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Review Article

Intrinsic Disorder in Nuclear Receptor Amino Termini: From Investigational Challenge to Therapeutic Opportunity

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Abstract. Epidermal keratinocytes form an effective renewable barrier to surface assaults and desiccation of underlying tissues through a tightly controlled program of regeneration and terminal differentiation which is significantly impacted by the activity of several members of the nuclear receptor (NR) superfamily. As such, there is significant interest in physiological and pharmacological control of select NRs. NRs are usually considered quintessential examples of constrained structure-function relationships among protein families because of amino acid identity and sequence subserving physical requirements inherent to a relatively centrally-located DNA-binding domain and carboxyl-terminal ligand-recognition domain which together lead to agonist-activated gene expression. Nevertheless, across the superfamily the amino terminus of many NR is an often-critical contributor in degree of receptor-dependent transcriptional activity despite little in apparent sequence similarity that might be instructive in understanding this ability. By looking beyond shared strict amino acid sequence identity, a number of investigations are revealing the "unstructured"-function consequences of this disparity. Significant correlations between *in silico* and *in vitro* biophysical assessments are highlighting the shared trait of the unstructured nature or intrinsic disorder (ID) of NR amino termini and related functional consequences. Rather than the limited protein sequence variation-on-a-theme seen for zinc fingers (DNA binding) or a hydrophobic pocket (ligand binding), these amino-termini show sequence order diversity but often strikingly shared amino acid composition profiles not supporting a one-sequence–one-structure conformation. In this review, we look to integrate amino-termini ID reported in the literature, or predicted here, for select keratinocyte-expressed NR. As evidenced by success in drug targeting the amino-terminus of the androgen receptor, increased appreciation of amino-termini structure - or unstructure - might provide better understanding of NR function in general and possible future investigations on pharmacologic control over keratinocyte regeneration and/or differentiation.

Keywords: Intrinsically Disordered Proteins, Intrinsically Disordered Regions, Nuclear Receptors, Differentiation, Keratinocytes

1. Introduction: Overview of Keratinocyte Differentiation and Key NR Players

The upper, cellular layer of the skin, the epidermis, is a dynamic, renewing, stratified epithelium capable of protecting the underlying tissues from their desiccation as well as invasion and infiltration of surface microbes and toxins, respectively. Following the infrequent replication of a resident stem cell population [1], daughter cells transiently amplify, cease mitosis, and leave their attachment to underlying connective tissue. As maturation proceeds, these post-mitotic keratinocytes then take on ever-more superficial positions while undergoing significant cellular remodeling. Although more likely a true continuum than separate stages,

there are four histologically recognized epidermal cell layers [2]: basal, as defined by mitotic capacity and integrin-mediated connection to the basal lamina; spinous, the next upper layer characterized by formation of new and numerous desmosome cell-cell junctions; granular, with the hallmark of layer-specific keratohyalin granules and initiation of nuclear degradation; and finally, squamous, at the interface with the environment, providing a physiologically inert layer made of highly protective, flattened cell products, with a totally degraded nucleus and filled with mostly insoluble, cross-linked proteins surrounded by extracellular lipids [3, 4]. In addition to this classically recognized physical protection ability, the lower, metabolically active layers are being increasingly appreciated for their role as signal producers and mediators in inflammatory pathways [5, 6]. Throughout this layer-to-layer progression there is sequential and selective expression of genes for structural proteins and metabolic enzymes courtesy of a tightly-controlled transcriptional program in part impacted (Figure 1) by several nuclear receptors (NR). These NR along with the coordinated influence of other transcription factors ([7] for review) lead to renewal of the protective skin surface every approximate 4 weeks [8, 9], depending on body site.

There is rich, varied, and extensive research literature on epidermal keratinocyte NR (here referring to "nuclear hormone receptor", "nuclear receptor", "steroid receptor", or "steroid hormone receptor"), epidermal responses, and the small diet-derived or steroid hormones that would much later be recognized as NR agonists. Pioneering work [10, 11] by W. Montagna with testosterone in the late 1940s and H.B. Fell with vitamin A in the 1950s established the potency of these compounds in affecting differentiation and replication in the epidermis that would move to the molecular biology era with the demonstration of their cognate NR in keratinocytes many decades later [12–14]. The human genome NR superfamily seems to be recently steady in its 48 members [15, 16] across seven subfamilies (including the NR0 subfamily of DAX and SHP1) with varying and for some NR underexplored effect in skin keratinocytes. Throughout this review, our NR inclusion (Figure 1) has been guided by one or more criteria of reported expression abundance, physiological or clinical ligand effect, and/or exemplar of possible future foci for keratinocyte investigations [17–31].

This consideration brings to the forefront the retinoid X receptor alpha (RXRα, NR2B1) [32] and several of its heterodimerization partners such as retinoic acid receptors (RARα and RARγ, NR1B1 and NR1B3, respectively), peroxisome proliferator-activated receptors (PPARα, δ/β, and γ, NR1C1, 2, and 3, respectively), liver X receptors (LXRα and β, NR1H3 and NR1H2, respectively), and separately the homodimeric steroid receptors for glucocorticoid (GR, NR3C1), mineralocorticoid (MR, NR3C2), and androgen (AR, NR3C4) hormones. Importantly, we note that these NR, while representative of those with epidermal keratinocyte relevance, are certainly not wholly exhaustive.

2. Lessons Learned from Exemplar Ligand and NR Studies

The functional impact of NR mentioned above does vary in regards to keratinocyte regeneration and differentiation [28, 33] but nevertheless illustrates the potential influence of overall NR function and need to understand what is driving their individual activity. For instance, consequences of agonist deprivation are difficult to achieve to 100% by inhibition of endogenous ligand production or dietary restriction. As such, genetic null mutation of the cognate NR (gene knockout) can be employed. However, in some cases these results can be at partial odds with what might be predicted as the converse of experimental agonist excess. For example, there are several examples of the enhancement or suppression, respectively, by retinoid removal or add-back/excess, of overall differentiation or differentiation-dependent gene expression in skin keratinocytes [34–40]. However, in mouse models with keratinocyte-specific loss of RARγ (NR1B3) or RXRα (NR2B1), by far the predominant retinoic acid receptors in epidermal keratinocytes [41], there are some seemingly more limited effects [42–44] than might be expected from these ligand studies. An important key to resolving these differences is consideration of developmental stage of the subject keratinocytes and that cultured adult human cells might respond differently than fetal newborn mouse epidermis. For instance, Chapellier [45] and colleagues concluded that mouse RXRα was "dispensable" for fetal keratinocyte development but necessary as the heterodimer partner for the many NR1 subfamily members (see above) that regulate terminal differentiation in adult epidermis. In mice with either RARγ or RXRα keratinocyte-specific knockout, skin barrier function, which is established late in embryogenesis, was only modestly reduced in newborn mice which nevertheless could survive to adulthood [44]. Redundancy for retinoid signaling via the low-level keratinocyte-expressed RARα may have only been minimally involved given the normal histological appearance of epidermis from RARγ germline-null mice that also had keratinocyte-specific RARα knockout [46]. In contrast, keratinocyte loss of RXRα led by 7 weeks postnatal to a hyperproliferative epidermis. By week 16, the usually basal cell-restricted keratin 5 protein was detected in suprabasal positions along with detection of keratin 6 protein, an indicator of hyperproliferative keratinocytes. Thus, receptor loss was indicating at least some positive involvement in keratinocyte maturation not immediately expected given the differentiation suppression seen with retinoid exposure, at least from supra-physiological experimental levels.

Complex and sometimes confounding ligand and receptor effects in epidermal keratinocytes also come from the NR3 subfamily. Given the clinical importance of glucocorticoids

NR	RAR		PPAR			LXR		RXR	GR	MR	AR
	α	γ	α	δ/β	γ	α	β	α			
SG	MED	MAX	MIN	MIN	MAX	MAX	MAX	MAX	MAX	MED	MIN
SS	MED	MAX	MAX	MED	MAX	MAX	MED	MAX	MED	MAX	MED
SB	MAX	MED	MAX	MAX	MIN	MAX	MIN	MAX	MED	MAX	MAX

Figure 1: **Qualitative overview of relative levels for individual NR in keratinocyte differentiation by epidermal layer.** SB, stratum basale; SS, stratum spinosum; and SG, stratum granulosum are the living layers of the epidermis. Nuclei are degraded by the time cells mature to the upper-most layer, the stratum corneum, and so it is not included. MIN, minimum; MED, medium; and MAX, maximum, reflect levels of staining within levels detected for that NR. Relative comparisons of levels should be made within columns only, not NR to NR across columns especially considering that different representative references [17–27] were consulted for individual NRs, utilizing epidermal tissues derived from different body sites, sun-exposed versus sun-protected regions, unfixed and differently fixed samples, and/or native or antigenretrieved sections.

in numerous inflammatory skin diseases, and the potential for activation overlap of glucocorticoid receptor (GR, NR3C1) and mineralocorticoid receptor (MR, NR3C2) by cortisol, there is continuing interest [47] in improving mechanistic understanding of these NR in keratinocyte physiology. While clearly there are multiple cell types in skin as an organ and especially the appearance of infiltrating immune cells to the epidermis during inflammation, there is definitive evidence that many of the beneficial as well as possibly detrimental epidermal effects of corticosteroids come from receptor signaling intrinsic to the keratinocyte [28, 37]. For instance, despite the anti-inflammatory tissue benefit of glucocorticoids, there is a suppression of keratinocyte replication leading to eventual epidermal thinning with chronic use [48–50]. This interestingly might be countered by purposeful introduction of agonists for other, predominant keratinocyte NR, e.g., PPAR [51]. Interpretation, and ideally counteracting the glucocorticoid detrimental effect at the receptor level, is complicated by differential and overlapping expression of GR and MR during keratinocyte maturation along with the potential for shared activation by the same agonist [47]. To decipher this, Pérez and colleagues have intensively investigated the consequences of individual and dual GR and MR keratinocyte-specific deletion [52–55]. In brief, loss of either GR or MR from the epidermis showed widely-different, receptor-associated consequences. GR-null keratinocytes exhibited delayed fetal skin barrier development, increased cell replication, and decreased expression of late suprabasal differentiation-dependent proteins. In contrast, such NR loss-associated effects were very much reduced in MR-null epidermis. In both cases, adult epidermis showed minimal difference to controls but both were hyper-responsive to phorbol-induced inflammation again emphasizing receptor role intrinsic to the keratinocyte as opposed to local or recruited inflammatory cells. Interestingly, keratinocyte-specific dual loss of GR and MR, when studied both with *in vivo* mouse responses and *in vitro* cultured cells derived from knockout animals, showed defects beyond what might have been expected from a phenotype additive of the two individual NR losses. Thus, while there are distinct GR and MR roles to be played in keratinocytes, there is likely some functional synergy between the two. In turn, this suggests the basic research and clinical application need of better separating ligand-induced effects, possibly by looking for targets outside the LBD-ligand interaction.

3. NR Subdomains: Considering More than Classic Structure-Function

Although not exhaustive of all NR studies on epidermal keratinocytes, and clearly extending to many other tissues that are physiologically responsive to the diverse ligands of NRs, the above RAR, RXR and GR, MR studies suggest additional consideration of NR activation outside of their hallmark function as agonist-dependent transcription factors. NR function [15, 16, 56], including their transcriptional activation of target genes, is classically mapped through A/B – E/F subdomains (although F is not present in all receptors) from amino-to carboxyl-termini ends (Figure 2).

This subregion emphasis was initiated early on from the success of experiments swapping the same subdomain across different NR as well as the biophysical understanding of some individual subdomain's function from its recombinant expression and crystallization for X-ray studies. Thus, we have extensive appreciation for the high degree of amino acid conservation in content and position for the C region, or DNA-binding domain (DBD), such as its containing two zinc fingers, dependent on key cysteine and intervening

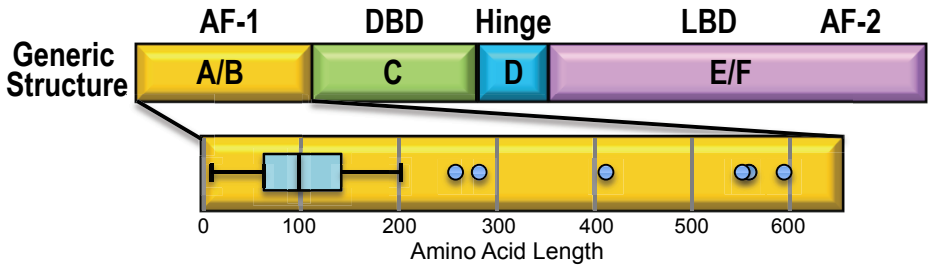

Figure 2: **NR domain representation.** Generic schematic of NR domains, not to scale. Subdomains A/B – E/F are labeled with associated features above schematic: AF-1, activating function 1; DBD, DNA binding domain; LBD, ligand binding domain; AF-2, activating function 2. The extreme length diversity of the A/B region for 41 NR (8 to 599 amino acids as retrieved from Prosite) across the superfamily is indicated by a box-and-whisker plot superimposed on an expanded generic A/B subregion below the full-length receptor schematic. For the A/B regions lengths, the central two quartiles (plotted "box") cover a length range of 63-140 amino acids (to-scale region lengths indicated by 100 unit vertical marker lines). The first and fourth quartiles (right and left "whiskers", respectively), extending from 8-62 and 141-201. Beyond the fourth quartile are 5 "outlier" lengths, mostly from the steroid NR3 group (blue circles ranging from 259-599).

amino acid residues for interaction with and specific recognition of the target DNA sequence. Likewise, the E region, comprising the ligand/hormone binding domain (LBD) and often the largest of the subdomains, has prompted intense study not only as an obvious pharmacologic target but also because of numerous functions mapped here beyond ligand interaction. Participation in NR hetero- or homo-dimerization interaction, with other transcription factors either activating or repressive, and in agonist-dependent transcription activation function (AF-2) have been documented across the E region length. These functions stem in part from retention of some key residues across family members. Additionally, important are the homologous helices providing a shared three-dimensionally ordered structure across the extensive carboxyl terminus domain to allow for interaction with hydrophobic ligands and as an interface for protein-protein interaction. The often relatively short D or hinge region between the DBD and LBD is highly variable in sequence across NR but for some very likely contributes to their intra- and inter-molecular activities. Though mentioned lastly here in this abbreviated subdomain overview, the amino termini of NR display significant amino acid sequence diversity and length variation (Figure 2) across the superfamily, reflected in part by the A/B designation. Nevertheless, the agonist-independent transcriptional activation function (AF-1) contained here for most NR highlights an important addendum to the sometimes agonist-centric interpretation of NR knockout or ligand studies. The sequence dissimilarity here also lends to variation in the type of post-translational modification that can occur and in turn, affect AF-1 and promoter activation [57, 58]. Structural studies for A/B lag behind those for C and E regions in part because A/B sequence diversity might suggest that it would be difficult extrapolate across different NRs the kind of generic lessons learned from the more-conserved structural features of DBD and LBD in the superfamily. This is further compounded by relatively unstructured A/B regions which because of amino acid sequence, shared or not across NR, are not favorable to the 3-dimensional qualities that otherwise successfully drive crystallization for X-ray analysis. Nonetheless, by looking beyond shared strict amino acid sequence identity, a number of investigations are revealing the "unstructured"-function consequences of this disparity. Significant correlations between *in silico* and *in vitro* biophysical assessments are highlighting the shared trait of the unstructured nature or intrinsic disorder (ID) of NR amino termini and related functional consequences.

4. Shift in Protein-Structure Paradigm: Intrinsic Disorder

Over the last two decades, the rigid "lock and key" paradigm of protein structure-function relationships has been challenged by the characterization of numerous proteins as intrinsically disordered (i.e., natively unfolded, intrinsically unstructured) [59–61]. Proteome analysis has revealed that greater than 30% of eukaryotic proteins feature intrinsically disordered regions (IDR) [62], a fraction enriched relative to bacteria and archaea. Across the eukaryotic proteins with IDR, there are a number of critical roles in which they function. These roles are generally categorized as molecular recognition, molecular assembly, entropic chain activity, and protein modifications [63]. The diversity of these functions as well as the relative abundance of IDR suggests an overall importance to diverse biological outcomes. This is most clear when looking at the role of various IDP in human diseases. As reviewed in [64], there are clear examples of IDR which are found within key proteins associated with human pathologies (e.g., p53, BRCA-1 in cancers, thrombin in cardiovascular disease, tau in Alzheimer's disease, among others). Aside from these established proteins, analysis of datasets of proteins related to human diseases have revealed abundance of predicted regions of disorder among proteins related to cancer and cardiovascular disease [64].

IDP are unique due to their lack of set rigid structure which is then associated with a set function. Instead, intrinsically disordered proteins (IDP) or protein IDR are defined by a dynamic nature which allows for transition between four distinct conformations, ranging from disordered random coils to folded, globular proteins with secondary structure [65]. Although structured proteins and IDP differ in their overall conformation, an underlying driver for their structure, or lack thereof, is amino acid composition. IDP and IDR are defined by a decreased abundance of hydrophobic (Ile, Leu, Val) and aromatic amino acids (Trp, Phe, Tyr) which promote stable, hydrophobic cores. Instead, IDP and IDR are enriched for polar, charged amino acids (Gly, Gln, Ser, Arg, Glu and Lys) which are unfavorable for spontaneous, stable, folded conformations [66], resulting in flexible, dynamic protein chains. This fundamental characteristic is one example of a commonality between IDP which is used to perform predictive assessments of whether a protein is disordered or not [67, 68]. The use of predictive algorithms, which are trained on varying length IDR, has been widely used by the IDP field [69], particularly due to development of databases continuously updated with experimentally verified IDP/IDR such as the pioneering DisProt [70] and more recently developed IDEAL [71]. These databases allow for *in silico* structural analysis of yet to be characterized IDR or IDR which lack crystal structure due to the inherent difficulties of unstructured protein X-ray crystallography.

Proteins regions featuring structural flexibility allow for a number of advantages with cellular signaling regulation and control. More specifically, flexibility within IDR allows for increased associations via short interaction motifs found within regions of disorder referred to as molecular recognition features (MoRFs) [72], with increased specificity but low affinity. These regions have been shown to fold upon binding with a partner protein, adopting secondary structure. A unique feature of this folding upon binding is the potential for multiple, distinct folded conformations depending on the associated binding partner [73]. These interactions are dynamic with increased rates of association and dissociation, allowing for finer tuned signaling regulation and fast responses [74]. Increased surfaces within IDR allow for increased post-translational modification (PTM), another tuning control for IDR activity and binding [75]. PTMs (e.g., phosphorylation and acetylation) promote differential surface charges leading to altered primary and secondary structure, including promoting increased or decreased overall order within an IDP/IDR through enhanced intra-molecular folding as well as increased protein-protein interaction induced folding [76]. The frequent occurrence of phosphorylation within any one NR amino terminus and across NR of different types [57, 58, 77, 78] may be facilitated by disorderprovided accessibility for kinases. Interestingly, acetylation is often mapped to the D or hinge region (Figure 2) which may also be an IDR. Working in series with increased PTMs, IDP are believed to be regulated through alternative splicing, a common feature of IDP [79]. It is thought that cell-specific alternative splice events may promote specific PTMs, allowing for an IDP or IDR within proteins to be destined to perform specific functions within specific cell-types [80, 81]. These features coupled with increased surface protein-protein interaction motifs mediating dynamic binding activity results in proteins which are promiscuous binding partners, diverse in their function, and adaptable in their role.

5. A Primer on Protein "Unstructure" and Relevance to NR A/B domains

The concept of diverse proteins contributing to or being wholly responsible for the equally diverse but highly specific biological activities ranging from DNA synthesis to antibody antigen recognition is rooted in the summary "structure-function" and its visualization as finely-shaped locks with reciprocally pre-formed keys. Function then inherently becomes dependent on newly translated proteins assuming and maintaining one, and likely only one, three-dimensional spatial arrangement of any subdomains derived from the primary amino acid sequence. This idea is reinforced by presence of key positional residues (e.g., cysteines for zinc fingers in DBD) or general conservation of sequence for similar secondary structure (e.g., recurring helices along the length of LBD) when homologues of the same or similar proteins are viewed across species. This conceptual view was certainly productive in the molecular level studies of NR DNA-binding and ligand-binding domains. For instance, recombinant expression and crystallization of the DBD and LBD, alone or less frequently as contiguous constructs, led to numerous, elegant, and seminal descriptions of protein-DNA interaction at response elements as well as protein-protein interaction among NR dimers [82, 83] which have been previously reviewed [84–86]. Striking, however, is that many fewer biophysical assessments have been on full-length NR. This was in part because of purposeful design to isolate DBD or LBD inherent functions or because of technical complications from the additional length such as on crystallization of samples destined for X-ray analysis. Rastinejad and colleagues have worked to overcome these challenges [86–88] in the study of full-length NR in dimers. In this respect, one of the perceived technical hurdles, unstable three-dimensional structure, or intrinsic disorder, present in the NR A/B subdomain, may itself be reflecting particular biological contribution to NR activity regardless of the fact that it can frustrate efficient crystallization or visualization of that region if a crystal is obtained. Indeed, despite purposeful inclusion of the AF-1-containing amino-termini for each full-length partner in a PPARγ-RXRα heterodimer, the A/B region was not visualized for either partner in the crystal structure; these termini were described as "intrinsically flexible" from hydrogen/deuterium exchange mass spectrometry [88]. Such flexibility/disorder could allow

access to A/B amino acids for post-translational modifications or interaction with multiple and differing NR coactivator proteins which individually or together could contribute to ligand-independent AF-1 potency.

Interestingly, in an RXRα-LXRγ X-ray structural study intended to recapitulate several of the actors present in a productive NR dimer transcriptional event (both heterodimer partners, peptide fragment of an NR coactivator, consensus DNA response element, and agonist ligands), successful expression of full-length NR was stymied. Although starting with cloned full-length RXRα, degradation during bacterial expression or purification led to a yield of truncated receptor fragments. Eventually it was a partial RXRα used for crystallography studies encompassing positions 98–462, meaning about three-quarters of the A/B region was missing. Parallel challenges were experienced with LXRγ and positions 72–461 were present in the final construct meaning an A/B truncation of 85%. While not directly attributing the full-length expression difficulties to the A/B intrinsic disorder, the authors did note in passing the NR dimers for their studies lacked "parts of the unstructured amino-terminal domain" [89]. Nevertheless, these investigators succeeded in better informing spatial understanding of a NR heterodimer on its cognate DNA response element, especially in regards to inter-molecular interactions of heterodimer partners. Here they noted possible consequences to positioning of RXRα DBD (C) and LBD (E) subdomains because of the flexibility of the intervening hinge region (D) that like the A/B region also often shows a high predicted degree of intrinsic disorder. Just as there was structural impact from inclusion of the flexible D region, it is tempting to consider what effect successful presence of the A/B region might have had in the context of interaction among heterodimers, DNA response element, and agonist. Thus, despite NRs usually being considered quintessential examples of constrained structure-function relationships among protein families because of highly regimented amino acid identity and sequence, there is building interest in what we refer to here as unstructure-function, a consequence or perhaps biological benefit of intrinsic disorder.

6. Example NRs and Consideration of ID in their Amino-terminal Region

Some potential conformational characteristics of ID seem tailor-made to the functional advantage of the NR A/B region. IDP interaction with binding partners, though of low affinity, can be of high specificity [90–93] and the low proportions of hydrophobic amino acid residues can lend to a more-open conformation increasing surface for interactions with partner proteins. These potential conformational traits can come at a cost. For instance, ID can result in a shortened protein half-life due to increased access for proteases [60, 94]. However, for the A/B region, this structural flexibility seems mostly advantageous allowing for interaction with a diversity of NR coregulatory proteins impacting overall NR function [95, 96]. Additionally, an unstructured conformation provides for ready access to A/B amino acids permitting post-translational modification, especially the commonly-occurring phosphorylation [57, 58, 77, 78], of key residues thus governing the region's activating function 1 (AF-1) [97, 98]. IDP and IDR have such biophysical traits in part because of their amino acid sequence (lower complexity due to amino acid repeats) and overall content (preferential presence or exclusion of certain amino acids). Recognition of such traits has led to the development of several *in silico* platforms [99–101] assessing amino acid physicochemical properties (e.g., frequency of occurrence, nature of side chain) and predicting degree of disorder from this and/or against datasets of empirically determined protein structures. For the A/B ID considerations presented here, we examined the NR superfamily for general trends (Figures 3 and 4) and then the cohort of NR of biological interest in keratinocytes to see if these trends held for these specialized cells (Figures 5 and 6).

Compositional bias, i.e., relative overall representation of each amino acid across a region of interest such as NR A/B, provides a snapshot overview of ID possibility. For instance, protein regions with a paucity of hydrophobic R group amino acids may be less likely to fold to limit exposure to an aqueous environment. Reciprocally, numerous charged hydrophilic residues may facilitate an unstructured conformation. In ID terms, this describes, respectively, below average occurrence of order-conferring residues, e.g., cysteine, isoleucine, tryptophan, and phenylalanine (some with characteristic hydrophobic R groups) and over-representation of leucine, glutamine, glutamic acid, and proline (some with charged R groups). Average residue occurrence is defined by comparison against the Swissprot database which is referenced as an unbiased distribution of amino acid usage [67]. Amino acid content (Figure 3) for A/B regions of 36 NR A/B regions, as assessed with Composition Profiler [68], showed preference for residues trending with or exceeding that characteristic of disorder, i.e., underutilization of "order-promoting" residues (e.g., cysteine, tryptophan, tyrosine, isoleucine) and enrichment of "disorder-promoting" residues (serine, glutamine, proline).

Notably, the under-representation (Figure 3) of hydrophobic residues (isoleucine, tryptophan, and phenylalanine) is consistent with the idea of reduced or absent folding driven to bury hydrophobic residues away from an aqueous environment. The high positive fractional difference value for proline, exceeding that for the DisProt reference calculation, is particularly striking given its strong secondary structure-disrupting qualities [67].

In silico assessments for ordered versus disordered proteins vary in the range of criteria examined to assign a protein to either group. The charge-hydropathy plot, uses just those two criteria, mean net charge and mean normalized hydropathy (Kyte-Doolittle) for each submitted sequence.

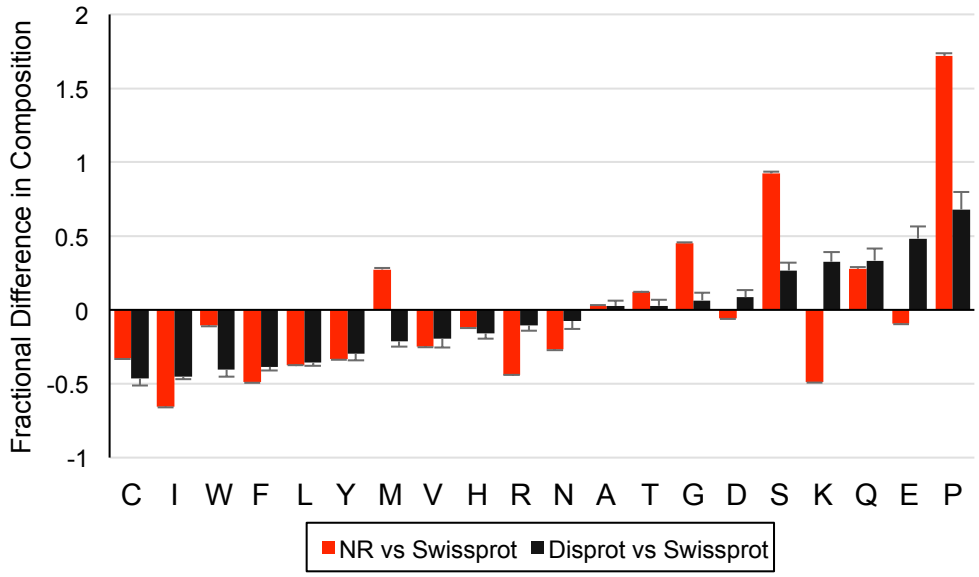

Figure 3: **Compositional bias of amino acids consistent with disorder within NR A/B region.** Preferential occurrence of amino acids in NR A/B regions calculated with Compositional Profiler and represented on the y-axis as the fractional difference. Calculations were done with the experimentally-defined IDP cohort Disprot [68, 70] and Swissprot databases to establish trend differences as (Comp_Disprot minus Comp_Swissprot) / Comp_Swissprot and (Comp_NRA/B minus Comp_Swissprot) / Comp_Swissprot, where Comp_NR A/B is the content of individual amino acids across 36 NR, and Comp_Swissprot is content of individual amino acids across in the reference Swissprot database. Calculation values below zero or over zero show under-representation or increased usage, respectively, in IDP/IDR. Amino acids are arranged on the x-axis by rank difference Disprot versus Swissprot comparison. Compositional profiler, CH and CDF plots were generated using NR N-term sequences from UniProt (https://www.uniprot.org).

A relative, but not absolute, boundary is generated from reference datasets of ordered and disordered proteins [102, 103] as determined from multiple criteria beyond charge and hydropathy. Notably, even these reference proteins themselves show a range and significant overlap when assessed by only these two measures. The greater the distance any one protein plots from the boundary, the greater the propensity for the entirety of the submitted sequence to be completely ordered or disordered (Figure 4). Like the reference proteins, the NR show a disorder/order overlap and range from extreme left (mostly disordered) to right of the relative boundary line. Although to the right of the boundary line, the average value for the NR assessed is in the overlap area reflecting that as a cohort of sequences there are mixtures of order and disorder.

Another holistic view of individual residues contributing to a protein's or region's disorder comes from the cumulative distribution function (CDF) analysis [104]. Here, a propensity for disorder or order (x-axis abscissa) at each residue is predicted via PONDR-VL-XT and then plotted as their cumulative frequency (y-axis ordinate). PONDR residue scores of 0.5 and above are indicative of disorder. Slow curve rise, or relatively convex shape, for any protein reflects cumulatively few residues (y-axis) with relatively low disorder (low PONDR score x-axis). In contrast, proteins that are highly structured throughout their length show a rapid curve rise (high frequency of low PONDR scores) reflecting that extensive numbers of individual residues are accounted for before reaching relative threshold disorder score e.g., 0.5. The shared nature of disorder for the A/B region is reflected in the curve established by the averaged cumulative frequency of scores across all the sequences considered (Figure 5, red line in all panels); it is convex in shape and well under the boundary reference for ID (Figure 5, heavy black line in all panels). Within receptor subsets, the trend of disorder along the N-terminus is seen clearly for the retinoid receptors (Figure 5A) and is consistent with that generated by the LXRs, PPARα and δ/β, and the steroid receptors (Figure 5B-D). For the 11 keratinocyte-expressed NR A/B regions assessed there is a CDF range with all but one (PPARγ) (Figure 5C) remaining below the commonly used reference boundary indicating their amino-terminal disorder. Although there are no hallmark conserved positional residues or those that would lead to shared structure, like the zinc fingers and alpha helices of the DBD and LBD, respectively, the disorder of the A/B region seems to be a conserved feature across the NR superfamily, possibly similar to that described for a large number of protein families [105, 106]. Krasowski and colleagues [107] have specifically assessed ID of domains across hundreds of vertebrate and invertebrate NR providing insight on correlates of evolution and ID that can affect expected function of these regions, such as coregulator protein binding of AF-1. Considering the boundary line and experimentally derived NR amino-terminal structural data, it is informative to call out PPARγ (Figure 5C). Compared to other NR A/B regions as well as the averaged value, PPARγ

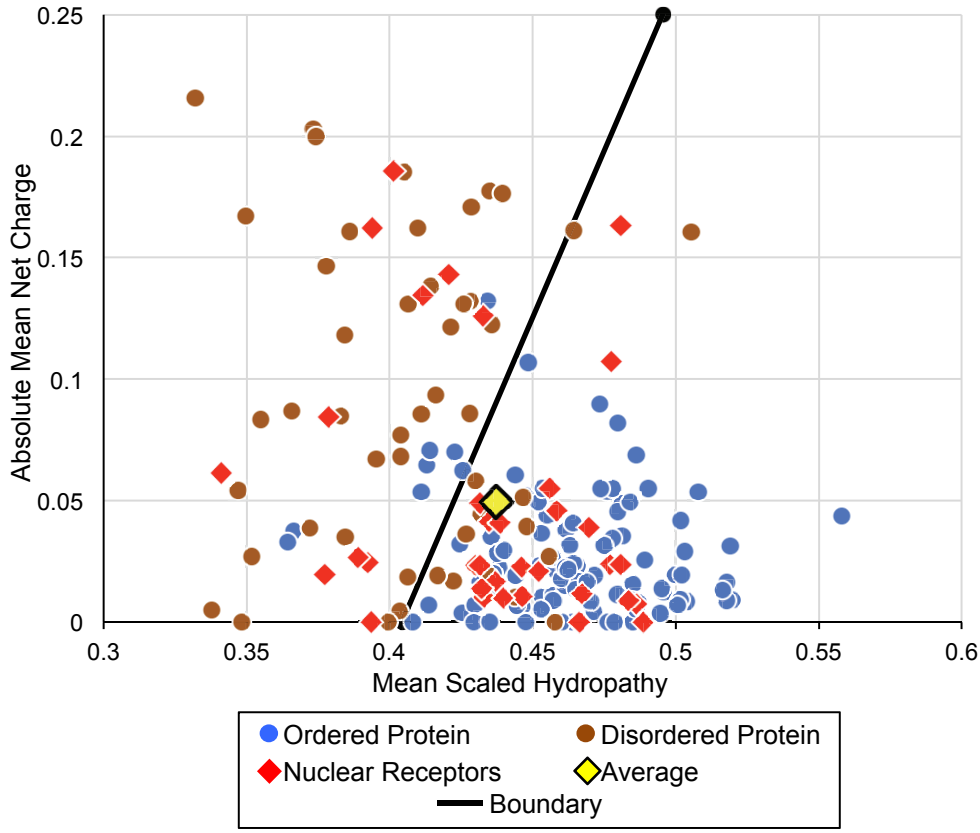

Figure 4: **Charge-hydropathy (CH) plot of NR A/B region.** Net charge versus mean scaled hydropathy (derived at http://www.pondr.com/) is plotted for NR A/B regions (red diamonds) against data sets for a reference boundary (black line), disordered proteins (relatively lower hydropathy, higher net charge; brown circles mostly occurring left of boundary), and ordered proteins (relatively higher hydropathy, lower net charge; blue circles occurring mostly to right of boundary). Bolded yellow diamond represents average A/B value.

rises faster and then straddles the reference boundary (Figure 5C). Nevertheless, what is predicted here by CDF as limited disorder agrees with the A/B regions not being visualized for either partner in a PPARγ-RXRα heterodimer crystal structure; these termini were described as "intrinsically flexible" [88].

RXRα is an ideal candidate to explore disorder within the A/B region because it is the obligatory partner with NR1 members in heterodimers both for keratinocytes and numerous other differentiating cell types. Although, as noted above, its role can be very dependent on cell type and even the developmental stage of the same cell. Nevertheless, while not specifically studied biophysically in terms of disorder, several features of the RXRα A/B are likely impacted by it shows ID assessments for some individual, keratinocyte-expressed NR starting with RXRα. PONDR analysis gives an order-disorder prediction for every residue along a submitted protein length showing that for RXRα and its heterodimer partners PPARδ/β and RARγ there are relatively contiguous regions of disorder along these amino termini again consistent with the individualized CDF plots for these A/B regions (Figure 6, upper three panels).

Taking analysis of RXRα further, there is good agreement along differently trained predictors that the vast majority of its A/B would be disordered (Figure 6, top panel). Considering then the accessibility to amino acids for post-translational modification disorder is expected to confer, it is especially intriguing to see that residues serine 32 and threonine 82 where phosphorylation inhibits transactivation for RXR partners [95] occur in regions of predicted high disorder. However, such consequences may be very cell- and gene promoter-dependent reflecting the combinatorial effect of A/B disorder, post-translational modification, and availability of other factors e.g., NR coactivators [95, 108]. Other effects of phosphorylation, including degradation of RXR, have been comprehensively reviewed [109]. The latter is particularly interesting as we note that an open conformation characteristic of IDP/IDR is often more susceptible to protease attack. Rochel and colleagues [110] have recently addressed the question of disorder for the amino-terminal domain of RXRα with nuclear magnetic resonance (NMR) spectroscopy and small-angle X-ray scattering (SAXS) to characterize its solution behavior. Consistent with *in silico* predictions, this region is highly flexible as expected for an IDR, even when the receptor was positioned on its DNA

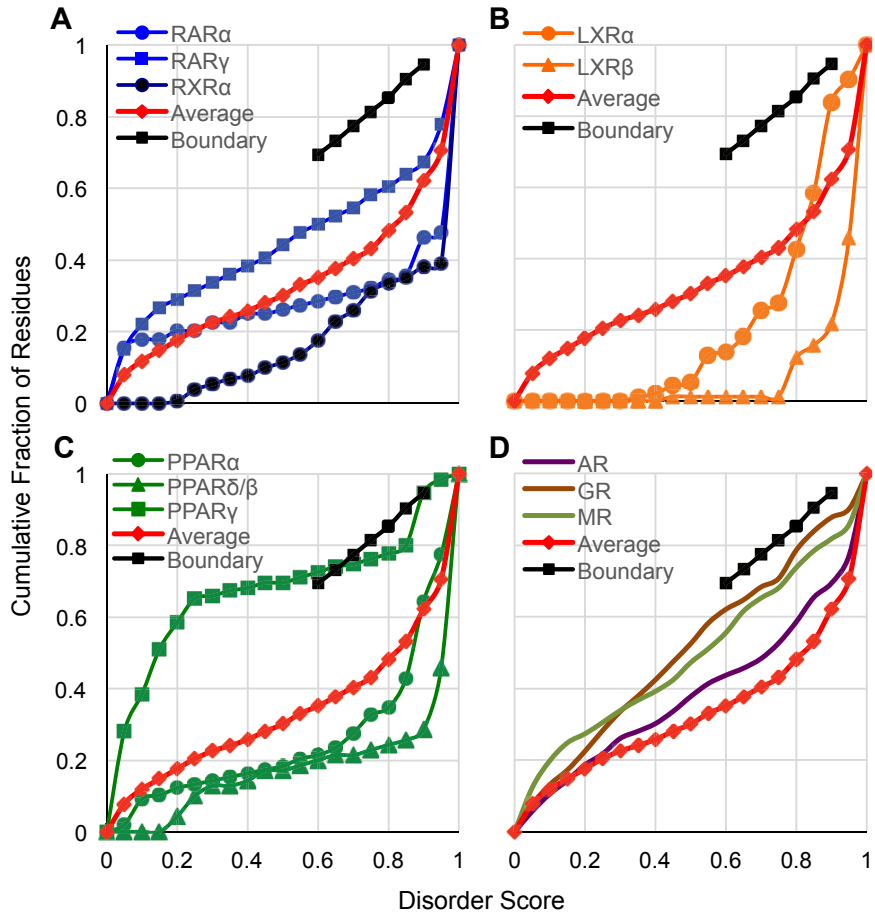

Figure 5: **Cumulative distribution function (CDF) curves for disorder analysis of NR A/B regions.** The CDF curve reports the frequency of predicted ordered to disordered scores for amino acids along the length of the submitted protein sequence from PONDR VL-XT. Curves (derived at http://www.pondr.com/) for A/B regions of keratinocyte-expressed NR, calculated in the context of the full-length NR, are presented above. Peptide lengths predicted to be disordered have CDF lines with low cumulative disorder values (ranged 0.0–1.0) over much to most of the x-axis. NR A/B domains are presented for separate groups with **(A)** retinoid receptors (RARs and RXR), **(B)** liver X receptors (LXRs), **(C)** peroxisome proliferator activated receptors (PPARs) and **(D)** steroid receptors. The average CDF analysis for the 11 keratinocyte-expressed NR across the four panels is plotted in each panel as bolded red line. The order-disorder boundary (bold black line, upper right each panel) is from previously described datasets [102]. Ordered proteins plot above the boundary and disordered proteins below. Simons and colleagues have previously comprehensively reviewed amino-terminal domain ID [31, 135] especially in regards to A/B allosteric interaction with other subdomains as highlighted for proteins in general by Berlow and coworkers [77]. Emphasis on steroid hormone receptor A/B regions can be found in [136, 137].

response element *in vitro*. Such studies provide important advancement to understanding the functionality of full-length NR especially in terms of inter-domain effects, regulation by post-translational processing, and interactions with A/B region-targeting NR coregulators may be impacted by region disorder.

While we have focused composition and sequence analysis on human RXRα, it is likely these considerations will translate to other mammalian species. Phosphorylation in the mouse RXRα A/B region is needed for activity with heterodimer partner RARγ in the retinoid-sensitive F9 embryonic carcinoma system for transcription of select gene subsets [109, 111, 112]. As disorder considerations for human RXRα may extend to other species, there is also a correlate that ID study in other species' RXR-related NR may be instructive for future human RXRα disorder-specific studies. For instance, *in silico* and biophysical assessment specifically for disorder [113, 114] has shown arthropod amino-terminal domain of Ultraspiracle (Usp), the homologue of mammalian RXR [115] to be an IDR. Like human RXRα, insect Usp-NTD sequence contains numerous charged groups and has a low content of hydrophobic amino acid residues which are expected to promote an open unstructured conformation.

The disorder trend for NR A/B regions continues for GR although representative of a distinct NR subfamily (NR3). As might be expected from their different training datasets, the extent of disorder predicted by the different

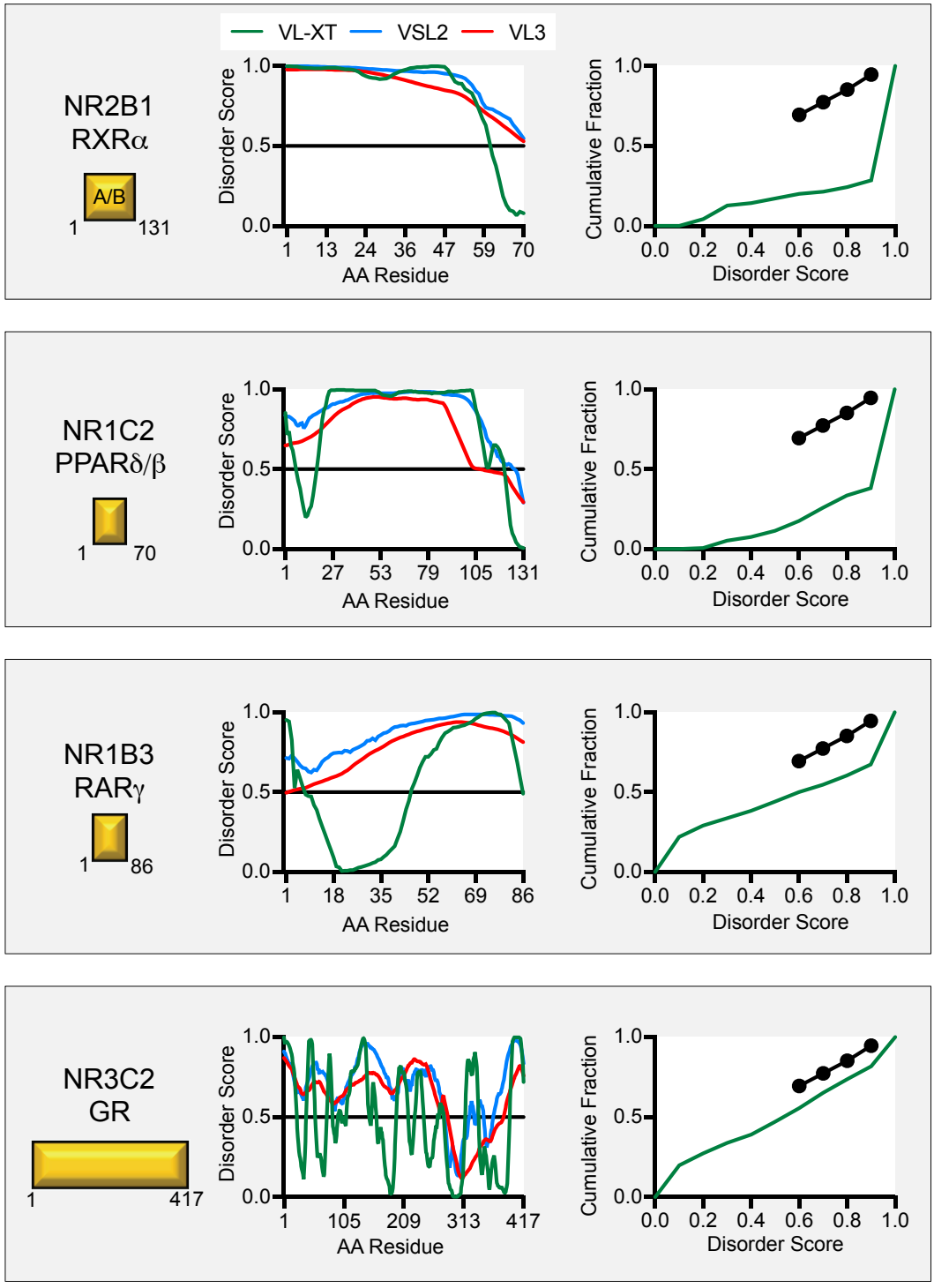

Figure 6: **PONDR and CDF assessment for select keratinocyte-relevant NR.** NR are referred to by their standardized and common nomenclature. A to-scale stick figure is provided under each name for the A/B region as retrieved from prosite.expasy.org/. Full-length UniProt deposits for indicated NR were submitted to PONDR and re-graphed here for the indicated NR along their A/B length only (left panel graphs) and cumulative fraction of disordered residues (right graph panels, calculated as in Figure 5). For PONDR assessments, full-length NR sequences were used and data for N-term regions was extracted from that analysis to accommodate any influence of subsequent residues.

algorithms does vary in some stretches of amino acids within the amino terminus. Unlike VLS2 and VL3, VL-XT examines protein termini and internal regions separately. In Figure 6 this only applies to the amino termini as the carboxyl regions plotted here were assessed in the context of their contiguous DNA-binding domain. As each predictor is assessing different criteria of amino acid content and/or length, there may be some disagreement in output especially at the start of plot lines (such as for RXRα, RARγ, and GR). This becomes more obvious for the greater number of amino acids assessed for GR. VSL2 and VL3 are neural network predictors for both short and long disordered regions and long regions, respectively. Additionally, the training dataset differ from VLXT which, though tending to under-represent longer disordered regions, does highlight potential protein-protein interaction sites [116]. Thus, there appears to be multiple short regions of disorder for GR via VXLT, in contrast to the longer regions from the VSL2 and VL3 plots. Such disparity is not uncommon especially when comparing the predictors across longer protein lengths [117]. Nevertheless, in sum, the differently-based predictors show fair to significant agreement of disorder for NR A/B regions independent of NR A/B region length (relatively short for RXRα, PPARδ/β, RARγ or long lengths for GR).

7. Conclusions and Implications from Considering A/B Region Intrinsic Disorder

Numerous cell types, like the epidermal keratinocytes we highlighted above, are physiologically responsive to diet-derived and endogenously-produced NR ligands integrally involved in their replication and differentiation. In turn, this has led to pharmacologic targeting either with agonists or antagonists depending on the desired outcome of NR function. While the activation function (AF-1) of the NR amino-terminus has long been recognized as contributory to overall function, its biophysical properties have slowed characterization of its structure – or lack of structure – and how this may be part of its addition to target-gene transcriptional activation. Although not yet studied across many NR, the unstructure, or intrinsic disorder, of the A/B region is being more-often directly experimentally addressed with that data corroborating several *in silico* platforms for the prediction of disorder. In turn, these combined approaches are increasing our overall comprehension of NR control of cell and organism physiology by offering new insight as to the amino-terminal conformational requirements for, and functional consequences of, post-translational modifications and interaction with NR coregulators.

Taking a cue from LBD searches for pharmacologic ligands to clinically control NR, and with recent insight accruing from ID studies of amino-terminal regions, there is seminal work being done in the exploration of small molecule regulators of NR A/B despite the often-assumed challenges ID may bring to drug discovery. In work possibly predictive for other NR, there are exciting developments with drug targeting of the AR A/B region driven in large part because of AR, and especially the amino terminus of AR, stimulating growth of androgen-independent cancerous prostate epithelial cells [118, 119]. Targeting A/B of normal sequence and variant constitutively active AR, some lacking a LBD [120] and often associated with prostate cancer, offers a new avenue of therapy. Briefly, the last several years have seen reporting of at least three examples of small molecule inhibitors for AR A/B AF-1 activity, EPI-001, niphatenones, and sintokamides [121–125]. Their discovery, in spite of ID in this region, is exceptionally promising given the progression to clinical trials for the EPI-001 pro-drug, marking it as the first drug to target the intrinsic disorder of an NR A/B region. These advances, along with drug-targeting for other pathology-associated IDP, are likely to be transformational for prostate cancer and other IDR/IDP-associated disease states [126, 127]. This progress [118] with antagonist drug discovery for AR A/B will likely spur drug discovery investigations based on ID in other NR.

Interestingly, back-to-back publications [30, 128] have recently reviewed not only AR ID but also ID of other possible regulators of androgen signaling. Combined with AR A/B AF-1-targeting, this could expand therapeutic application to other androgen-responsive cells. Potential benefit to blocking androgen reduction of scalp hair follicle growth and stimulation of sebocyte cell secretion are well-established. However, interfollicular keratinocytes also express AR [14, 27] and are the cells responsible for the majority of skin barrier function. Recent evidence from mouse models suggests that blocking AR activity facilitates skin wound healing [129, 130]. The combination of non-LBD AR targeting and easy topical access to target cells could be of particular advantage in cutaneous medicine.

As with synthetic ligands for NR LBD not all showing complete selectivity for LBD of only one NR, there seems to be the parallel challenge for NR A/B region drug discovery. For instance, the AR A/B-targeting niphatenones inhibit its AF-1 function but also bind to GR AF-1, decreasing overall GR activity; they appear to have no effect on PR [122]. EPI-001 physical binding, or at least functional effect, has been mapped within the AR A/B though cell type may also be a determinant in its binding site [123, 131, 132]. Though specific to AR among steroid hormone receptors, EPI-001 is also described for selective modulation of PPARγ, again possibly reflecting some effect of cell context [132]. Antagonizing AF-1 contribution to gene transcription by classic sex steroid NRs has obvious and immediate possible clinical translation for growth inhibition of the AR- and ER-associated cancers. For some NR, there may be benefit from the reciprocal approach of small molecule facilitation of NR A/B activity. Retinoids in various forms are key to chemotherapy for various dermatologic and non-dermatologic benign hyperproliferative and malignant

neoplastic pathologies [133, 134]. However, their use is often limited by metabolic inactivation or dose toxicity. It is exciting to speculate regarding a next-gen class of "A/Binoids", a chemical class not for targeting the RAR or RXR LBD but the amino terminal regions and thus possibly negating some of the current therapeutic retinoid limitations. Whether this comes to pass or not, clearly, the challenges of studying, and possibly co-opting for therapeutic benefit, the ID nature of NR A/B domain are being met and, in many cases, conquered.

Competing Interests

The authors declare no competing interests.

Author Contributions

RA and BJA reviewed the literature and wrote the manuscript.

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