The role of the FACT complex in differentiation of multipotent stem cells

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I hereby declare that the PhD thesis entitled “The role of the FACT complex in differentiation of multipotent stem cells” was written independently and a significant portion was taken from the article entitled as “Histone Chaperone SSRP1 is Essential for Wnt Signaling Pathway Activity During Osteoblast Differentiation. *STEM CELLS*. 2016. doi: 10.1002/stem.2287” written by me with no other sources and aids than quoted.

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List of publications

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List of abbreviations

%	percentage
(NH4)2SO4	ammonium sulfate
µg	microgram
µl	microliter
µM	micromolar
18s rRNA	ribosomal rna with sedimentation rate 18s
ACTB	coding gene for beta- actin
Adip	adipocyte
ADP	adenosine diphosphate
ALPL	alkaline phosphatase, liver/bone/kidney
ANOVA	analysis of variance
AP1	activator protein 1
APC	adenomatous polyposis coli
APS	ammonium persulfate
ASF1	Anti Silencing Function 1
ATF4	activating transcription factor 4
AXIN2	axis inhibition protein 2
BGP	β-glycerophosphate
BGS	bovine growth serum
BMP	bone morphogenetic protein
bp	base pair
BAF	Brg1- or Brm-associated factors
bam	binary version of sam files
bigwig	binary version of wiggle files
BSA	bovine serum albumin
C/EBPβ	CCAAT/enhancer binding protein (c/ebp), beta
C2C12	mouse myoblast cell line
CAF1	chromatin assembly factor 1
cAMP	cyclic adenosine monophosphate
CBP	camp-response element-binding protein (creb) binding protein
cDNA	complementary dna
CHAF1A	chromatin assembly factor 1 subunit a

CHD	chromodomain helicase dna binding protein
ChIP	chromatin immunoprecipitation
CID	c terminal intrinsically disordered domain
CK2	casein kinase 2
CO2	carbon dioxide
CoA	conenzyme a
COL1A1	collagen, type 1, alpha 1
COL1A2	collagen, type 1, alpha 2
CTD	carboxy-terminal domain
DAPI	4',6-diamidino-2-phenylindole
DD	dimerization domain
DEPC	diethylpyrocarbonate
Diff	differentiated
DMEM	dulbecco- minimum essential medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
Dsh	dishevelled
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediamainetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FACT	facilitates chromatin transcription
ES	enrichment score
exp.	expression
FBS	fetal bovine serum
FGF	fibroblast growth factor
FRET	fluorescence resonance energy transfer
FRV alkaline solution	fast red violet alkaline solution
Fzd	frizzled
GO	gene ontology
GR	glucocorticoid receptor
GSK-3β	glycogen synthase kinase-3β

H1	histone 1
H2A	histone 2a
H2B	histone 2b
H3	histone 3
H4	histone 4
H2Bub1	histone h2b monoubiquitination at lys 120
H3K27me3	histone h3 trimethylation at lys 27
H3K36 me	histone h3 methylation at lys 36
H3K4me	histone h3 trimethylation at lys 4
H3K56 ac	histone h3 acetylation at lys 56
H3K9me3	histone h3 trimethylation at lys 9
hADF	human adult dermal fibroblasts
Her2/neu	human epidermal growth factor receptor 2, erb-b2, c-erbb2
hFOB	human fetal osteoblast
HIRA	hir (histone cell cycle regulation defective) homolog a
HMG	high-mobility group
HNRNPK	heterogeneous nuclear ribonucleoprotein k
hr	hour
HRP	horseradish peroxidase
HSC70	heat shock 70kda protein
IAA	iodacetamide
IBMX	isobutyl-methyl-xanthine
IBSP	bone sialoprotein
IDD	intrisically disordered domain
IgG	immunoglobulin g
INO80	dna helicase ino80
ISWI	imitation switch family
JDP2	jun dimerization protein 2
K_2HPO_4	dipotassium phosphate

KCl	potassium chloride
KH_2PO_4	monopotassium phosphate
LRP	low density lipoprotein receptor-related protein
MAPK	mitogen-activated protein kinases
MD	mid domain
Mef2	myocyte enhancer factor-2
MEM	minimum essential media
mg	milligram
MgCl2	magnesium chloride
Mi-2/NURD	mi-2 nucleosome remodeling deacetylase complex
Min.	minute
ml	mililiter
mM	millimolar
MSC	mesenchymal stem cell
MSX2	msh homeobox 2
MYH1	myosin, heavy chain 1
MyoD	myogenic differentiation
MYOG	myogenin (myogenic factor 4)
Na_2EDTA	disodium ethylenediaminetatraacetate
NaCl	sodium chloride
NCBI	national center for biotechnology information
Nap1	nucleosome assembly proteins 1
NaN_3	sodium azide
NaH_2PO_4	sodium dihydrogen phosphate
NEM	n-ethylmaleimide
NES	normalized enrichment score
Nhp6	non-histone chromosomal protein 6

$NiCl_2$	nickel(ii) chloride
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated b-cells
NP-40	nonidet p40
NTD	n terminal domain
NuRD	nucleosome remodeling deacetylase
NURF	nucleosome-remodeling factor
OA	osteoactivin
OB	osteoblast
OPG	osteoprotegerin
OPN	osteopontin
OSX	osterix
p	probability
P/S	penicillin/streptomycin
p300	e1a binding protein p300
pAdj	adjacent p value
Paf1	polymerase associated factor
PARP1	poly (adp-ribose) polymerase 1
PBS	phosphate buffered saline
PC1	principle component 1
PC2	principle component 2
PCA	principle component analysis
PCAF	p300/cbp-associated factor
PCR	polymerase chain reaction
PDK4	pyruvate dehydrogenase kinase 4
PH1	pleckstrin homology domain 1
PH2	pleckstrin homology domain 2
PKC-δ	protein kinase c delta

Pob3	pol1 binding
PPARG	peroxisome proliferator-activated receptor gamma
P-TEFb	positive transcription elongation factor-b
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real-time pcr
RASD1	ras, dexamethasone-induced 1
RGB	red-green-blue
RIPA	radioimmunoprecipitation assay
Rel.	relative
RNA	ribonucleic acid
RNAP II	rna polymerase ii
RNA-seq	sequencing of rt-transcribed rna
RPKM	reads per kilo base per million mapped reads
RNF20	ring finger protein 20
RNF40	ring finger protein 40
RPLP0	ribosomal protein, large, p0
RSPO1	r-spondin-1
RT	room temperature
Rtt109	regulator of ty1 transposition 109
Rtt101	regulator of ty1 transposition 101
RUNX2	runt-related transcription factor 2
RXR	retinoid x receptor
SD	standard deviation
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SET2	set domain-containing

SFRP1	secreted frizzled-related protein 1
SFRP2	secreted frizzled-related protein 2
SFRP4	secreted frizzled-related protein 4
siCont	negative control sirna
siRNA	small interfering rna
SP7	sp7 transcription factor or osterix
DLX5	distal-less homeobox 5
sec	second
SPT16	suppressor of ty homolog 16
SPT4	suppressor of ty homolog 4
SPT5	suppressor of ty homolog 5
SUPT6H	suppressor of ty homolog 6 (human)
SSRP1	structure specific recognition protein 1
STAT5A	signal transducer and activator of transcription 5a
SUPT16H	suppressor of ty homolog 16 h
SWI/SNF	switch/sucrose nonfermentable
SWR1	wi2/snf2-related 1
Taq	thermus aquaticus
TBP	tata-binding protein
TE	tris-edta
TBST	tris-buffered saline and tween 20
TCF/LEF	t-cell factor/lymphoid enhancer factor
TEMED	tetramethylethylenediamine
TFIIA	transcription factor ii a
TFIIB	transcription factor ii b
TFIIE	transcription factor ii e
TFIIH	transcription factor ii h

TGF-β	transforming growth factor beta
Tris	tris(hydroxymethyl)aminomethane
TSS	transcription start site
U	unit (enzyme activity)
U20S	u-2 osteosarcoma
UbM	ubiquitination machinaries
Undiff.	undifferentiated
UTR	untranslated region
VDR	vitamin d receptor
Vs.	versus
WB	western blot
WIF 1	wnt inhibitory factor 1
WISP2	wnt1 inducible signaling pathway protein 2
w/v	weight per volume
Wt	wild type

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Abstract

Cellular differentiation is accompanied by dramatic changes in chromatin structure which are associated with the activation of lineage-specific transcriptional programs. Facilitates Chromatin Transcription (FACT) is a histone chaperone complex which is important for chromatin-associated processes such as transcription, DNA replication and DNA repair. However, the role of FACT during differentiation of undifferentiated or stem-cell like cells has not yet been elucidated. We investigated the role of the FACT component Structure Specific Recognition Protein 1 (SSRP1) in adipocyte and osteoblast differentiation. Depletion of SSRP1 in human mesenchymal stem cells (hMSC) elicited lineage-specific effects where the adipocyte-specific genes *PPARG, RASD1* and *PDK4* were significantly increased while markers of osteoblast differentiation markedly decreased. Consistently, Oil Red O staining was increased during adipocyte differentiation while alkaline phosphatase staining was decreased in osteoblast differentiation following knockdown of SSRP1.

Osteoblast differentiation plays a pivotal role in maintenance of bone homeostasis important for different bone-associated diseases including age-related bone loss. Thus this study was further focused on the molecular regulation of SSRP1-mediated effects on osteoblast differentiation. Transcriptome-wide RNA-seq revealed a specific enrichment of down-regulation of the canonical Wnt signaling pathway following SSRP1 depletion in osteoblasts. Furthermore a number of biological processes important for osteoblast differentiation including glycosylation, cell-cell contact, adhesion, extra cellular matrix, ossification, osteoblast differentiation, bone and skeletal development were affected by SSRP1 knockdown. In addition a significant nuclear co-localization of SSRP1 and β-catenin was observed where depletion of SSRP1 diminished accumulation of active β-catenin in the nucleus. Together, our data suggest a previously unknown specific role for SSRP1 in promoting the activation of canonical Wnt signaling during lineage-specific differentiation.

1. Introduction

1.1 Chromatin organization

The genomic DNA in the nucleus of an eukaryotic cell is packaged into a highly ordered chromatin structure (Tremethick, 2007). The basic unit of chromatin is the nucleosome containing approximately 147 bp of DNA wrapped around an octamer containing two of each of histones H2A, H2B, H3 and H4. The histones H3 and H4 form a tetramer (H3-H4)2 whereas H2A and H2B present as a dimer. Condensation into polynucleosomal arrays is aided by the peripheral linker histone H1 and other nucleosome-associated factors, including histone chaperones (McBryant et al., 2010; Tremethick, 2007; Williams and Tyler, 2007; Woodcock and Ghosh, 2010) (Figure 1). This compact architecture of chromatin provides a fundamental barrier to restrict the normal cellular processes such as gene expression, DNA repair and DNA replication. This barrier could affect the transcription machinery in two levels. (i) RNA polymerase cannot pass through the template strand of DNA, which requires an open chromatin; (ii) it restricts the access of transcriptional activators and general transcription machineries to their specific DNA sequences within the upstream regulatory region and core promoter (Oike et al., 2014). Thus it is necessary to open or disassemble the chromatin during transcription with the equal importance to close it back or reassemble once the required process is done. Growing evidence suggest that epigenetic mechanisms play an essential role in regulating chromatin dynamics by two distinct ways: using histone modifications and chromatin remodeling (Berger, 2007).

N-terminal and C-terminal tails of the individual histones are subjected to a wide range of post-translational covalent modifications, including acetylation, phosphorylation, methylation, monoubiquitination, sumoylation, formylation, crotonylation, ADP ribosylation and so on. These play a pivotal role in controlling chromatin remodeling and regulating gene transcription (Bernstein et al., 2007; Campos and Reinberg, 2009; Clapier and Cairns, 2009; Jenuwein and Allis, 2001; Kouzarides, 2007; Weake and Workman, 2008; Workman and Kingston, 1998). Notably, these modifications are reversible.

Chromatin remodelers usually function as complexes that change nucleosome structure (e.g. by forming a DNA-loop or sliding a nucleosome) in an ATP- dependent manner. There are several chromatin remodeling complexes have been identified including SWI/SNF, ISWI, INO80, SWR1, NURD/Mi2/CHD and NURF (Clapier and Cairns, 2009).

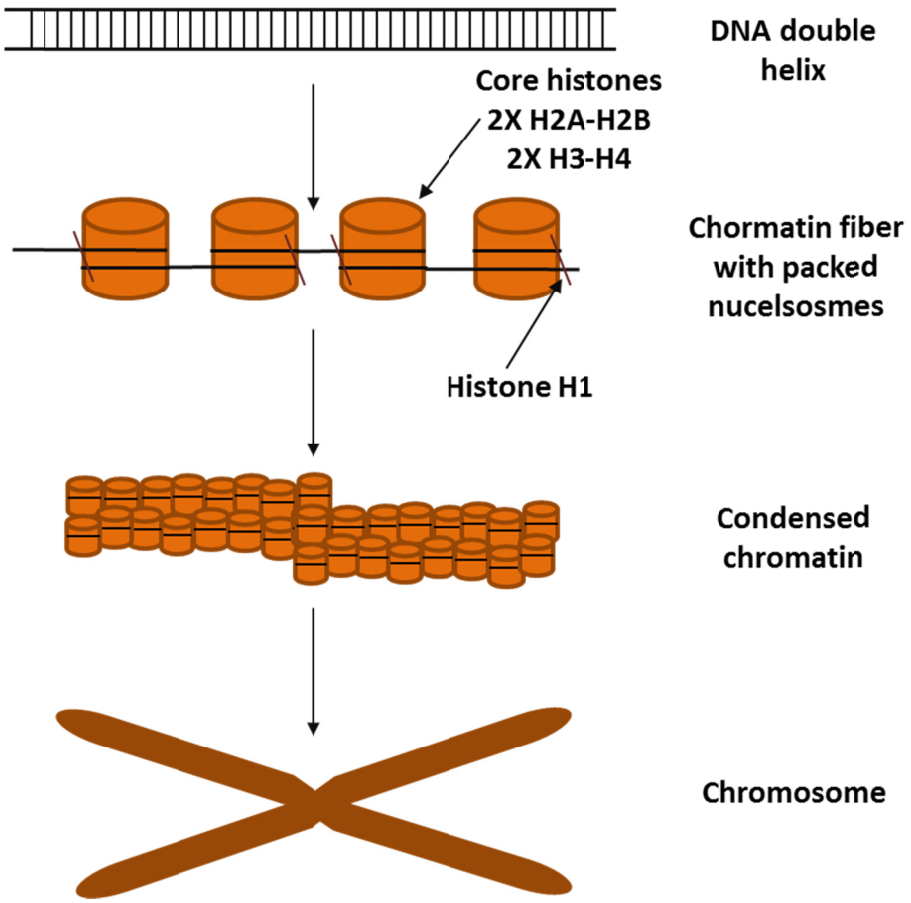


Figure 1. Basic organization of chromatin. The double stranded linear DNA gets folded around the octamer of histones approximately 2 times. The histone octamer contains 2 copies of each of the histones H2A, H2B, H3 and H4. Another histone called H1 acts as a seal and tighten the binding between histones and DNA. Thus form nucleosome, the basic structural unit of chromatin. Chromatin fibers with packed nucleosomes get folded. Finally higher ordered chromosome is formed by the highly folded nucleosomes.

In addition to ATP-dependent chromatin remodeling and histone modifications histone chaperones are shown to be involved in the dynamic changes of chromatin architecture during transcription and other DNA-associated processes. These along with ATP-dependent remodeling complexes guide the assembly of histones from the newly synthesized and recycled histones in a stepwise manner and the disassembly

of chromatin (Clapier and Cairns, 2009; Ransom et al., 2010). The highly acidic nature of histone chaperone proteins allow them bind directly to the basic histones and aid in directing histone deposition, exchange and eviction during nucleosome assembly and disassembly (Akey and Luger, 2003; Eitoku et al., 2008; Park and Luger, 2008).

1.2 Histone chaperones: Modulators of chromatin architecture

Since the complex higher order structure of chromatin provides a fundamental barrier to the different DNA-associated processes including transcription, replication, recombination and DNA damage responses, it is critical to modulate the chromatin structure for proper cellular growth, differentiation and development. Several regulators which play key role in dynamic remodeling of the chromatin structure are histone modifiers, histone variants, chromatin remodelers and histone chaperones. Moreover studies also showed the interplay between these key regulators which ultimately lead to the more dynamicity in the accessibility of the chromatin (Avvakumov et al., 2011; Niederacher et al., 2011; Venkatesh and Workman, 2015).

Histone chaperones are histone-interacting proteins which are involved in the storage, transport, nucleosomal assembly and disassembly of the histones thus modulating the architecture of the chromatin (D'Arcy et al., 2013; de Koning et al., 2007; Venkatesh and Workman, 2015). They usually bind to the non-chromatinized histones, crucial for nucleosome formation. The association of histones with their chaperones could prevent the histone from non-specific binding to nucleic acids and other proteins. Moreover, it could also stabilize the histone oligomers in the free and soluble state outside of the nucleosomes (Burgess and Zhang, 2013; Elsässer and D'Arcy, 2013). The specificity in binding of histone chaperones to histones differs. Some of the histone chaperones can bind to H2A-H2B whereas others are specific to H3-H4. Nucleosome-Assembly Protein 1 (Nap1) can bind to H2A-H2B dimer in the cytoplasm, shuttle them into the nucleus which then can assemble into the nucleosome (Miyaji-Yamaguchi et al., 2003; Mosammaparast et al., 2002). The histone chaperones Anti-Silencing Function 1 (ASF1) and the Chromatin Assembly Factor 1 (CAF1) can deposit H3-H4 tetramers during replication (Winkler et al., 2012). Interestingly a single histone chaperone can bind to all the histones, for example Facilitates Chromatin Transcription (FACT). Moreover, some histone

chaperones such as CAF1 and HIRA are highly specific to histone variants H3.1 and H3.3 respectively (Tagami et al., 2004). Suppressor of Ty Homolog 6 (SUPT6H) and FACT modulate nucleosomal remodeling during transcription (Duina, 2011).

Unlike ATP-dependent chromatin remodelers histone chaperones destabilize the chromatin by using spontaneous DNA movement around the dyad axis (Hondele et al., 2013). Number of studies also showed the cooperation between the histone chaperones and different chromatin remodelers for the dynamic remodeling of chromatin structure (Kuryan et al., 2012; Lorch et al., 2006; Swaminathan et al., 2005).

Histone chaperones also play an important role in transcription specific histone post translational modifications. H3K56 acetylation is mediated by Regulator of Ty1 Transposition 109 (Rtt 109) which is important for histone exchange and subsequent transcription initiation and elongation in yeast (Schneider et al., 2006). However, Rtt 109 can not directly acetylate H3K56 within the nucleosome. It needs the assistance of the histone chaperone ASF1 which presents H3K56 in a structural conformation favorable for the effective acetylation (Kolonko et al., 2010; Tsubota et al., 2007). Another well studied example is the trimethylation of H3K36 mediated by the histone methyltransferase SET2. Importantly, the histone chaperone Spt6 is required to make different regions of the nucleosome accessible for this trimethylation (Du and Briggs, 2010; Du et al., 2008).

1.2.1 The histone chaperone FACT

The human FACT complex is a heterodimeric protein composed of two subunits: Structure Specific Recognition Protein 1 (SSRP1) and Suppressor of Ty Homolog 16 (SUPT16H) (Orphanides et al., 1998, 1999; Reinberg and Sims, 2006). In yeast the homolog of SSRP1 is known as Pob3-Nhp6. This is a highly conserved histone chaperone among eukaryotes which has been shown to play roles in overcoming chromatin barriers during transcription and replication and is also important for assembling and maintaining nucleosomes (Brewster et al., 1998, 2001; Wittmeyer and Formosa, 1997). Initially, FACT was identified as a factor which allows RNA Polymerase (RNAP II) to passage through the DNA template during transcription elongation, thus named as “facilitates chromatin transcription”

(Orphanides et al., 1998, 1999; Reinberg and Sims, 2006). Moreover, FACT is essential for the viability of a range of organisms including yeast and mice (Formosa, 2008; Lejeune et al., 2007; Van Lijsebettens and Grasser, 2010; Winkler and Luger, 2011).

1.2.1.1 Structure of FACT complex

SUPT16H/Spt16

SUPT16H contains four domains termed as N-terminal domain (NTD), the dimerization domain (DD), the middle domain (MD), and C-terminal domain (CTD) (Figure 2) (Keller and Lu, 2002; Tsunaka et al., 2009; VanDemark et al., 2006). The NTD of SUPT16H is a highly conserved domain across several species. Interestingly, NTD was shown to be nonessential for yeast viability as well as nucleosome binding (O'Donnell et al., 2004; Stuwe et al., 2008; VanDemark et al., 2008). Moreover, the NTD of yest Spt16 displays an aminopeptidase-like "pita bread" fold. However, no peptidase activity has been detected in Spt16 or FACT due to the missing critical peptidase active site residues (Stuwe et al., 2008; VanDemark et al., 2008). The NTD of Spt16 is capable of specific binding to H3 and H4 N-terminal tails but not with H2A-H2B dimer (Stuwe et al., 2008). However, the proteolytic hydrolysis of all histone tails significantly affected the affinity of FACT complex to nucleosome (Stuwe et al., 2008; VanDemark et al., 2008) indicating the crucial importance of the NTD of Spt16 as a binding partner of the histone tails. One study reported that the NTD of Spt16 from *S. Pombe* did not show binding to H3-H4 N-terminal tails, rather higher binding affinity was reported to H4 tail only (Stuwe et al., 2008). Interestingly in yeast, a functional interaction was found between Spt16 NTD and the C-terminal docking domain extension of H2A (VanDemark et al., 2008). Thus it appears that the Spt16 NTD has different affinities to each histone which could lead to the nucleosomal reorganization through the destabilization of dimer-tetramer interaction.

The DD of SUPT16H is implicated in the heterodimerization with SSRP1 (Keller and Lu, 2002). This domain is suggested to be partially unfolded in metazoans and yeast, which is stabilized by the neighboring middle domain. Notably, the middle domain of both SUPT16H/Spt16 and SSRP1/Pob3 shares some sequence homology (Winkler and Luger, 2011).

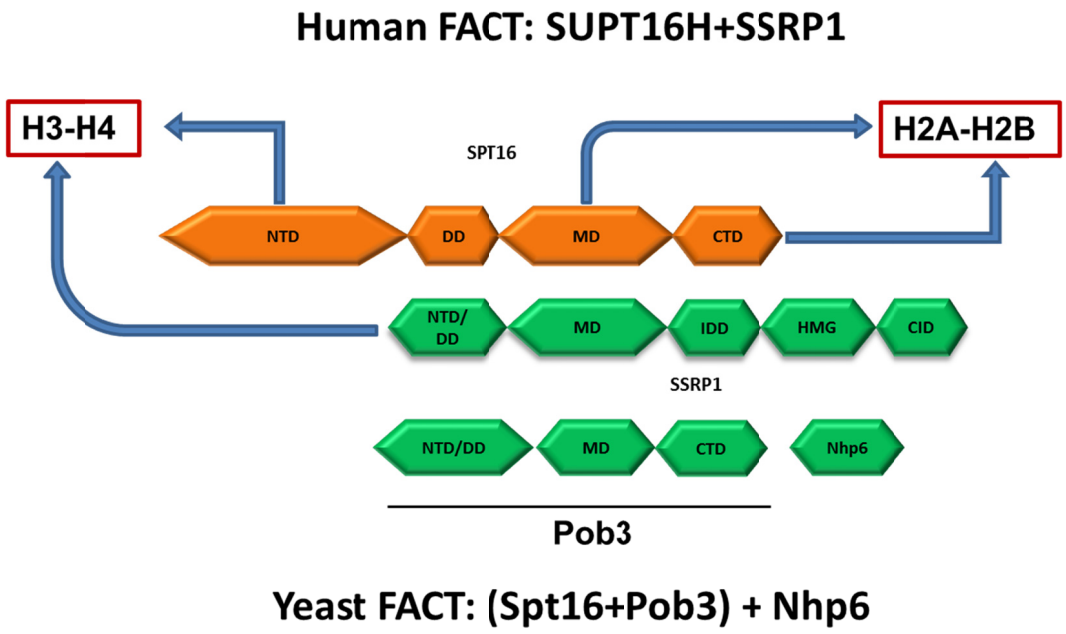


Figure 2. Structural composition of human and yeast FACT components. Human FACT composed of SUPT16H and SSRP1. SUPT16H is conserved in yeast and human. SSRP1 has difference between yeast and human. Yeast homolog of SSRP1 is composed of two separate subunits: Pob3 and Nhp6. The dimerization is formed between DD of SUPT16H and NTD/DD of SSRP1/Pob3. CTD and MD of Spt1616 can bind to H2A-H2B dimer, whereas Spt16 NTD can bind to H3-H4 tetramer. SSRP1 can also bind to H3-H4 tetramer (Figure modified from Winkler and Luger, 2011).

However, the function of middle domain of SUPT16H is poorly known. A recent report revealed that Spt16 middle domain can bind to H2A-H2B dimer in a fashion similar to full length Spt16 in *Chaetomium thermophilum*, consistent also in human. These findings suggest a conserved binding module of the middle domain wiith H2A-H2B (Hondele et al., 2013).

The highly acidic CTD of Spt16 carries regions having similarity to other histone chaperone molecules (Philpott et al., 2000). Moreover, deletion of Spt16 CTD is lethal in yeast (Belotserkovskaya et al., 2003; Evans et al., 1998; Schlesinger and Formosa, 2000). The Spt16 CTD contains 37 out of 75 residues with a negatively charged aspartic or glutamic acid; whereas in human and *Drosophila* 41 of 75 residues are negatively charged. Moreover, higher eukaryotes contain a significant proportion of positively charged arginine and lysine residues though the function is not known yet (Winkler and Luger, 2011). One of the important

features of CTD in human SUPT16H is that it binds to the H2A-H2B dimers (Belotserkovskaya et al., 2003).

SSRP1/Pob3-Nhp6

The human SSRP1 consists of five domains which are NTD/DD, MD, Intrinsically Disordered Domain (IDD), High Mobility Group 1(HMG-1) and C-terminal Intrinsically Disordered Domain (CID) (Figure 2) (Tsunaka et al., 2009; VanDemark et al., 2006).

The yeast homolog of SSRP1 has two proteins in a complex namely Pob3 and HMG domain containing protein Non-Histone Protein 6 (Nhp6) (Brewster et al., 2001). The Pob3 protein has some structural similarities with SSRP1 by containing the NTD/DD, MD and an intrinsically disordered CTD (VanDemark et al., 2006). The SSRP1/Pob3 NTD has been implicated in the heterodimerization with the DD of SUPT16H/Spt16 (Keller and Lu, 2002; O'Donnell et al., 2004). Moreover the first 111 of 220 residues in Pob3 NTD/DD contins a single pleckstrin homology (PH) domain which is characterized by a range of ligand binding properties including proteins or small peptides and lipids as well (VanDemark et al., 2006; Winkler and Luger, 2011).

The middle domain of Pob3 is also a well characterized domain which has double PH domain termed as PH1 and PH2, both of which are homologous to the PH domain of Pob3 NTD/DD. However, PH2 of Pob3 MD is more similar to Pob3 NTD/DD compared to PH1 (VanDemark et al., 2006). Moreover, Spt16 MD also contains a tandem PH domain which is similar in sequence with Pob3 MD (Winkler and Luger, 2011). The Spt16 MD was also reported to bind to the H3-H4 histones in addition to H2A-H2B binding, whereas Pob3 MD can bind only to H3-H4 histones (Formosa, 2012; Winkler and Luger, 2011; Yang et al., 2016). Interestingly, a recent report showed the DNA binding properties of Pob3 MD. In human and other higher eukaryotes, SSRP1 contains a C-terminal HMG-1 domain which has the significant binding affinity to DNA. However, in yeast, the HMG-1 domain represented as a separate protein called Nhp6a/b. This domain assists the FACT to recognize DNA in nucleosome and thus helps in the positioning and reorganization of the chromatin (Yang et al., 2016). Moreover there are two intrinsically disordered domains (CID) in SSRP1, whose function remains elusive. Interestingly, a very recent study reported

the CTD of Pob3 which has similarity with the CID of SSRP1 that can bind to H2A and H2B (Hoffmann and Neumann, 2015).

1.2.2 Function of FACT complex in chromatin remodeling

The FACT complex is well established in their role in reorganizing chromatin structure to make it more accessible. Currently there are two models available to explain the action of FACT on chromatin remodeling termed “dimer eviction” and “global accessibility” model (Figure 3).

In the dimer eviction model, the FACT complex binds to the surface of DNA in a canonical nucleosome, thus leading to the bending of DNA. Binding and bending of DNA thus results in a more open and less stable nucleosome which is then reorganized by the FACT components through the disruption of the nucleosome due to the dissociation of a single H2A-H2B dimer. Here a hexasome of histones containing a H3-H4 tetramer and a H2A-H2B monomer is left on the DNA. Furthermore, the histone components are tethered in a way so that they are not lost from comparatively open form of chromatin (Belotserkovskaya et al., 2003; Orphanides et al., 1999; Reinberg and Sims, 2006). In the global accessibility model, the nucleosome is represented already in an equilibrium state between the canonical and the less stable reorganized form. Then, FACT can bind to the open state preferentially. According to the global accessibility model, binding of FACT to the relatively open state can prolong the duration of the accessibility of the chromatin in the reorganized and less stable state (Formosa, 2008; Xin et al., 2009). Both models were depicted based on the previous observations. For instance, when FACT components were incubated with immobilized nucleosome for one hour, around half of the H2A-H2B dimers were lost (Belotserkovskaya et al., 2003). In another study histones were crosslinked to prevent the H2A-H2B dimer expulsion which resulted in the ability of FACT to promote transcription (Orphanides et al., 1999). A study with single molecule Fluorescence Resonance Energy Transfer (FRET) experiments support the global accessibility model, where the nucleosome was found in an equilibrium state between the closed and a relatively opened state with the displaced H2A-H2B dimer (Böhm et al., 2011).

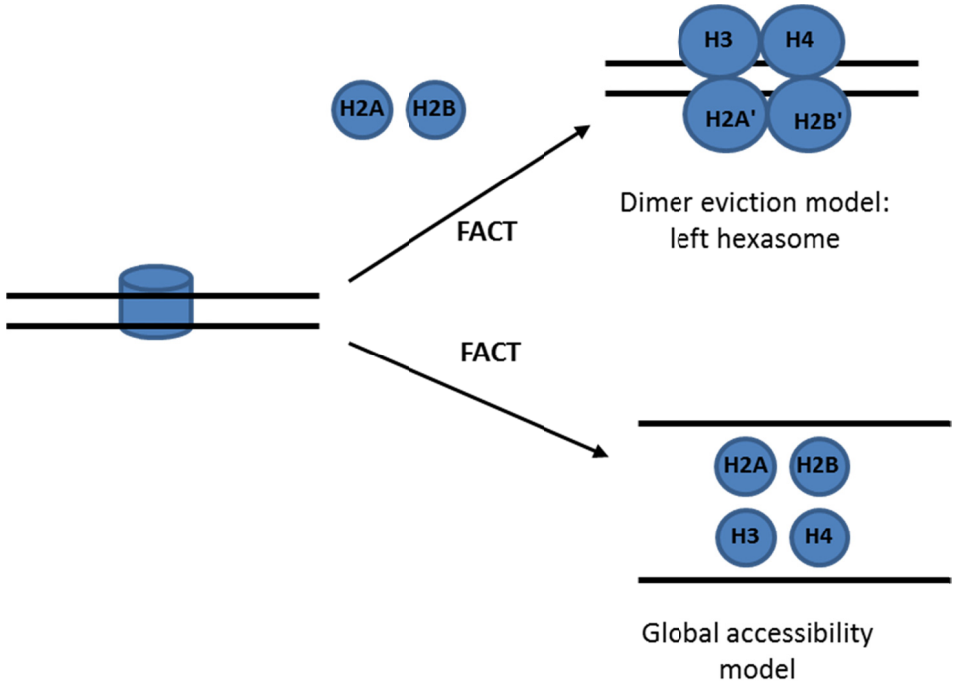


Figure 3. Functional model of FACT for nucleosome reorganization. In dimer eviction model, FACT could bind to the H2A-H2B histones through the CTD of SPT16 thus displace the dimer from the nucleosome, leave the unstable hexasome containing H3-H4 tetramer and H2A-H2B monomer indicated as H2A' and H2B'. SPT16 also re-place the dimer after the required process is done. In global accessibility model FACT binds to the histones, drive the loosening of contacts between the histones and DNA as well. However, FACT holds the histones so that they are not lost.

1.2.3 FACT in transcription

The FACT complex is widely studied to play an important role in aiding transcription. Interestingly a number of evidences suggest that FACT functions in every stage of transcription including initiation, elongation and termination.

1.2.3.1 FACT in transcription initiation

During transcription initiation, formation of the initiation complex is one of the crucial prerequisites which require the access of different factors to the chromatin. Evidences support the role of FACT as a histone chaperone in overcoming the nucleosomal barrier.

Recruitment of FACT at the promoter region with TATA box could facilitate the binding of TATA-box binding protein (TBP) which further allows the recruitment of TFIIA, TFIIB and RNAP II, thus forming an effective transcription initiation complex

(Biswas et al., 2005). Consistently loss of Spt16 minimized TBP, TFIIB and PolII binding at the promoter region (Mason and Struhl, 2003). Moreover, FACT was reported to play an important role in the fidelity of transcription initiation (Mason and Struhl, 2003). Functional inactivation of Spt16 through conditional mutations led to the increased Pol II density, transcription and TBP occupancy at the 3' regions of some coding genes, which suggests the role of FACT to prevent cryptic transcription(Carvalho et al., 2013). Furthermore Nhp6 was reported to promote TBP binding to RNAP II. Nhp6 also displayed involvement during transcriptional initiation through Pol III. Another histone chaperone ASF1 was demonstrated to bind in the upstream of *HO* promoter along with yeast FACT (Takahata et al., 2009).

1.2.3.2 FACT in transcription elongation

The role of FACT in DNA transcription elongation is well established. FACT was initially purified as a factor being capable of promoting RNAP II elongation through nucleosomes (Orphanides et al., 1998). Later more evidences supported the direct participation of FACT in elongation(Belotserkovskaya et al., 2003; Hsieh et al., 2013; Orphanides et al., 1999; Wada et al., 2000). FACT was also showed to be interacting with a number of transcription elongation factors. For example, the yeast FACT subunits Spt16 and Pob3 were shown to interact with Spt4, Spt5, Spt6 and polymerase associated factor 1 (Paf1) complex (Krogan et al., 2002; Lindstrom and Hartzog, 2001; Lindstrom et al., 2003; Sims et al., 2004; Squazzo et al., 2002). In addition, a Chromodomain Helicase DNA Binding Protein 1 (CHD1) also showed interactions with FACT both in mammalian cells and yeast (Krogan et al., 2002; Simic et al., 2003). In yeast, Chd1 is recruited to the open reading frame along with FACT and several other elongation factors. Specifically SSRP1 was reported for its colocalization and interaction with CHD1 (Kelley et al., 1999). After transcription initiation RNAP II pauses or stops transcribing at 15 to 45 nucleotides after TSS which is denoted as promoter proximal pausing of RNAP II. Interestingly, FACT promotes the release of pause 45 nucleotide position by disrupting the H3-H4:DNA contacts (Hsieh et al., 2010, 2013; Ujvári et al., 2008).

Studies suggest that FACT is recruited to the transcription complex by other transcription factors. Now the question comes how FACT is recruited to transcription machineries. Since FACT interacts with a number of general transcription factors

including TFIIE, TFIIH, it is possible that FACT can have an indirect interaction with RNAPII (Belotserkovskaya et al., 2004; Lindstrom and Hartzog, 2001; Squazzo et al., 2002). Moreover, FACT can also bind to single stranded DNA which is generated during transcription (Belotserkovskaya et al., 2004). Pavri et al., (2006) showed an interesting finding about the role of FACT in transcription where they reported a functional interaction between FACT, PAF and H2B monoubiquitination (H2Bub1) which represents active transcription elongation. They showed that H2B monoubiquitination by the E3 ubiquitin ligase RNF20/RNF40 facilitates the function of FACT to evict H2A-H2B dimer thus disrupt H2A-H2B:DNA contacts and as a consequence RNAP II get access to DNA (Pavri et al., 2006).

1.2.3.3 Fact in transcription termination

The role of FACT in transcription termination is not well understood. FACT and other transcription factors which run along with RNAP II have been found to dissociate at two distinct sites near to the 3' end indicating an ordered manner of termination (Mayer et al., 2010). Notably FACT becomes separated at the first dissociation site. When histone H3 was mutated there was an accumulation of FACT and RNAP II at this site (Duina et al., 2007; Lloyd et al., 2009; Myers et al., 2011). Moreover FACT can have an interaction with H3, but the role of this interaction in transcription is yet to be elucidated.

1.2.4 Regulation of FACT

The FACT complex was shown for its potential effects in regulating several factors involved in different DNA-associated processes including replication, transcription, repair and recombination. However, FACT complex can also be regulated by other factors. Phosphorylation of Spt16 restricts FACT to bind to the nucleosome in response to genotoxic stress (Huang et al., 2006). Phosphorylation of SSRP1 by Casein Kinase 2 (CK2) affects its nucleosomal DNA binding (Keller and Lu, 2002; Keller et al., 2001). FACT can also undergo to the post-translational modifications which further regulates its activity. In yeast, Spt16 can be ubiquitinated by the E3 ubiquitin ligase Rtt101 which assist its recruitment into the origin of replication (Han et al., 2010).

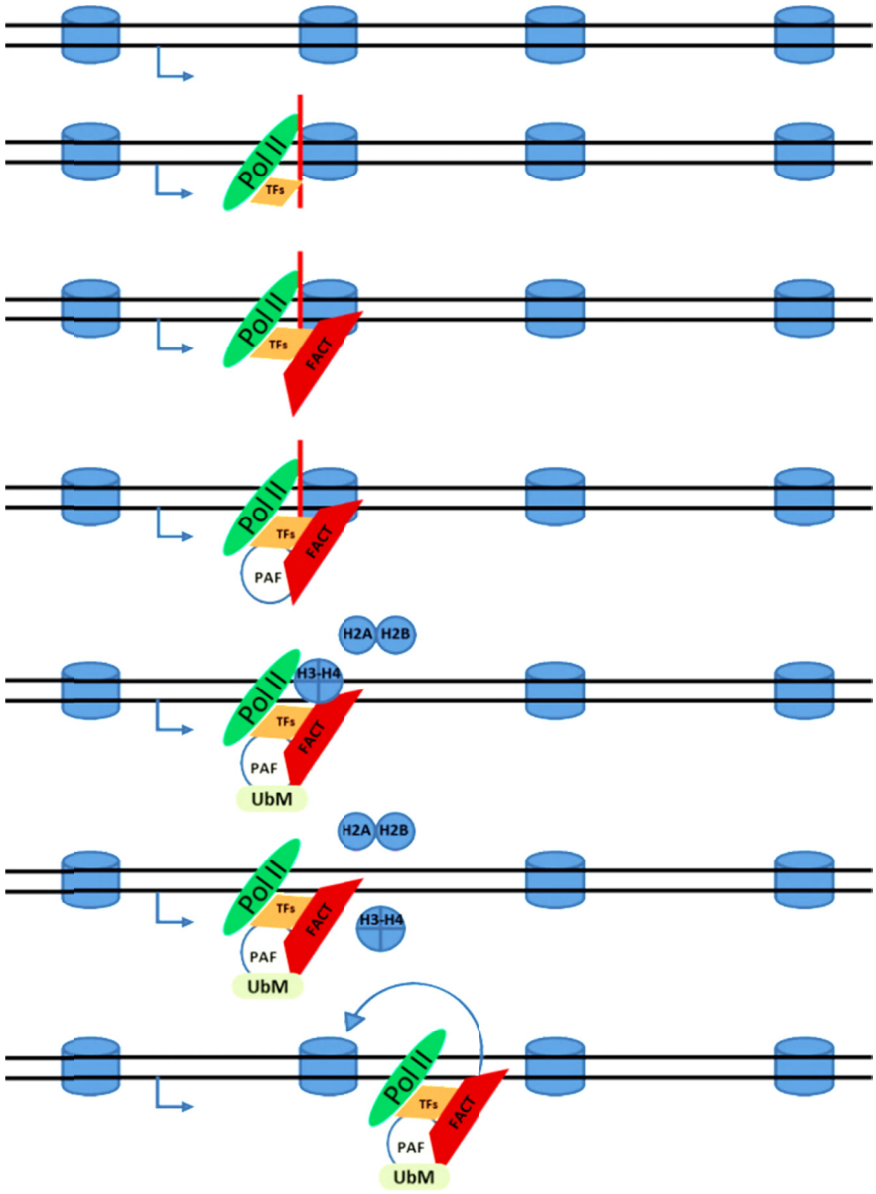


Figure 4. Schematic representation of the transcriptional events facilitated by FACT. During transcription initiation RNAP II (indicated as Pol II in figure) along with transcription factors (TFs) faces the nucleosomal barrier and halts at the first nucleosome, FACT is recruited. FACT recruits PAF and ubiquitination machinaries (UbM) which lead to the ubiquitination of H2B, thus facilitates FACT to displace the H2A-H2B dimer, the Pol II run through the DNA. However these events happen in the first stop at 15 nucleotides. In the second stop at 45 nucleotides FACT assists in displacing H3-H4 tetramer, promotes elongation steps.

1.3 Bone

Bone is a highly specialized connective tissue with the extraordinary capacity for growth, regeneration, and remodeling throughout life. Bone consists of organic extracellular matrix hardened by the inorganic minerals. The regions between the organic and inorganic portions are filled with two main types of components: (i) the outer hard cortex and (ii) the inner spongy like substances (Clarke, 2008). Bone performs several crucial functions comprise: (i) structural and mechanical support to the body, (ii) protects the vital inner organs, (iii) key component in the locomotive system, (iv) maintains the mineral homeostasis, and (v) primary sites for the formation of blood cells (Lee et al., 2007; Long, 2012).

1.3.1 Bone cells

The bone contains three major types of cells. These are osteoblasts, osteocytes and osteoclasts which are responsible for production, maintenance, and resorption of bone respectively (Figure 5) (Clarke, 2008; Sims and Martin, 2014). Osteoblasts, the chief bone forming cells are originated from a common progenitor cell with adipocytes, bone marrow derived mesenchymal stem cells (MSCs). The sequential process of new bone formation involves (i) recruitment of osteoblasts precursor to the site of osteoid deposition, (ii) precursors start differentiating towards osteoblasts, (iii) synthesizing first the organic matrix (i.e. the osteoid) and (iv) contributing to its mineralization. After all these processes are accomplished, the majority of osteoblasts enter into apoptosis. The rest of the cells are entrapped in the mineralized extra cellular matrix (ECM) as terminally differentiated mature osteoblasts which are termed as osteocytes. A part of the survived cells can also reside in the covering of the bone surface as bone-lining cells (Bonewald, 2011; Bonewald and Dallas, 1994; Imai et al., 1998).

Osteoclasts are multinucleated cells which originate from the hematopoietic stem cell precursor along the myeloid differentiation lineage. These cells are mainly responsible for the degradation of bone. During the process of bone resorption osteoclasts attach to the bone surface, isolate the area, cause acidification and finally disperse the inorganic ECM. Different proteases also come into action to dissolve the organic part of bone (Arai et al., 1999; Kikuta and Ishii, 2012).

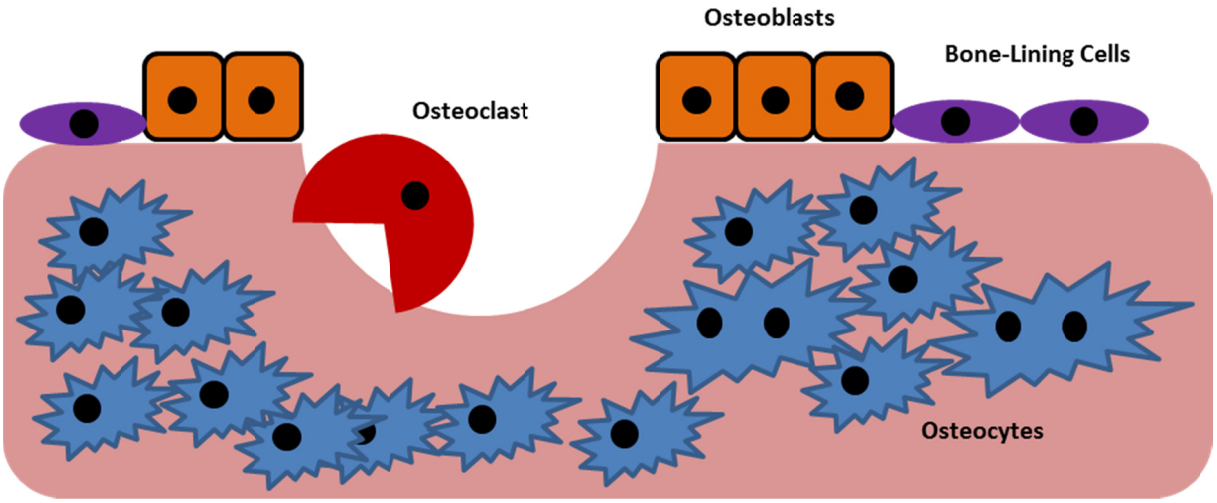


Figure 5. Bone cells in maintenance of bone homeostasis. Osteoblasts are the major bone forming cells originated from the mesenchymal progenitor. Matured osteoblasts reside in the bone as osteocytes and bone-lining cells. Osteoclasts are responsible for degradation of bone. All together these cells maintain dynamic bone remodeling. An imbalance or defect in the bone remodeling process consequence in different bone-related pathological condition including osteoporosis.

In normal physiology, bone formation and resorption are tightly regulated processes for continuous remodeling important for bone homeostasis. Any imbalance between these processes result in pathological conditions including osteoporosis, osteopetrosis, osteopenia etc. Moreover, these conditions could be the result of other diseases such as cancer and autoimmune diseases. As a common progenitor hMSCs can be differentiated into multiple cell lines. However differentiation to adipocyte and osteoblast has a reciprocal regulation whose imbalance results in different pathological conditions. For instance, increased marrow adipocity has been reported in bone loss diseases including osteoporosis and age related bone loss (Caplan, 1991; Horwitz et al., 1999, 2005; Justesen et al., 2001; Kawai et al., 2012; Meunier et al., 1971; Moerman et al., 2004; Pino et al., 2012).

1.3.2 Osteoblast differentiation

Osteoblasts, mainly originated from the mesenchymal progenitors are the major bone making cells which undergo through a sequential process to form bones. Multiple transcriptional cascades as well as signaling pathways need to be regulated

for this specific lineage fate. Overall osteoblast differentiation can be discussed in the following points (Figure 6).

(I) Lineage commitment: This is the very first step where MSCs become committed to be differentiated into osteoblast. The committed cells are called osteoprogenitors. There is very limited knowledge about the circumstances that induce osteoblast lineage commitment from the multipotent stem cells. Even there is not much difference between MSCs and osteoprogenitors (Franceschi, 1999). However, several transcription factors and mediators start their action here including *RUNX2* and *SP7, DLX5* and *MSX2* (Ducy et al., 1997; Komori, 2006; Komori et al., 1997; Nakashima et al., 2002a; Otto et al., 1997).

(II) Proliferation and matrix formation: In the next phase osteoprogenitors are developed into pre-osteoblasts. ECM synthesis also begins in this step. Important feature of this step is to express collagen type 1 (*COL1A1*) and bone sialoprotein (*IBSP*). Still *RUNX2* and *SP7* are expressed (Delorme et al., 2009).

(III) Matrix maturation and mineralization: Pre-osteoblasts are then differentiated into mature osteoblasts with the enhanced expression of *ALPL*. The ECM also matures in this step to make contact with the neighboring cells. Importantly mineralization also happens during this step (Aubin, 2001; Choi et al., 2010; Traianedes et al., 1993).

(IV) Mature bone tissue: Last stage of bone formation. A subset of mature osteoblasts is entombed within the bone matrix to form osteocytes. A portion also resides on the surface as inactive bone-lining cells. The rest of the matured osteoblasts undergo programmed cell death (Bonewald, 2011). In mammalian bone, osteocytes are the most abundant cellular component accounting for 95% of total cells. As terminally differentiated cells, osteocytes play a vital role in mediating communication with neighboring cells, translating mechanical stimuli to the biochemical signals for formation or resorption of bone. Recently their endocrine functions have also been reported expressing fibroblast like growth factor 23 and other factors important in phosphate homeostasis (van Bezooijen et al.,

2004; Keller and Kneissel, 2005; Long, 2012; Powell et al., 2011; Robling et al., 2008).

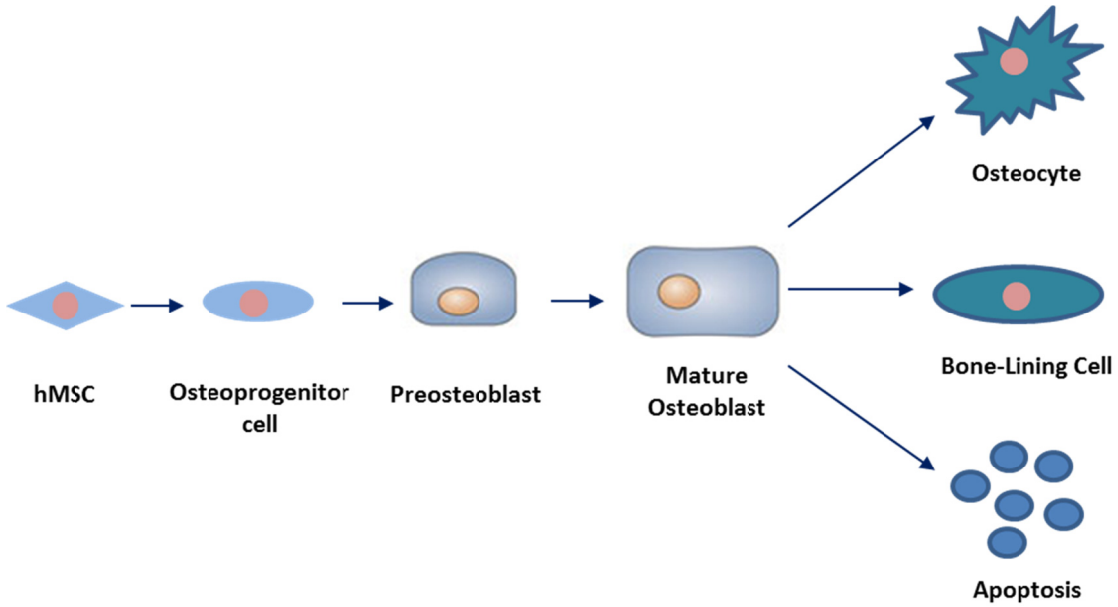


Figure 6. Sequential steps of osteoblast lineage cell differentiation. Human mesenchymal cells differentiate into committed osteoprogenitor cells which then give rise to preosteoblast. Preosteoblast cells develop ECM components and later differentiate into mature osteoblast which has three distinct fates; most of the mature osteoblasts are entrapped into bone matrix called osteocytes, some reside on the surface of bone as bone-lining cells, a large portion goes through apoptosis.

1.3.3 Regulation of osteoblast differentiation

Osteoblast differentiation is a multi-dimensional process controlled by several growth factors, cytokines, hormones and components of ECM contributing to the signaling cascades, transcription factors and signaling pathways.

1.3.3.1 Regulation by transcription factors

Transcription factors are specifically involved in different stages of osteoblast differentiation. The first transcription factor come in consideration is SOX9. It has been reported to play role in the lineage commitment of hMSCs towards chondrocytes and osteoblasts, thus separating these two lineages from other cellular fates. Sox9 is indispensable for chondrocyte differentiation whereas the role in osteoblast differentiation is not well known (Akiyama et al., 2002; Akiyama‡ et al., 2005; Bi et al., 1999, 2001; Kist et al., 2002). *RUNX2* is the master transcription

factor in regulation of osteoblast differentiation. Homozygous deletion of *RUNX2* in mice resulted in complete loss of osteoblast activity (Komori et al., 1997; Otto et al., 1997). Moreover deletion of RUNX2 carboxy terminal domain responsible for nuclear targeting signal resulted in the identical RUNX2-null mice phenotype (Choi et al., 2001). Additionally *RUNX2* was reported to be essential for induction of the major bone matrix related genes (Ducy et al., 1999). Interestingly most of the osteoblast differentiation related signaling pathways are targeted at RUNX2 (Komori, 2006). However *RUNX2* is the early expressed gene which gradually decreases with the maturation of osteoblasts while other genes come into action such as *osterix (SP7)* and *β-catenin* (Komori, 2006, 2010; Maruyama et al., 2007; Nakashima et al., 2002b). SP7 is another important transcription factor essential for proper osteoblast differentiation. Deletion of SP7 resulted in the complete lack of osteoblasts in mouse embryos. Importantly SP7 was reported downstream to RUNX2 as deletion of *RUNX2* also abolished SP7 (Nakashima et al., 2002b).

Other transcription factors have been found to be crucial for osteoblast differentiation include activating transcription factor 4 (*ATF4*) and activator protein 1 (AP1) family (Wagner, 2002; Yu et al., 2005). ATF4 is a member of the basic Leucine zipper (bZIP) family of transcription factors which plays important roles in mature osteoblasts. Misregulation of ATF4 activity has been linked with the skeletal abnormalities (Elefteriou et al., 2006; Yang et al., 2004). AP1 family members also have been shown to affect the osteoblast differentiation and bone formation (Eferl et al., 2004; Wagner, 2002).

1.3.3.2 Regulation by signaling pathways

Several signaling pathways have been implicated in the tight regulation of osteoblast differentiation. Among them Wnt signaling has emerged as one of the major pathways in regulating osteoblastogenesis, bone and skeletal development. It also plays important role in bone formation and resorption.

Wnt-signaling occurs in three different ways; i) Canonical or Wnt β-catenin pathway ii) Non canonical and iii) Wnt-calcium pathway (Baron and Kneissel, 2013). Among these, the canonical pathway has emerged as one of the important regulators of bone homeostasis. In the canonical pathway, when there is no

stimulation, β-catenin resides in the inactive complex along with GSK-3β, AXIN, and APC which later become phosphorylated by GSK-3β and thus targeted for polyubiquitination by proteasome-mediated degradation. In response to appropriate stimuli Wnt ligands bind to the receptor [Frizzled (Fzd) and either Low-density lipoprotein receptor-related proteins (LRP5 or LRP6)]; β-catenin becomes activated by coming out of the inactive complex. The activated β-catenin then translocate into the nucleus where they bind to the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor, thus activate expression of the genes required for bone formation. Wnt signaling can be controlled in different stages of the pathway. For example different Wnt ligands such as Wnt2, Wnt3a, Wnt 11, Wnt 10b etc. can induce the activation whereas Wnt antagonists such as DKK/Kremen can inhibit the pathway (MacDonald et al., 2009; Yavropoulou and Yovos, 2007). The phosphorylation of β-catenin can be blocked by the cytoplasmic protein Dishevelled (Dsh) which inhibits GSK-3β in response to the Wnt ligands binding to the receptors such as FZD, LRP5 or LRP6 (van Amerongen and Nusse, 2009; Baron and Kneissel, 2013; Kikuchi et al., 2009; Kohn and Moon, 2005).

Studies showed that activation of the Wnt-signaling by the canonical Wnt ligand Wnt10b or inhibition of GSK-3β activity enhanced osteoblast differentiation (Bennett et al., 2005, 2007). Moreover stimulation of the canonical Wnt-signaling through Wnt3a-LRP5 activation led to the increased ALPL expression and decreased lipid droplet formation in hMSC. Conversely these effects were reversed when LRP5 was inactivated (Qiu et al., 2007). Similar results regarding LRP5 activity were also observed in vivo systems. A high bone mass or increased trabecular bone volume and decreased fat within the marrow were observed as a consequence of gain of mutations in LRP5 in humans (Boyden et al., 2002; Little et al., 2002; Qiu et al., 2007). An opposite phenomena was observed in response to the loss of functions in LRP5 which promoted osteoporosis characterized by the decreased bone and increased intramedullary fat (Gong et al., 2001; Qiu et al., 2007).

Beside canonical Wnt signaling, noncanonical pathway has also been reported for regulation of bone formation and development (Baron and Kneissel, 2013). Noncanonical ligand WNT7b was reported to stimulate osteoblast differentiation through G protein–linked protein kinase C δ (PKC-δ) signaling (Tu et

al., 2007). Another ligand Wnt5a can induce osteoblast differentiation over adipocyte differentiation (Takada et al., 2007). Wnt Inhibitory Factor 1 (WIF 1) can inhibit osteoblast differentiation (Cho et al., 2009). Moreover, activation of noncanonical receptor ROR1 and ROR2 can induce bone formation and skeletogenesis (Maeda et al., 2012). Interestingly some components of the Wnt signaling can control both canonical and noncanonical pathway. These include *RSPO1, SFRP1, SFRP2, SFRP4* (Baron and Kneissel, 2013).

Apart from Wnt signaling other important signaling pathways involved in regulation of osteoblast differentiation and bone formation include TGF-β, BMP, Notch, Hedgehog, and Fibroblast Growth Factors (FGF). These pathways have been shown to act independently as well as to interact with each other for the complex regulation of osteoblast differentiation, bone and skeletal development (Guo and Wang, 2009; Lin and Hankenson, 2011; Rahman et al., 2015).

1.3.3.3 Epigenetic regulation of osteoblast differentiation

Epigenetic mechanisms bring changes in gene expression due to the modifications on DNA and chromatin without alteration in DNA sequences. The orchestrated regulation of gene expression during differentiation requires a complex interplay between transcription factors and epigenetic mechanisms which together direct lineage-specific gene expression (Håkelien et al., 2014). Epigenetic mechanisms including post-translational histone modifications, DNA methylation and changes in chromatin structure by chromatin remodelers and histone chaperones, are required for the proper regulation of gene expression during osteoblast differentiation (Gordon et al., 2015; Hemming et al., 2014; Karpiuk et al., 2012; Wei et al., 2011).

Acetylation and methylation are the most well studied post-translational histone modifications in osteoblast differentiation and bone development. Methylation of histones at specific position can activate or repress osteoblast differentiation. H3K4 trimethylation or H3K36 methylations were reported to be the active marks whereas H3K27 trimethylation act as an inactive mark for osteoblast differentiation (Hassan et al., 2007, 2009; Lee et al., 2014; Leventopoulos et al.,

2009). Moreover a number of demethylases can demethylate the histones thus altering their role in regulation of osteoblast differentiation.

In addition to histone methylation, acetylation and deacetylation of histones have also been demonstrated to be important in the regulation of osteogenic genes. Several histone acetyltransferases such as p300, CBP, PCAF were implicated as a direct or co-regulator of a number of bone-related genes including *RUNX2* through histone acetylation (Sierra et al., 2003). Both promoter and coding region of *BGLAP* gene was found to be enriched in H3 and H4 acetylated marks during the proliferative period of osteoblast differentiation (Montecino et al., 1999; Shen et al., 2003). Interestingly, histone acetylation is often associated with a comparatively open chromatin and active transcriptional state while histone deacetylation is involved with chromatin condensation and gene silencing.

Apart from the post-translational histone modifications, DNA methylation is an important epigenetic regulator for osteoblast differentiation. Notably significant hypermethylation is associated with chromatin condensation which can repress the gene expressions (Villagra et al., 2002). Methylation at the *ALPL* gene was found to be a major regulator for controlling its expression. Evidences came from the DNA demethylating agents, while used increased the significant expression of *ALPL* during osteoblast differentiation in osteogenic and non-osteogenic condition. Accordingly, very low level of methylation was found in *ALPL* expressing osteoblasts while in osteoclast it was found in hypermethylated state (Delgado-Calle et al., 2011; El-Serafi et al., 2011; Locklin et al., 1998; Vaes et al., 2010). The changes in methylation status was also observed in osteocalcin gene during osteoblast differentiation in response to stimulation of differentiation (Arnsdorf et al., 2010; Villagra et al., 2002). The other osteoblast differentiation marker genes whose regulation is also changed due the methylation are *OPG, OPN, OSX* etc (Arnsdorf et al., 2010; Delgado-Calle and Riancho, 2012; Lee et al., 2006).

1.4 Role of histone chaperones in cellular differentiation

Histone chaperones are the proteins having the abilities to execute nucleosome reorganization which could play crucial role during cellular differentiation. Importantly studies have been carried out to elucidate the role of histone chaperones

in stem cell differentiation to specific lineages. Some examples have been listed below:

- The histone chaperones HIRA and ASF1 showed specific functions during osteoblast and myoblast differentiation from C2C12 cells. Both of the histone chaperones were maintained throughout the differentiation stages for myoblast differentiation whereas they were diminished half or more during osteoblast differentiation (Song et al., 2012b). Two previous studies also reported the importance of HIRA and ASF1 in myoblast differentiation were mediated by the regulation of master regulator *MyoD* and *Mef2* expression (Yang et al., 2011, 2011).

- Histone chaperone SUPT6H was found to play important role during myogenesis by assisting in the removal of repressive epigenetic mark H3K27me3. SUPT6H could facilitate the proper engagement of demethylase KDM6A (UTX), which in turn mediated demethylation of H3K27me3, thus activated the expression of muscle genes including the key myoblast specific gene *MyoD* (Wang et al., 2013).

  A connection between H2Bub1 and SUPT6H in regulating osteoblast and adipocyte differentiation from hMSC was reported by Bedi et al., (2014). Knockdown of SUPT6H decreased the level of H2Bub1 and also negatively affected osteoblast and adipocyte differentiation, depicting the crucial role of SUPT6H in hMSC differentiation which could be partly mediated by H2Bub1 (Bedi et al., 2015). Consistently, the role of H2Bub1 in cellular differentiation of hMSC was demonstrated by a previous study (Karpiuk et al., 2012).

- CHAF1A is another histone chaperone was reported to inhibit neuronal differentiation and to promote aggressive neuroblastoma with the overall poor prognosis by regulating H3K9me3 (Barbieri et al., 2014).

Above discussion shows a clear connection between the histone chaperones, their role in chromatin modifications and consequences in the regulation of cellular differentiation.

1.5 Aim of the study

Several studies have shown the pivotal role of the histone chaperone FACT in different DNA-associated processes including transcription, replication, DNA repair and recombination. Higher expression of FACT components was reported in undifferentiated or poorly differentiated cells, stem cells or stem cell-like cells whereas the adult mammalian tissues contain very low level of FACT. However the core functions of FACT components during differentiation are largely unknown. We hypothesized that FACT components play an important role in cellular differentiation of multipotent stem cells. Specifically we aimed to investigate the role of the FACT component SSRP1 in adipocyte and osteoblast differentiation considering the significant reciprocal balance of these two cellular fates in development process. To address this hypothesis we depleted SSRP1 in hMSCs and examined the influence on differentiation potency. Considering the importance of osteoblast differentiation in bone homeostasis, we further aimed to extend the study to osteoblast differentiation. High-throughput RNA sequencing in human Fetal Osteoblast (hFOB) was used to identify the transcriptome-wide effects of SSRP1 on osteoblast differentiation. In addition dual luciferase reporter assays was used to test the role of SSRP1 in Wnt-signaling pathway activity.

2. Materials

2.1 Technical equipment

Agarose gel chamber	Harnischmacher Labortechnik, Kassel
Balance	Sartorius AG, Göttingen
Bandelin Sonoplus Sonicator	Bandelin electr. GmbH & Co. KG, Berlin
Biological Safety Cabinet “Hera Safe”	Thermo Fisher Scientific, Waltham, USA
Bioruptor	Diagenode, Belgium, Europe
Centrifuge (Megafuge 1.OR)	Thermo Fisher Scientific, Waltham, USA
Centrifuge 4°C (5417R)	Eppendorf AG, Hamburg
Centrifuge 4°C (Fesco 21)	Thermo Fisher Scientific, Waltham, USA
C1000TM Thermal Cycler	Bio-Rad Laboratories GmbH, München
CFX96TM Optical Reaction Module	Bio-Rad Laboratories GmbH, München
Counting chamber (Neubauer)	Brand GmbH & Co. KG, Wertheim
Confocal microscope LSM510 META	Carl Zeiss MicroImaging GmbH, Göttingen
5100 Cryo 1°C Freezing Container	Thermo Fisher Scientific
Electrophoresis & Electrotransfer Unit	Bio-Rad Laboratories GmbH, München
Freezer -20 °C	Liebherr GmbH, Biberach
Freezer -80 °C (Hera freezer)	Thermo Fisher Scientific, Waltham, USA
Freezer -150°C (MDF-C2156VAN)	Panasonic, Kadoma, Japan
Gel Imager “Gel iX imager”	Intas Science Imaging GmbH, Göttingen
Incubator (bacteria)	Memmert GmbH & Co. KG, Schwabach
Incubator (cell culture) “Hera cell 150“	Thermo Fisher Scientific, Waltham, USA
Luminometer „Centro LB 960“	Berthold Technologies, Bad Wildbad
Magnet stirrer “MR3001”	Heidolph GmbH & Co. KG, Schwabach
Microscope Axio Scope A1	Carl Zeiss MicroImaging GmbH, Göttingen
Microscope “Axiovert 40 C“	Carl Zeiss MicroImaging GmbH, Göttingen
Microwave	Clatronic International GmbH, Kempen
Nano Drop® ND-1000 Spectrophotometer	Peqlab Biotechnology GmbH, Erlangen
OptiMax X-ray Processor	Typon Medical, Krauchthal
pH meter inoLab®	WTW GmbH, Weilheim
Pipette Aid® portable XP	Drummond Scientific Co., Broomall, USA
Pipettes “Research” Series	Eppendorf AG, Hamburg
Power supply “Power Pack P25T”	Biometra GmbH, Göttingen Material
Qubit® 2.0 Fluorometer	Invitrogen GmbH, Karlsruhe
Refrigerator	Liebherr GmbH, Biberach
Scanner Epson 1680	Seiko Epson, Suwa, Japan
Shaker “Rocky”	Schütt Labortechnik GmbH, Göttingen
Table centrifuge (VWR Mini Star)	Korea
Test tube rotator	Schütt Labortechnik GmbH, Göttingen
Ultrapure Water System “Aquintus”	MembraPure GmbH, Bodenheim
Vacuum pump (BVC Control)	Vacuubrand GmbH and Co. KG,

	Germany
Vortex-Genie 2	Electro Scientific Industr. Inc., Portland, USA
X- Ray Cassettes	Rego X-ray GmbH, Augsburg

2.2 Consumable materials

Cellstar 6-, 12-, 24-well cell culture plates	Greiner Bio-One GmbH, Frickenhausen
Cellstar PP-tube 15 and 50 ml	Greiner Bio-One GmbH, Frickenhausen
Cellstar tissue culture dish 100×20 mm	Greiner Bio-One GmbH, Frickenhausen
Cellstar tissue culture dish 145×20 mm	Greiner Bio-One GmbH, Frickenhausen
Cell scraper (16 cm, 25 cm)	Sarstedt AG & Co., Nümbrecht
Cryo TubeTM Vial (1.8 ml)	Thermo Fisher Scientific, Waltham, USA
Falcon® assay plate, 96 well	VWR Int., LLC, West Chester, USA
Gel blotting paper (Whatman paper)	Sartorius AG, Göttingen
Glass coverslips (18 mm)	Gebr. Rettberg GmbH, Göttingen
HybondTM-PVDF Transfer Membrane	GE Healthcare Europe GmbH, München
Microtube 0,5 ml, 1.5 ml, 2 ml	Sarstedt AG & Co., Nümbrecht
Microtube 1.5 ml, conical	VWR International GmbH, Darmstadt
96 Multiply® PCR plate white	Sarstedt AG & Co., Nümbrecht
96-well Multiplate PCR plate white (low)	Bio-Rad Laboratories GmbH, München
NORM-JECT Syringes of different volume	Henke Sass Wolf GmbH, Tuttlingen
Parafilm® “M”	Pechiney Plastic Packaging, Chicago, USA
Petri dish 92×16 mm	Sarstedt AG & Co., Nümbrecht
Pipette tips	Greiner Bio-One GmbH, Frickenhausen
Pipette filter tips	Sarstedt AG & Co., Nümbrecht
Protan® Nitrocellulose transfer membrane	Whatman GmbH, Dassel
Syringe filter, CA-membrane, 0,20 µm	Sartorius AG, Göttingen
Ultra low attachment plates	Corning Life sciences, NY, USA
X-ray films “Super RX”	Fujifilm Corp., Tokyo, Japan

2.3 Chemicals

2.3.1 General chemicals

Acetic acid	Carl Roth GmbH & Co. KG, Karlsruhe
Acetone ROTISOLV	Carl Roth GmbH & Co. KG, Karlsruhe
Adefodur WB developing concentrate	Adefo-Chemie GmbH, Dietzenbach
Adefodur WB fixing concentrate	Adefo-Chemie GmbH, Dietzenbach
Adenosine Triphosphate (ATP)	Sigma-Aldrich Co., St. Louis, USA
Agarose	Biozym Scientific GmbH, Hessisch Oldendorf
Albumin Fraction V (BSA	Carl Roth GmbH & Co. KG, Karlsruhe
Ammonium persulfate (APS)	Carl Roth GmbH & Co. KG, Karlsruhe
Ammonium sulfate $(NH_4)_2SO_4$	Carl Roth GmbH & Co. KG, Karlsruhe
Antibiotic-Antimycotic	Life Technologies, Carlsbad, USA
Aprotinin	Carl Roth GmbH & Co. KG, Karlsruhe
Bromophenol blue	Sigma-Aldrich Co., St. Louis, USA
Calcium Chloride ($CaCl_2$)	Carl Roth GmbH & Co. KG, Karlsruhe
Chloroform	Carl Roth GmbH & Co. KG, Karlsruhe
Co-precipitant Pink	Bioline, Luckenwalde
Colorless co-precipitant	Bioline, Luckenwalde
Crystal violet	Sigma-Aldrich Co., St. Louis, USA
DePeX mounting media	VWR International GmbH
Diethylpyrocarbonate (DEPC)	Carl Roth GmbH & Co. KG, Karlsruhe
Dimethyl sulfoxide (DMSO)	AppliChem GmbH, Darmstadt
Dithiothreitol (DTT)	Carl Roth GmbH & Co. KG, Karlsruhe
DMEM, no Phenol Red	Life Technologies, Carlsbad, USA
DMEM/F-12, no Phenol Red	Life Technologies, Carlsbad, USA
dNTPs	Carl Roth GmbH & Co. KG, Karlsruhe
Ethanol absolute	Th. Geyer GmbH & Co. KG, Renningen
Ethidium bromide	Carl Roth GmbH & Co. KG, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH & Co. KG, Karlsruhe
Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N′*,*N′*-tetraacetic acid (EGTA)	Carl Roth GmbH & Co. KG, Karlsruhe
EDTA disodium salt (Na_2 EDTA)	Carl Roth GmbH & Co. KG, Karlsruhe
Fetal Bovine Serum (FBS)	Thermo Scientific HyClone, Logan, USA
Formaldehyde	Sigma-Aldrich Co., St. Louis, USA
Glycerol	Carl Roth GmbH & Co. KG, Karlsruhe
β-Glycerolphosphate disodium salt hydrate (BGP)	Sigma-Aldrich Co., St. Louis, USA
Glycine	Carl Roth GmbH & Co. KG, Karlsruhe
Glycylglycine	AppliChem GmbH, Darmstadt
Hydrochloric acid (HCl)	Carl Roth GmbH & Co. KG, Karlsruhe
Insulin	Sigma-Aldrich Co., St. Louis, USA
Isopropanol	Carl Roth GmbH & Co. KG, Karlsruhe
Magnesium chloride ($MgCl_2$)	Carl Roth GmbH & Co. KG, Karlsruhe
Magnesium sulfate	AppliChem GmbH, Darmstadt

MEM, no Glutamine, No Phenol Red	Life Technologies, Carlsbad, USA
Methanol	Carl Roth GmbH & Co. KG, Karlsruhe
M-MuLV Reverse Transcriptase Reaction Buffer	New England Biolabs, Frankfurt am Main
N-ethylmaleimide (NEM)	Sigma-Aldrich Co., St. Louis, USA
Nickel Chloride ($NiCl_2$)	Sigma-Aldrich Co., St. Louis, USA
NonidetTM P40 (NP-40)	Sigma-Aldrich Co., St. Louis, USA
Oil Red O	Sigma-Aldrich Co., St. Louis, USA
Opti-MEM	GIBCO®, Invitrogen GmbH, Darmstadt
PBS tablets	GIBCO®, Invitrogen GmbH, Darmstadt
Pefabloc SC Protease Inhibitor	Carl Roth GmbH & Co. KG, Karlsruhe
Penicillin-Streptomycin solution	Sigma-Aldrich Co., St. Louis, USA
Potassium acetate	Carl Roth GmbH & Co. KG, Karlsruhe
Potassium chloride (KCl)	AppliChem GmbH, Darmstadt
Potassium dihydrogen phosphate	Carl Roth GmbH & Co. KG, Karlsruhe
Potassium hydrogen phosphate	Carl Roth GmbH & Co. KG, Karlsruhe
RNase inhibitor	New England Biolabs, Frankfurt am Main
RNAiMAX	Invitrogen GmbH, Karlsruhe
Roti®-Phenol	Carl Roth GmbH & Co. KG, Karlsruhe
Rotiphorese® Gel 30	Carl Roth GmbH & Co. KG, Karlsruhe
Rotipuran® Chloroform	Carl Roth GmbH & Co. KG, Karlsruhe
Rotipuran® Isoamylalcohol	Carl Roth GmbH & Co. KG, Karlsruhe
SepharoseTM CL-4B	GE Healthcare, Uppsala, Sweden
Skim milk powder	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium acetate	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium azide	AppliChem GmbH, Darmstadt
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium citrate	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium deoxycholate	AppliChem GmbH, Darmstadt
Sodium Dodecylsulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium Fluoride (NaF)	AppliChem GmbH, Darmstadt
di-Sodium hydrogen phosphate dihydrate	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium pyruvate (Na-Pyr)	GIBCO®, Invitrogen GmbH, Darmstadt
SYBR Green I	Roche Diagnostics GmbH, Mannheim
TEMED	Carl Roth GmbH & Co. KG, Karlsruhe
α,α-Trehalose Dihydrate	USB Corporation, Cleveland, USA
Tris	Carl Roth GmbH & Co. KG, Karlsruhe
Triton X-100	AppliChem GmbH, Darmstadt
TRIzol® Reagent	Invitrogen GmbH, Karlsruhe
Trypsin-EDTA (0.05%)	GIBCO®, Invitrogen GmbH, Darmstadt
Tween-20	AppliChem GmbH, Darmstadt
Xylene	Carl Roth GmbH & Co. KG, Karlsruhe

2.3.2 Differentiation chemicals

Ascorbic acid	Sigma-Aldrich Co., St. Louis, USA
Calcitriol (1α,25-dihydroxy Vitamin D3)	Cayman chemicals, Ann Arbor, USA
Dexamethasone	Sigma-Aldrich Co., St. Louis, USA
ß-Glycerolphosphate (BGP)	Sigma-Aldrich Co., St. Louis, USA
Insulin	Sigma-Aldrich Co., St. Louis, USA
Isobuthylmetylxantine (IBMX)	Sigma-Aldrich Co., St. Louis, USA
Troglitazone	Sigma-Aldrich Co., St. Louis, USA

2.4 Kits and reagents

Alkaline phosphatase leukocyte kit	Sigma-Aldrich Co., St. Louis, USA
Bioanalyzer DNA High sensitivity kit	
Immobilon Western Chemiluminescent HRP Substrate	Millipore, Billerica, USA
LipofectamineTM 2000	Invitrogen GmbH, Karlsruhe
Lipofectamine RNAiMAX	Invitrogen GmbH, Karlsruhe
PageRulerTM Prestained Protein Ladder	Fermentas GmbH, St. Leon-Rot
PureYieldTM Plasmid Midiprep	Promega GmbH, Mannheim
QIAprep® Spin Miniprep Kit	Qiagen GmbH, Hilden
Qubit dsDNA HS assay	Invitrogen GmbH, Karlsruhe
SuperSignal® West Femto Maximum	Thermo Fisher Scientific, Waltham, USA

2.5 Nucleic acids

2.5.1 siRNA oligonucleotides

Target gene siRNA target sequence source

siRNAs	**Number**	**Catalog No.**	**Sequence**	**Source**
SSRP1	01	D-011783-01	GAUGAGAUCUCCUUUGUCA	Thermo Scientific Dharmacon
SSRP1	02	D-011783-03	GACUUAAACUGCUUACAAA	Thermo Scientific Dharmacon
SSRP1	03	D-011783-04	GCAAGACCUUUGACUACAA	Thermo Scientific Dharmacon
SSRP1	04	D-011783-	GAGGGAGGAGUACGGGAAA	Thermo Scientific

		17		Dharmacon
Luciferase GL2 Duplex	-	D-001100-01-20	CGUACGCGGAAUACUUCGA	Thermo Scientific Dharmacon

For transfections, the Dharmacon siRNAs (#1 - #4) were pooled in a 1:1:1:1 ratio.

2.5.2 Quantitative PCR (qPCR) primers

All the primers were designed using a primer designing tool from NCBI: (www.ncbi.nlm.nih.gov/tools/primer-blast/) in a 5' to 3' direction. The primers were ordered from Metabion AG, Martinisried and Sigma Aldrich, Hamburg.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Source
36B4	GATTGGCTACCCAACTGTTG	CAGGGGCAGCAGCCACAAA	Fritah et al., 2005
ALPL	TGGGCCAAGGACGCTGGGAA	AAGGCCTCAGGGGGCATCTCG	Karpiuk et al., 2012
AXIN2	ATTTCCCGAGAACCCACCGCCT	GGCTGTGGCGGCTCTCCAAC	Hossan et al., 2016
BGLAP	CACTCCTCGCCCTATTGGC	CTTGGACACAAAGGCTGCAC	Hossan et al., 2016
BMP4	GGAGCTTCCACCACGAAGAA	GGAAGCCCCTTTCCCAATCA	Hossan et al., 2016
DKK1	CGGGCGGGAATAAGTACCAG	TTTGCAGTAATTCCCGGGGC	Hossan et al., 2016
HNRNPK	ATCCGCCCCTGAACGCCCAT	ACATACCGCTCGGGGCCACT	Karpiuk et al., 2012
LEF1	GACGAGATGATCCCCTTCAA	CGGGATGATTTCAGACTCGT	Hossan et al., 2016
LRP4	ATAAGAAAGAGGGAGGGCCTGA	TGAGGTCATCCCACTCAGCA	Hossan et al., 2016
PDK4	TTCACTCCGCGGCACCCTCA	TCGGAGCAGAGCCTGGTTCCG	Karpiuk et al., 2012
PPARG	ACCTCCGGGCCCTGGCAAAA	TGCTCTGCTCCTGCAGGGGG	Karpiuk et al., 2012
RASD1	CAAGACGGCCATCGTGTCGCG	GCTGCACCTCCTCGAAGGAGTCG	Karpiuk et al., 2012
RUNX2	TCGGAGAGGTACCAGATGGG	CATTCCGGAGCTCAGCAGAA	Hossan et al., 2016
SP7	TTCTGCGGCAAGAGGTTCACTC	GTGTTTGCTCAGGTGGTCGCTT	Hossan et al., 2016
SSRP1	AAAGGTTCCATGAATGATGGTC	GCCTGGATGTTGTCCACTTT	Hossan et al., 2016
TCF7L2	CCTGGCACCGTAGGACAAAT	GGAACCTGGACATGGAAGCA	Hossan et al., 2016
VDR	TGGAGACTTTGACCGGAACG	GGGCAGGTGAATAGTGCCTT	Hossan et al., 2016
WISP2	CATGCAGAACACCAATATTAAC	TAGGCAGTGAGTTAGAGGAAAG	Johnsen et al., 2009
WNT11	GCCAAGTTTTCCGATGCTCC	GACACCCCATGGCACTTACA	Hossan et al., 2016
WNT2	GGATGCCAGAGCCCTGATGAATC	GCCAGCCAGCATGTCCTGAGAGT	Hossan et al., 2016

2.6 Proteins

2.6.1 Enzymes and substrates

Coelenterazine	Promega Corp. Madison, USA
Coenzyme A (CoA)	Sigma-Aldrich Co., St. Louis, USA
D-Luciferin	Synchem UG & Co. KG, Felsberg /

	Altenburg, Germany
Proteinase K	Invitrogen GmbH, Karlsruhe
Reverse Transcriptase (M-MuLV)	New England Biolabs, Frankfurt am Main
RNase A	Qiagen GmbH, Hilden
Taq DNA Polymerase	Prime Tech, Mink, Belarus

2.6.2 Antibodies

2.6.2.1 Primary antibodies

Following antibodies and dilutions were used for Western Blot (WB) and Immunofluorescence (IF) staining.

Target Protein	Clone	Cat No.	WB	IF	Source
β-actin	I-19	sc1616	1:5000	-	Santa Cruz
Active-β-catenin	D13A1	8814S	-	1:300	Cell Signaling
HSC70	B-6	sc7298	1:25000	-	Santa Cruz
SSRP1	3E4	609802	1:1000	1:300	Biolegend

2.6.2.2 Secondary antibodies for WB

Name	Dilution	Catalog No.	Source
Goat anti-mouse IgG-HRP	1:5000	sc-2005	Santa Cruz
Goat anti-rabbit IgG-HRP	1:5000	sc-2004	Santa Cruz

2.6.2.3 Secondary antibodies for IF

Name	Dilution	Reference No.	Source
Alexa Fluor®594 anti mouse-IgG	1:500	A11005	Life Technologies, Carlsbad, USA
Alexa Fluor®488 anti mouse-IgG	1:500	A11008	Life Technologies, Carlsbad, USA

2.7 Plasmids

Following plasmids were used in this study

Name	Vector	Working Concentration	Remarks	Source
super-TOP-FLASH	pTAluc	900 ng	Wnt reporter (8X TBE)	Veeman et al., 2003
super-FOP-FLASH	pTAluc	900 ng	Wnt reporter negative Control (8X mut TBE)	Veeman et al., 2003
pRenilla	pRL-CMV	100 ng	Luciferase control	Promega, Mannheim, Germany
β-Catenin S33Y	pCI-neo	500 ng	Mutated beta catenin	Kendziorra et al., 2011
pCI-neo	pCI-neo	500 ng	Empty vector	Promega(cat #E1841)

2.8 Cells

2.8.1 Human cell lines

Human fetal osteoblasts (hFOB1.17)	Prof. T. Spelsberg, Mayo Clinic, USA
Human mesencymal stem cells (hMSC)	Prof. M. Kassem, Odense University Hospital, Denmark
U20S	Prof. T. Spelsberg, Mayo Clinic, USA

2.8.2 Wnt3a producing cells

L-Cells	Prof. Tobias Pukrop, UMG, Göttingen
L-Cells Expressing Wnt3a	Prof. Tobias Pukrop, UMG, Göttingen

2.9 Buffers and solutions

RIPA buffer

PBS	1X
NP-40	1%
Sodium deoxycholate	0.5%
SDS	0.1%

TBS-T 10X (pH 7.6)

Tris	0.1 M
NaCl	1.5 M
Tween-20	0.5%

Western salts 10X

Tris	0.25 M
Glycine	0.86 M
SDS	0.7 M

PBS-T 10X (pH 7.4)

NaCl	0.73 M
KCl	0.027 M
$NaH_2PO_4 * 7H_2O$	14.3 mM
KH_2PO_4	14.7 mM
Tween-20	1%

PBS for cell culture

1 PBS tablet per 500 ml distilled H2O

PBS-T

PBS including 0.1% (w/v) Tween-20

PCR-Mix 10X

Tris-HCl (pH 8.8)	750 mM
(NH4)2SO4	200 mM
Tween-20	0.1%

RT-PCR master mix

PCR-Mix 10X	1X
MgCl2	3 mM
SYBR Green	1:80000
dNTPs	0.2 mM
Taq-polymerase	20U/ml
Triton X-100	0.25%
Trehalose	300 mM

Blocking solution

Skim Milk	5%
TBST	1X

Cell culture freezing medium

DMEM	42%
FBS	50%
DMSO	8%

Protease, phosphatase and deubiquitinase inhibitors

Pefabloc	1 mM
Aprotinin/Leupeptin	1 ng/µl
BGP	10 mM
NEM	1 mM
IAA	10 mM
$NICl_2$	1 mM

DMEM/F12 cell culture medium

Phenol red-free high-glucose DMEM/F12 with 10% FBS, 1× Penicillin/Streptomycin

DMEM cell culture medium

Phenol red-free, high-glucose DMEM, 10% BGS, 1× Penicillin/Streptomycin

MEM cell culture "normal" medium: phenol red-free, high-glucose MEM, 10% BGS, 1× Antibiotic-Antimycotic solution

MEM cell culture "adipocyte" medium: phenol red-free, high-glucose MEM, 15% FBS, 1× Antibiotic-Antimycotic solution, 10 nM dexamethasone, 0.45 mM isobutyl-methyl-xanthine, 2 µM insulin, 10 µM Troglitazone.

MEM cell culture "osteoblast" medium: phenol red-free, high-glucose MEM, 10% FBS, 1× Antibiotic-Antimycotic solution, 10 nM dexamethasone, 10 mM ß-glycerol phosphate (BGP), 0.2 mM ascorbic acid, 10 nM calcitriol.

Ascorbic acid stock solution (1000x)

0.2 M ascorbic acid in sterile water

Calcitriol stock solution (1000x)

10 µM calcitriol in 100% DMSO

Dexamethasone stock solution (1000x)

100 µM dexamethasone in 100% EtOH

β-glycerol phosphate (BGP) stock solution (100x)

1 M BGP in sterile water

IBMX stock solution (100x)

0.45 M isobutyl-methyl-xanthine in 100% EtOH

Troglitazone stock solution (1000x)

10 mM Troglitazone in 100% DMSO

6x Laemmli buffer

Tris (pH 6.8)	0.35 M
Glycerol	30%
SDS	10%
DTT	9.3%
Bromophenol Blue	0.02%

SDS separating gel (X%)

Acrylamide	X%
Tris-HCl (pH8.8)	375 mM
SDS	0.1%
APS	0.1%
TEMED	0.04%

SDS stacking gel (5%)

Acrylamide	5%
Tris-HCl (pH6.8)	125.5 mM
SDS	0.1%
APS	0.1%
TEMED	0.1%

TAE buffer (50x)

Tris	2 M
Acetic acid	1 M
EDTA	0.1 M

Transfer buffer

10X Western Salts	10%
Methanol	15%

Firefly buffer (pH 8.0)

Glycylglycine	25 mM
K_2HPO_4	15 mM
EGTA	4 mM

Renilla buffer (pH5.1):

NaCl	1.1 M
Na_2EDTA	2.2 mM
K_2HPO_4	0.22 M

Firefly working reaction buffer

Per 10 ml firefly buffer following reagents were added

$MgSO_4$	15mM
ATP pH 7.0	4 mM
DTT	1.25 mM
CoA	0.1 mM
Luciferin	80 µM

Renilla working reaction buffer

Per 10 ml renilla buffer following reagents were added

BSA	0.5 mg/ml
NaN_3	1.5 mM
Coelenterazine	1.5 µM

3. METHODS

3.1 Cell culture

3.1.1 Culture conditions for hMSC, hFOB and U2OS

hMSCs were cultured in low glucose Minimum Essential Media (MEM) without glutamine and Phenol Red supplemented with 10% FBS, 1× Antibiotic-Antimycotic at 37°C with 5% CO_2. To induce adipocytic differentiation cells were cultured in presence of 15% FBS, 10 nM dexamethasone, 0.45 mM isobutyl-methyl-xanthine, 2 µM insulin, 10 µM Troglitazone and 1× Antibiotic-Antimycotic solution. For osteoblast differentiation the cells were cultured in the media contained 10% BGS, 10 nM dexamethasone, 10 mM ß-glycerol phosphate (BGP), 0.2 mM ascorbic acid, 10 nM calcitriol and 1× Antibiotic-Antimycotic solution.

hFOB 1.17 cells were cultured in high glucose, phenol red free DMEM/F-12 supplemented with 10% Fetal Bovine Serum (FBS) and 1× Antibiotic-Antimycotic at the permissive temperature 34°C with 5% CO_2 supply. For induction of the differentiation to osteoblasts cells were cultured in the same medium with the osteoblast differentiation factors at 39°C (restrictive temperature) with 5% CO_2.

U2-OS cells were cultured in high glucose, phenol red free DMEM supplemented with 10% FBS and 1× Penicillin/Streptomycin at 37°C with 5% CO_2.

3.1.2 Cell culture for obtaining Wnt3a containing conditioned media

L-cells and L-cells expressing Wnt3a protein were used to obtain the conditioned medium. For maintaining the cells in the culture phenol red-free, high-glucose DMEM supplemented with 10% FBS, 1% sodium pyruvate and 1X Penicillin/Streptomycin media was used at 37°C with 5% CO_2.

For obtaining the conditioned medium, cells were split into 1:10 in 15 mL culture medium using 20 cm culture plate. The cells were grown for 4 days to attain an approximate confluency. Then the medium were taken off and filtered using 0.20 µm sterile filters. These were considered as the first batch of the conditioned medium. The cells were cultured for another 3 days and then the second batch of the

conditioned medium was collected. The first and second batch were then mixed as 1:1 ratio from the respective plates. These medium contained Wnt3a protein.

3.1.3 siRNA mediated reverse transfection

siRNA mediated reverse transfection was performed using Lipofectamine TM RNAiMAX according to the manufacturer's instructions. For transfection in 6-well plate 30 pmol corresponding siRNAs were used in 500 µl Opti-MEM along with 5 µl of LipofectamineTM RNAiMAX. Then the mix was incubated in room temperature for 20 minutes. In the meantime, cells were trypsinized and counted using Neubauer counting chamber. Then, approximately 250,000 cells were used for the transfection in 6-well plate for hMSC and U2-OS, whereas 300,000 cells were used in case of hFOB. During transfection the media without antibiotic was used. After 24 hours of transfection the media with antibiotics was used along with the respective differentiation media. For trnasfcetion into 12 well as well as 24 well plate, the transfection reagents were scaled and calculated accordingly.

3.1.4 Plasmid DNA transfection

For transfections into 24-well format approximately 1.9 µg of total plasmids DNA were diluted in 50 µl of OptiMEM media. In a separate tube, 3 µl of Lipofectamine TM 2000 were diluted in a total volume of 50 µl of OptiMEM. Both solutions were incubated at room temperature (RT) for 15 min followed by mixing and additional incubation for 20 min at RT. After that a total mix of 100 µl was added to the cells grown in media without antibiotics. 5 h after transfection the cells were washed with PBS and antibiotic containing media was added.

3.1.5 Cell proliferation assay

Cell proliferation assay was performed by crystal violet staining. Cells were washed twice with 1X PBS followed by incubation in crystal violet staining solution containing 0.1% (w/v) crystal violet, 10% (v/v) formaldehyde for 10 min at RT. Then the cells were washed gently with distilled water. Finally cells were kept for drying and the whole plate was scanned using Epson 1680 in 300 dpi resolution at 48-bit RGB color.

3.2 Molecular Biology

3.2.1 RNA isolation

RNA isolation was performed using QIAzol® reagent according to the manufacturer's instructions. Cells were washed twice with PBS, lysed by adding 500 µl of QIAzol® reagent to each well (6-well format) and scraped into 1.5 ml tubes. 100 µl of chloroform was added to the samples followed by vortexing for 20 sec and then centrifuged at 10,000g for 20 min (4°C). The aqueous phase was collected into a fresh 1.5 ml tube and chloroform extraction was performed again followed by 2 h or overnight isopropanol precipitation at -20°C. After that, samples were centrifuged at maximal speed of 12,000g for 30 min (4°C), pellets were washed twice with 70% ethanol, dried on vacuum concentrator and re-dissolved in 40 µl of DEPC water. RNA concentration was measured using a NanoDrop.

3.2.2 cDNA synthesis

For DNA synthesis 1 µg of total RNA was mixed with 2 µl of 15 µM random primers and 4µl of 2.5 mM dNTP mix and incubated 5 min at 70°C. After that 4 µl of reverse transcription master mix containing 2 µl 10x reaction Buffer, 10 units of RNAse Inhibitor, 25 units of Reverse Transcriptase and 1.625 µl of DEPC water were added to each sample. cDNA synthesis was performed at 42°C for 1 h followed by enzyme inactivation for 5 min at 95°C. Finally, samples were brought to 50 µl volume by adding DEPC water.

3.2.3 Quantitative real-time PCR

For quantitative real-time PCR 1 µl of cDNA sample was used in a final reaction volume of 25 µl. A PCR reaction contained 75 mM Tris-HCl (pH 8.8), 20 mM (NH4)2SO4, 0.01% Tween-20, 3 mM MgCl, 200 µM dNTPs, 0.5 U/reaction Taq DNA Polymerase, 0.25% Triton X-100, 1:80,000 SYBR Green I, 300 mM Trehalose and 30 nM primers.

The two-step PCR protocol used was setup as follows:

95° C – 2 min

95° C- 15 Sec
60° C- 1 min } X 40

The PCR reaction was followed by a melting curve analysis from 60°C to 95°C with read every 0.5°C.

cDNA samples were quantified using a standard curve made from all cDNA samples. Quantified samples were normalized to 36B4 (RPLPO) or HNRNPK as an internal reference gene. The expression levels were determined relative to the vehicle treated control sample and expressed as “relative mRNA expression”. ANOVA-single factor test was used for analyzing statistical significance.

3.2.4 RNA-sequencing and analysis

RNA-sequencing was performed at the sequencing facility by Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany.

RNA integrity was checked using Bioanalyzer 2100 (AgilentTechnologies, Palo Alto, CA, http://www.agilent.com), and 1 µg of total RNA was prepared for sequencing using the NEBNext Ultra RNA Preparation kit for Illumina (New England Biolabs) according to the manufacturer’s instructions. 300 bp size range was confirmed using Bioanalyzer. Then, amplification and cluster generation of these samples were done using cBot with 50 cycles and 50 bp single end sequencing was performed on an Illumina HiSeq 2500. Fastq files were used for mapping to the human reference transcriptome (UCSC HG19) using Bowtie 2 (version 2.1.0). Read counts for each sample and gene were counted using a custom Ruby script. Differential expression was analyzed by DESeq (version 1.14.0 (Anders and Huber, 2010). R-based heatmap.2 in gplots package was used for generating heatmaps. Threshold levels for fold changes in upregulated and downregulated genes were log2FC ≥ 0.585 and logFC ≤ -0.515. Adjusted p value (pAdj) was calculated according to Benjamini Hochberg. pAdj ≤ 0.05 was used as the significance level. The online gene ontology (GO) resource DAVID (Huang et al., 2009, 2009) was used to analyze the SSRP1-dependent genes with the cutoff values as used for

generating heatmap. A list of Wnt signaling genes was made from KEGG (Kanehisa and Goto, 2000; Kanehisa et al., 2016) and NetPath (Kandasamy et al., 2010) database. Each of the genes from the gene list was checked for their log2fold expression changes in RNA-seq data with a significance level pAdj ≤ 0.05. Then a heatmap was generated by heatmap 2.

The Wnt-signaling pathway map was drawn by the CellDesigner software (Funahashi et al., 2003, 2008). Numerical values of log-fold changes were used from RNA-seq data. The threshold levels were from +1 [log2(2) = 1] to -1 [log2(0.5) = -1]. The expression values below -1 or above +1 were assigned for the most intense color codes. The nodes were colored in Cytoscape (Shannon et al., 2003) using BiNoM plugin.

3.2.5 Dual luciferase assay

For dual luciferase reporter assay, the hFOB or U2-OS cells were transfected with control and SSRP1 siRNAs as described in section 3.1.3. 24 h after transfection the media was changed with antibiotic. After 48 h the cells were cotransfected with Super-TOP- or Super-FOP-FLASH plasmids (Veeman et al., 2003) (900 ng per well) containing eight copies of a wild-type or mutated TCF/Lef binding site. An internal control plasmid expressing Renilla luciferase (100 ng) (pRL-CMV; Promega) was also cotransfected at the same time. Where indicated, cells were additionally transfected with a control vector (pCI-neo; Promega) or a vector expressing a constitutively active form of β-catenin containing an S33Y mutation (Kendziorra et al., 2011). Then after 4 h of plasmids DNA transfection the cells were washed once with 1XPBS followed by the media change with antibiotic. Additionally, Wnt3a containing conditioned media was diluted with normal cell culture media as 3:1 and then was used in the indicated wells to induce Wnt-signaling pathway. The dual luciferase reporter assay was performed as described by Dyer et al., (Dyer et al., 2000). However, the measurement setup was as follows:

- Dispense > Injector, Volume (100 µl), Speed (Middle), by plate/by well.
- Delay > 2 s
- Measurement > 10 s

3.3 Protein biochemistry

3.3.1 SDS-PAGE

SDS-PAGE was used for separating proteins using denaturing agent SDS (sodium dodecylsulfate) on a polyacrylamide gel upon electrophoresis (Laemmli, 1970). Proetin samples were prepared by cell lysis in RIPA buffer containing the protease inhibitors 1 mM Pefabloc, 1 ng/µl Aprotinin/Leupeptin, 10 mM BGP and 1 mM NEM. Genomic DNA was sheared by sonication where samples were sonicated for 10 sec at 10% power using a tip sonicator. Before loading, protein samples were boiled in Laemmli buffer for 10 min at 95°C and then subjected to SDS-PAGE. The composition of stacking and resolving gel is described in section 2.8. Polyacrylamide gels were run in SDS running buffer at 20 mA/gel.

3.3.2 Western blot analysis

After electrophoresis, proteins of interest were separated and detected according to their molecular weight using specific antibodies (Towbin et al., 1979). Separated proteins were transferred to PVDF nitrocellulose membranes at 100 V using transfer buffer for 90 min, depending on the size of the protein. The membranes were incubated in 1X TBST 5% (w/v) milk for 1 h to block non-specific antibody binding. Afterwards the membranes were incubated for 1 h at room temperature or overnight at 4°C in the same blocking buffer containing the respective primary antibodies, diluted as described in the antibody table (2.6.2.1). After that the membranes were washed twice with 1X TBS-T followed by the incubation for 1 h with the corresponding horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies in appropriate dilutions. Further the membranes were washed twice by 1X TBS-T and the HRP signals were detected using enhanced chemoluminescence and exposed to X-ray films.

3.3.3 Immunofluorescence

For immunofluorescence staining, the cells grown on chamber slides, washed with 1X PBS and fixed with 4% paraformaldehyde for 15 min at RT. Cells were washed twice with 1X PBS and permeabilized using 0.1% Triton X-100 for 10 min. After washing twice with 1X PBS, cells were blocked with 10% FBS for 10 min

followed by overnight incubation with primary antibody dilution in 3% BSA. Next day, unbound antibodies were washed away by washing twice with 1X PBS and incubated with Alexa-488 or Alexa-594 conjugated secondary antibodies. Cells were washed twice with 1XPBS and stained for DAPI and mounted with coverslips using mounting medium. Images were taken using LSM 510 META confocal microscope and analyzed using the LSM Image Browser.

3.4 Staining

3.4.1 Alkaline phosphatase staining

Osteoblast differentiation efficiency was determined by staining for alkaline phosphatase activity. Staining was performed with alkaline phosphatase kit for leukocytes according to manufacturer's instructions. All steps were performed at RT. First, cells were washed with PBS and fixed for 30 sec with Citrate fixing solution containing (for 98 ml): 66 ml acetone, 25 ml Citrate solution and 8 ml 37% formaldehyde. After fixation, cells were washed three times with distilled water and incubated in dark for 15 min with diazonium salt followed by rinsing with distilled water and drying. Diazonium salt preparation: 1 ml of FRV-Alkaline solution was mixed with 1 ml of sodium nitrate solution and incubated for 2 min. Then 45 ml of distilled water and 1 ml of Naphtol AS-BI Alkaline solution were added to the mix. Diazonium salt was prepared freshly before each staining. Microscopic images were taken by a Zeiss Axiovert 40 using 10X magnification.

3.4.2 Oil Red O staining

Presence of lipid droplets indicative for adipocyte differentiation efficiency were detected by Oil Red O staining. All steps were performed at RT. Oil Red O working solution was prepared by mixing 3 parts of Oil Red O stock solution (300 mg/ml of Oil Red O powder in 99% isopropanol) with 2 parts of distilled water and incubated for 10 min followed by filtration. Cells were washed with PBS, fixed with 10% formaldehyde for 30 min and incubated with 60% isopropanol for 5 min. Then, cells were stained with Oil Red O working solution for 5 min in dark followed by rinsing with distilled water and drying. Pictures of the stained plates were taken under light microscope using 20X magnification.

4. Results

Stem cells and stem cell like cells which can differentiate into multiple cell lineages undergo constant and dynamic changes of the native chromatin structure where different chromatin remodeling factors including histone chaperones could be an essential part. Though the knowledge on functions of histone chaperone during cellular differentiation is limited, but it has been started to be revealed. For instance, the histone chaperone ASF1A was shown to be required for maintenance of pluripotency and cellular reprogramming (Gonzalez-Muñoz et al., 2014) for human adult dermal fibroblasts (hADFs). Another report showed the histone chaperone SUPT6H in regulating muscle gene expression and cellular differentiation through demethylation of H3K27me3 marks (Wang et al., 2013). The fundamental functional aspects of histone chaperones in dynamic remodeling of chromatin suggest their essential role in cellular differentiation. It is likely they could also promote lineage specificity from a specific stem cell where differentiation to one cell type could be conducted through the inhibition of differentiation to the other types. Indeed, recent evidences suggest the lineage specific function of some of the histone chaperones. HIRA and ASF1 were reported to be diminished more than 50% during osteoblast differentiation from C2C12 cells, while they were maintained through the entire period of myotube differentiation (Song et al., 2012b). Interestingly, suppression of the CAF-1 promoted the enhanced conversion of B cells into macrophages and fibroblasts into neurons (Cheloufi et al., 2015). CHAF1A is a component of CAF-1, which was reported to block the differentiation of embryonal neural crest differentiation which ultimately results to the pathogenesis of high-risk neuroblastoma (Barbieri et al., 2014). Overall it is apparent that modulating chromatin state through the histone chaperones could be an important aspect of cellular differentiation or lineage specificity. In this study we investigated the lineage specific role of the histone chaperone component SSRP1.

4.1 SSRP1 depletion enhances adipocytes differentiation

In order to understand the effects of SSRP1 on adipocyte differentiation potency of hMSC we performed siRNA mediated knockdown followed by a 7 days of differentiation toward adipocytes by the differentiation mix containing dexamethasone, insulin (inhibitor of lipolysis), isobutylmethylxantine (enhancer of

cAMP signalling) and troglitazone (agonist of PPARG receptor). The smart pool siRNA was used to perform SSRP1 knockdown. The knockdown efficiency was tested in protein as well as mRNA level (Figure 7A, 7C).

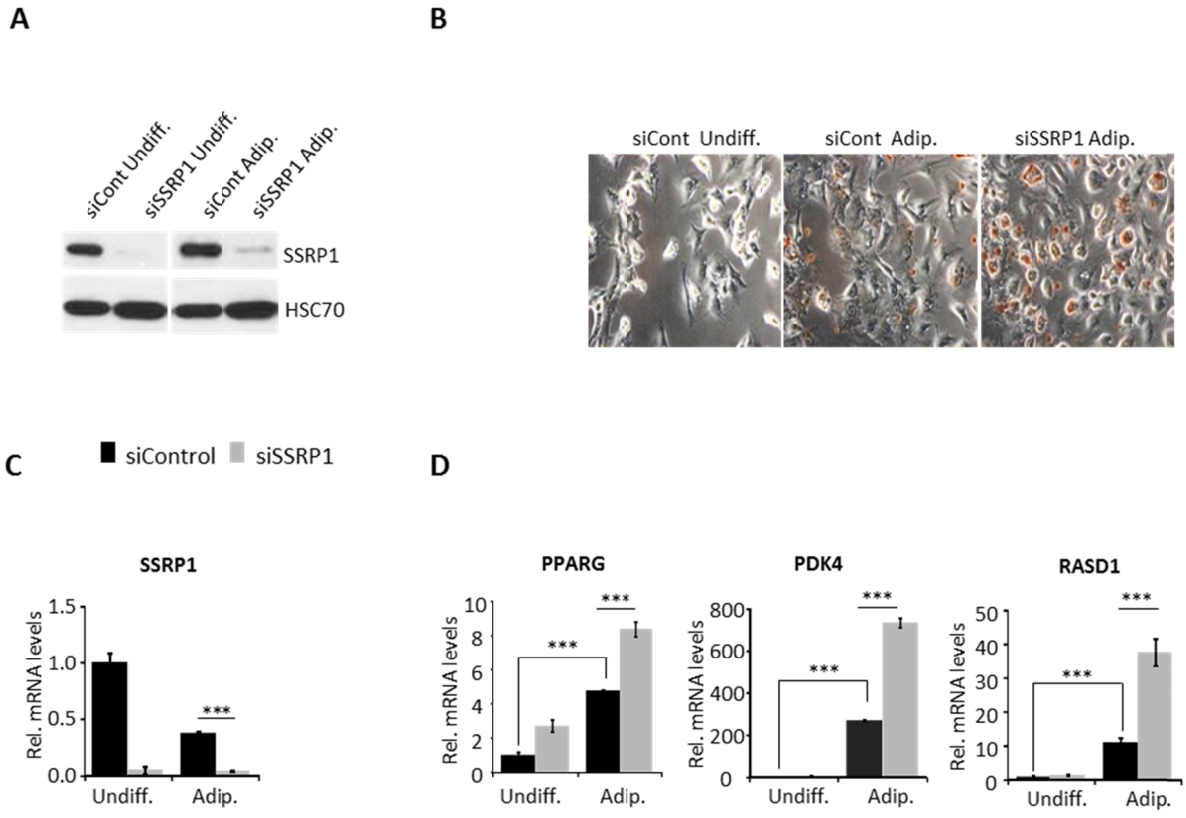


Figure 7. SSRP1 depletion promotes adipocyte differentiation. (A) Human Mesenchymal Stem Cells (hMSCs) were transfected with control or SSRP1 siRNAs followed by an induction of differentiation into adipocytes (Adip.) for 7 days. Undifferentiated state is labeled as Undiff. Protein extracts were analyzed by Western blotting using the antibodies against SSRP1 and HSC70 showing the knockdown of SSRP1. (B) Adipocyte differentiation was verified using Oil Red O staining after transfection of control and SSRP1 siRNAs. (C, D) Real time PCR analyses were performed for the gene expression of *SSRP1* (to show effective knockdown) and adipocyte differentiation specific genes including *PPARG, PDK4* and *RASD1*. Transcript levels were normalized to an internal reference gene *hnRNPK* and denoted relative to the undifferentiated (Undiff.) control. Data are represented as mean ± SD (n = 3). ***p ≤ 0.001. Microscopic images were taken using a Zeiss Axiovert 40 using 20X magnification.

The effective knockdown of SSRP1 promoted adipocytes differentiation confirmed by Oil Red O staining and gene expression study for the adipocyte differentiation marker genes (Figure 7B, 7D). Oil Red O staining demonstrate the lipid droplets or neutral fats in the cultured cells or tissue section. The basic principle of staining solution is based on its greater solubility of the dye in the neutral fats or

lipid droplets than the solvent used to dissolve the dye. Thus when there are lipid droplets present in the cells dye dissolve there and turn into red color. We found an increased number of red droplets indicative for the presence of fat (Figure 7B). Further the gene expression study using commonly used adipocyte differentiation marker genes such as *PPARG, PDK4, RASD1* also showed a significant increase in their expression following SSRP1 knockdown and adipocyte differentiation for 7 days (Figure 7D). Notably, the nuclear receptor peroxisome proliferator activated receptor gamma (*PPARG*) is one of the key regulators of adipocyte differentiation (Chawla et al., 1994) both in vitro and in vivo (Barak et al., 1999; Rosen et al., 1999). Moreover increased *PDK4* expression was reported to be correlated with higher *PPARG* expression (Holness et al., 2012). *RASD1* is another important protein to promote adipogenesis confirmed in both in vivo and in vitro (Cha et al., 2013; Karpiuk et al., 2012; Kemppainen and Behrend, 1998)

4.2 SSRP1 depletion decreases osteoblast differentiation

Next, we investigated the effects of SSRP1 on osteoblast differentiation. For this purpose, we used hMSC, performed siRNA mediated knockdown and induced osteoblast differentiation for 7 days with the differentiation mix containing calcitriol (vitamin D, nuclear receptor ligand) dexamethasone (glucocorticoid receptor agonist), ascorbate (vitamin C, required for collagen synthesis) and β-glycerophosphate (required for mineralization of collagen matrix). The efficient knockdown was demonstrated by western blot using antibody against SSRP1 (Figure 8A). Alkaline phosphatase staining was used as readout for differentiation efficiency (Figure 8B). Alkaline phosphatase is a well-known marker for osteoblast differentiation and is required for the mineralization and calcification. Thus the osteoblasts expressing alkaline phosphatase can convert certain chemicals into violet color indicative for the differentiation efficiency.

The finding was further confirmed by the gene expression analysis using qRT-PCR. Some of the key osteoblast differentiation marker genes such as *ALPL, BGLAP, RUNX2* and *SP7* were examined. All of them showed a significant decreased expression following SSRP1 knockdown (Figure 8C). Together these data demonstrate a role of SSRP1 in osteoblast differentiation.

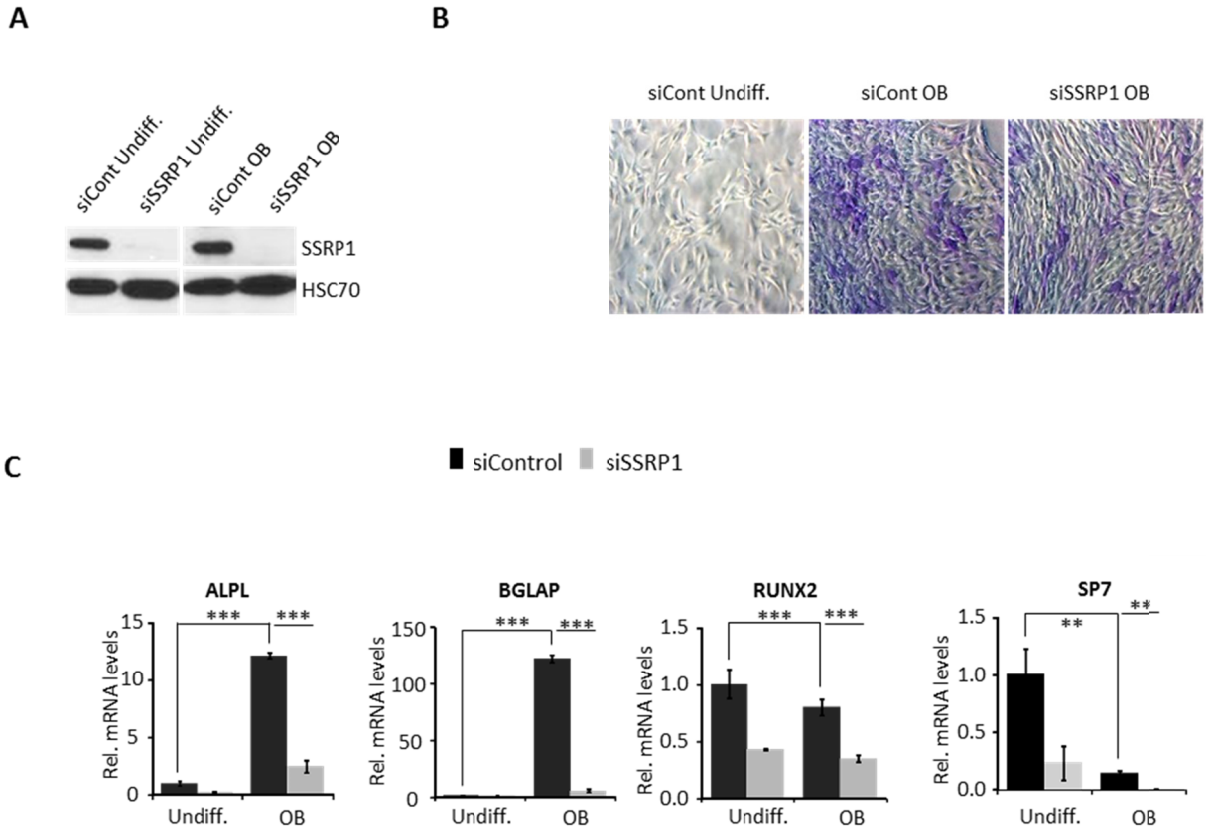


Figure 8. SSRP1 depletion decreases osteoblast differentiation. (A) hMSCs were transfected with control or SSRP1 siRNAs followed by an induction of differentiation into osteoblast (OB) for 7 days. Undifferentiated state is labeled as Undiff. Protein extracts were analyzed by Western blotting using the antibodies against SSRP1 and HSC70 showing the knockdown efficiency of SSRP1. (B) Osteoblast differentiation was verified using Alkaline Phosphatase staining after transfection of control and SSRP1 siRNAs. (C) Real time PCR analyses were performed for the gene expression of osteoblast differentiation specific genes including *ALPL, BGLAP, RUNX2* and *SP7*. Transcript levels were normalized to an internal reference gene hnRNPK and denoted relative to the undifferentiated (Undiff.) control. ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05. The data are represented as mean ± SD (n = 3). Microscopic images were taken using a Zeiss Axiovert 40 using 10X magnification.

4.3 SSRP1 knockdown alters cell morphology

While performing experiments for demonstrating the role of SSRP1 in adipocytes and osteoblasts differentiation, we observed an altered morphology of the hMSC cells in undifferentiated state after 24 h of SSRP1siRNA mediated transfection. The cells displayed an elongated shape with multiple nuclei. Given the fact that hMSCs have the capacity to differentiate into a number of cell lineages we hypothesized the elongated cells may differentiate to the myoblast lineage. Indeed hMSCs can also be differentiated into muscle cells under specific conditions. The microscopic images of the observed phenotype are displayed on figure 9A. Further

we used 10% horse serum to the normal culture media and incubated the cells in incubator for 7 days, then checked the expression of different myoblast differentiation-specific genes including *MYOG, MYH1* and *MEF2A*. The elevated expression of *MYOG* and *MYH1* were observed in SSRP1 depleted undifferentiated state. Nevertheless, knockdown of SSRP1 on the mentioned myoblast specific genes were surprisingly blocked under myoblast differentiation conditions (Figure 9B).

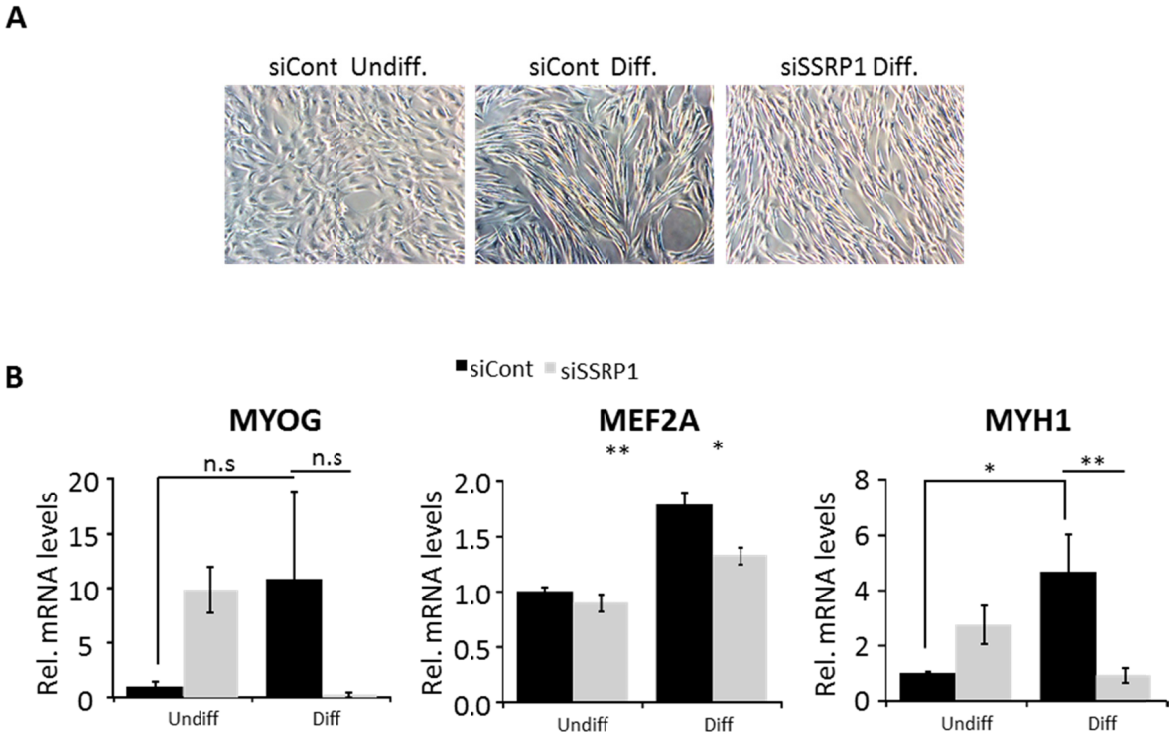


Figure 9. SSRP1 depletion affects myoblast phenotype. (A) hMSCs were transfected by control and SSRP1 siRNAs followed by differentiation for 7 days using 10% horse serum. Undifferentiated state is labeled as Undiff. Microscopic images were taken using a Zeiss Axiovert 40 using X10 magnification. (B) Real time PCR analyses were performed for the gene expression of myoblast differentiation specific genes including *MYOG, MEF2A* and *MYH1*. Transcript levels were normalized to an internal reference gene hnRNPK and denoted relative to the Undiff control. ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05. The data are represented as mean ± SD (n = 3).

4.4 SSRP1 affects osteoblast differentiation in hFOB cells

The human fetal osteoblast (hFOB 1.19) cell line has been used widely to investigate osteoblast differentiation and function in vitro. hFOB also shares some common features resemble to hMSCs (Panaroni et al., 2014). Both of the cell lines have the capacity to proliferate and differentiate into osteoblasts under the specific culture conditions. Moreover, hFOB has the characteristics of osteoprecursor cells

that are more advanced in their osteogenic differentiation compared to hMSCs, making hFOB as an interesting biological tool to study bone development as well different bone-related diseases (Krattinger et al., 2011; Panaroni et al., 2014). Thus, we investigated whether SSRP1 also influences osteoblast-specific gene expression in hFOB cells. At first, we examined alkaline phosphatase activity of hFOB cells after 7 days of differentiation following siRNA-mediated knockdown of SSRP1. Consistent with the previous results in hMSC, hFOB cells also showed a drastic decrease in alkaline phosphatase activity (Figure 10B).

We also examined the mRNA expression level of different osteoblast specific genes including *ALPL, RUNX2* and *VDR* following siRNA transfection and differentiation to osteoblasts (Figure 10C). We found a marked decrease in the expression level of *ALPL, RUNX2* and *VDR* following SSRP1 depletion and osteoblast differentiation.

4.5 SSRP1 knockdown shows dramatic and specific changes in gene expression during osteoblast differentiation

Next, we wanted to determine the extent to which SSRP1 directs transcriptional reprograming during osteoblast differentiation. We performed transcriptome-wide RNA sequencing in hFOB cells following SSRP1 depletion. At first we checked the variability between the samples of different experimental conditions. Principle component analysis (PCA) was used to measure the variability within and between the samples of each condition. 2 replicates of each of the 3 biological conditions termed as undifferentiated control (UND_ConSi), osteoblast differentiated control (OST_ConSi) and osteoblast differentiated SSRP1 knockdown (OST_SSRP1) were used for high throughput sequencing. Figure 11 A shows the distance between the samples of each condition and Figure 11B shows the heatmap which were generated from the log2-transformed data. The results showed minimal or no variance between the samples of respective conditions.

Then we investigated the impact of SSRP1 depletion on osteoblast differentiation. We produced a heatmap to show the pattern of differentially expressed genes during osteoblast differentiation. The selection criteria of the genes were based on the fold of regulation. Threshold levels for fold changes in

upregulated and downregulated genes were log2 FC ≥ 0.585 and log2 FC ≤ -0.515 respectively.

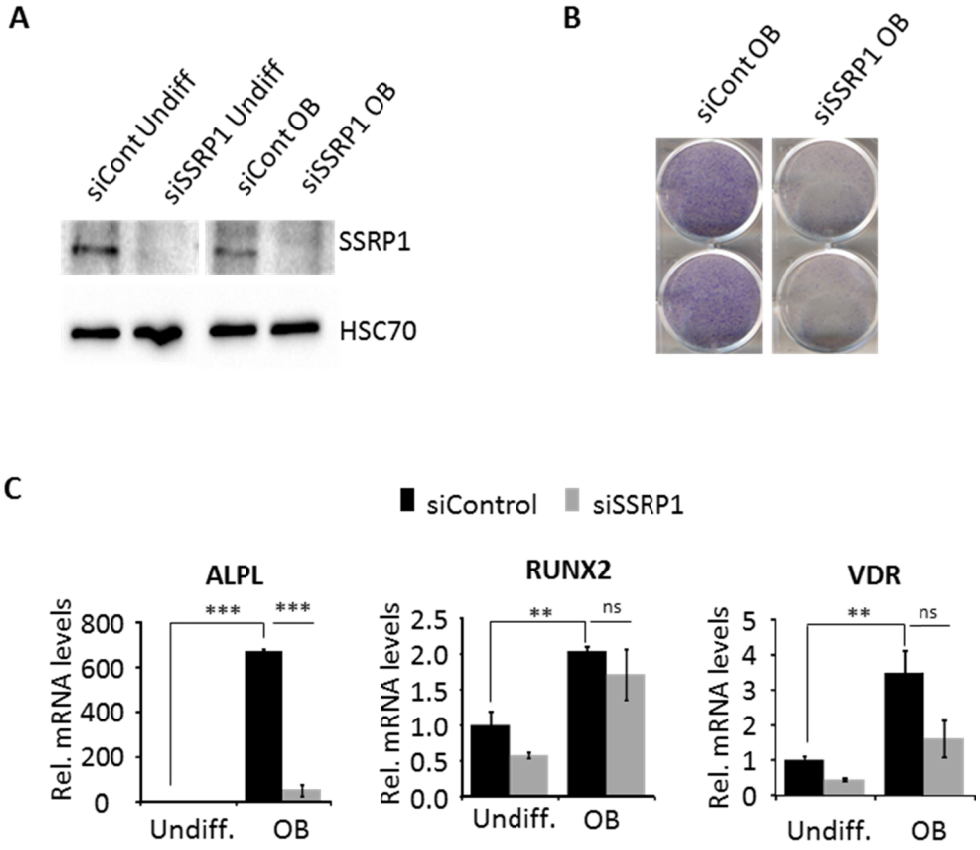
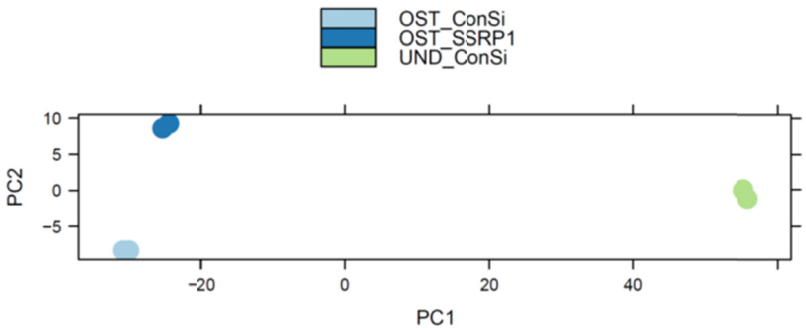


Figure 10. SSRP1 depletion decreases osteoblast differentiation in hFOB. (A) hFOBs were transfected by control and SSRP1 siRNAs followed by an induction of differentiation into osteoblast (OB) for 7 days. Undifferentiated state is labeled as Undiff. Protein extracts were analyzed by Western blotting using the antibodies against SSRP1 and HSC70 showing the knockdown efficiency of SSRP1. (B) Osteoblast differentiation was verified using Alkaline Phosphatase staining after transfection of control and SSRP1 siRNAs. An Epson 1680 scanner was used to scan the plate using 300 dpi resolutions at 48-bit RGB color. (C) Real time PCR analyses were performed for the gene expression of osteoblast differentiation specific genes including *ALPL, RUNX2* and *VDR*. Transcript levels were normalized to an internal reference gene RPLP0 (36B4) and denoted relative to the undifferentiated (Undiff.) control. ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05, n.s non significance. The data are represented as mean ± SD (n = 3).

Adjusted value (pAdj) was calculated according to Benjamini Hochberg. pAdj ≤0.05 was used as the significance level. We observed a block in the changes of gene expression following SSRP1 depletion.

A



B

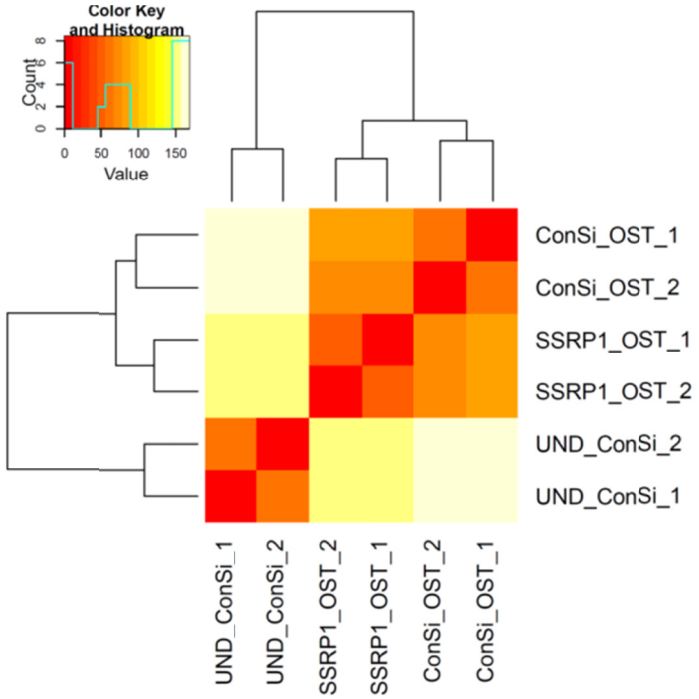


Figure 11. Plots showing the variances between the replicates. (A) PCA plot displaying the distances between samples of each condition. The whole transcriptome corresponding undifferentiated and osteoblast differentiated genes after transfections with control and SSRP1 is represented as dot plot. Each dot shows one of two RNA-seq samples of corresponding experiment conditions. Normalized gene expression counts were calculated for the respective samples and conditions and then plotted as principle component 1 and 2 (PC1 and PC2). (B) Heatmap generated from the normalized gene counts to show the correlation between the replicates of each condition. The highly saturated red color indicates higher correlation.

The genes which were expressed during osteoblast differentiation were largely inhibited in this induction/repression by SSRP1 depletion (Figure 12). This result shows a significant change in the gene expression profile during osteoblast differentiation following SSRP1 depletion.

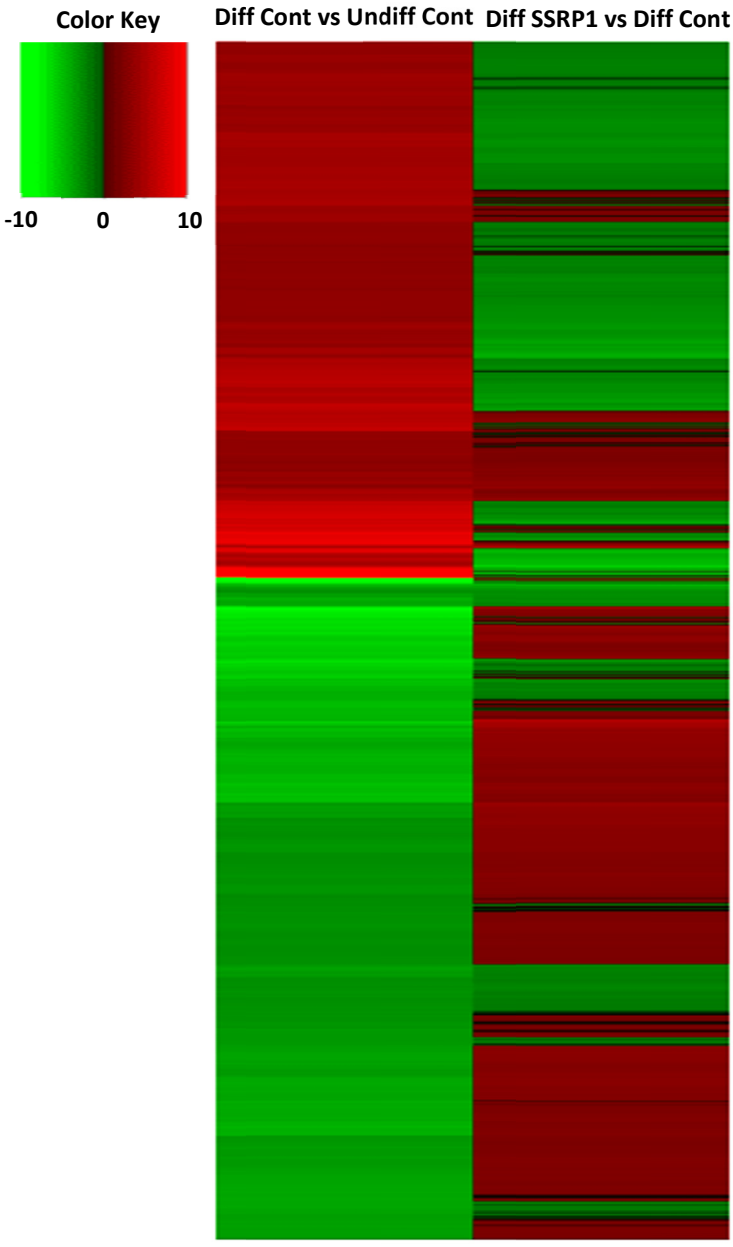


Figure 12. SSRP1 depletion displayed opposing effects in gene expression during OB differentiation. Heatmap analysis of RNA-seq data denoting log2-fold changes in gene expression in differentiated control-transfected hFOB cells vs. undifferentiated cells (left) or differentiated SSRP1-depleted cells vs. differentiated control transfected cells (right).

4.6 Gene ontology (GO) analysis reveals impact of SSRP1 on bone-related important biological processes

Next we directed to find out the biological processes that are affected by SSRP1 knockdown during osteoblast differentiation. For this purpose we used the online GO software DAVID. Importantly we found a number of biological processes and pathways that are enriched following SSRP1 depletion (Table 1). Important

biological processes include glycoprotein and glycosylation, extracellular matrix, cell-cell adhesion, ossification, osteoblast differentiation and osteogenesis, bone and skeletal system development, Wnt-signaling pathways etc. Notably all of these processes and pathways are crucial for osteoblast differentiation to bone development at different stages. For example, glycoprotein and glycosylation of membrane proteins playing role in signal transduction. Proteins of the extracellular matrix are critical for cell to cell contact as well as for maturity of the osteoblasts.

4.7 Wnt signaling pathway as a target of SSRP1

4.7.1 SSRP1 mediates transcriptional regulation of Wnt signaling pathway genes

One of the striking outcomes of GO analysis was the enrichment of genes involved in Wnt signaling (Table 1), a central pathway controlling osteoblast differentiation (Baron and Kneissel, 2013) whose activity was perturbed following SSRP1 depletion. Then we generated a heatmap to show the changes in gene expression pattern which displayed the Wnt signaling pathway genes were expressed during osteoblast differentiation whose activity was inhibited after SSRP1 knockdown (Figure 13). These results clearly show an involvement of SSRP1 with Wnt signaling pathway where SSRP1 could regulate the pathway activity. Therefore we verified the changes in gene expression of several key Wnt signaling genes as well as Wnt target genes including *AXIN2, BMP4, DKK1, LEF1, LRP4, TCF7L2, WNT2, WNT11* and *WISP2*. A number of these genes showed a significant decrease in expression following SSRP1 depletion (Figure 14). In addition, the effects of SSRP1 depletion on the Wnt-signaling pathway during osteoblast differentiation was further verified using a custom-made Wnt-signaling pathway map (Figure 15).

All together we show an essential need of SSRP1 for the functional activity of the Wnt signaling pathway which could be disrupted by the depletion of SSRP1. Notably Wnt signaling plays a pivotal role during osteoblast differentiation and bone development. Thus these results indicate that previously observed effects of SSRP1 on osteoblast differentiation could be due to SSRP1-mediated regulation through Wnt-signaling.

Table 1: SSRP1 dependent biological processes revealed by GO analysis. The table lists GO terms in biological processes deregulated by SSRP1 knockdown. Important biological processes required for osteoblast differentiation are marked as blue. Log2 FC ≤ -0.515 and pAdj ≤ 0.05 were the threshold level for SSRP1-mediated downregulated genes.

Biological Process	Count	P Value	Enrichment Score
Signal	168	4.6E-24	**16.08**
Glycoprotein	185	1.2E-17	
Glycosylation site:N-linked (GlcNAc.)	177	3.0E-16	
Extracellular region	110	4.7E-15	
Disulfide bond	121	1.0E-9	
Extracellular region part	74	2.1E-17	**12.16**
Extracellular matrix	39	4.0E-14	
Extracellular matrix	31	1.7E-13	
Proteinaceous extracellular matrix	36	5.3E-13	
Extracellular matrix part	15	2.1E-6	
Cell adhesion	39	3.4E-6	**4.85**
Biological adhesion	39	3.4E-6	
Cell adhesion	24	2.5E-4	
Ossification	12	1.2E-4	**3.31**
Osteogenesis	6	1.9E-4	
Bone development	12	2.1E-4	
Skeletal system development	20	3.3E-4	
Osteoblast differentiation	5	1.9E-2	
Wnt signaling pathway	9	9.4E-3	**1.77**
Wnt signaling pathway	10	1.1E-2	
Frizzled cysteine-rich domain	4	1.3E-2	
Wnt receptor signaling pathway	9	1.7E-2	
Frizzled related	3	6.6E-2	

In addition, BMP4 is a component of the bone morphogenetic protein (BMP) signaling pathway which plays vital role during osteoblast differentiation along with Wnt and other signaling pathways.

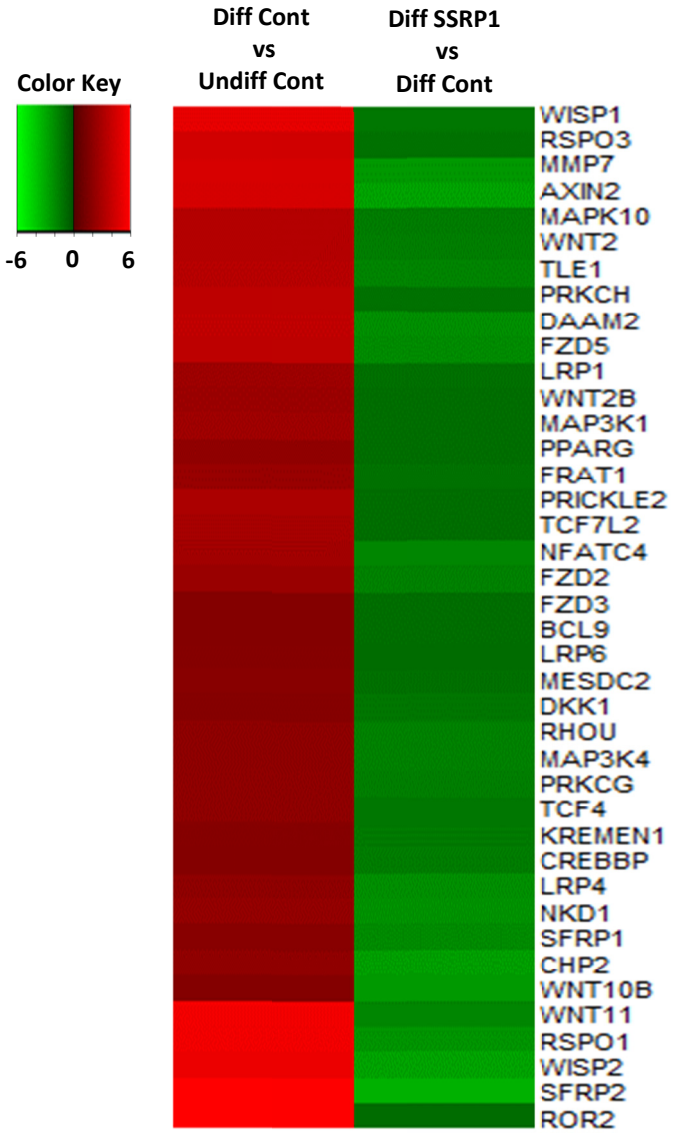


Figure 13. SSRP1-mediated changes in Wnt signaling gene expression. Heatmap analysis of RNA-seq data denoting log2-fold changes in gene expression in differentiated control-transfected hFOB cells vs. undifferentiated cells (left) or differentiated SSRP1-depleted cells vs. differentiated control transfected cells (right). Threshold levels were determined as described in section 4.5 for figure 12.

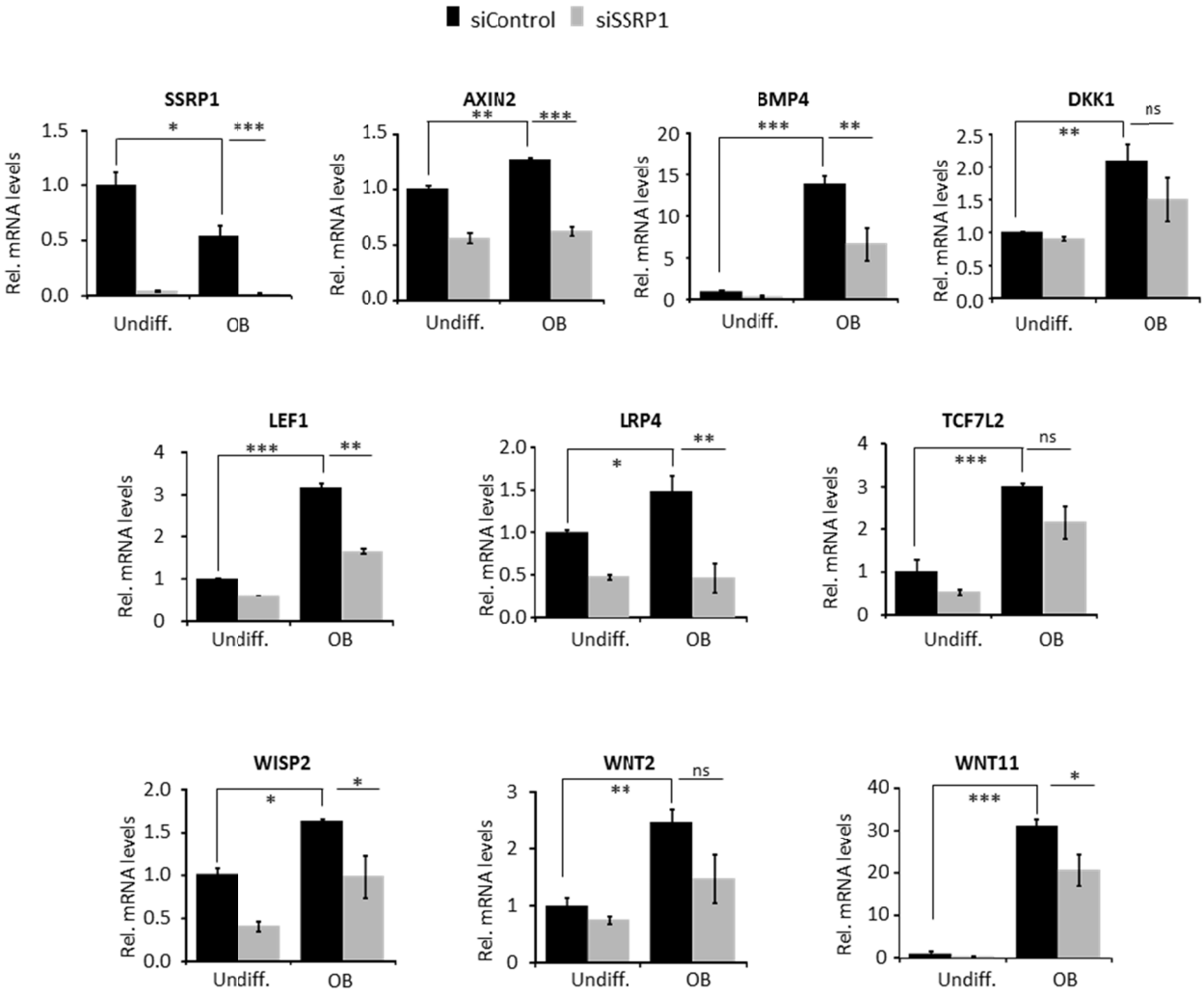
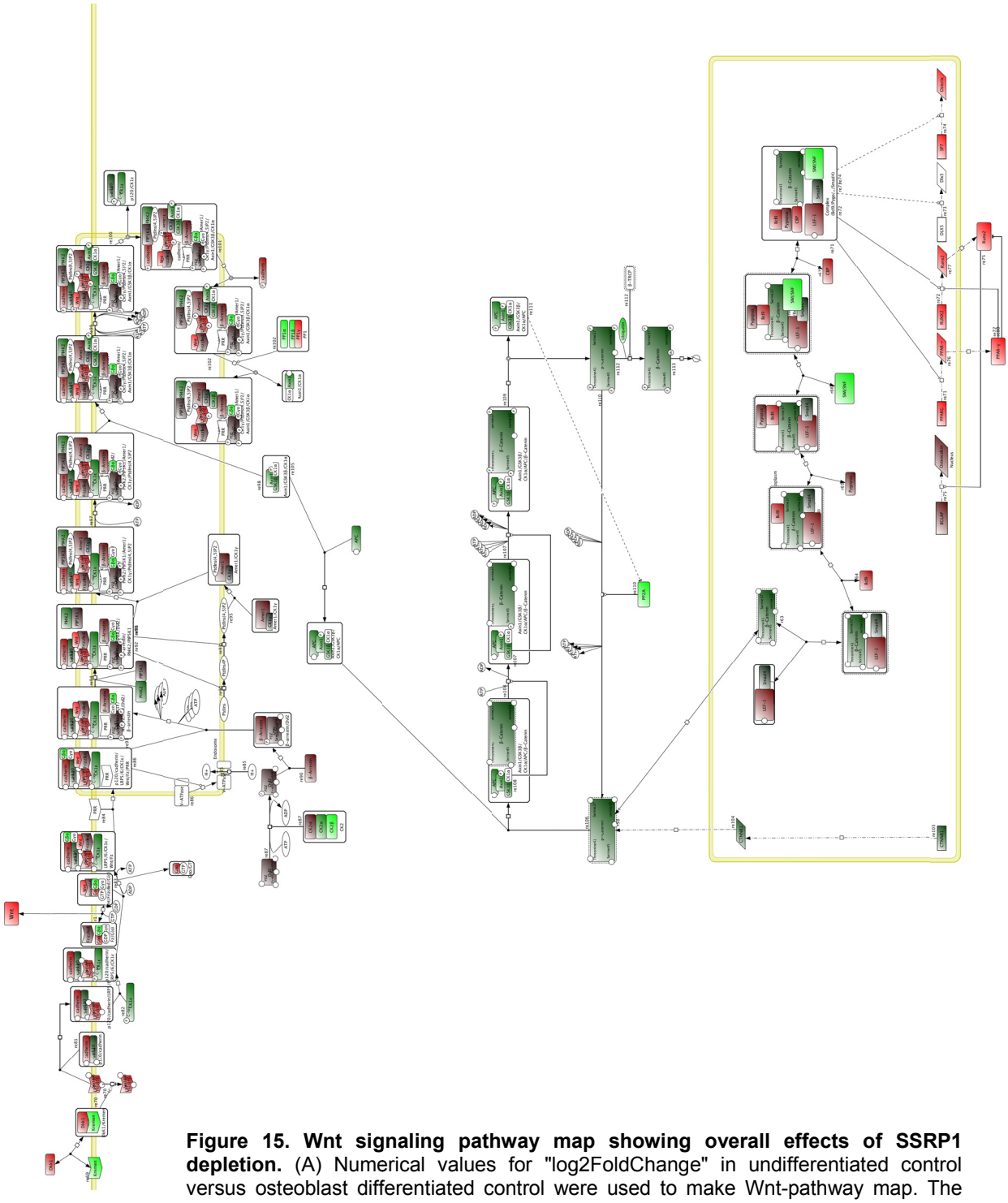


Figure 14. Verification of SSRP1-dependence for Wnt target gene activation during osteoblast differentiation. hFOB cells were transfected with control or SSRP1 siRNAs followed by osteoblast differentiation induction for 7 d. Real time PCR analyses were performed for the expression of SSRP1 and the Wnt pathway target genes *AXIN2, BMP4, DKK1, LEF1, LRP4, TCF7L2, WISP2, WNT2, WNT11*. Transcript levels were normalized to *RPLP0* and shown relative to the undifferentiated (Undiff) control. Mean ± SD (n = 3). ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05, ns: non significant. Undiff., undifferentiated; OB, osteoblast differentiated.

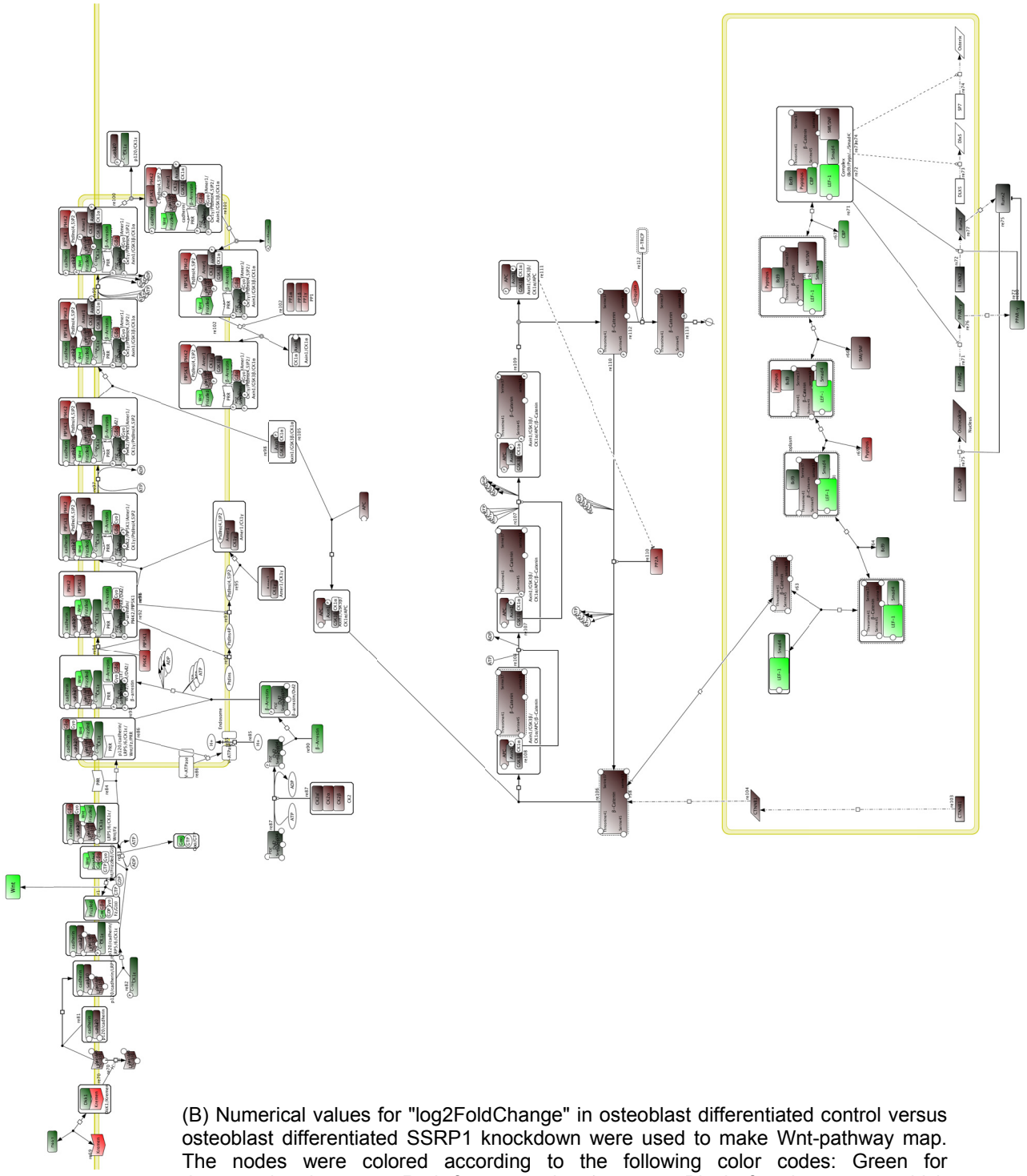
4.7.2 SSRP1 affects Wnt signaling pathway activity

To further verify whether SSRP1 has effects on the Wnt-signaling pathway activity we performed super-TOP/super-FOP FLASH dual luciferase reporter assay on hFOB and osteosarcoma cell line U2OS. Osteosarcoma is a childhood bone cancer with deregulation of cell cycle and differentiation (Sadikovic et al., 2010).



A

Figure 15. Wnt signaling pathway map showing overall effects of SSRP1 depletion. (A) Numerical values for "log2FoldChange" in undifferentiated control versus osteoblast differentiated control were used to make Wnt-pathway map. The nodes were colored according to the following color codes: Green for downregulated genes; Red for upregulated genes; Black for the genes whose expression were not changed; White for the genes which could not be placed in the map. Continued in the next page.



B

(B) Numerical values for "log2FoldChange" in osteoblast differentiated control versus osteoblast differentiated SSRP1 knockdown were used to make Wnt-pathway map. The nodes were colored according to the following color codes: Green for downregulated genes; Red for upregulated genes; Black for the genes which expression were not changed; White for the genes which could not be placed in the map. All nodes having expression values below -1 or above 1 have been assigned the most intense colors (intense green for values below-1 and intense red for values above 1). -1 and 1 were selected as thresholds because these values correspond to a doubling of one quantity relative to the other (log2(2)=1, log2(0.5)=-1).

Several genes necessary for late or terminal osteoblast differentiation are overexpressed in osteosarcoma (Haydon et al., 2007; Sadikovic et al., 2010; Wagner et al., 2011). Considering these points we used U2OS osteosarcoma cells in addition to hFOB for further verification of the SSRP1 involvement in the Wnt signaling pathway.

The super-TOP FLASH reporter construct contains 8 optimal copies of TCF/LEF sites upstream of a thymidine kinase minimal promoter that in response to activation of Wnt pathway β-catenin bind to TCF/LEF transcription factor and induces transcription of luciferase reporter gene. In contrast super-FOP FLASH contains mutated copies of the TCF/LEF binding sites. Thus β-catenin does not have the binding partners which result in inhibition of luciferase reporter gene used as a negative control (Figure 16A, 16B). We performed siRNA-mediated knockdown of SSRP1 followed by Wnt3a treatment for induction of Wnt-signaling. We observed consistent results from both the cell lines where SSRP1 depletion led to a significant decrease in Wnt3a-induced reporter activity following SSRP1 knockdown (Figure 16C, 16D). These results further confirm the critical necessity of SSRP1 for the transcriptional activity of Wnt signaling pathway during osteoblast differentiation.

4.7.3 SSRP1 is required for nuclear translocation of active β-catenin

Given the fact that SSRP1 was required for Wnt pathway activity and also exerted control on different components of the pathway including ligands, receptors, mediators, regulators and targets as well, we next examined whether SSRP1 depletion affected nuclear translocation of β-catenin. We therefore performed immunofluorescence analyses for SSRP1 and active (non-phosphorylated) β-catenin in U2OS cells following SSRP1 knockdown. Here we also induced Wnt signaling pathway using Wnt3a containing conditioned media.

We observed a significant colocalization of SSRP1 and β-catenin in the nucleus following Wnt3a treatment which was disrupted after SSRP1 depletion. Importantly, SSRP1 depletion resulted in a reduction of active-β-catenin in the nucleus to levels found in uninduced control cells (Figure 17A). Protein analyses by western blot also showed decreased levels of active β-catenin following SSRP1 depletion and Wnt3a induction (Figure 17B).

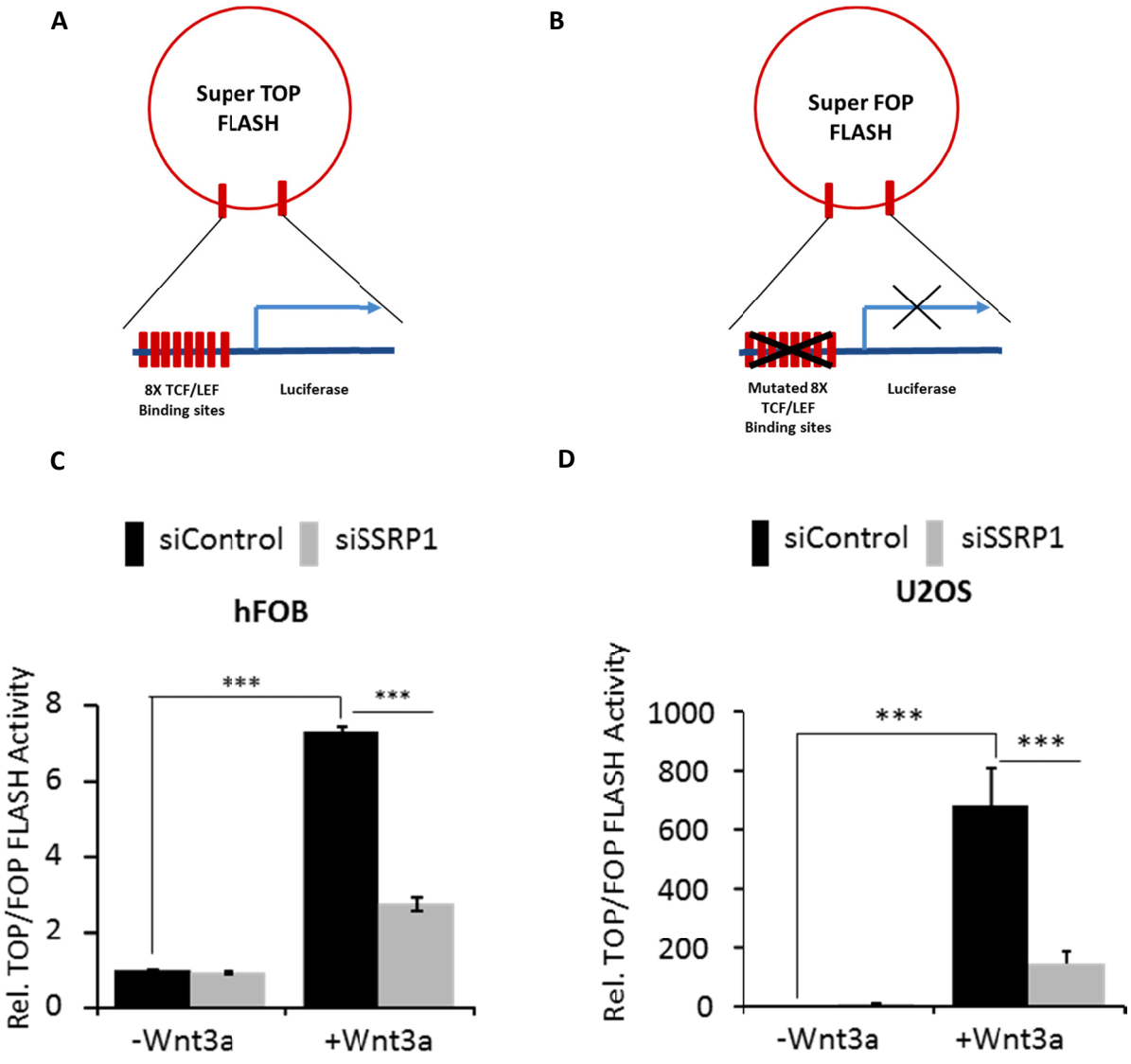


Figure 16. SSRP1 regulates Wnt signaling pathway activity. (A, B) The super-TOP FLASH and super-FOP FLASH systems with 8X TCF/LEF binding sites and mutated TCF/LEF binding sites respectively. hFOB (C) and U2OS (D) cells were transfected with control and SSRP1 siRNAs. After 36 h, cells were transfected with super-TOP- or super-FOP-FLASH constructs together with an internal Renilla luciferase expression vector. Cells were cultured for an additional 24 h under stimulation with Wnt3a or control conditioned media. Wnt signaling pathway activity is shown as relative firefly luciferase activity normalized to Renilla luciferase activity and shown relative to the control condition (cells treated with conditioned medium from non-Wnt3a-expressing L-cells). The negative control (FOP-FLASH activity) is shown as a black dotted line. ***p ≤ 0.001, **p ≤ 0.01. Data are represented as mean ± SD (n = 3).

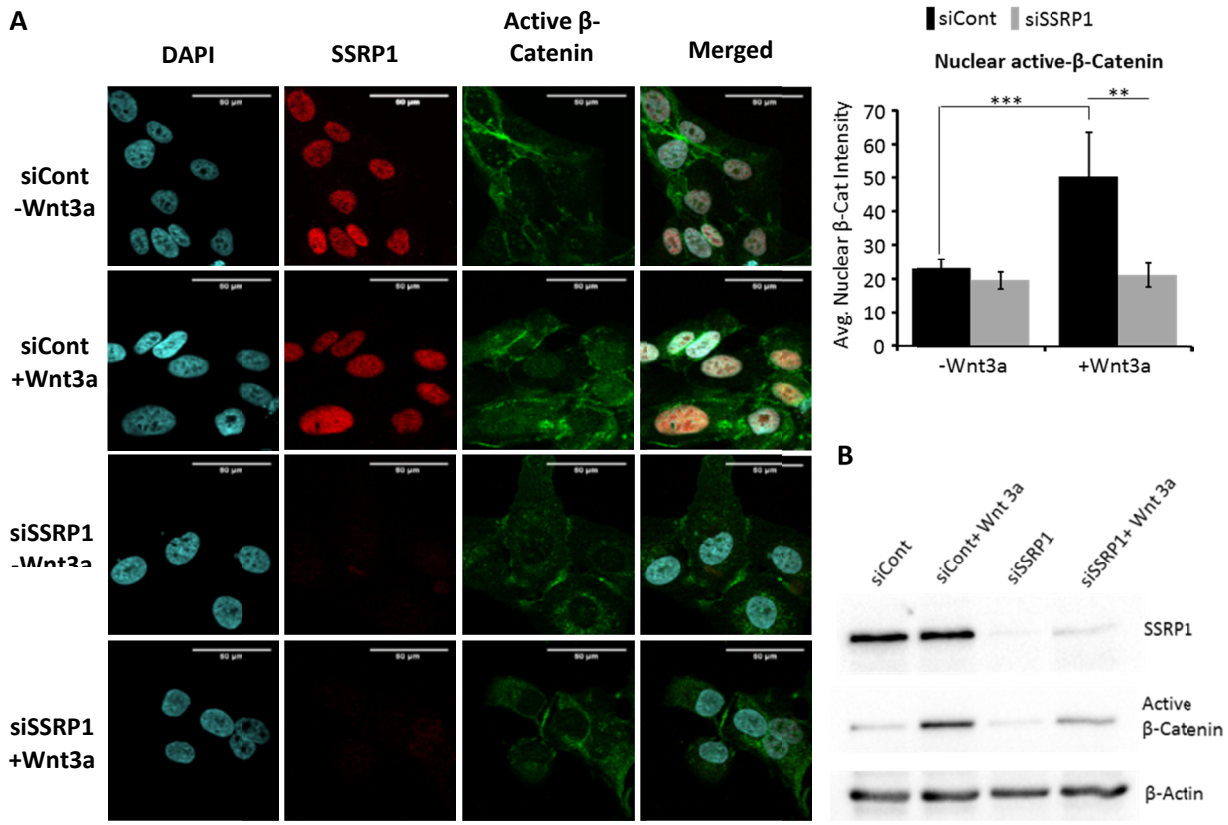


Figure 17. SSRP1 expression is required for nuclear translocation of active β-catenin. (A): Immunofluorescence staining was performed for anti-SSRP1 (red) and anti-active β-catenin (green) in U2 OS cells. Following siRNA-mediated knockdown of SSRP1, cells were treated for 48 h with Wnt3a-containing conditioned media. DAPI (blue) was used as the nuclear stain. Images are shown at a 63X magnification. The intensity of active β-catenin in the nucleus was quantified using Image J software (right panel). ***p ≤ 0.001, **p ≤ 0.01. (B): Protein extracts were isolated and analyzed by Western blotting using the antibodies against SSRP1, active β-catenin and β-actin showing the knockdown efficiency of SSRP1 as well as the expression of active β-catenin.

4.7.4 SSRP1 is required for β-catenin activity in the nuclues

We observed that SSRP1 colocalized with active β-catenin in the nucleus. Then we sought to test if, in addition to its requirement for Wnt signaling pathway activity and β- catenin translocation to the nucleus, whether SSRP1 may also be required for β-catenin activity in the nucleus. Therefore, we performed additional super-TOP-/super-FOP FLASH reporter assays in U2OS cells following the depletion of SSRP1 (Figure 18) in conjunction with the expression of a constitutively active (non-phosphorylatable) form of β-catenin (S33Y). Importantly, SSRP1 depletion also

resulted in decreased TOP-FLASH activity driven by the overexpression of constitutively active β-catenin (Figure 18). Thus, these results establish an important role of the human FACT component SSRP1 in the regulation of multiple steps of the Wnt-signaling pathway during osteoblast differentiation.

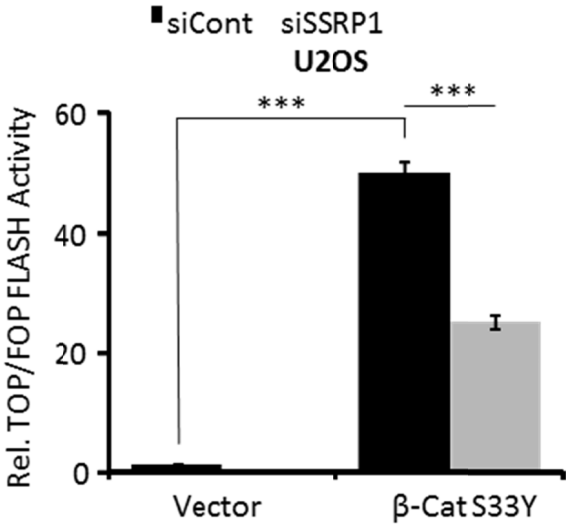


Figure 18. SSRP1 regulates β-catenin activity of the Wnt signaling pathway. The requirement of SSRP1 for β-catenin activity was further verified by TOP-/FOP-FLASH dual luciferase reporter assay as in Figure 17 following co-transfection of either a plasmid expressing a constitutively active β-catenin mutant (S33Y) or a control plasmid (pCI-neo). Luciferase activity was measured and was plotted relative to Renilla luciferase and compared to the empty vector control condition. Mean values ± SD (n = 3) and ***p ≤ 0.001, **p ≤ 0.01.

4.8 SSRP1 siRNA stringency test in U2OS cells

siRNAs have been widely used as a tool for gene inactivation in basic research and therapeutic applications. But one major caveat is sequence specific off-target effects for which siRNA pools are being used. Since we used the smart pool which has all the single siRNAs for SSRP1 in 1:1 ratio, next we tested the precision of smart pool and other single siRNAs in U2OS cells both in protein and gene expression levels (Figure 19). We conducted siRNAs mediated knockdown of SSRP1 and then we analyzed the protein as well as gene expression levels (Figure 19B, 19A). We found a marked decrease in the expression level of osteoblast specific genes (*ALPL, COL1A1*) and Wnt pathway genes (*AXIN2, BMP4, WISP2*) after siRNA mediated knockdown of SSRP1using individual siRNAs (Figure 19A). Additionally we also performed a dual luciferase reporter assay using super-TOP FLASH system along with Wnt signaling induction by Wnt3a (Figure 19C). Here we

also observed a marked decrease in the luciferase activity following Wnt3a treatment. From the observed data of single siRNA experiment on U2OS we could conclude that the effects were specific and consistent with smart pool.

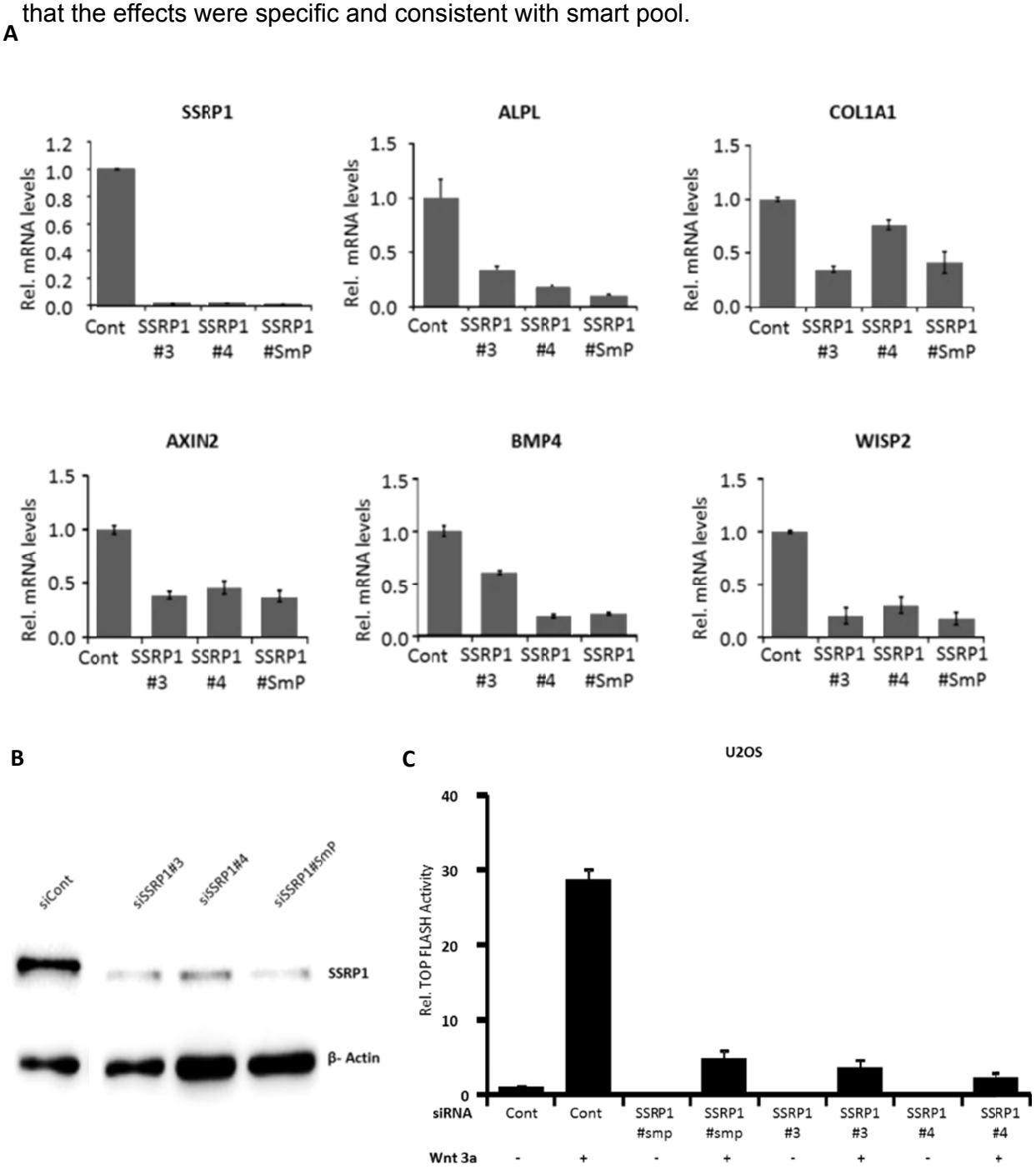


Figure 19. Single siRNA of SSRP1 phenocopies smart pool affects. (A) U2OS cells were transfected using single siRNAs of SSRP1 corresponding number 3 (SSRP1 #3) or 4 (SSRP1 #4) or smart pool (#SmP). (A) Gene expression study was carried out by real time PCR to check the efficient knockdown of SSRP1, osteoblast specific genes and Wnt pathway related genes. (B) Knockdown efficiency was also confirmed by Western blotting for the antibodies against SSRP1 and β-actin. (C)

U2OS cells were transfected with control or single/smart pool SSRP1 siRNAs. After 36 h, cells were transfected with super-TOP FLASH constructs together with an internal *Renilla* luciferase expression vector. Cells were cultured for an additional 24 h under stimulation with Wnt3a or control conditioned media. Wnt signaling pathway activity is shown as relative firefly luciferase activity normalized to Renilla luciferase activity and shown relative to the control condition (cells treated with conditioned medium from non-Wnt3a-expressing L-cells). Here, SSRP1 # smp means SSRP1 smart pool siRNA, #3 and #4 means SSRP1 siRNA number 3 and 4.

5. Discussion

Cellular differentiation is a tightly regulated process involving multiple orchestrated control mechanisms. These mechanisms exert critical role in the emergence of different cell types from a particular stem cell. Nevertheless one of the important factors which control the regulation of cell differentiation is the highly compact and condensed structure of chromatin, which provides a fundamental barrier for all the DNA associated processes essential for differentiation. Therefore alteration of chromatin architecture by various chromatin modifying factors is essential for cell differentiation.

The histone chaperone FACT was reported to be highly expressed in the undifferentiated, less differentiated or stem cell like cells compared to the adult mammalian cells (Garcia et al., 2011, 2013). However, the specific role of the FACT complex in cellular differentiation or lineage specific cell fate decision is still unknown. Our studies suggest that FACT component SSRP1 plays a role in regulating lineage specific cellular differentiation towards adipocyte and osteoblast through affecting the transcription components of these two processes. Moreover we demonstrate a novel link between SSRP1 and Wnt signaling which is essential for regulating osteoblast differentiation.

5.1 SSRP1 elicits lineage specificity between adipocyte and osteoblast

5.1.1 SSRP1 regulates adipocyte differentiation

hMSCs are multipotent stem cells which can differentiate into a number of cell lineages including adipocytes, osteoblast, chondrocyte, myoblast and neurons. Nevertheless the exact mechanisms how the lineage specificity is determined by the cells still unknown. The nuclear receptor Peroxisome Proliferator-Activated Receptor Gamma (*PPARγ* or *PPARG*) is one of the master regulators for adipocyte differentiation and adipogenesis which becomes activated by a series of transcriptional events. Upon stimulation by 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone induction of CCAAT/enhancer binding protein-β (CEBPB) and -δ (CEBPD) happens. Mitogen Activated Protein Kinase (MAPK) and Glycogen Synthase Kinase-3β (GSK-3β) play an essential role in the hyperphosphorylation

and activation of CEBPB, which increases the DNA binding affinity of both CEBPs, then leads to the expression of *PPARG* as well as *CEBPA*, which subsequently induces the expression of other genes necessary for adipocyte differentiation. Increased *PDK4* expression was found to be correlated with the enhanced expression of *PPARG* (Holness et al., 2012). In another study, overexpression of *RASD1* enhances expression of *PPARG, CEBPA* as well as other adipocyte differentiation marker genes (Cha et al., 2013). It is likely that the changes in the gene expression pattern during adipocyte differentiation require dynamic remodeling of chromatin. ATP-dependent chromatin remodeling enzymes SWI/SNF were reported to be required for the activation of *PPARG* preceded by preinitiation complex formation and recruitment of SWI/SNF on *PPARG* promoter along with TFIIH and other general transcription factors (Salma et al., 2004). Jun Dimerization Protein 2 (JDP2) was reported to have the histone chaperone activity and suppresses adipocyte differentiation through inhibiting the acetylation of H3 in the promoter of *CEBPD* (Nakade et al., 2007). In this study, we show that the depletion of the histone chaperone protein SSRP1 promoted adipocyte differentiation. The expression of key adipocyte differentiation marker gene *PPARG* is significantly increased following SSRP1 knockdown and adipocyte differentiation. We also observed that the expression of other adipocyte differentiation genes including *PDK4* and *RASD1* were increased. Thus SSRP1 can regulate adipocyte differentiation by regulating the expression of key differentiation markers. However, the exact mechanisms how SSRP1 could affect adipocyte differentiation is broadly unknown. It could be possible that when SSRP1 is present it occupies the DNA in a way which blocks the binding of other chromatin remodelers essential for adipocyte differentiation, thus SSRP1 depletion facilitates the accessibility of those factors to chromatin resulted in an enhanced adipocyte differentiation.

5.1.2 SSRP1 promotes osteoblast differentiation

One major focus of this study was to find out the role of the histone chaperone SSRP1 in osteoblast differentiation. Osteoblast differentiation is an important process to establish a fine tune balance between bone formation and resorption in normal physiological condition where dynamic remodeling of chromatin structure is required for the expression or suppression of a number of genes regulating these

processes. The role of histone chaperones in different DNA associated processes are well established (Song et al., 2012b). Importantly, in our study, we observed a significant effect on the expression of the key osteoblast differentiation marker genes such as *ALPL, RUNX2, BGLAP* and *SP7* following SSRP1 depletion. This supports the transcriptional regulation of osteoblast differentiation specificity by SSRP1. The presence of SSRP1 could be essentially needed for facilitating the expression of the key osteoblast specific genes. Thus a loss of SSRP1 results in the deregulation of the expression of these genes leading to the inhibition of osteoblast differentiation.

5.1.3 SSRP1 and transdifferentiation

Another interesting cellular phenotype we observed from hMSC following SSRP1 knockdown was very elongated multinucleated cells resembles to muscle cells. Thus we hypothesized that transdifferenitation could be promoted in the absence of SSRP1. Since hMSCs are adult stem cells and having the ability to differentiate into a number of cell lines including muscle cells, thus it is likely that the cells could transdifferentiate into myoblast following SSRP1 knockdown. But our data regarding the gene expression studies did not further support transdifferentiation aspect.

5.2 Biological processes regulated by SSRP1

In our studies, gene ontology analyses of the transcriptome-wide RNA-seq data revealed various important biological processes affected by SSRP1 depletion during osteoblast differentiation (Table 1). These include glycoprotein/glycosylation, extracellular matrix and cell-cell adhesion, proteinaceous extracellular matrix, ossification, bone and skeletal system development etc. which have a significant influence during different stages of osteoblast differentiation.

Among these, glycosylation of extracellular protein, glycoprotein and extracellular protein contribute to the formation of extracellular region important for cell-cell interaction, adhesion, maturity and mineralization of the osteoblast differentiated cells (Clarke, 2008).

The bone ECM is composed of proteins such as collagen, fibronectin, laminin, vitronectin, osteopontin and osteonectin (El-Amin et al., 2003). Mathews et al., (2012)

studied the significance of some of the ECM proteins such as collagen type1, fibronectin, laminin and vitronectin in regulating the proliferation and osteogenic differentiation of the bone marrow derived hMSCs. They found the induced transcription of various osteoblast differentiation specific genes like *ALPL* following the treatment of ECM proteins. Overall, they showed that ECM proteins can induce osteoblast differentiation (Mathews et al., 2012). Studies also showed the essential functions of integrin in regulating osteoblast differentiation (Gronthos et al., 1997; Xiao et al., 2002).

Thus, it is apparent that the cellular microenvironment is crucial for cell-cell interaction, adhesion, maturity and signal transduction in order to respond to different environmental and biochemical stimuli which collectively results in regulation of proliferation and differentiation of osteoblasts from the progenitor or stem cells. Any effect which mediates an imbalance in the expression of ECM proteins would significantly affect the osteoblast differentiation. Similar effects would be expected if changes occur in glycosylation or glycoproteins. SSRP1-mediated effects on different osteoblast-related important biological processes indicate the transcriptional siginifcance of FACT complex in regulating the expression of a large number of genes involved in different processes necessary for maintaining extracellular microenvironment and signalings.

5.3 SSRP1 promotes osteoblast differentiation by regulating Wnt-signaling

The major finding of our GO analyses was the effect of SSRP1 depletion on Wnt-signaling (Table 1). Notably, Wnt-signaling is crucial for osteoblast differentiation and bone development and canonical Wnt-signaling posesses its potential role in bone homeostasis (Kanehisa and Goto, 2000). Moreover, non-canonical pathways are also important during bone homeostasis. In our studies, the expression of number of canonical Wnt-signaling genes was inhibited following SSRP1 depletion. Further verification of genes including *AXIN2, BMP4, DKK1, LEF1, LRP4, TCF7L2, WNT2, WNT11,* and *WISP2* in mRNA levels showed a significant decrease (most cases) in their expression following SSRP1 knockdown during osteoblast differentiation. Importantly, the effect was observed on Wnt targets (e.g., *BMP4, WISP2*), central mediators (*LEF1, TCF7L2, and LRP4*) and regulators (*DKK1,*

WNT2, and *WNT11*). This finding is important since activation of Wnt-signaling plays a pivotal role in enhancing osteoblast differentiation and represents a potential target for osteoanabolic therapy (Baron and Kneissel, 2013).

In addition, an effect on nuclear translocation of active-β-catenin following SSRP1 depletion and a significant co-localization of SSRP1 with active-β-catenin (Figure 17) suggests an inactivation of the canonical Wnt-signaling pathway following SSRP1 depletion. These findings indicate that SSRP1 may essentially interact with the transcription factors and other mediators of the Wnt signaling which lead to the expression of the genes necessary for osteoblast differentiation. Indeed canonical Wnt signaling was reported to induce key osteoblast differentiation specific genes including *RUNX2, SP7* etc (Gaur et al., 2005; McCarthy and Centrella, 2010). Thus removal of SSRP1 could result in the disruption of interaction complex, led to the inhibition of osteoblast differentiation specific gene expressions.

Apart from the Wnt-signaling, bone morphogenetic protein (BMP) signaling also plays a critical role in bone formation and skeletal development (Sanchez-Duffhues ez al., 2015). SSRP1knockdown-mediated reduced expression of *BMP4* suggests a link between SSRP1, BMP and Wnt signaling pathways. Interestingly, the interaction between Wnt/β-catenin and BMP signaling during osteoblast differentiation and bone formation is known (Lin et al., 2011; Zhang et al., 2013). Moreover, Wnt/β-catenin pathway was found as an upstream of the BMP (Zhang et al., 2013) signaling which means that the activation of canonical Wnt-signaling could result in induction of BMP signaling. In contrast, BMP signaling was also found to stimulate Wnt/β-catenin pathway (Zhang et al., 2013). Here, we show that SSRP1 depletion resulted in a significant decrease of a BMP signaling gene *BMP4*. Thereby we predict that SSRP1 has effects on Wnt/β-catenin pathway which in turn bring changes in BMP signaling pathway or vice versa. Moreover, TGF-β and Notch pathways also possess a positive role in osteoblast differentiation. Various studies also reported the interplay between Wnt, BMP, TGF-β, Notch and Hedgehog signaling pathways during osteoblast differentiation. Though we did not focus on pathways, it is possible that changes in Wnt-signaling and or BMP-signaling via SSRP1 also affect multiple signaling pathways necessary for osteoblast differentiation.

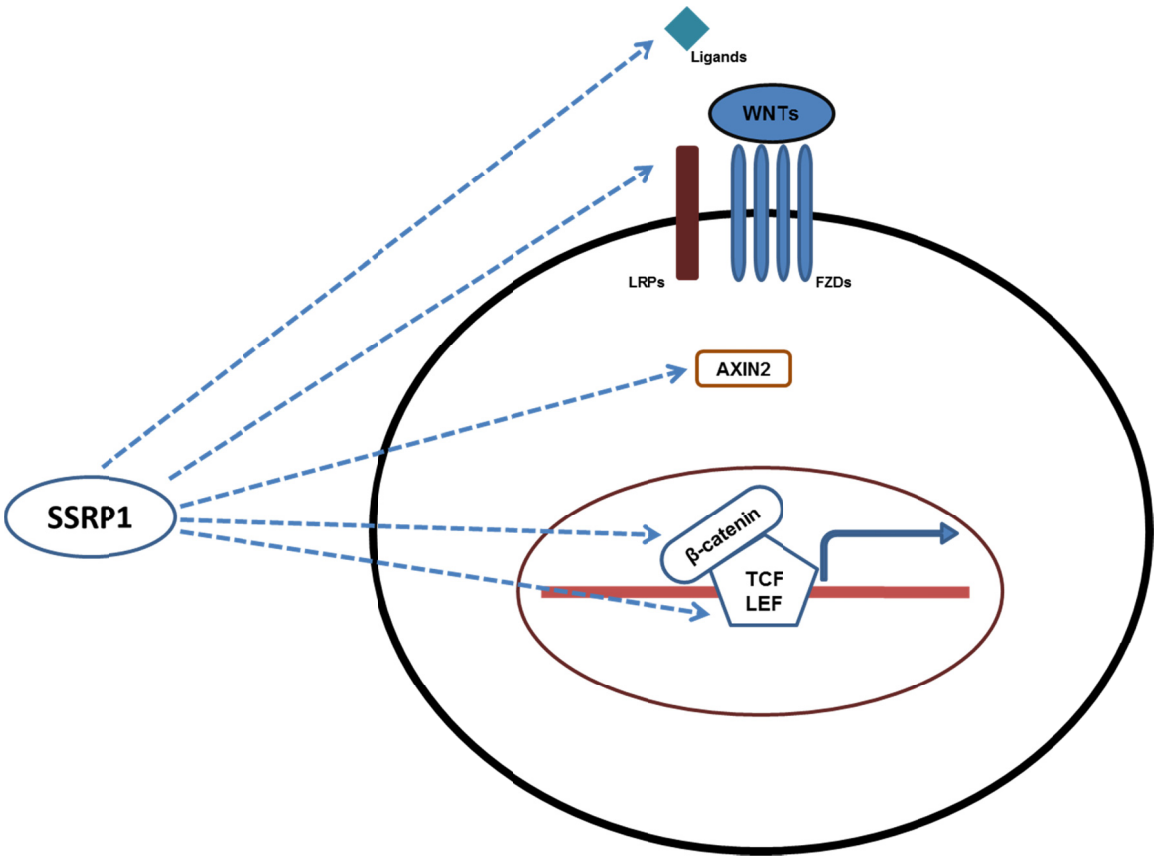


Figure 20. SSRP1 regulates Wnt signaling pathway. SSRP1 governed affects are implemented on different components of the Wnt signaling pathway including regulators (*DKK1, WNT2,* and *WNT11*), central mediators (*LEF1, TCF7L2, LRP4*) as well as targets (e.g., *BMP4, WISP2*).

5.4 Regulation of lineage specificity by SSRP1

Since we observed SSRP1-mediated lineage specificity from hMSC towards adipocyte or osteoblast, the question arises how SSRP1 plays role in determination of the cell specificity. This could be due to the changes in signaling and chromatin structure-associated events.

5.4.1 Signaling control aspects

Our study demonstrates that SSRP1 control the lineage-specific differentiation to adipocytes or osteoblasts. Interestingly previous studies showed the crucial importance of canonical Wnt-signaling pathway in regulating the switches between adipocyte or osteoblast differentiation.

Activation of canonical Wnt-signaling stimulates osteoblastogenesis over adipogenesis by inhibiting the expression of key adipocyte differentiation specific

genes *PPARG* and *CEBPA*. The role of Wnt10b in inhibiting adipocyte differentiation is well studied, while activated Wnt10b induced osteogenesis by blocking adipogenesis (Kang et al., 2007; Liu et al., 2009; Ross et al., 2000). Cawthorn et al., demonstrated that Wnt6 and Wnt10a as additional Wnt family members which could show similar effects like Wnt10b during osteoblast and adipocyte differentiation. Activation of Wnt10a or Wnt6a showed suppressed adipogenesis and increased osteogenesis (Cawthorn et al., 2012). Moreover, Wnt3a mediated stimulation also inhibited adipogenesis through the suppression of *PPARG* and *CEBPA* gene expression (Byun et al., 2014; Kawai et al., 2007; Park et al., 2015). Consistently, inactivation of canonical Wnt/β-catenin pathway inhibited osteoblast differentiation and facilitated adipocyte differentiation (Bennett et al., 2002; Laudes, 2011; Ross et al., 2000). Interestingly, loss of β-catenin function from preosteoblasts behaved as a switch for changing osteoblasts to adipocytes (Song et al., 2012a). Furthermore, the transcription factor *PPARG* is a prime inducer of adipogenesis that inhibits osteogenesis. When adipocyte differentiation was prompted by *CEBPB* and *PPARG* there was an extensive downregulation of the nulear β-catenin which suggest a *CEBPB* and *PPARG* mediated control on canonical Wnt-signaling that blocks osteogenesis (Moldes et al., 2003). Overall, these observations clearly show a reciprocal regulation between adipocyte and osteoblast fate deteremination. SSRP1 mediated regulation on Wnt signaling and BMP signaling pathways can significantly control this reciprocal relationship where the presense of SSRP1 promotes osteoblast differentiation over adipocyte differentiation.

5.4.2 Chromatin-associated aspects

Transcription is highly assisted by alterations in the chromatin structure so that it becomes more accessible for different molecular machineries essential to mediate cellular differentiation and proliferation. Thus, reorganization of chromatin by histone chaperone SSRP1 may be essential for facilitating gene expression including the key osteoblast differentiation-specific genes as well as Wnt-signaling and target genes which promotes osteoblast differentiation and suppresses adipocyte differentiation. More importantly, specificity of the histone chaperones and other chromatin remodelers could also be a limiting point for cellular fate decision (Pedersen et al., 2001; Salma et al., 2004).

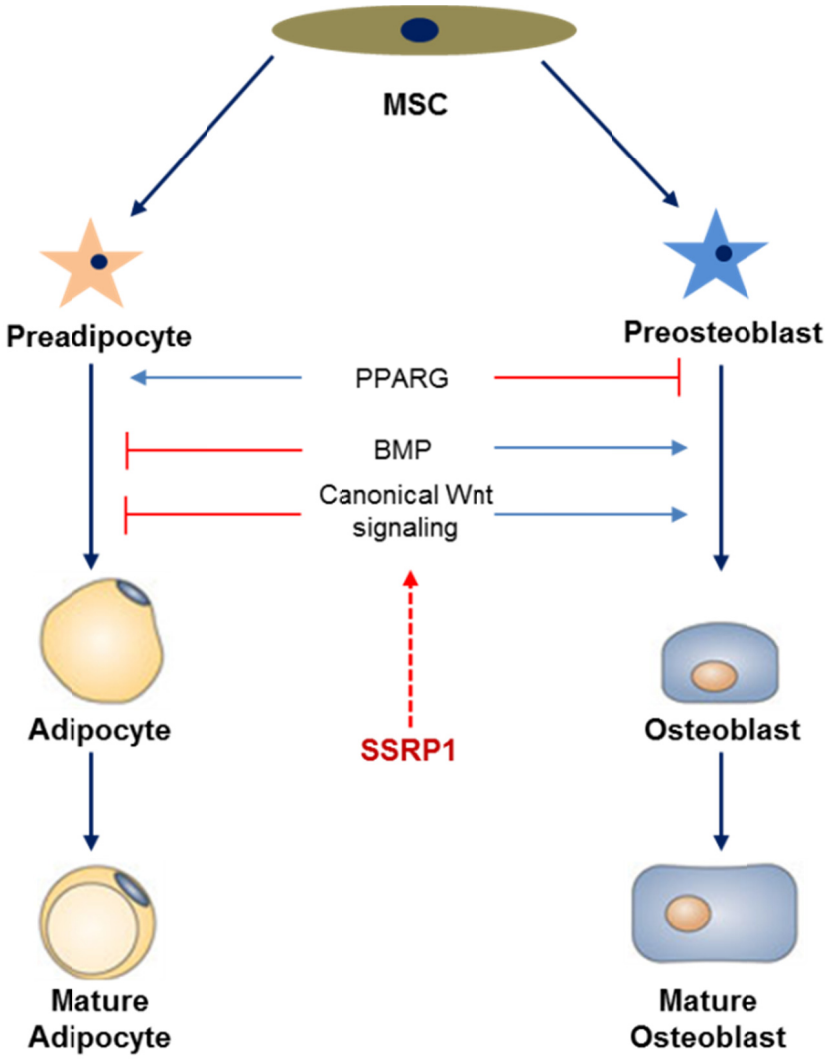


Figure 21. SSRP1 mediates lineage determination of hMSC. hMSCs are multipotent stem cells capable of differentiating into distinct cell fates. Multiple signaling pathways and transcription factors are involved in this reciprocal regulation. SSRP1 plays an essential function during osteoblast differentiation by regulating the key Wnt signaling pathway and associated targets whereas lack of SSRP1 facilitates adipocytes differentiation.

Furthermore, the chromatin remodeling by ATP dependent chromatin remodeler SWI/SNF was reported to be necessary for promoting activation of early stage gene expression during adipocyte differentiation. Thus, it could be possible that SSRP1 is specifically needed for osteoblast differentiation by exhibiting its histone chaperone activity. When SSRP1 is less abundant, this could promote the activity of other histone chaperones or chromatin remodelers necessary for adipocyte differentiation.

The spatial and temporal binding of histone chaperones to the chromatin may also determine the cell fate decision depending on cellular and environmental stimuli as well as local and global arrangement of the chromatin. We checked the available SSRP1 occupancy and observed that some osteoblast differentiation marker genes possess binding of SSRP1 on the transcribed regions whereas Wnt signaling genes show SSRP1 occupancy from transcribed region to transcription termination sites (data not shown). Importantly for adipocyte differentiation, formation of early and late enhancer complexes along with transcription factors C/EBPB/D, GR, STAT5A (early enhancer complex); PPARG:RXR, CEBPA (late enhancer complex) are very crucial for the activation of *PPARG* promoter and subsequent activation of other downstream gene expression (Siersbæk et al., 2012). We did not find SSRP1 occupancy in the enhancer region of these key adipocyte differentiation specific genes (data not shown). This could suggest that the direct transcriptional role of SSRP1 could be implemented on osteoblast-specific genes. The lack of SSRP1 occupancy in the enhancer regions of the important adipocyte differentiation specific genes indicate that absence of SSRP1 might be necessary for adipocyte differentiation.

5.5 Translational significance of this study

Initially FACT complex was thought as a ubiquitously expressed housekeeping factor which is not associated with any disease (Singer and Johnston, 2004). However, recently higher expression of FACT complex was found to be associated with undifferentiated, stem cells or stem cell-like cells and poorly differentiated aggressive cancer (Gasparian et al., 2011; Koman et al., 2012; Garcia et al., 2013). Importantly FACT complex was found to be highly expressed in a number of tumor types including breast, gastrointestinal neoplasms, colorectal neoplasms, intestinal and digestive system neoplasms and osteosarcoma. Notably, there is an inhibitor available for FACT called Curaxin or CBL0137 which is currently in Phase I trial and showed to possess potential effect on glioblastoma, neuroblastoma, pancreatic cancer, Her2/neu positive mammary carcinomas (Dermawan et al., 2016; Carter et al., 2015; Burkhart et al., 2014; Koman et al., 2012).

Our findings provide an important insight into the molecular function of the histone chaperone SSRP1 during osteoblast differentiation by controlling the Wnt signaling pathway and exhibiting a fine-tune over cell fate determination and lineage-specific differentiation. Thus SSRP1 could be a potential target for treating cancers where FACT complex is highly expressed. Moreover, a number of diseases including colorectal cancer and age related bone loss shows dysregulated Wnt signaling pathway which can also be targeted for treatment through inhibition of SSRP1. Interestingly both of the FACT components were found to be higher in colorectal cancer cells (data not shown). Furthermore osteosarcoma is a bone related childhood cancer with highly proliferative undifferentiated or less differentiated malignant cells where osteoblast-specific genes expression is also disrupted. SSRP1 inhibition could also be an important therapeutic approach for osteosarcoma.

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Curriculum Vitae

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Education

Ph.D, 2014 – 2016

Genes and Development program under Göttingen Graduate School for Neurosciences, Biophysics, and Molecular Biosciences (GGNB), Georg-August-Universität Göttingen, Germany.

Ph.D, 2012-2014, Department of Tumor Biology, Center of Experimental Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

Master of Science, 2006-2007 in Biochemistry and Molecular Biology. Examination held in 2010; Department of Biochemistry & Molecular Biology; Faculty of Biological Sciences; Jahangirnagar University, Savar, Dhaka-1342, Bangladesh.

Bachelor of Science (Honors), 2002-2006 in Biochemistry and Molecular Biology Examination held in 2008; Department of Biochemistry & Molecular Biology, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh.

Current employment

1. German Academic Exchange Service (DAAD) Doctoral Fellowship

Georg-August-Universität Göttingen, Germany.

2. Faculty Member (Lecturer), Department of Biochemistry and Molecular Biology, Jahangirnagar University, Savar, Dhaka 1342, Bangladesh (December, 2011 – till to date; On study leave).

Awards and scholarships

German Academic Exchange Service (DAAD) PhD Fellowship, 2012-2016

Awarded by “Jahangirnagar University Merit Scholarship” in the year of 2006-2007, based on M.S. result.

Awarded by “Jahangirnagar University Merit Scholarship” in the years of 2003, 2004, 2005 and 2006 based on the results of each year during the entire period of B.Sc. program.

Awarded by “Bangladesh Jute Mills Corporation Scholarship” in the years of 2003, 2004, 2005 and 2006. This scholarship is provided as a subsidy to the children of the employees of Bangladesh Jute Mills Corporation (BJMC) on the basis of merit.

Research experiences

Ph.D (2012-Present)

Cellular Differentiation and Epigenetics, University Medical Center Hamburg-Eppendorf, Georg-August University, Goettingen, Germany.

Supervisor: Prof. Dr. Steven A. Johnsen

Project: The role of the FACT histone chaperone complex in cellular differentiation

Project Trainee, M.Sc. Dissertation, 2009-2010

Dept. of Biochemistry and Molecular Biology, Jahangirnagar University, Dhaka, Bangladesh.

Supervisor: Prof. Dr. Sohel Ahmed

Project: Dynamics of Distribution and Prevalence of Antibiotic Resistant Bacteria in the Waste-Water Samples along the Main Sewerage Line at the Central Cattle Breeding and Dairy Farm of Bangladesh.

Laboratory skills

Molecular Biology: Plasmid isolation, cloning techniques, RNA isolation from cells, PCR, Quantitative PCR, Chromatin Immuno Precipitation and sequencing (ChIP-seq), Library preparation for Illumina, chromatin fractionation, Dual luciferase reporter assay.

Cell culture: Maintenance of cell lines and transfection methodologies. MTT cytotoxicity assay and proliferation assays.

Protein chemistry: Coimmunoprecipitation, SDS-PAGE, 2D gel electrophoresis, Protein isolation from cells, Protein Quantification, Western blotting.

Staining: Immunofluorescence staining, Proximal ligation assay.

Microscopy and Image analysis: Confocal microscopy, Image J software, LSM image browser.

Microbiology: Bacterial cell culture, Isolation and characterization of bacterial strains from clinical and environmental specimens.

Bioinformatics: RNA-seq and ChIP-seq analysis tools elementary level.

Language skills

Bengali : Mother tongue

English: Good

German: Basic