these previous observations, along with the observation of increased vacuolated cells in HepSHP, it would be interesting to study the possibility of critical molecular crosstalk between SHP and HABP1 in regulating autophagy in HepG2 cells. Thus, the models

utilized herein for this study include HepG2, HepSHP and HepR21 cell lines described under 'Materials and Methods' section.

Results

SHP overexpressing HepSHP cells exhibit increased frequency of vacuolation

During the morphological examination of HepSHP cells under the phase contrast microscope, vacuolated cells were observed to be present more frequently as compared to HepG2 cells. Hence, an equal number of HepG2, HepSHP and HepR21 cells were separately seeded, onto glass coverslips and further processed for hematoxylin-eosin staining. Brightfield images were then acquired and the vacuole frequency was assessed from at least 10 different fields for all the three cell lines. **Fig. 41A** shows the hematoxylin-eosin stained HepG2, HepSHP and HepR21, where the vacuolated cells are indicated with blue arrows. The vacuole frequency of the three different cells lines are shown in a histogram that are statistically analyzed **(Fig. 41B).** A significantly elevated incidence of vacuolation in HepSHP (~18%) was revealed as compared to its parental counterpart HepG2 (~8.7%), while HepR21 cells showed a significant decline in vacuoles (~5%).

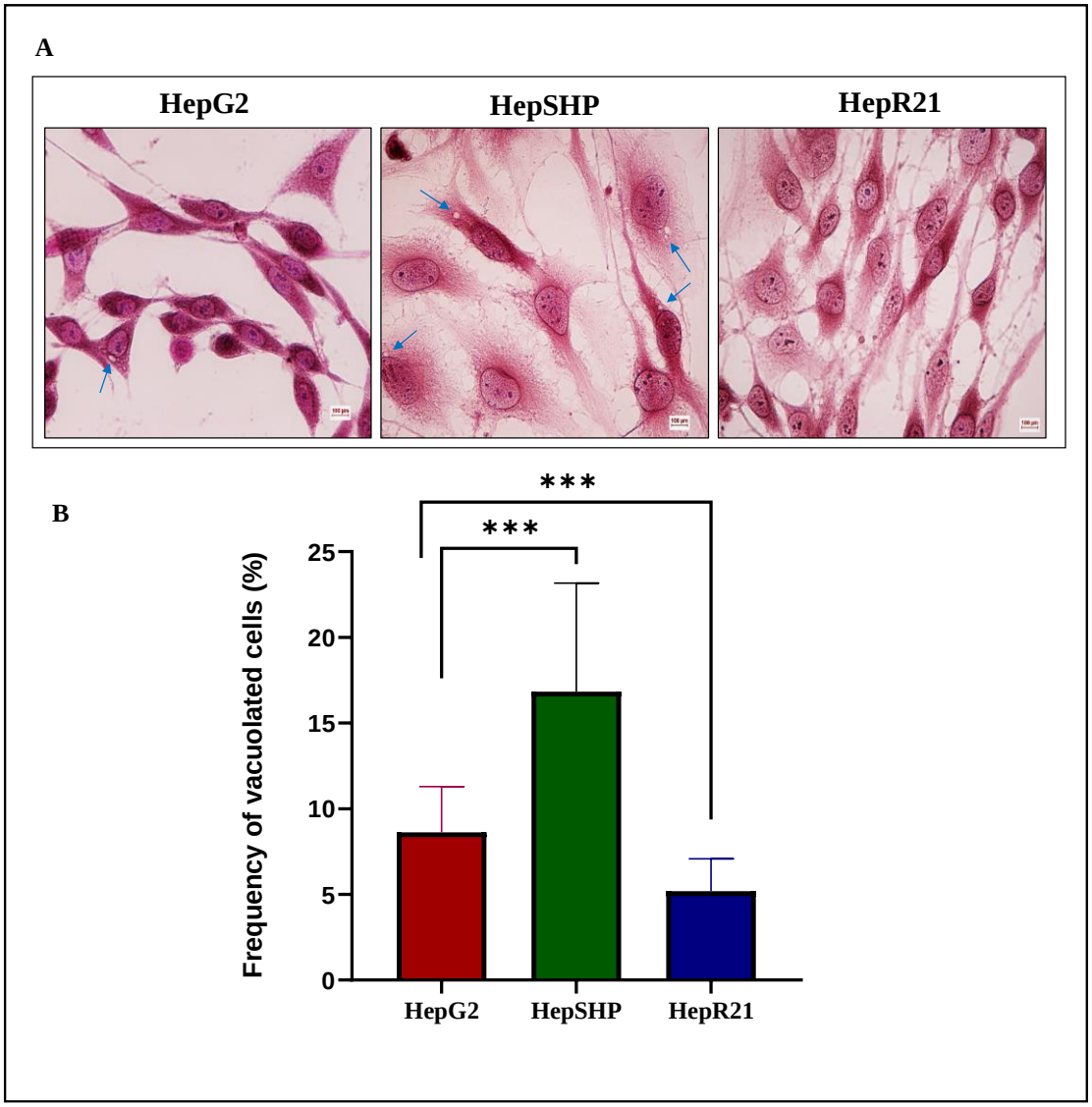


Figure 41: Increased incidence of vacuolation in HepSHP cells compared to HepG2 and HepR21 cells. ***(A)*** *Hematoxylin-Eosin staining of HepG2, HepSHP and HepR21 cells shows numerous conspicuous vacuoles in the HepSHP cells, indicated by blue arrows* ***(B)*** *Upon quantification of vacuoles from 10 different fields for each cell line, significantly elevated percentage of vacuolated cells were observed for the HepSHP cell line as compared to HepG2 and HepR21 cells. Statistical analysis of significance was done by Single factor one-way ANOVA (***p<0.001).*

Overexpression of SHP in HepG2 cells leads to upregulation of autophagy marker MAP-LC3-II

Autophagy is a complex and coordinated process of digestion of cytoplasmic components with the help of a wide array of proteins [337]. MAP-LC3 is a unique modifier protein and autophagosomal marker which associates with the membrane of autophagosomes [338]. MAP-LC3 exists in two forms LC3-I (18 kDa), which is found

in the cytoplasm and LC3-II (16 kDa), which is membrane-bound and is converted from LC3-I (18 kDa), to initiate formation and lengthening of the autophagosome. It differs from LC3-I as it is covalently modifies with lipid extensions (lipidation). MAP-LC3 is the most widely used reliable biomarker to detect autophagy [339]. The morphological observation of increased vacuolation in HepSHP, which is a characteristic of induction of autophagy, prompted us to study the levels of MAP-LC3. Immunoblotting of the whole cell lysates of HepG2, HepSHP and HepR21 cells exhibited significant upregulation of the autophagic marker MAP-LC3-II. The fold change in MAP-LC3-II was ~1.8 in HepSHP cells as compared to its parent cell line HepG2 after normalization with the housekeeping gene β-actin (**Fig. 42A and B)**. Decreased proteolysis in HepR21 is evident from the significantly downregulated expression of MAP-LC3-II, in comparison to its expression in HepG2 cell line. Subsequent, immunocytochemical analysis revealed distinctively increased punctate staining of MAP-LC3, for autophagic vacuoles in HepSHP as compared to HepG2 (**Fig. 42C**) while HepR21 cells showed a fewer number of puncta concomitant with the immunoblotting. Thus, both immunoblotting and cytochemical staining for MAP-LC3 of HepG2, HepSHP and HepR21 cells concurs with the observed vacuole frequency indicating that the vacuoles in HepSHP are autophagic in nature.

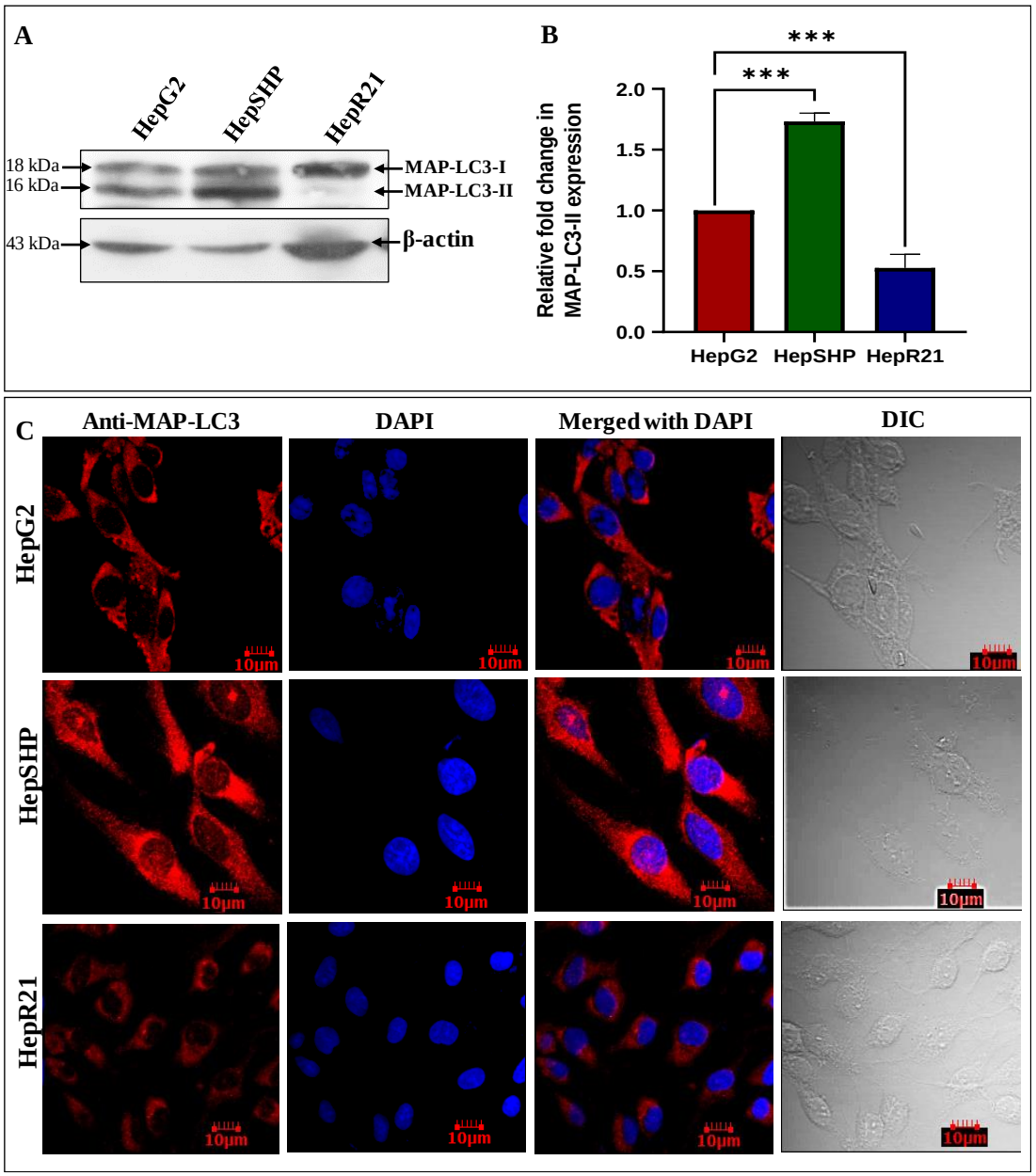


Figure 42: Enhanced expression of autophagic marker MAP-LC3-II in stable HepSHP cell line. ***(A)*** *Whole cell lysates of HepG2, HepSHP and HepR21 were subjected to immunoblotting with MAP-LC3 and β-actin.* ***(B)*** *Significantly higher expression of MAPLC3-II (~1.8 fold) was observed in the HepSHP samples compared to HepG2 cells. Fold changes were calculated taking β-actin as the loading control, using ImageJ and expressed as mean ± standard deviation (SD) of observations in triplicate (n = 3). Statistical analysis of significance was done by single factor one-way ANOVA (***p<0.001).* ***(C)*** *Immunocytochemical analysis revealed a typical punctate staining of MAP-LC3 for autophagic vacuolation. The higher expression of MAP-LC3 in the HepSHP cells corroborates with the immunoblotting data and the highly vacuolated cells.*

SHP colocalizes with HABP1

In order to examine for any potential interaction between SHP and HABP1, colocalization studies were performed with HepG2, HepSHP and HepR21. For the first study, cells grown on coverslips were fixed and further processed for immunocytochemical staining to examine for endogenous localization and expression of SHP and HABP1 using respective antibodies. Both the proteins were observed to be primarily localized in the cytoplasmic compartments by confocal microscopy (Olympus model IX71). A small fraction of SHP was also observed to be localized in the nucleus in the HepSHP cells, which was otherwise absent in both HepG2 and HepR21. As expected, increased SHP expression in HepSHP cells and increased HABP1 expression in HepR21 cells were also evident from the confocal microscopic images **(Fig. 43A)**. To assess colocalization, Pearson's correlation coefficient values were analyzed for all the three cell lines which revealed a highly significant colocalization of SHP and HABP1 in the HepSHP and HepR21 cell lines as compared to their parental counterpart, HepG2 **(Fig. 43B)**.

Further, we also examined the colocalization of the SHP and HABP1 in live cell studies. Live cell study was performed in COS-1 cells. The cells were transiently cotransfected with RFP-SHP and HABP1-GFP (p32) and were allowed to express the protein for 24 hours post-transfection. Previous reports suggest that HABP1 localizes into multiple compartments of the cells, depending on the cell type and physiological conditions. Earlier RFP-SHP was observed to localized into the cytoplasmic compartment of the cell and HABP1-GFP reportedly localizes in the mitochondrial compartment. Interestingly, both the proteins were observed to be colocalize at the nuclear periphery, which is also a characteristic of mitochondrially localized proteins **(Fig. 43C)**.

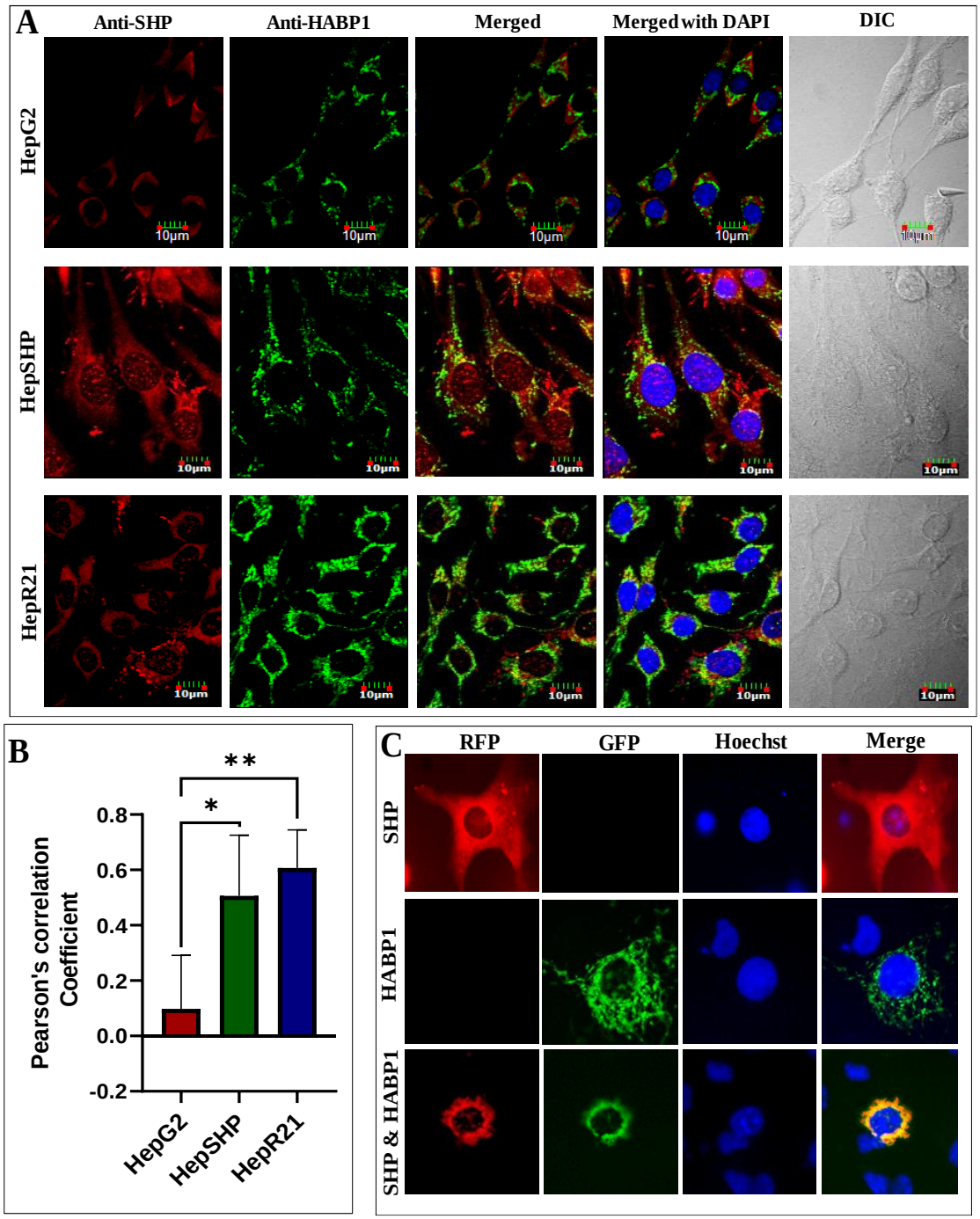


Figure 43: Colocalization of SHP and HABP1. ***(A)*** *Immunocytochemistry of HepG2, HepSHP and HepR21 cells with the primary anti-SHP (Rabbit) and anti-HABP1 (Mouse) antibody followed by Cy3 and FITC conjugated secondary antibody respectively.* ***(B)*** *Pearson's correlation coefficient (PCC) analysis and quantification of images of colocalized Cy3-SHP and FITC-HABP1 in cells.* ***(C)*** *SHP and HAPB1 colocalized at the nuclear periphery when observed in live cells, upon expression with transient cotransfection of RFP-tagged SHP and GFP-tagged HABP1 plasmids in COS-1 cells.*

SHP co-immunoprecipitates with HABP1 suggesting physical interaction between two proteins

To validate the possibility functional interaction between SHP and HABP1, COS-1 cells were co-transfected with RFP-SHP and HABP1-GFP plasmids. After 24 hrs of the expression period, both the cotransfected and untransfected control COS-1 cells were lysed. Immunoprecipitation was performed by treating the lysates with anti-HABP1 polyclonal antibody as described in the 'Materials and Methods'. After incubating for binding, Protein-G beads were collected and washed three times using chilled PBS to remove the non-specific binding and to clear the cell lysates. The samples were boiled in lysis buffer and the bound proteins were resolved on a 10% SDS-PAGE. The RFP-SHP band was detected in the immunoblot using anti-SHP antibody (**Fig. 44A, Blot 1**). Further, to confirm the presence of HABP1 the immunoblot was also detected by using anti-HABP1 antibody **(Fig 44A, Blot 2)**.

Further, *in silico* approach was applied to identify potential amino acid residues responsible for protein-protein interactions, using the crystal structure of HABP1 (C1QBP or p32, PDB: 3RPX) and modeled structure of SHP as previously mentioned in 'Chapter-2'. The detailed protocol of modeling and *in silico* analysis is mentioned under 'Material and Methods'. The protein-protein docking study predicted the binding affinities for the interacting partners SHP and HABP1 **(Fig 44B & Table 9)**. Further, amino acid residues which are critical for intermolecular interactions were predicted using a mutational scan of SHP with HABP1 and the binding affinity of the individually replaced amino acids as mentioned in **Table 9.** As per analysis, wild-type SHP has the highest binding affinity for HABP1.

Table 9: Protein-Protein docking and their estimated binding affinities as determined by ClusPro server tool.

Protein-1	Protein-2	Binding affinity
HABP1	**SHP-WT**	**-1055.4**
HABP1	SHP-R85D	-623.2
HABP1	SHP-I240D	-678.2
HABP1	SHP-D243A	-650.2
HABP1	SHP-R57D	-732.1

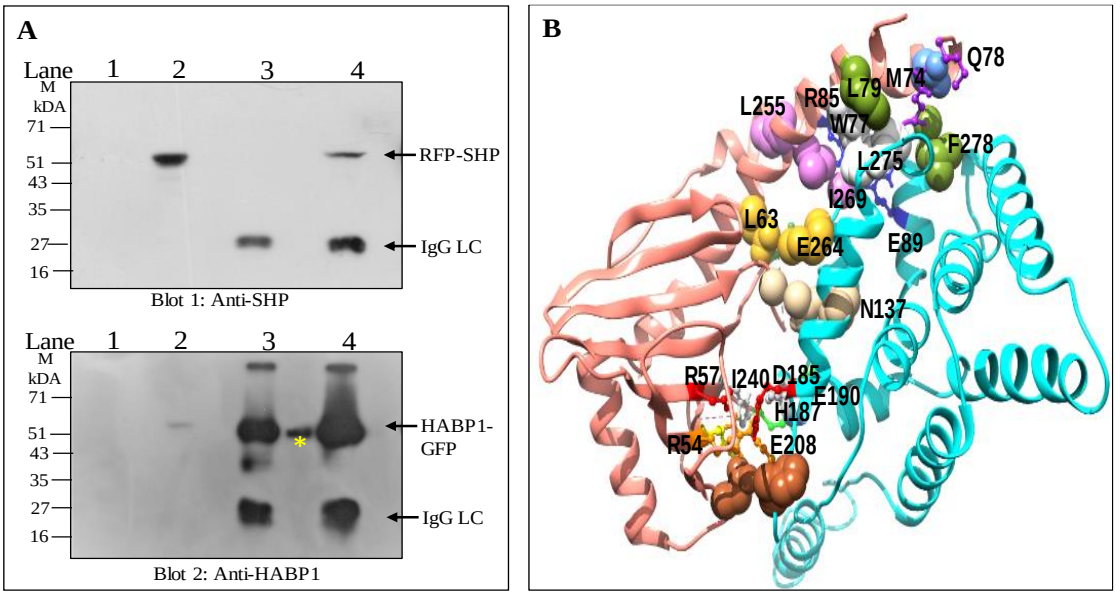


Figure 44: SHP and HABP1 physically interacts with each other. ***(A)*** *Immunoblot of immunoprecipitated SHP using anti-SHP antibody was performed using standard protocol described under 'Materials and Methods'. In blot 1, lane 1 is COS-1 cell lysate, lane 2 consists of lysate of COS-1 cells co-transfected with RFP-SHP and HABP1-GFP, lane 3 corresponds to untransfected COS-1 cells lysate precipitated with Protein-G beads; while lane 4 constitutes the cell lysate from COS-1 cells co-transfected with RFP-SHP and HABP1-GFP immunoprecipitated with anti-HABP1 antibody and protein G. Positive band corresponding to RFP-SHP in lane 4 in blot 1 indicates that SHP is co-immunoprecipitated with HABP1, suggesting a physical binding between the two. Immunoblot 2 was probed with anti-HABP1 to detect for the presence of HABP1.* ***(B)*** *In silico protein-protein interaction of SHP and HABP1. In the image, cyan color represents the amino acid residues of SHP and salmon color stands for HABP1 amino acid residues. The amino acids interacting at both interfaces are marked in the image. M = Standard markers.*

Discussion

In this study, increased vacuole frequency has been observed in SHP overexpressing HepSHP cells compared to the parental cell line HepG2. The numerous conspicuous vacuoles present in HepSHP cells have been verified to be autophagic in nature through the upregulation of autophagic marker MAP-LC3-II which has been observed by immunoblot and immunocytochemical analyses. Overexpression of autophagic modulator, HABP1 in HepG2 cells has already been previously reported to increase cellular proliferation and tumorigenicity with decreased proteolysis and increased level of HA [330]. In the first chapter, we have reported the growth pattern of HepSHP cells, which shows a much slower growth rate than that of the HABP1 overexpressing HepR21 cells. As mentioned above, in this chapter, we have observed

a significantly higher incidence of proteolysis compared to HepR21 cells invoked by the augmented levels of autophagy in HepSHP cells. As revealed from the *in silico* docking results, wild type SHP has a high binding affinity with HABP1 and colocalization experiments also indicated a significant amount of colocalization between SHP and HABP1 in HepSHP and HepR21 cell lines. Intense levels of colocalization were also observed when both SHP and HABP1 were transiently transfected in COS-1 cells. Co-immunoprecipitation studies further validated the occurrence of physical interactions between SHP and HABP1. It is imperative to mention here that, transient overexpression of HABP1 in COS-1 cells leads to the generation of autophagic vacuoles and disassembly of f-actin networks, a precondition for programmed cell death [340]. Stable overexpression of mature HABP1 in normal fibroblasts (F-HABP07) leads to depletion of the HA pool upon generation of excess ROS ensuing autophagy [331,332]. The overexpressed HABP1 has been observed to be mainly localized in the mitochondria and a further increase in ROS production in these cells results in apoptosis, subsequent to autophagic flux [332,333]. SHP has also been reported to localize in mitochondria, wherein it interacts with Bcl-2, thus interrupting the Bcl-2/Bid binding and causing cytochrome c release resulting in apoptosis [341]. SHP overexpression upon AHPN (a synthetic ligand of SHP) treatment leads to overexpression of DNA damage sensor, poly(ADP-ribose)polymerase-1 [PARP-1] [341]. PARP-1 overexpression has also been correlated with an increased level of autophagy as a DNA damage response, although the regulatory mechanism behind induction of autophagy by PARP-1 is still to be ascertained [342]. Induction of SHP has been reported to inhibit the growth of peritoneal pancreatic tumor implants, suggesting its tumor suppressive effect [325].

One of the emerging functions of SHP is its regulatory control over inflammatory signals [194]. Microbial attack stimulates toll-like receptor (TLR) signaling, the crucial innate immune response, which elicits proinflammatory cytokine release through canonical NFκB pathway. SHP has been identified by Yuk et al., [194], as a novel endogenous inhibitor of NF-kB in TLR signalling to maintain immune balance in organisms. TLR signalling, apart from inducing cytokines, also stimulate AMP-activated protein kinase (AMPK) in macrophages. AMPK activation, in turn, has been elucidated to induce SHP expression. SHP in turn physically interacts with p65 and TNF receptor-associated factor 6 (TRAF6) of the canonical NF-kB pathway ensuing downregulation of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) [220].

AMPK, which acts as an energy and metabolic sensor is activated in a low-energy state when the AMP/ATP ratio increases [343]. Active AMPK results in the repression of mTOR activity, thereby favouring catabolic pathways via stimulation of autophagy [344]. AMPK drives activation of SHP which interacts to limit nuclear translocation of NF-kB, while SHP-TRAF6 binding inhibits ubiquitination of TRAF6 [220]. Inhibition of TRAF6 ubiquitination is of significance since it reportedly leads to induction of autophagy [345] and TRAF6 binding with Beclin1 critically regulates Beclin1 mediated autophagy [346]. Beclin1 reportedly has two TRAF6 binding motifs, and K63-linked ubiquitination of Beclin1 by TRAF6 essentially regulates TLR4 stimulated autophagy in macrophages [347]. All this information thus correlates with the enhanced expression of SHP with the induction of autophagy, as observed in the present study.[345]

HABP1, also known as the receptor for the globular head of the complement component (gC1qR), that interacts with c1q to initiates the classical complement cascade. It has been implicated in downregulation of TLR4-induced IL-12 in human monocytes [331]. The gC1qR–TLR crosstalk has been suggested to maintain homeostasis regulating T-cell immunity, under physiological conditions [348]. Literature thus suggests existence of several pathways or factors which are functionally associated with both, SHP and HABP1.

The present study for the first time, reports the novel interaction between SHP and the multifunctional protein HABP1 and the preliminary data suggests a regulatory role of both the proteins in induction of autophagy in hepato-carcinoma cell lines. As already mentioned, HABP1 nowadays is considered as a tumour biomarker, since it has been clinically observed to be overexpressed in several types of cancers and its levels correlate with poor prognosis in patients [331]. Latest studies on SHP postulates about its activity as a tumour suppressor in liver cancer [13]. This is supported by the observation of spontaneous development of hepatoma upon deletion of SHP gene [212]; while SHP overexpression inhibits hepatocyte proliferation and induces apoptosis [195]. Moreover, the expression of SHP in human HCC samples has been observed to be highly reduced due to promoter hypermethylation [243].

Further studies are necessary to ascertain the status of autophagy and tumorigenicity by stable expression of HABP1 in SHP overexpressing and SHP overexpression in HepR21. This would give better insights into the molecular crosstalk between HABP1 and SHP in the regulation of autophagy and tumorigenicity.

Summary & Conclusions

Nuclear Receptors (NRs) play pivotal roles in all major events of life, including reproduction, development and immunity, metabolism and homeostasis. Their dysfunction can exert a wide range of metabolic diseases including obesity, diabetes, inflammation, circadian rhythm, endocrine cancers (breast/prostate/endometrial cancers) etc. [349]. Hence, they are one of the major target areas for modern therapy and research [38,42,350]. In view of their importance and ability to respond to small lipophilic ligands and synthetic compounds, NRs have emerged as potential therapeutic targets in physiology [3].

The field of' 'Nuclear Receptor biology' is widely studied under the umbrella of' 'NR superfamily' based on the resemblance of their modular structure [3]. In 1996, David Moore's laboratory identified SHP as an atypical nuclear receptor devoid of the most conserved DNA binding domain [9]. SHP appeared to be a key transcription regulator, whether permissively or by conditions, by regulating the transactivation of target genes [351]. In the current context, SHP, which is one of the 48 key members of the human NR superfamily, appears to have immense relevance in normal physiological functions and dysregulation. Its apparent functional cross-talk and transcriptional regulation of several other NRs appears to give this emerging receptor a central stage. Initially, it was hypothesized to be a repressor or negative regulator for many of the nuclear receptors and some other non-NR proteins [13].

In recent years it is observed that during cell division, many transcription factors, including several nuclear receptor, remain associated with the mitotic chromatin [36,352]. This association of a transcription factor with mitotic chromatin is shown to be at the promoter sites of the target genes [120,353]. These promoter associated factors are functionally silenced during mitosis but are activated again in the progeny cells [354]. This phenomenon of retention and transmission of transcription status from progenitor to progeny is referred to as 'mitotic gene-bookmarking' or 'genomic bookmarking' [35]. However, insights into this phenomenon are still at an emerging stage and await extensive investigations.

The location and translocation of transcription factors to specific cellular compartments is considered to be one of the most important events in the proper functioning of a living cell. In context to this, the influence of SHP on the functioning of other NRs during interphase and mitosis can be a decisive factor which has not yet been investigated extensively during cell cycle. Therefore, in this study we have sought

to investigate the role of SHP during interphase and mitosis with specific reference to PXR and ERα dynamics and transcription function.

Overall, we have made an in-depth analyses of receptor subcellular localization, dynamics, mitotic gene bookmarking as well as the influence of SHP on the transcription function of its interacting partners. In addition, we have also attempted to investigate the molecular basis of SHP on the alteration of cell morphology and associated autophagic events.

The key findings from the first chapter of this study are highlighted underneath.

- For a sensitive detection of SHP, a polyclonal antibody against full-length human SHP was raised in New Zealand white female rabbit. The specificity of the antibody was determined by indirect-immunodetection and western blot analysis. By immunocytochemistry, the wild type SHP was found to be localized predominantly in the nucleus with a small fraction of the receptor observed in the cytoplasm.
- A stably expressing human SHP cell line in HepG2 was generated that served as a useful tool to examine the influence of SHP on its heterodimeric partner nuclear receptors or vice versa. In addition, the cell line provided a platform to examine the SHP-mediated cellular and functional events.
- In order to study the subcellular trafficking of the receptor in live cells, RFP-tagged SHP was constructed that showed a cytoplasmic shift in comparison to its GFP-tagged counterpart. This gave us a suitable working tool for the study of receptor-dynamics in live cells.
- Subcellular distribution profile of RFP/GFP-SHP was observed across different cell lines. RFP-SHP was 60-70% predominantly cytoplasmic in HepG2, COS-1 and HEK293T cell lines while GFP-SHP was localized predominantly in the nuclear compartment (~60%) in the same cell lines.
- Nucleocytoplasmic shuttling and dynamic movement of receptors are critical for NRs for accomplishing their transcriptional regulatory functions. *In silico* predictions showed that SHP lacks classical Nuclear Export Signals (NES) sequence(s). In addition, it did not exhibit the presence of a typical NR-like

Nuclear Localization Signal (NLS) which is generally present in DBD-hinge regions of the receptors.

- Leptomycin B is an established potent inhibitor of classical leucine-rich NES export pathway. In our study we confirmed no effect of LMB on the subcellular localization of SHP [87]. However, GFP-tagged-p65, which has classical leucine-rich NES, was able to accumulate in the nucleus in the presence of LMB. This implied that unlike p65, subcellular localization of SHP is operated independent of classical NLS/NES signals.

In the second part of our study, we investigated the influence of SHP on i) subcellular localization and receptor dynamics ii) mitotic gene bookmarking and iii) transcriptional functions of its interacting partners. The major findings are highlighted here.

- To reveal the influence SHP on key NRs, SHP was coexpressed with either PXR, ERα or HNF4α1. When co-expressed with these receptors, SHP was observed to efficiently shift from its cytoplasmic residency to the nuclear compartment. This shift was in accordance to the normal localization of the co-expressed partner. Similarly, though only partially, presence of CAR also shifted SHP localization from cytoplasm to nucleus. On the contrary, some other NRs like RXRα, AR and DAX-1 did not appear to interact with SHP in live cells and therefore did not exhibit altered subcellular localization. This change in SHP localization with the co-expressed receptor implied existence of intermolecular interactions between SHP and selective interacting nuclear receptors.
- In addition, the transcriptional activity of PXR, ERα, CAR and RXRα, were evaluated with their responsive promoter-reporter constructs in the absence and presence of coexpressed SHP. It was observed that coexpressed SHP reduced the transactivation of interacting NRs significantly. The transcriptional repression of other transcription factors by SHP may have an important implication in the physiological homeostasis. This implies that SHP mostly inhibits the transcriptional function of NRs. Overall, it is reasonable to conclude

that the intermolecular interactions between SHP and the partners results in repressive promoter activity.

- Mitotic chromatin binding of NRs may be i) constitutive, ii) ligand-mediated or iii) partner induced. Therefore, to examine the influence of other mitotic chromatin-binding proteins on mitotic chromatin association of SHP, cotransfection studies were conducted by coexpressing SHP with PXR. When expressed alone, SHP did not associate with mitotic chromatin throughout the mitotic stages. Contrary to our expectations, SHP when coexpressed with PXR impeded the mitotic chromatin binding property of latter. Similarly, another mitotic chromatin associating receptor ERα, which binds to mitotic chromatin in ligand-induced manner was also altered by co-expressed SHP. From the observations it was evident that the abrogation of PXR and ERα from the mitotic chromatin could be attributed to the intermolecular interactions between the receptors. Overall, this implies a potential role of SHP in regulation of 'gene-bookmarking' events in the life of the cell.

- Detailed studies with isolated individual domains of PXR indicated PXR-DBD is the minimal essential domain to interact with SHP. This interaction with the partner shifts SHP towards the nucleus, albeit to different extent. Similarly, a detailed study with ERα indicated that ERα-LBD is the minimal domain which is crucial for interaction with SHP. This observation with respect to differential requirements for interacting domains appears to be intriguing.

- It was observed that the interaction of SHP with PXR or ERα is the primary reason for the shifting of SHP from cytoplasm from nucleus. Further, the critical amino acid residues in SHP that influenced the binding and stability of the receptor-receptor interactions were identified by *in silico* analysis. Subsequently, observations were confirmed by the live cell imaging. After mutation of these critical amino residues in SHP its interactions with both PXR or ERα were observed to be dramatically reduced as compared to interactions with the wild-type SHP.

- It was observed that E204R, W206H and R216E mutations in SHP residues result in loss of intermolecular interaction with partner PXR. Similarly, SHP

mutations at E154R, N176D, R216E and K225A highlighted the involvement of these critical amino acid residues for interaction with ERα.

In the final part of this study, we generated and utilized a stable cell line, HepSHP to further analyze other intramolecular and intracellular factors regulating SHP functions using different approaches. Some of the findings from this part have been highlighted underneath.

- Interestingly, an increased vacuole frequency has been observed in the SHP overexpressing HepSHP cells compared to its parental cell line HepG2. The numerous conspicuous vacuoles present in HepSHP cells were verified to be 'autophagic' through the upregulation of autophagic marker MAP-LC3-II which has been observed by immunoblot and immunocytochemical analyses.
- The increased vacuole count in HepSHP cell line indicated progression towards autophagy, which correlated with significant increase in autophagy marker LC3-II level in HepSHP cell line.
- The morphological differences in HepSHP cell line, when compared to its parental cell line, indicated that the proliferation rate of HepSHP is compromised indicating that SHP may have a significant role in proliferation induced cancer.
- HABP1 is a multifunctional hyaluronan binding protein, which is reported to be involved in some of the key cellular pathways. The present study reports novel interactions between SHP and the multifunctional protein HABP1/p32.
- We confirmed intermolecular interactions between SHP and HABP1 by co-immunoprecipitation assays supporting our observations. Overall, involvement of SHP-HABP1 interactions in regulation of autophagy and tumorigenicity is suggested.

In summary, we report that SHP has intermolecular interactions in various platforms with some of the nuclear receptors. We observed that SHP displaces a major fraction of mitotic chromation associated PXR and ERα. This is viewed as a unique action by SHP as it is devoid of a typical DNA binding domain. The abrogation of

PXR and ERα from the mitotic chromatin suggests the potential role of SHP in regulating the event of 'gene-bookmarking' in the life of a cell. Overall, evidences from transcription function data reasonably concludes that intermolecular interactions between SHP and the partner PXR or ERα may result in repressive promoter activity of the latter two during the interphase. In depth observations identified the DBD of PXR and LBD of ERα as the minimal domain required for intermolecular interactions with SHP. In addition, essential amino acid residues of SHP were also predicted using *in silico* methods and the findings were subsequently confirmed by live cell experiments with PXR and ERα. Parallel to in depth molecular analysis, we also assign a new function to SHP in cell autophagy and involvement in the maintenance of cell homeostasis. We also observed the inter-relationship between SHP and a multi-functional protein HABP1. This observation suggests a regulatory role for both the proteins in the induction of autophagy in hepatocarcinoma cell lines. In conclusion, we propose that SHP primarily serves as a 'transcriptional modulator' for its interacting transcription factors but may have other roles beyond this primary function.

Future Perspectives

Future perspective

The structurally and functionally unique nuclear receptor SHP appears to mediate many important cellular and physiological roles in the life of a cell including cell proliferation, tumor suppression, inflammation, autophagy, homeostasis etc. Present study suggests that some of the NRs/non-NRs transcription factors may be a crucial potential interacting partners of SHP. Previous reports suggested that SHP adopts different modes of interaction with its partner proteins. However, broadly it appears to act as a major 'transcriptional corepressor' in diverse metabolic process. SHP's novel transcriptional corepressive ability offers investigators and pharmaceutical companies with an invaluable clinical tool to regulate metabolism. However, SHP does not have the most conserved DBD domain but has ligand binding domain (LBD) that allows for modulation of SHP by natural or synthetic ligands. The literature has shown that ligands of FXR (GW4064) and RXRs / RARs (retinoic acid) induce the expression of SHP. Of particular interest, some synthetic retinoids such as AHPN and 3-Cl-AHPC are documented to be the modulators of SHP's expression through direct SHP or indirect LRH1 bindings on the SHP-promoter. Nonetheless, till date no SHP bonafide ligand has been established. The regulatory mechanisms for SHP are still ambiguous, awaiting further investigation to highlight its clinical relevance. In future, a comprehensive study of SHP's ligand-binding domain will help unravel the discovery and precise synthesis of SHP activators and inhibitors that can become an imminent treatment for life-threatening metabolic diseases such as liver cancer, diabetes, and obesity. Further work is expected provide insights not only into the disease mechanisms triggered by SHP but also much needed therapeutic interventions.

Approximately 15% of the US Food and Drug Administration (USFDA) licensed drugs for clinical sale are used to target NRs and provide huge scope for exploring new molecules and improving upon existing ones [355]. The approach to develop NR selective ligands/modulators is an emerging field for treatments for many important disease conditions. In this context, the study of SHP and its interacting partners under different disease conditions, and the effects of their specific ligand/modulators, may pave the way for a new area known as 'NR-medicine'. Also, since SHP functions in a *hog-tie* manner, during cell cycle as well as during 'mitotic gene-bookmarking', many of the important issues need to be resolved related to relevance of 'cellular transcriptional memory' in erosion, retention and execution of cellular functions. The

rapid advancement of this field is evident from the fact that we still have many more aspects that need to be investigated by targeting these receptors for the treatment of various metabolic disorders. The continued identification of specific endogenous ligands and synthetic modulators of SHP could provide new horizons for advanced therapeutic intervention.

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Publications

Published (Research article, Book Chapter & Review)

- A Comprehensive Analysis and Prediction of Sub-Cellular Localization of Human Nuclear Receptors. Mathew, Sam P, Keshav Thakur, **Sudhir Kumar**, Ashutosh S Yende, Shashi Kala Singh, Amit K Dash, and Rakesh K Tyagi. Nuclear Receptor Research, 5: 1–20 (2018) (Research Article)
- Androgen receptor signalling by growth factors in androgen independent prostate cancer: recent advances and emerging perspective (Subodh Kumar, Sanjay Kumar, Keshav Thakur, **Sudhir Kumar**, Gargi Bagchi, Rakesh K. Tyagi) Updates on Integrative physiology & amp; comparative endocrinology by Press and publication division BHU Varanasi. ISBN: 81-85305- 72-2 (2016) (Book Chapter)
- The Constitutive Androstane Receptor (CAR): a nuclear receptor in health and disease. (Amit K Dash, Ashutosh S Yende, **Sudhir Kumar**, Shashi K Singh, Deepak Kotiya, Rana Manjul and Rakesh K. Tyagi J Endocrinol Reprod. 18(2): 59 – 74) (2015). (Review)

Manuscript under preparation

- Incidence of increased autophagy in mifepristone treated in vitro and in vivo polycystic ovarian models and its reversion upon thymoquinone treatment (Paramita Saha*, **Sudhir Kumar***, Kasturi Datta, and Rakesh K. Tyagi) (Under communication) (* First equal authors)
- Intermolecular interactions of nuclear receptor SHP with PXR and ERα alters receptor localization and mitotic gene bookmarking functions (**Sudhir Kumar**, Ramachandran Vijayan, Amit K Dash and Rakesh K Tyagi) (Ready for communication)
- Mechanism of action of breast cancer drug fulvestrant (ICI182780): evidence from live cell imaging (Under preparation)

Conference attended

- Stable integration of atypical nuclear receptor SHP in liver cell line & its characterization; **Sudhir Kumar**, Amit K. Dash Rakesh K. Tyagi, ISIPCE-2016, Banaras Hindu University Varanasi (Poster)

- Stable integration of atypical nuclear receptor SHP into human liver cell line and its characterization' **Sudhir Kumar**, Amit K. Dash Rakesh K. Tyagi. at the National Science Day Celebration at Jawaharlal Nehru University Convention Centre, 28th February, 2017 (Poster)

- Functional studies on atypical nuclear receptor SHP with its key heterodimeric partners; **Sudhir Kumar**, Rakesh K. Tyagi, International Congress of Cell Biology (ICCB) 27th to 31st January 2018, CCMB Hyderabad (Poster)

- Role of atypical nuclear receptor SHP with its partners PXR and ERα; **Sudhir Kumar**, Rakesh K. Tyagi. at the National Science Day Celebration at Jawaharlal Nehru University Convention Centre, 28th February, 2018 (Poster)

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Research Article

A Comprehensive Analysis and Prediction of Sub-Cellular Localization of Human Nuclear Receptors

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Abstract. The Nuclear Receptor (NR) superfamily comprises of conserved ligand-modulated intracellular transcription factors which in the presence of their cognate ligands activate a plethora of signaling networks, thereby commencing their respective transcription functions. All NRs are nuclear when liganded or active. However, their localization may differ between nucleus and cytoplasm when unliganded or inactive. NRs control a majority of physiological processes in body ranging from metabolism to reproduction and development. Hitherto, in case of humans, 48 NRs have been identified which are localized either in cytosolic, nuclear or both compartments of the cell. Sub-cellular localization of proteins has great relevance in relation to their function. However, specific sub-cellular localization patterns of human NRs are clouded with ambiguity and are mostly ridden with controversy, with only a few of them being well-studied and established under specific physiological conditions. In the present study, we attempted to bridge the gap and attempted to draw conclusions in relation to sub-cellular localization of human NRs based on published experimental data and by *in-silico* prediction methods. This comprehensive analysis may not only be useful to draw conclusions on their control of physiological processes but may also open new avenues towards understanding of the molecular basis of NR-mediated diseases attributed to their mislocalization and malfunctioning.

Keywords: Nuclear Receptors, sub-cellular localization, *in-silico,* ngLOC, Hum-mPLoc 3.0

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1. Introduction

Nuclear receptors (NRs) are transcription factors which on activation by physiological stimuli, bind to the specific DNA sequences and bring about regulation of complex biological pathways [1]. These receptors function alongside other proteins to regulate the expression of specific target genes, thereby effectively controlling vital cellular functions such as development, homeostasis and metabolism in an organism.

NRs constitute a large superfamily of evolutionarily-conserved proteins. The NR superfamily can be broadly categorized into four subfamilies based on their DNA-binding properties and dimerization preferences. Class I receptors include steroid hormone receptors, such as GR, MR, PR, AR, ER etc., which act as ligand-induced homodimers and bind to the half-sites of target DNA oriented as inverted repeats. Class II consists of receptors which heterodimerize with RXR such as VDR, RAR, TR etc., and bind to the direct repeat half-sites. Class III and class IV receptors are orphan receptors where class III receptors bind to the direct repeat as homodimers while class IV receptors typically bind to extended core sites as monomers [1, 2].

Androgen Receptor Signaling by Growth Factors in Androgen-independent Prostate Cancer: Recent Advances and Emerging Perspectives

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Abstract

Androgens control the growth and differentiation of the prostate cell by activation of the androgen receptor (AR). The ligand-activated AR contributes to not only the normal functioning of prostate gland but also the development of prostate cancer. The inevitable transition of prostate cancer from an androgen-dependent to androgen-independent state that occurs over time is a clinical challenge in the field of prostate cancer treatment. The present article summarizes updates on mechanisms by which androgen-independent prostate cancer (AIPC) develops. An analysis of different signaling pathways and signaling cross-talks that induce prostate cancer progression has been presented to enhance our in-depth understanding of the role of these pathways in AIPC and open new avenues toward its therapy.

Keywords: Prostate cancer, androgen receptor, growth factors, cell signaling

J Endocrinol Reprod 18 (2014) 2 : 59 - 74 **Review Article**

The Constitutive Androstane Receptor (CAR): a nuclear receptor in health and disease

Amit K. Dash, Ashutosh S. Yende, Sudhir Kumar, Shashi Kala Singh, Deepak Kotiya, Manjul Rana and Rakesh K. Tyagi

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Summary

Constitutive Androstane Receptor (CAR, NR1I3), a member of the nuclear receptor superfamily of transcription factors, has emerged as one of the key regulators of the drug and xenobiotic metabolism. The unique feature that separates CAR from other members of the superfamily is that it remains active in the absence of ligand and is further regulated by activators. From its first isolation in 1994, a number of studies related to its distribution, characteristics, functions, and relation to other members of the superfamily have been conducted that place it centrally, governing many key events of the body. Human CAR is expressed relatively higher in liver and epithelial cells of the small intestine villi and less in heart, muscle, kidney, brain and lung. Though there are some controversies regarding its subcellular localization in different cell lines, in general, the subcellular localization of CAR is reported to be predominantly cytoplasmic, in complex with co-chaperone partners HSP90 and CCRP (cytoplasmic CAR retention protein). To execute transcription functions, nuclear translocation is a prerequisite event for a NR, including CAR. In this context, existence of two pathways is suggested, i) direct mechanism of action; and ii) indirect mechanism of action that is governed via nuclear translocation of CAR. Additionally, existence of species-specific differences in its modulation with ligands acting either as an agonist, antagonist or inverse agonist is also apparent. Like the other xenobiotic receptor PXR, CAR also functions as an alternative 'xenosensor' to defend the body against persistent chemical insults. It responds to diverse array of chemically distinct compounds, including endobiotics and xenobiotics, to regulate the clearance of noxious chemicals and toxic metabolites in liver and intestine via induction of genes involved in their metabolism. The usefulness of targeting CAR in metabolic diseases including bilirubinemia, obesity, type 2 diabetes mellitus, atherosclerosis, preeclampsia, hypertension, cholestasis and also in liver cancer is being extensively studied in animal models. However, to determine the human relevance it requires further investigation. Though a large number of natural and synthetic compounds act as modulators of CAR, designing new derivatives with defined therapeutic benefit need to be investigated. The purpose of this review is to highlight the general aspects of nuclear receptor CAR, its mechanism of action and importance in human health and disease.

Key Words: Nuclear receptors, Transcription factors, Constitutive Androstane Receptor, Xenosensor, Drug metabolism, Metabolic diseases, Sub-cellular localization, Cancer.

Introduction

Nuclear Receptors (NRs) belong to a superfamily of phylogenetically-related proteins comprised of 48 members in humans. They act as transcriptional switches by responding to their cognate ligands including various hormones, vitamins, lipids, steroids, etc., and share a general modular structure (Mangelsdorf et al., 1995; Nuclear Receptors Nomenclature Committee, 1999; Burris et al., 2012). The members of this superfamily have a central DNA binding domain (DBD), also termed 'C region' which is highly conserved in sequence. There is a highly variable region on the amino-terminal to the C region called region A/B which contains the activation function 1 (AF-1) whose function (transcriptional activity) is independent of the presence of ligand. On the carboxy-terminal to the DBD, another conserved region is found, which is termed as the ligand binding domain (LBD) or E region and contains the activation function 2 (AF-2) whose action (transcriptional activity) is ligand-dependant. This region is responsible for recognition and binding of the specific ligands. There is a comparatively shorter region which connects C and E regions, called the hinge region or region D. On the extreme carboxy terminal to the LBD, some receptors may contain a region of unknown function called F region (Burris et al., 2012) (Fig. 1).

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