

Multiple functions and essential roles of nuclear receptor coactivators of bHLH-PAS family

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Classical non-peptide hormones, such as steroids, retinoids, thyroid hormones, vitamin D₃ and their derivatives including prostaglandins, benzoates, oxysterols, and bile acids, are collectively designated as small lipophilic ligands, acting via binding to the nuclear receptors (NRs). The NRs form a large superfamily of transcription factors that participate virtually in every key biological process. They control various aspects of animal development, fertility, gametogenesis, and numerous metabolic pathways, and can be misregulated in many types of cancers. Their enormous functional plasticity, as transcription factors, relates in part to NR-mediated interactions with plethora of coregulatory proteins upon ligand binding to their ligand binding domains (LBD), or following covalent modification. Here, we review some general views of a specific group of NR coregulators, so-called nuclear receptor coactivators (NRCs) or steroid receptor coactivators (SRCs) and highlight some of their unique functions/roles, which are less extensively mentioned and discussed in other reviews. We also try to pinpoint few neglected moments in the cooperative action of SRCs, which may also indicate their variable roles in the hormone-independent signaling pathways.

Key words: nuclear receptors, coactivators, bHLH-PAS family

Nuclear receptor coactivators (NRCs) or steroid receptor coactivators (SRCs) are broad/wide (specific) group of transcription-regulating nuclear proteins, first discovered as auxiliary factors, recruited by members of the nuclear receptor (NR) superfamily. The three basic family members, SRC-1 (NCoA1), SRC-2 (NCoA2/Grip1/Tif2), and SRC-3 (NCoA3/CIP/AIB1/ACTR/RAC3/TRAM-1), belong to the structurally homologous p160 family of coactivators. The common feature of this family is the presence of basic-helix-loop-helix (bHLH) and period-aryl hydrocarbon receptor nuclear translocator protein (Arnt)-Single-minded protein (PAS) domains in their N-terminal region (Figure 1). The bHLH-PAS region facilitates DNA binding, protein-protein interactions with other coregulators and transcrip-

tion factors (TFs) and contains a canonical nuclear localization signal (Awais et al. 2007; Chang and Wu 2012; Zelenko et al. 2012; Endler et al. 2014). More centrally located is a serine/threonine-rich domain, the phosphorylation status, which influences the SRC activity. Following is a receptor and TF interaction domain, containing two or three LXXLL motifs (so-called NR-boxes) that facilitate NR binding. Near the C-terminus, there are two activation domains (AD1 and AD2) that interface with other coregulators, such as CBP, p300, CARM1, and PRMT1. Although the activities of CBP and p300 are consistent with the accepted functions of a coactivator, these proteins are now considered to be ubiquitous integrative components of virtually every eukaryotic transcription complex (Black et al. 2006; Pugh 2006; Tyteca et al.

Figure 1. Schematic presentation of various bHLH-PAS proteins demonstrated in eukaryotic metazoans, including ARNT, Tango, AhR, AhRR, HIF-1, Sima, Trachealess, Single-minded, BMAL, and all three SRCs. Note substantial size differences between the superfamily members, whereas all they share N-terminally located bHLH and PAS domains; majority has twin-PAS consisting PAS-A and PAS-B domains. Several members display Gln- and Ser-rich motifs in their C-terminally positioned regions.

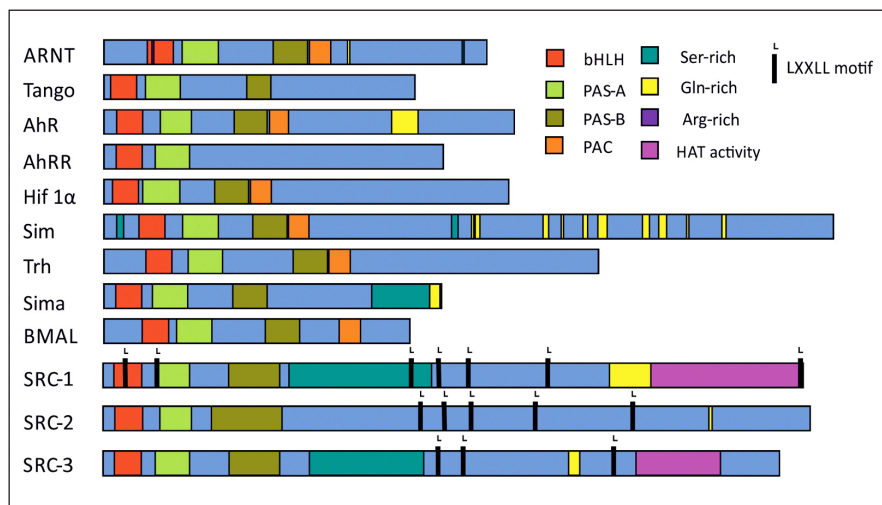


Figure 2. Crystallographic 3D structure of bHLH domain of human Upstream Stimulatory Factor protein (1AN4.pdb) composed of several α -helices X-ray diffracted at 2.9Å resolution.

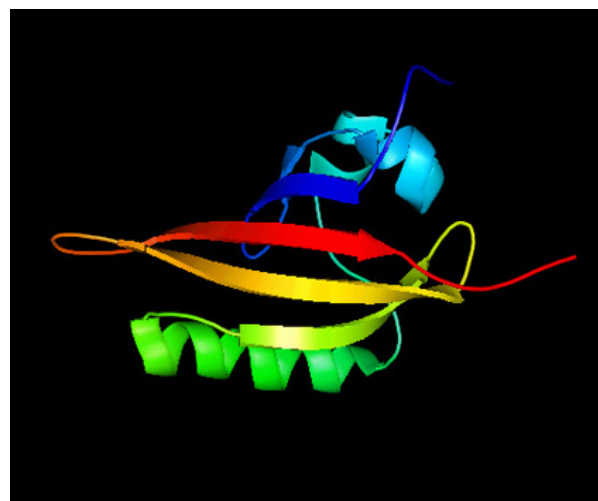


Figure 3. The 3D structure of PAS domain of human ARNT (HIF1 β) protein (1X00.pdb) obtained by NMR. Note the sandwich made four antiparallel β -sheets (blue, red, yellow, light green) and a single longer α -helix (green) at the bottom.

2006). The SRCs are involved in the regulation of almost all the aspects of the gene expression, including transcriptional initiation, cofactor recruitment, elongation, RNA splicing, post-translational modification (PTM) of NRs/coregulators, and chromatin components (Dasgupta et al. 2014).

In fact, both bHLH and PAS proteins are classes of their own. The bHLH is a protein structural motif (Figure 2) that identifies a family of metazoan transcription factors (Murphy et al. 2007; Weber et al. 2014; Stashi et al. 2014), characterized by two α -helices connected by a loop. Transcription factors

bearing this domain are dimeric, each with one helix containing basic amino acid residues that facilitate DNA binding. One helix is usually smaller and due to the flexibility of the loop allows dimerization by folding and packing against another helix. The larger helix typically contains the DNA-binding regions. The bHLH proteins usually bind to a consensus sequence called an E-box, CANNTG (Chaudhary and Skinner 1999). The canonical E-box is CACGTG (palindromic), however, some of the bHLH transcription factors, notably those of the bHLH-PAS family, bind to the related non-palindromic sequences, which are simi-

lar to the E-box. Transcription factors that belong to the bHLH family play fundamental role in numerous developmental programs, including neurogenesis, myogenesis, hematopoiesis, sex determination, etc. (Littlewood and Evan 1994; Farah et al. 2000; Hjalt 2004; Jones 2004; Stashi et al. 2014; D'Rozario et al. 2016). On the other hand, the PAS domain (Figure 3) is present in many signaling proteins, found in a large number of organisms from bacteria to humans, where it functioning as a signal sensor composed of several antiparallel β -sheets forming a bunch or sandwich (Ponting and Aravind 1997; Hefti et al. 2004; Stashi et al. 2014). Many PAS-domain proteins, which are involved in variety of activities, including neural development, circadian rhythms or dioxin and other toxins binding, often detect their signal by a way of an associated cofactor. The mammalian bHLH-PAS proteins, notably of SRC/NRC family, have regularly two PAS domains of approximately 50 to 70 amino-acid residues (known as PAS A and PAS B), separated by about 80 to 150 residues that are poorly conserved. The existence of cofactors, termed co-activators or adapters, was first suggested by a transcriptional squelching between the progesterone and estrogen receptors (Hong et al. 1997; Shibata et al. 1997; Li and Chen 1998; Leo and Chen 2000). Corepressors have also been postulated to contribute to the silencing function of an unliganded thyroid hormone receptor (TR). Onate et al. (1995) have cloned the first functional coactivator, termed steroid receptor coactivator 1 (SRC-1) that appears to be a general coactivator for several steroid receptors tested, which enhances transactivation of steroid hormone-dependent target genes. Subsequently, more co-activators have been reported, including the SRC-1 related proteins, TIF2 and GRIP1, and other putative and unrelated co-activators, such as ARA70, Trip1, RIP140, and TIF1 (Nagy et al. 1999; Hsu et al. 2003; van de Wijngaart et al. 2006, 2012; Dasgupta et al. 2014; Stashi et al. 2014). In addition, another co-activator CREB-binding protein (CBP) has been shown to enhance the steroid receptor-dependent target gene transcription. CBP and SRC-1 interact and synergistically enhance the transcriptional activation by the ER and PR. Therefore, a ternary complex (consisting of liganded steroid receptor dimer, SRC-1, and CBP) must be form to achieve an increase in the rate of hormone-responsive gene transcription.

How hormonal signaling works

NRs are unique transcription factors composed of three modulatory domains: N-terminal transactivation domain (NTD), highly conserved and ordered

DNA binding domain (DBD), and C-terminally positioned also highly ordered ligand binding domain (LBD). NRs can be categorized not only based on the nature of their DNA binding sites, but also the fact whether they form homodimers, such as the androgen receptor (AR), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), estrogen receptor (ER), and progesterone receptor (PR) or heterodimers, such as the retinoic acid receptor (RAR), thyroid hormone receptor (TR), ecdysteroid receptor (EcR), farnesoid X receptor (FXR), liver X receptor (LXR), peroxisome proliferator-activated receptors (PPARs), and vitamin D₃ receptor (VDR). Heterodimerizing group of receptors requires one of the three isoforms of the universal and promiscuous dimerization partner, retinoid X receptor (RXR), a natural ligand, which is the 9-*cis* retinoic acid (Norman 1992; Kastner et al. 1995; Mouchon et al. 1999; Laffitte et al. 2000; Wolf 2000; Pogenberg et al. 2005). Generally, after transportation through the cell membrane, ligand is taken up into the pocket of NR LBD (LBP). Ligand binding by NR is considered as an initial step in the hormone action (Yamamoto 1985; Evans 1988; Chen and Evans 1995; Mangelsdorf et al. 1995; Nagy et al. 1999; King-Jones and Thummel 2005; O'Malley 2007; Heldin et al. 2016). It is an allosteric mechanism, which allows conformational change and dimer formation, then leading, among others, also to accessibility of coactivators and release of corepressor proteins if previously complexed with NR. The ligand bound receptor can not only dimerize, but it also binds to its cognate DNA, so called hormone-response element (HRE) from where it regulates the gene transcription (Hong et al. 1997; Mouchon et al. 1999; Klinge et al. 2004; Heldin et al. 2016).

The LBD domain of NRs forms a defined globular structure, in which eight to twelve α -helices, as well as 1 to 4 β -sheets are arranged together in an antiparallel three-layered sandwich (Figure 4). All so far obtained crystal structures of NR LBDs indicate that the ligands are completely engulfed inside LBP in the interior of the protein. As documented by numerous X-ray crystallographic studies, the ligand binding is accompanied by a transition from the unbound to bound states what means that the C-terminal helix 12 (H12) moves from an open to a closed conformation in a manner termed as "mousetrap" model (Parker and White 1996; Wurtz et al. 1996). This conformational change results in a completion of the AF-2 pocket (contributed by helices H3, H4/5, and H12) that forms a conserved surface exposing hydrophobic groove (Darimont 2003). Coactivators interact with this AF-2 groove of NRs through a highly con-

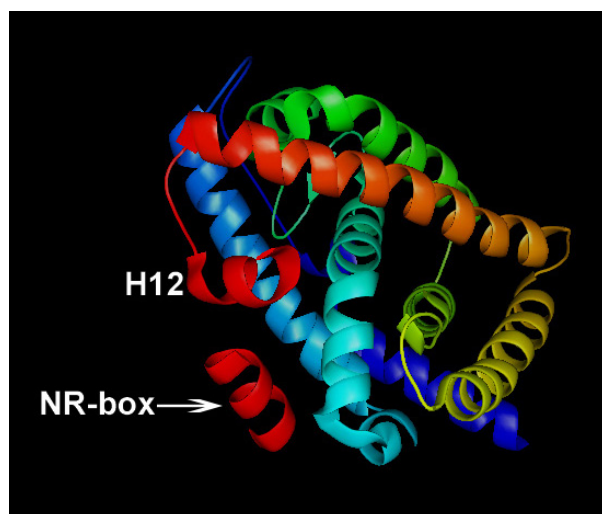


Figure 4. Rendered image of the X-ray crystallographic 3D structure of a single LBD of RAR (3KMR.pdb) diffracted at 1.8Å resolution. Note the indicated position of helix H12 and LXXLL peptide (NR-box) co-crystallized in the vicinity with a charge clamp of RAR (both red).

served LXXLL consensus sequence (where L stands for leucine and X is any amino acid) also called NR-box, which bind as amphipathic α -helices with the hydrophobic L residues, arranging themselves into three hydrophobic subpockets found in the AF-2. In the absence of a bound ligand, H12 is positioned such that the moieties, forming the AF-2, are distorted and exposed as a corepressor-binding interface or simply not binding any coregulator (Shibata et al. 1997; Leo and Chen 2000; Dutertre and Smith 2003; Mita et al. 2010; Demizu et al. 2013). By other words, ligand binding results in a repositioning of H12 in the LBD thus enabling the recruitment of SRC coactivators. SRCs interacting with NRs via NR-boxes facilitate the transcription via histone acetylase (HAT) activity intrinsic to SRC (Spencer et al. 1997; Torchia et al. 1997; Utley et al. 1998; Sterner and Berger 2000; Sachs and Shi 2000; Sachs et al. 2001; Black et al. 2006; Pugh 2006; Tyteca et al. 2006). In addition, the activation domain 1 (AD1) of SRCs binds to the C-terminal SRC interaction domain (SID) of p300 as well as its homolog, the cAMP response element-binding (CREB) protein (CBP) (Li and Chen 1998; Sheppard et al. 2001; Dutertre and Smith 2003; Ding et al. 2015). The p300/CBP complex plays an important role in the transcription process through connecting other transcriptional activators, basal transcription factors, chromatin remodeling proteins, and HATs to the transcriptional machinery (Zhou et al. 1998;

Vo and Goodman 2001; van de Wijngaert et al. 2012; Zelenko et al. 2012; Wang et al. 2014).

Numerous structural and *in silico* studies on NRs have documented the importance of the SRCs-NRs interaction for hormonal signaling. For example, comparison of farnesoid X receptor (FXR) LBD simulations with and without ligand-LBD interaction has revealed that the H11-H12 loop is fundamental in stabilizing H12 to be able to bind NR box of SRC (Costantino et al. 2005; Mackinnon et al. 2014). In the AR, a conserved proline at position 892, located at the H12, has been shown to be critical for the proper function as mutation of this residue, rendered the receptor inactive due to a distorted, unstructured, and misplaced H12 thus blocking interaction with SRC's NR box (Elhaji et al. 2006). Furthermore, mutation of residue 887 in H11 of AR results in H12 being locked in the active conformation leads to antagonist resistance (Bisson et al. 2007). The role of H3 in the allosteric movement of H12 has been demonstrated via mutations of residue 280, a residue that does not directly interact with the ligand but modulates the coactivator binding. In the TR, mutations of residue 280 significantly reduce the overall ligand binding affinity from 29% (I280M) to 100% (I280R and I280K) (Souza et al. 2011). Molecular dynamic (MD) simulations suggest that the mutations indirectly block either the coactivator or corepressor activity or both. Indeed, both the I280R and I280K mutations were found to form salt bridges between the E457 in H12 and LBP, stabilizing H12 in a conformation that may prevent either coactivator or corepressor binding. On the other hand, the I280M mutation blocked the corepressor binding and it appears that enhances the coactivator affinity, suggesting stabilization of H12 in agonist conformation." Moreover, mutation of residue 774 in H5 of the AR has been shown to sufficiently reduce AR ligand binding activity to cause complete androgen insensitivity syndrome (CAIS) (Wu et al. 2003; Mackinnon et al. 2014). Temperature-dependent MDs data have demonstrated that this thermolabile mutant prompted significant structural distortion above 20°C that induces H1-H2 loop rearrangement followed by an H4-H5 loop distortion and a subsequent H5 rearrangement, indicating that a temperature-dependent mechanism is involved in the development of CAIS.

This type of studies was able to provide deeper insights into the understanding of the dynamics of allosteric consequences (population of NR conformational ensembles) of ligand binding to NRs. MD simulations of a GR-antagonist complex have shown that while the antagonist can be sterically compatible with an H12 agonist position the ligand serves

to induce rapid H12 conformational rearrangements (Presman et al. 2010). These observations have suggested that the ligand does not induce one particular receptor conformation but instead changes the dynamic equilibrium of H12 that results in several conformational states. Similar behavior has also been reported for PPAR γ bound to either of the agonist MRL20 or MRL24. Using a combination of nuclear magnetic resonance (NMR), hydrogen-deuterium exchange (HDX) and molecular docking Hughes et al. (2012) have found that ligands sample multiple binding modes, in which the most populated binding mode occurs between 62% (MRL24) and 91% (MRL20) of the time. In an alternative approach, using ancestral steroid NR reconstruction nanosecond MD simulations has been shown that the protein-ligand complex's ability of sampling a huge number of conformational microstates could be impeded by the introduction of an excess of interaction partners (Harms et al. 2013). Interestingly, subtle changes in the structure of the NR, via the introduction of two mutations, resulted in transitions among frustrated ensembles of suboptimal states that resulted in a 70 000-fold reduction in specificity (Mackinnon et al. 2014).

Originally simple scheme becoming more complicated

The above described scheme of ligand and NR actions was recognized and supportingly constructed over two decades. In the recent years, it is facing to several new challenges, which on the one side complicate the original simple view, but on the other side it provides more comprehensive characteristics of the entire system helping to explain several previously not understood observations. In fact, since the beginning to make this notion simpler, it should be said that previous static view nowadays start to pre-occupy more dynamic contours. As indicated above, upon binding of the ligand to the LBP, the spatially distinct AF-2 coactivator binding site, is formed via a reorganization of the surface-exposed hydrophobic cleft that involves H3, H4-5, and H12 (He et al. 2000; Darimont 2003; Osguthorpe and Hagler 2011; Osguthorpe et al. 2012; van de Wijngaart et al. 2012). However, employment of several modern techniques, including fluorescence spectrometry, absorption and emission spectroscopy, and calorimetry that collectively can be named as molecular dynamics (MD), have revealed that ligand binding to AR led to a bi-phasic receptor rearrangement that involves population of a molten globule-like intermediate state, caus-

ing a substantial rearrangement of the AF-2 binding surface (Jasuja et al. 2009; Zakharov et al. 2011). Further analysis of the communication between the AR with ligands and coactivators by dynamic cross-correlation maps from MD simulations and thermodynamic data has revealed a bidirectional effect on binding at the AR ligand and AF-2 sites (Xu et al. 2011; Buzon et al. 2012; Mackinnon et al. 2014; Gallastegui et al. 2015). Binding of the dihydrotestosterone to the AR LBD resulted in the opening of the LBD. What become curious, however, is that the recruitment of the SRC coactivator to AR has to be found to also enable the opening of the LBD pocket. In addition, nanosecond time-scale MD simulations have revealed that a second coactivator peptide was able to bind along H3 of the FXR and the peptide position was modified by the binding of the ligand and the first coactivator (Costantino et al. 2005). Fidelak et al. (2010) have used normal mode of PPAR γ analysis to show the existence of a network of conformations derived from low-frequency collective motions that couples different physiologically important interfaces of the LBD. In these simulations, they have observed that mutation of the H12 Y473 residue was sufficient to disrupt full agonist binding by uncoupling H12 from H3, H4/5 and H10/11 and thus indicated hydrophobic cleft misformation (Fidelak et al. 2010). On the case of ERs, we can document an example of a synergism between transactivation function 1 (AF-1) and AF-2 of ER α mediated by steroid receptor coactivator protein-1, which requires AF-1 α -helical core for a direct interaction between the N- and C-terminal domains. This is functionally achieved by bridging coactivators, such as CEBP binding protein/p300 and members of the p160 subfamily, such as SRC-1. Glutathione-S-transferase pulldown assays have demonstrated that the AF-1 core is able and sufficient for the ER α N-terminal region to interact with SRC-1. In the presence of the ER α ligand-binding domain an enhancement of this recruitment has been observed, being dependent on the direct interaction between the N-terminal B domain and the ligand-binding domain (Metivier et al. 2001).

In order to identify the underlying mechanism of coactivator binding, Carlsson et al. (2005) have used a series of 10 ns MD simulations of the wild type (wt) GR as well as its A571M, A573Q and A571M/A573Q mutations. They have found that the mutations conferred an important shift in both the structural and dynamic properties of the GR LBD. When the biological activity increase was induced via mutation, the distance between the H12 and the N-terminal-facing aspect of H3 decreased and this trend was also found

between H3 and the C-terminal-facing part of H11. Moreover, H4 was seen to move closer to H12, whereas H5 increased its distance from H12. Combination of these structural shifts has resulted in a modified binding cleft for the coactivator peptide. Using correlation analyses for each residue, they detected an overall reduction of 10–25% in backbone fluctuation associated with increased biological activity (from wt to the double mutant). This effect has been found to be most pronounced in the loops preceding the H3 and H12 helices, where a 40–70% reduction was observed. Together, they concluded that the mutation-induced rearrangement of the AF-2 helices facilitates coactivator binding by lowering energetic costs for receptor coactivator complex formation, and the reduced fluctuations that improved steric interaction may improve binding affinity through lowered entropic costs for binding. Yet, despite the evidence in favor of cofactor pocket remodeling, they point out that the low nano-second timescale they employed does not permit the incorporation of slow protein events such as folding or interdomain movements. Perhaps, the most interesting observation they have found is that “in all simulations of wt and mutant models, the H12 remains in the canonical agonist position and displays low Ca fluctuations in both the apo- and holo-structure model”. They have concluded that the fluctuation of H12 residues is virtually uncorrelated to biological activity in the four complexes investigated. They attribute the difference in their simulation with other results that correlate differences in H12 position and dynamics with roles in activity due to the presence of the C-terminal coil that precedes the GR H12 (a feature that has been absent in other NRs such as AR), which they speculate immobilizes H12.

Where the SRCs act

Originally, each individual SRC has been identified as coactivator of a particular NR. More specifically, SRC-1 has been shown to interact with ER and PR to regulate uterine function, with loss of SRC-1 decreasing uterine growth during the development (Walsh et al. 2012). Moreover, SRC-1 is an important regulator in the progression of endometriosis (Han et al. 2012). In the breast cancer, SRC-1 plays a role of potential oncogene associated with poor outcome prognosis, with roles in cell migration and metastasis (Qin et al. 2009). In the case of SRC-2, its loss in mice (*Ncoa2^{-/-}/Ncoa2^{-/-}*) affects the whole-body physiology with growth retardation and reduced adiposity. Defects in the spermatogenesis and testicular degeneration have been observed in adult male *Ncoa2^{-/-}*

Ncoa2^{-/-} mice (Gehin et al. 2002). SRC-2 also plays a role in the cancer development. It is amplified in the prostate cancer. Translocations of SRC-2 with monocytic leukemia, zinc-finger protein (MOZ) have been found in cases of acute myeloid leukemia (Shiota et al. 2009; Taylor et al. 2010). Similarly, SRC-3 ablation in mice (*Ncoa3^{-/-}/Ncoa3^{-/-}*) results in a decreased growth, development, and reduction of circulating estrogen that delays the puberty onset (Xu et al. 2000). As an oncogene, SRC-3 has been extensively studied in various cancers, but mostly, as an amplified gene, in breast cancer with defined molecular roles in cell growth, epithelial-mesenchymal transition (EMT), and Her2 signaling (Osborne et al. 2003; Xu et al. 2009). Similarly to SRC-1, SRC-2 functions as either a coactivator or a corepressor for GR (Gupte et al. 2013). When SRC-2 is recruited directly to the p65 subunit of nuclear factor κ B (NF- κ B) and activator protein 1 (AP-1), it acts as a corepressor for GR and decreases gene expression in the inflammatory response (Gupte et al. 2013). The interplay of SRC-2 with both coactivator and corepressor for GR mediates inflammatory and metabolic signaling (Uhlenhaut et al. 2013). SRC-3 coactivates the progesterone receptor (PR) in the pituitary cells. This interaction is increased by gonadotropin releasing hormone (GNRH) or progesterone treatment (An et al. 2006). Loss of SRC-3 in females increases the anxiety responses, suggesting a dynamic interplay between the SRCs and the regulation of stress responses (Stashi et al. 2013). SRC-3 expression in the brain changes by the age, being higher in young (i.e. 25 week old) and lower in old male mice (i.e. 70 week old). Likewise SRC-3, in which expression also decreases in pluripotent cells after differentiation (Paramanik and Thakur 2011; Percharde et al. 2012). Each of the SRCs plays a prominent role in the hypothalamic-pituitary-adrenal-axis (HPA axis), with SRC-1 regulating CRH production and feeding behaviors in the hypothalamus. SRC-3 is being a PR coactivator for the gonadotropin α -subunit promoter. Within the adrenal glands, SRC-2 is a major regulator of the corticosterone synthesis. SRC-1 and SRC-2 also play an important regulatory role in the cholesterol-mediated signaling via the liver X receptor (LXR)/retinoid X receptor (RXR) heterodimer, which is important not only in lipid and xenobiotic metabolism, but also in the cholesterol regulation (Son and Lee 2010; Percharde et al. 2012). SRC-2 coactivation of LXR/RXR is crucial for the cholesterol biosynthesis in Sertoli cells of the testis and loss of SRC-2 leads to an accumulation of cholesteryl esters, which may explain the hypofertility in aging males (Mascres et

al. 2004). Inflammation and metabolism are inherently linked as NRs regulate gene programs that control both of these processes. SRC-1, together with other coactivators and NRs, contributes to cytokine-induced hepatic metabolic changes during the acute phase response (APR) (Wang and Burke 2008). SRC-1 expression decreases during the APR (Kim et al. 2007). Inflammation-induced APR alters metabolism with interleukin 1 (IL-1) or tumor necrosis factor (TNF) treatment decreasing LXR, RXR, PGC1- α , and SRC-2 expression, resulting in a blunted LXR-mediated APR (Wang 2005). Additionally, several cholesterol intermediates affect SRC-1 and SRC-2 binding to NRs. Both SRC-1 and SRC-2 show preferential recruitment to LRH-1 upon medium-chain phospholipid binding, but are inhibited by long-chain phospholipids (Musille et al. 2012). In steroid biosynthesis, protein inhibitor of activated STAT- γ (PIAS γ) can inhibit SRC-1 binding to LRH-1 on the *Cyp11a1* promoter through competitive binding, thereby decreasing steroid production (Hsieh et al. 2009). Similarly, binding of 24S-hydroxycholesterol inhibits SRC-2 complex formation with ROR α and ROR γ , thereby decreasing Bmal1 and Rev-erba expression, molecular components of the mammalian circadian clock (Wang et al. 2010). Thus, both SRC-1 and SRC-2 regulate cholesterol synthesis through coordination of LXR/RXR- and LRH-1-driven gene transcription.

The SRCs regulate diverse metabolic processes in several key tissues, including the brain, liver, heart, and skeletal muscle, as well as in brown and white adipose tissue. For example, SRC-2 coactivates the bile acid receptor, farnesoid X receptor (FXR), in the liver to promote transcription of BSEP, the gene encoding the bile salt exports pump transporter protein, which is essential for bile acid transport and dietary fat absorption. As a result, SRC-2 deletion increases fecal triglycerides but decreases circulating plasma triglycerides and hepatic bile acid accumulation with reduced expression of bile acid synthesis genes *Cyp7a1*, *Cyp8b1*, *Cyp7b1*, *Cyp27a1*, and *Ntcp* (Chopra et al. 2011). As observed in adipose tissue, hepatic SRC-3 expression increases upon high fat diet (HFD) feeding (Ma et al. 2011). Loss of SRC-3 protects against HFD-induced hepatic steatosis by reducing lipid accumulation and the accompanying inflammatory response (Stashi et al. 2014). From oncological point of view, members of the steroid receptor coactivator (SRC) family are over-expressed in numerous types of cancers. In particular, SRC-3 (often designated under name AIB1) has been recognized as a critical coactivator, associated with tumor initiation, progres-

sion, recurrence, metastasis, and chemoresistance, where it interacts with multiple nuclear receptors and other transcription factors to enhance their transcriptional activities and facilitate cross-talk between pathways that stimulate cancer progression. Because of its central role as an integrator of growth signaling pathways, the development of small molecule inhibitors (SMIs) against SRCs have a potential to simultaneously disrupt the multiple signal transduction networks and transcription factors involved in tumor progression.

Another level of complexity in this system can be achieved by posttranslational modifications (PTM) of proteins, specifically of SRCs. As an important consequence of their existence as multisubunit complexes, SRCs are able to transmit signals from the environment by supplying a variety of enzymatic activities at the promoters of the genes they regulate in an orderly way. For instance, the SRC-3 coactivator exists in a complex that includes kinases, ubiquitin ligases, ATPases, methyltransferases, acetylases and an alternative proteasome activator assembled together. Many other enzymatic capabilities reside in these coregulator complexes that have yet to be characterized (Jung et al. 2005), suggesting that still it is a lot work to do to be able to understand the full contributions of the coregulators provided for the regulation of transcription (Han et al. 2006). SRC-3 serves as a good example to demonstrate how a coregulator PTM code contributes to the biological complexity, signal integration, and the propagation of a biological program. Initially, SRC-3 is likely to be sumoylated (at amino acids 723 and 786) (Wu et al. 2007) and is hypophosphorylated at serine/threonine residues (Wu et al. 2004), existing as an inactive protein in its basal state. After phosphorylations at Ser 505/509 in a GSK3-dependent manner, SRC-3 becomes monoubiquitinated at amino acids 723 and 786 and is able to function as a potent and specific transcriptional activator (Wu et al. 2007). The ubiquitination sites are progressively polyubiquitinated during subsequent rounds of transcription, ultimately leading to its degradation by the 26S proteasome. Independent of this, other phosphorylation sites in SRC-3 are targeted by other kinases and are necessary for SRC-3 to form divergent multiprotein complexes and coactivate its full range of transcription factors (Wu et al. 2004). However, PTMs have yet another mode how to play; SRC-3 can be methylated or acetylated leading so to coregulator complex disassembly. This, along with proteasome-mediated degradation, can contribute to the coregulator component dynamics, including turnover (Lee et al., 2005; Feng et al., 2006).

Combined and non-hormonal actions of SRCs

As mentioned above, SRCs/NRCs have been originally identified and for a long time known as coregulators of NRs. Majority of the information come out from the *in vitro* studies, using stable cell lines, which naturally have their limitations. What makes SRCs even more interesting is the fact that they have also their own or NR-independent life. This property has not been comprehended adequately yet. In fact, it should not be surprising that due to their structural and domains' diversity, the SRCs display broader regulatory potential than that being associated with hormone signaling and NRs. This is prompting us to look at the SRCs family also as proteins with unexplored potential in developmental and broader comparative studies. Already early studies, using heterologous systems *e.g.* yeast two-hybrid and mammalian coexpression systems, have shown that the magnitude of SRC-2/GRIPI enhancement of liganded NR homodimer is dependent upon NR subtype and HRE configuration. For most HRE configurations, TR and RAR homodimers were essentially unresponsive or very weakly active in the absence of SRC-2/GRIPI, but SRC-2/GRIPI dramatically restored the ligand-dependent function of these NRs. Although, SRC-2/GRIPI exerted no significant effect on NR homodimers in the absence of their cognate ligands, it increased the transactivation of unliganded NR heterodimers (Walfish et al. 1997; Klinge et al. 2004), indicating that the transcription regulatory ability of SRC family members is their inherent property rather than being fully dependent on the liganded NR.

Another departure from the classical NR-SRC scheme can be seen in a modulation of ER α -mediated gene regulation by a multimeric chromatin complex involving the two receptors and the coregulator RIP140 by aryl hydrocarbon receptor (AhR) (Matthews et al. 2005; Madak-Erdogan and Katzenellenbogen 2012). In addition, ligand activated dioxin receptor itself (heterodimer composed of two bHLH-PAS proteins, AhR and ARNT1) recruits SRC-1 and or complex of ER α and SRC-1 to propagate dioxin receptor-mediated gene transcription (Mimura and Fujii-Kuriyama 2003; Beischlag et al. 2008; Endler et al. 2014). This type of interaction is often context- and/or enhancer-specific and can be dictated by the position of consensus hexanucleotide sequence known as the E-box within a target gene, such as *CYP1A1*. For instance, some of the dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, known as TCDD)-bound AhR/ARNT signals can

be mediated by GR/SRC-2 complex, rather than ER α and SRC-1 (Endler et al. 2014). The presence of SRC-1 or SRC-2 is essential for transcription initiation to unwind histone-bound DNA by exposing chromatin recognition sites to histone acetyltransferase (HAT) activity provided by SRC's HAT domain (Utley et al. 1998). Dutertre and Smith (2003) have provided evidence that interactions of SRC-1 or other p160/steroid receptor coactivators and CREB-binding protein (CBP) with ER α can be fully ligand-independent and regulated by phosphorylation sites (Ser104/106/118) in the A/B region of the ER α . Molnar et al. (2005) have identified some of the structural determinants of the agonist-independent association of human PPAR receptors with coactivators. Using PPAR γ as target, they found that four different amino acid groups contribute to the ligand-independent stabilization of helix H12 of the PPAR ligand-binding domain. These amino acid groups include: (i) Lys329 and Glu499, mediating a charge clamp-type stabilization of helix H12 via a SRC bridge; (ii) Glu352, Arg425, and Tyr505, directly stabilizing the helix via salt bridges and hydrogen bonds; (iii) Lys347 and Asp503, interacting with each other as well as contacting the SRC; and (iv) His351, Tyr(355), His477, and Tyr501, forming a hydrogen bond network. These amino acids are highly conserved within the PPAR subfamily, suggesting that the same mechanism may apply for all three PPARs. Phylogenetic trees of helix H12 amino acid and nucleotide sequences of that time crystallized NRs and all human NRs, respectively, indicated a close relationship of PPARs with constitutive androstane receptor and other constitutive active members of the NR superfamily. Taking together, the ligand-independent tight control of the position of the PPAR helix H12 provides an effective alternative for establishing an interaction with SRC proteins. The importance of a specific DNA sequence within HRE as an allosteric effector of NR action has been further documented by Klinge et al. (2004) for ER and its EREs. Alterations in ER conformation induced by binding to different ERE sequences modulate ER interaction with coactivators and corepressors. CHO-K1 cells transfected with ER α or ER β show ERE sequence-dependent differences in the functional interaction of ER α and ER β with SRC-1, SRC-2, SRC-3, then with ACTR, CBP, and steroid receptor RNA activator (SRA), as well as corepressors including NCoR and SMRT, and secondary coactivators such as coactivator associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase 1 (PRMT1). Their data

demonstrated that the ERE sequence impacts estradiol- and 4-hydroxytamoxifen-occupied as well as ligand-independent ER α and ER β interaction with coregulators, as measured by transcriptional activity in mammalian cells.

Sort of interstep between the hormonal actions and divergence from classical coactivators' involvement, can be seen in ability of some NR receptors to recruit non-SRC bHLH-PAS proteins instead of particular SRC or ramification of the original hormonal signaling towards dioxin pathway. One of the first such unexpected deviations has been described by Rushing and Denison (2002) for RAR and TR silencing mediator's interaction with the AhR, which, however, failed to repress AhR-dependent gene expression. Very interesting was an observation of Widerak et al. (2006) who have found that AhR activates the RAR α through SMRT antagonism. TCDD-bound AhR activated a subset of retinoid-dependent genes (tissue-transglutaminase, IGF binding protein-3, AhR) in MCF-7 breast cancer cells via stimulated transactivation by several class I heteromeric receptors (RAR and TR), while it antagonized homodimeric nuclear receptors (ER and PR). TCDD exerted a dose-dependent effect on a RAR α -dependent reporter gene expressed in MCF-7 cells. AhR has been shown to be involved in a mutual antagonism with RAR α corepressor SMRT. Ruegg et al. (2008) have described interaction of ER β with ARNT in place of SRC-1 or SRC-3. This interaction was dominant for ER β over ER α and mediated through A/B domain of ER β . In addition, this unusual biochemical behavior of ER β can result in alternative pathway mediating antiestrogenic effects of dioxin instead of estrogenic signaling.

On the other hand, perhaps complete absence of typical hormonal involvement in the SRC actions can be envisaged from the interaction of individual SRCs and related partners with non-NR proteins complexes. Nguyen et al. (1999) have found interaction of SRCs, ERAP140 and SMRT with AhR/ARNT complex via co-immunoprecipitation and gel mobility shift assays by their binding to ³²P-dioxin response element (DRE), documenting that dioxin signaling can utilize NR coactivators and/or corepressors for mediating own action. Currently, there is growing evidence, indicating that other bHLH-PAS pathways use SRCs or NR-machinery components for exerting their signaling (Xu and Li 2003; York and O'Malley 2010). This is best exemplified by components of the circadian rhythms machinery such as Cycle, Clock, BMAL, and Period. The circadian clock orchestrates physiological and

behavioral activities, including metabolism, neuronal activity, and cell proliferation in synchrony with the environmental cycle of day and night. BMAL1-Clock is a core transcription complex in the molecular circadian clock. Aryl hydrocarbon receptor nuclear translocator-like protein 1 is protein that in humans is encoded by the ARNTL gene also known as *Bmal1* and plays a key role as one of the positive elements in the mammalian autoregulatory transcription translation negative feedback loop (TTFL), which is responsible for generating molecular circadian rhythms. BMAL1 has also been identified as a candidate gene for susceptibility to hypertension, diabetes, and obesity (Pappa et al. 2013; Richards et al. 2014) and mutations in *Bmal1* have been linked to infertility, gluconeogenesis. BMAL1 binds with a second bHLH-PAS protein via the PAS domain, CLOCK to form a heterodimer in the nucleus (Huang et al. 2012). Via its bHLH domain, this heterodimer binds to E-box response elements (Wang et al. 2013) in the promoter regions of *Per* (*Per1* and *Per2*) and *Cry* genes (*Cry1* and *Cry2*) (Buhr and Takahashi 2013; Wang et al. 2013). This binding upregulates the transcription and translation of PER1, PER2, CRY1 and CRY2 proteins. After the PER and CRY proteins have accumulated to sufficient levels, they interact by their PAS motifs to form a large repressor complex that travels into the nucleus to inhibit the transcriptional activity of the CLOCK:BMAL1 heterodimer (Maywood et al. 2014). This inhibits the transcription of *Per* and *Cry* genes, and causes protein levels of PER and CRY drop. This complex transcriptional machinery is greatly mediated by interaction of BMAL/CLOCK heterodimer with SRC-1 or SRC-2 coactivators where no NRs are involved (Xu et al. 2009; Zhu et al. 2015; Liu et al. 2016; Morishita et al. 2016; Xiong et al. 2016).

LXXLL as NR boxes and non-NR boxes: their diversity in proteins and practical implications

As already indicated above, one of the key interaction, which is required for transduction of ligand-bound NRs to SRCs, is mediated via amphipathic α -helical NR boxes, containing LXXLL motifs recognizing AF-2 groove within NR LBD (Shibata et al. 1997; Darimont 2003; Dutertre and Smith 2003; Loinder and Soderstrom 2004; Stashi et al. 2014). However, these amphipathic α -helical LXXLL motifs participate in numerous different protein-protein interactions associated with different aspects of transcriptional regulation. These motifs are present in many other transcription factors and cofactors, me-

diating interactions that can activate or repress transcription. Some coactivators are bifunctional, containing LXXLL motifs and domains that bind them. For example, a PAS-B domain in SRC-1 interacts with a LXXLL motif in the C-terminal transactivation domain of signal transducer and activator of transcription factor 6 (STAT6), which is a transcription factor involved in regulating the response to interleukin-4 (Litterst and Pfitzner 2002). Unlike the LXXLL motifs of SRC cofactors, the K1 residue of the STAT6 motif is polar. The crystallographic 3D structure of this interaction shows PAS-B to adopt a mixed a/b structure with the α -helical STAT6 peptide binding in a shallow hydrophobic groove (Razeto et al. 2004). The 3D structure also reveals several mechanistic differences in the LXXLL motif-binding mode compared with previous, NR-based complexes. For example, PAS-B does not employ a “charge-clamp” mechanism to tether the LXXLL α helix. Furthermore, the K1 residue does not contribute to the complex interface (Razeto et al. 2004). Interestingly, although many of the residues lining the LXXLL-binding site of PAS-B are conserved throughout the NCoA family, only SRC-1 interacts with STAT6. Authors attribute this to a “surface complementarity” between SRC-1 PAS-B and STAT6.

CBP/p300 also possesses the capacity to recognize LXXLL motifs. The KIX domain of CBP interacts with a LXXLL motif in the transactivation domain of c-Myb, which like STAT6, contains a polar K1 residue that is not involved in the interaction (Zor et al. 2004). The c-Myb motif binds to the same interface on KIX as the phosphorylated kinase-inducible domain (pKID) of CREB (Radhakrishnan et al. 1997; Zor et al. 2004).

E-proteins are another family of transcription factors that interact with CBP/p300 via LXXLL motifs. E-proteins (e.g. E2A, HeLaE-box-binding (HEB) and E2-2) are involved in regulation of cell growth, differentiation, and apoptosis (Quong et al. 2002). In E-protein transactivation, CBP/p300 is recruited by a LXXLL motif in the N-terminal activation domain 1 (AD1) (Zhang et al. 2004). This functionally crucial interaction is inhibited in the presence of AML1-ETO, a fusion protein commonly associated with acute myeloid leukemia (AML). Zhang and co-workers (2004) have demonstrated that AML1-ETO specifically targets the E-protein LxxLL motif, thereby, directly preventing the binding of CBP/p300. AML1-ETO is a fusion protein that results from t(8;21) chromosomal translocations (Licht 2001). The LxxLL-binding activity of AML1-ETO was localized to the TATA-binding protein (TBP)-associated factor

(TAF) homology (TAFH; also called nervy-homology region 1, NHR1) domain from the fusion partner ETO (Zhang et al. 2004). The TAFH domain family – which has examples in several TAFs from transcription factor IID (TFIID), other ETO-related proteins and the *Drosophila* protein nervy (Kokubo et al. 1993; Kitabayashi et al. 1998; Davis et al. 1999) had been functionally annotated, albeit loosely, as a protein-protein interaction module. The results of Roeder and colleagues suggest the TAFH domain functions as one of a growing number of non-NR LXXLL-binding domains (Zhang et al. 2004).

The presence of TAFH domains in subunits of TFIID raises the possibility of direct regulation of the preinitiation RNA polymerase II complex by LXXLL-carrying proteins. Such a system has already been established for certain nuclear receptors that can interact with LxxLL motifs in Med1, a subunit of the mediator complex (Rachez et al. 2000). Currently, it remains to be established whether other members of the TAFH family are capable of interacting with LXXLL motifs. These observations raised the exciting possibility of using modified LxxLL-containing peptides as targeted inhibitors of the AML1-ETO-E-protein complex or other LXXLL interactions involved in disease. Potentially, such molecules would not only provide substantial insight into the functional importance of regulatory interactions involving these motifs, but might also represent promising therapeutic strategies.

Indeed, in several recent years, there are numerous reports describing new LXXLL peptide mimetics often called also SRC small molecule inhibitors (SMIs) or SRC small molecule stimulators (SMS), which either prevent or potentiates activity of individual SRCs. The first were benzodiazepine derivatives selectively acting as LXXLL peptide mimetic inhibitors of the interaction of VDR with coactivators (Mita et al. 2010), followed by 2-aminoisobutyric acid containing leucine-based stapled short helical peptides (Demizu et al. 2013). More recent are gossypol cardiac glycoside buffalin, and protein synthesis inhibitor verrucaric acid, both highly selective against SRC-3, although they show lowered activity also against SRC-1 (Wang et al. 2011, 2014; Yan et al. 2014). These inhibitors selectively promote the degradation of the SRC-3 protein, while affecting SRC-1 and SRC-2 to a lesser extent and having no impact on CARM-1 and p300 protein levels. Verrucaric acid has been found to be cytotoxic toward multiple types of cancer cells at low nanomolar concentrations, but not toward normal liver cells. Up to now, most targeted chemotherapeutic drugs have been designed largely to block a single

pathway at a time, but cancers frequently acquire resistance by switching to alternative growth factor pathways. These findings might represent promising development in a novel category of chemotherapeutic agents against SRC coactivators that sit at the nexus of the multiple cell growth signaling networks and transcriptional factors might be particularly effective drugs.

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