Biophysics of disordered nuclear receptors and their DNA binding regulation

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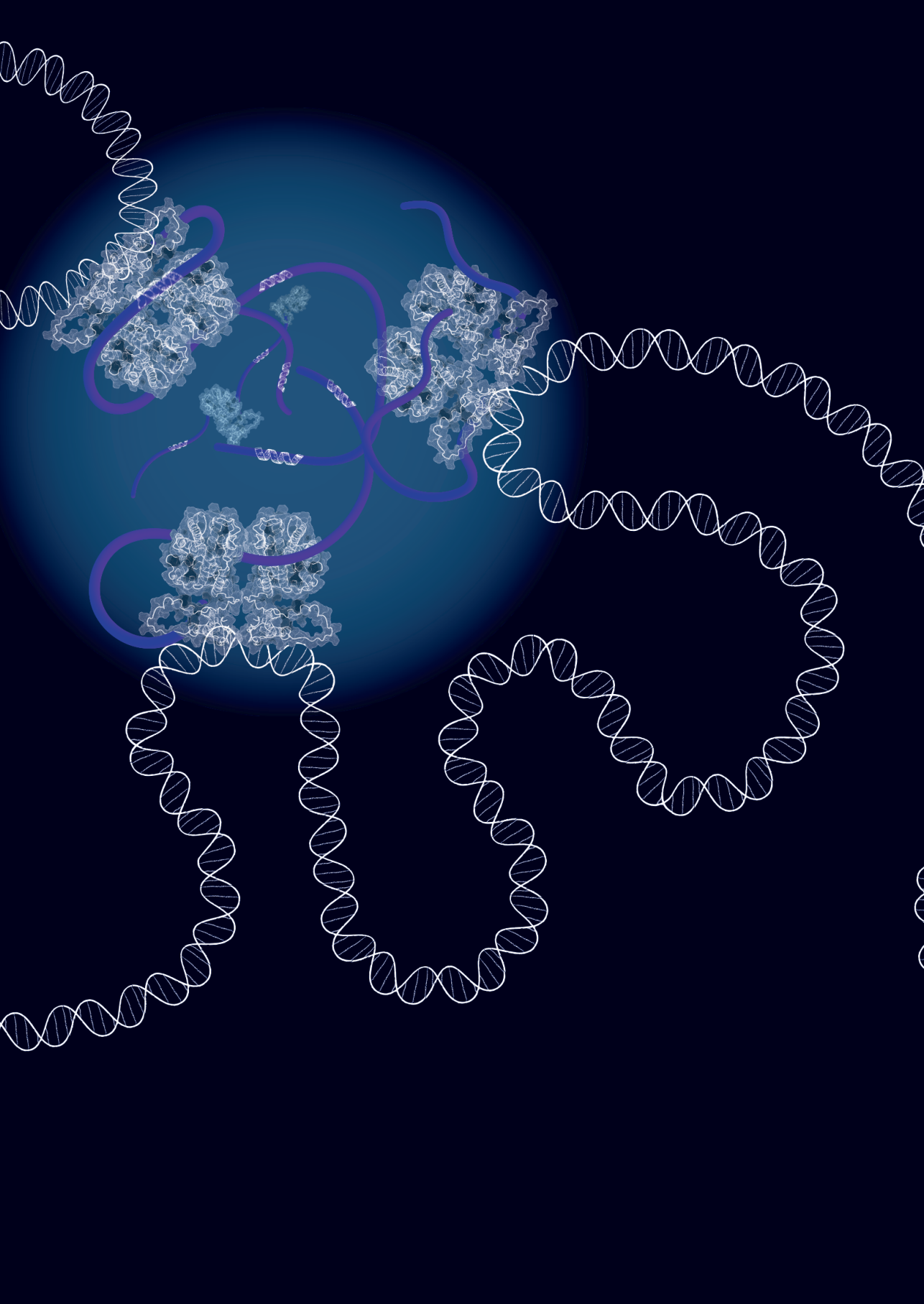
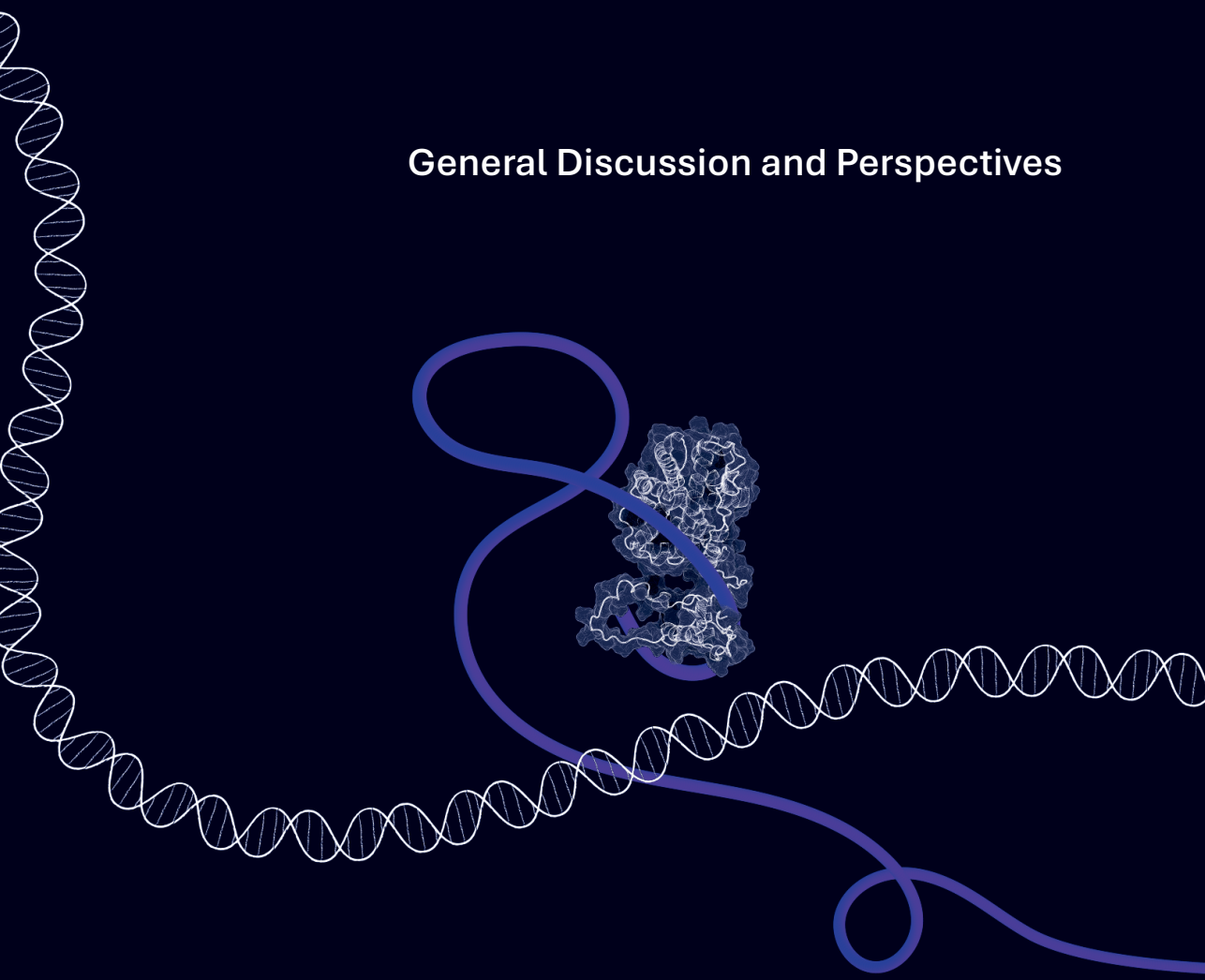


Chapter 6

General Discussion and Perspectives



Nuclear receptors constitute a large superfamily of (ligand-activated) transcription factors (TFs) that are involved in a wide variety of key physiological processes—spanning developmental regulation, reproductive health, metabolic homeostasis, inflammatory responses, cellular proliferation and differentiation[1–3]. The modular structure, dynamic conformational changes, interplay with co-regulatory proteins, post-translational modifications, and diverse ligand specificities enable them to precisely regulate a myriad of biological processes. Given their involvement in both normal physiology and various pathologies—including numerous cancers, inflammatory disorders, and metabolic diseases[4–6]—a deeper understanding of the structural and functional dynamics of nuclear receptors therefore represents high scientific and medical significance. Consequently, therapeutic developments targeting nuclear receptors are an area of intense research, and structural studies of individual domains have led to several approved drugs, often targeting the ligand binding domain (LBD)[7]. The inherent structural complexity of nuclear receptors, with the dynamic nature of their intrinsically disordered regions (IDRs) and a complex interplay of interdomain interactions, have made it difficult to recapitulate these results[7]. Furthermore, highly conserved sequence and structure[2] and the prevalence of splicing variants in nuclear receptors have hindered the successful development of selective compounds. Considerable focus has therefore been directed at the N-terminal domains (NTD) of nuclear receptors. The structural plasticity of these IDRs has challenged the structure–function paradigm of proteins but despite the lack of a stable 3D folded structure (*in vitro*), these regions are crucial in biological processes. Investigating the mechanisms by which these IDRs can (dys)regulate protein function is key to gaining a better understanding of their biological relevance and for drug discovery.

The aim of this thesis was to deepen our understanding of nuclear receptors, their DNA-binding mechanisms, and the role of the NTD. Using a combination of advanced molecular modelling, real-time single-molecule fluorescence microscopy and solution-based biophysical assays, we created reductionist *in vitro* and *in silico* models representative of two nuclear receptor classes, the steroid hormone-activated androgen receptor (AR) and the orphan Nur77 receptor. We have demonstrated that the complex interplay between the nuclear receptor IDRs, the well-structured domains and DNA has a considerable effect on the function of the protein. In addition to investigating the intradomain interactions within nuclear receptors, we generated the first structural atomistic model of pQ expanded AR-NTD. Despite the inherent complexity of modelling nuclear receptors and large IDRs, we have provided important new insights into the structural and functional implications of an aberrant mutation in the disordered NTD of AR.

Previously, we revealed a computational model of a wild-type AR-NTD[8], creating the first picture of a large IDR. This domain has eluded structural characterisation through experimental[2] and state-of-the-art AI approaches[9]. We showed that—consistent with select biophysical analyses—the NTD of AR can form collapsed and highly dynamic regional segregation, which we termed the N-terminal and C-terminal regions (NR and

CR respectively), and summarised the key interactions and mechanisms in the first full-length model of AR. We also introduced an update on the circuit topology[10] framework, specifically poised to capture the dynamic that intrinsic disorder possesses[11]. Those efforts had set the stage for the next chapter of AR biology as well as the study of intrinsic disorder in nuclear receptors. Building upon that, in **Chapter 2** we investigated the effects of the polyglutamine (pQ) expansion in AR-NTD, a mutation that causes spinal bulbar muscular dystrophy (SBMA, also known as Kennedy's disease). We found that this mutation leads to a global restructuring of the NTD driven by direct interactions of the glutamine residues. This not only affects the structural and topological dynamics of the domain, disrupting the previously identified regional segregation, but also affects the delicate balance of protein–protein interactions (PPIs) and oligomerisation dynamics. The results in this chapter identify several effects of pQ expansion that can plausibly disrupt cellular functioning. While pQ expansion is pathogenic in proteins like AR and Huntingtin, many native TFs, such as FOXP2, contain long glutamine-rich tracts without forming toxic aggregates. These proteins rely on intrinsic protective mechanisms to maintain solubility[12]. Throughout interphase, DNA binding—irrespective of sequence specificity—has a powerful solubilizing effect on these assembly-prone proteins. During mitosis, when most TFs are evicted from chromatin, this protective role is supplanted by widespread phosphorylation. Multiple mitotic phosphorylation events, particularly within concentrated phospho-patches, introduce negative charges that maintain solubility in the absence of DNA binding[12]. This reveals a dual-protection strategy, where TF solubility is ensured across the cell cycle. The pathology of diseases like SBMA may therefore arise not just from the pQ expansion itself, but from the failure of these intrinsic protective mechanisms to buffer against the increased propensity to aggregate. Our model shows that interactions between AR-NTD and the chaperone proteins HSP70 and HSP40 are disrupted after pQ expansion. Previously it was shown that these chaperones sequester and regulate the solubility and turnover of inactivated AR through interactions with AR-NTD[13]. Future experiments, for which our model can serve as a framework, will be critical to understand the pQ-expansion-driven effects on transcriptional regulation, and developing potential approaches to reverse the toxic effects.

Transcription—the vital biological process that reads out DNA to RNA—is governed by TFs such as nuclear receptors. These key regulatory proteins are responsible for turning genes "on" and "off" through specific interactions with nucleotide sequences within the genome[14,15]. TFs do this by searching along the DNA[16], in a process that must happen with high spatiotemporal precision to maintain cellular homeostasis and proliferation[17]. Moreover, in multicellular eukaryotes, most cells contain identical DNA sequences, and different cells within this organism express distinct sets of genes finely tuned to the developmental trajectory of the cell. The search for specific DNA motifs by transcription factors therefore presents a formidable challenge, due to the immense quantity of non-target DNA and the relatively small number of proteins. Advances in single-molecule fluorescence microscopy, biochemical and biophysical approaches have revolutionised our ability

to study nuclear biology processes, such as DNA repair, transcriptional regulation and replication[18–25]. In **Chapter 3**, **Chapter 4** and **Chapter 5** we used a combination of single-molecule fluorescence microscopy and bulk-phase biophysical assays (microscale thermophoresis) to assess the DNA binding capabilities of AR and Nur77. Our single-molecule fluorescence imaging approach revealed that the kinetics of both nuclear receptors on DNA follow a bi-exponential distribution, indicating that the DNA-bound populations are composed of two subpopulations with distinct kinetics. Decades of biochemical studies have reported similar distributions for other TFs, where the short-lived population was interpreted as non-specific interactions, while the longer-lived interactions described the interactions to protein specific motifs in promotor or enhancer regions[26–30], supported by evidence that the longer lifetime population was attenuated when the DBD regions were mutated[26,27,31–34]. AR binds to the androgen receptor element (ARE), a hexameric palindromic repeat, as a dimer, while for Nur77 there are two known motifs: the NGFI-β response element (NBRE) and the Nur-response elements (NurRE), to which it binds as a monomer and dimer respectively. In our single-molecule assay, these specific motifs were not present for AR, and for Nur77 only two NBREs were present along the λ-DNA. However, several partial ARE and NurRE sites were encoded, suggesting that partial sites may be sufficient to induce a population of longer-lived proteins. Interestingly, while we were not able to discern the binding of Nur77 to non-specific DNA, NBRE or partial motifs using our single-molecule approach, thermophoretic data revealed a sequence dependent affinity. It will be interesting to see what the effect of a full motif would be on the DNA residence time. Will a third population arise with even longer lifetimes, or are other factors necessary to fully stabilise the protein DNA complex? What is the role of the intrinsically disordered region on DNA binding?

Our full-length wild-type AR model suggested that DNA binding modulation of AR[35] can happen through differential interdomain interactions between the segregated regions of NTD and the DBD[8], while others have studied the role of IDR regions on transcription factor binding to DNA[36]. In **Chapter 3,** we therefore investigated the DNA binding capabilities of recombinantly expressed AR proteins, and the complex synergistic allosteric regulation of AR–DNA binding by the NTD subregions *in vitro*. We found that the NR subregion has no discernible effect on the affinity of an NTD-truncated-AR (Δ-NTD AR) to DNA, while the CR decreases the affinity in a concentration-dependent manner. Using single-molecule imaging, we directly observed a behavioural change that occurs when either NR or CR are present, demonstrated by the changes in rate constants and number of events. The addition of CR pushes the distribution towards the shorter lifetime and concurrently increases the dissociation rate constant, while NR causes a substantial increase in the rate constant of the longer lifetimes. For Δ-NTD AR with NR, we observed a similar number of molecules as for apo Δ-NTD AR, whereas with CR we observed few bound molecules The subregions added together exhibited a cooperative modulation of AR, which our computational model suggests could be through altering the conformational landscape. NR shows interactions with the binding function 3 (BF-3) surface, which plays a role in

dimerisation. The CR interaction were mapped to the activation function (AF-2), a pocket responsible for ligand-binding stabilisation. This suggests that NR impairs dimerisation, while CR-AR has a very low affinity for non-specific DNA, likely through deactivation of AR. An alternative explanation, as suggested by the computational full-length AR model[8] and the model in **Chapter 2**, is that NR inhibits dimerisation through the DBD by sterically blocking the dimerisation interface on the DBD, while CR-mediated interactions block the DNA-binding surface of the DBD. These results add previously underappreciated allosteric roles for IDR regions in nuclear receptors. IDRs are abundant in transcription factors[37–40], and the proportion of sequences predicted to be disordered in TFs is markedly higher than in other proteins (~55% vs ~25% respectively)[41]. IDRs play central roles in transcriptional regulation processes, with the conformational plasticity thought to make them more suited for post-translational modifications and co-factor interactions, and thus acting as a scaffold for higher-order structures. Our results here also support a model for direct intramolecular regulatory effect, with IDRs having concentration dependent effects. In normal physiological conditions, the NR, CR and NTD do not exist as separate proteins to the rest of AR in the cellular environment, and the NTD is present in equimolar quantities as the rest of the AR-domains.Because of our observations of concetration dependent effects, our model therefore not only sheds light on the allosteric role of IDRs in nuclear receptors, but it also opens potential perturbation strategies. This can have implications for new therapies. AR in aggressive castration resistant prostate cancer (CRPC) is characterised by an acquired resistance through alterations to the receptor itself. The occurrence of AR splice variants (AR-Vs) may provide a mechanism through which AR can evade androgen ablation therapies. AR-Vs lack part or the whole LBD of the full-length receptor, and are suggested to arise through alterations of splicing, mutations to the *AR* gene or non-coding and micro RNAs. One of the best characterised variants lacking the LBD is ARv7, which furthermore includes a hinge and a unique amino acid tail at the C-terminal. ARv7 is consistently present in the nucleus, constitutively active and abundantly present in clinical patient samples. Evidence shows that this splice variant is able to dimerise and enhance function of full-length (FL)-AR and bind to AREs[42,43], and independently from FL-AR can regulate turnover mediating enzymes that are upregulated in CRPC[44]. The exact mechanisms by which ARv7 is able to remain constitutively activated has been subject to debate. The model we developed in **Chapter 3** offers a plausible mechanistic basis for this phenomenon, suggesting that the NTD allosterically regulates DNA binding primarily through a pathway mediated by the LBD; consequently, the lack of this domain in ARv7 disrupts this regulatory pathway. This highlights the need for therapeutic approaches that target AR-Vs or AR through other means than the LBD. The work in **Chapter 3** highlights that NTD subregions can act as modulatory peptides through the DBD. Further work is required to elucidate specific amino acid motifs responsible for the effects on AR.

A significant amount of research has focussed on the activating aspect of nuclear receptors, but nuclear receptors have also been shown to repress transcriptional

activity. Nur77, for which no endogenous ligands are presently identified, is thought to exclusively be modulated through PPIs (as outlined in **Chapter 1**) and its signalling axes is intertwined with that of the glucocorticoid receptor (GR) along the hypothalamo-pituitary-adrenal (HPA) axis. Crosstalk between nuclear receptors is not unique to Nur77 and GR, and the aspects of the interplay on the signalling axes need to be considered for understanding and modulating nuclear receptor function[45]. Nur77 and GR interact through their DBDs and have been shown to co-repress each other, potentially through the formation of transcriptional inactive complexes with Brg1, Brm and HDAC2[46,47]. Whether an activated GR is sufficient of this repression remains unknown, and the mechanism behind this repression is unclear. In **Chapter 4**, we sought to determine the consequences on DNA-binding that happen when GR is introduced to Nur77. Our data shows that GR stabilises Nur77–DNA interactions, as evidenced by the decreased dissociation rate constants. This decrease mostly affected the short lifetime population, doubling the dwell-time. NurREs do not attract GR[47], and several partial glucocorticoid response elements (GREs), but no full-length GREs are present along our DNA construct. However, *in vitro* experiments have suggested that GR also binds to a negative GRE (nGRE) motif, which overlaps with the NBRE[48,49]. The different modes of interplay between nuclear receptors have been reviewed before[45], and based on this, analysis of *in cellulae* data and our results, we hypothesise that GR is sufficient in modulating Nur77, which it does so by physically sequestering Nur77 at (partial) GRE sites or the nGRE/NBRE motifs. It may be counterintuitive that decreased lifetimes on DNA lead to decreased transcriptional output. Other studies have unveiled that dwell time is a regulatory factor, influenced through several mechanisms including transcription factor concentration, co-regulators, DNA sequence and genome structure[50–54].

Despite the classification as an orphan receptor, multiple studies have identified small-molecule compounds that bind and may directly influence Nur77 activity. However, due to inconsistent results, there is no consensus regarding these compounds, and mechanistic information is incomplete. We investigated the effects of such compounds in **Chapter 5**. Cytosporone-B (Csn-B) is an octaketide extracted from *Dothiorella* sp. HTF3, an endophytic fungus. Csn-B was identified to bind to Nur77 at its LBD and activate Nur77 mediated processes[55]. Our results in **Chapter 5** add new mechanistic information regarding this activation. Firstly, we showed that Csn-B alters the kinetic landscape of Nur77–DNA interactions through the redistribution of the fast and slow dissociating populations, with the marked majority of events being fast paced. Concurrently, the dwell time for both kinetic populations bound to DNA increases. We had already attributed longer lifetimes with binding to (partial) DNA motifs, with 6 partial motifs of the NurRE and 2 full NBRE motifs contained on the λ-DNA construct. We therefore hypothesized that Csn-B induces a conformation of Nur77 that is more sequence stringent, likely towards its DNA motifs. To test this hypothesis, we tested Nur77–DNA binding against different DNA constructs with Nur motifs using thermophoretic assays. The bulk biochemical data revealed that Csn-B treatment attenuated interactions with non-specific and

NBRE DNA-motifs but strongly increased the affinity for the NurRE motif. A mechanism that may explain our observations is that Csn-B stabilises Nur77 into an oligomerised state. Ligand- and small-molecule modulator activation of nuclear receptors has been associated with a structural oligomerisation state into (homo- or hetero-) dimers or higher order structures[56–59]. Oligomerisation of nuclear receptors can occur through multiple domains[45], and while a wealth of information exists regarding the molecular determinants for DBD and LBD mediated oligomerisation[60–65], for the NTD this remains poorly characterised. Results from cell-based assays reveal that there is no difference between activation of gene on a NBRE or NurRE enhancer/promotor motif, suggesting that there may be another mechanism or factor at play here[55]. Further structural and functional studies should shed a light on the exact molecular mechanism through which Csn-B modulates Nur77.

The discrepancy between our *in vitro* results for the effects of Csn-B and *in cellulae* results of others highlight the unintended effect of ligands on different proteins. Synthetic ligands have widespread therapeutic potential and are already in use for a range of inflammatory and oncogenic pathologies. A significant limitation, however, is their propensity to induce adverse effects or therapeutic resistance, which is in part a direct consequence of low receptor selectivity leading to indiscriminate regulation of signal transduction pathways and transcriptional outcomes following off-target receptor binding[45]. In **Chapter 4** we describe the direct effect we (somewhat accidentally) observed of dexamethasone (Dex), a common synthetic glucocorticoid, on Nur77. This is a striking result, as studies looking at Dex modulation in cell culture-based assay, attributed these effects to the GR being involved in the regulation of Nur77 pathways. Systemic synthetic corticosteroids have been associated with a wide range of adverse effects[66], including an increased risk of secondary bacterial, viral, and opportunistic infections. Attenuated or inhibited Nur77 has been linked to (chronic) inflammatory dysfunction. Therefore, our observation that Dex significantly directly modulates Nur77—coupled with the widespread clinical use of Dex to treat inflammation[67]— stresses the critical need for further functional assays. Ultimately, these findings may prompt a re-evaluation of Dex in therapeutic regimens.

The work discussed above, which highlights a distinction between our *in vitro* findings and those from cellular assays, reflects an age-old dilemma in biology that is not unique to nuclear receptor dynamics. Cells are extreme physicochemical environments, with millions of proteins. Indeed, we have demonstrated that the folding dynamics of biomolecules are significantly altered when moving from dilute buffer to a cytosolic solution[68]. Given this complexity, the power of *in vitro* biochemical approaches, as presented in this thesis, is invaluable to establish the core biophysical variable properties and behaviours of the components in a well-defined milieu is invaluable. This quantitative baseline provides the foundation for the subsequent challenge of understanding how the function of these intrinsic properties leads to organised biological function.

One fundamental question in biology is still outstanding. How is the dense cellular space organised to spatiotemporally control complex biochemical reactions that are the basis of diverse cellular processes? Many of these processes are packaged in distinct subcellular organelles, such as the nucleus, endoplasmic reticulum or Golgi apparatus, which are clearly defined by the lipid bilayers surrounding these compartments. This physical separation between the interior and exterior of these organelles is impermeable to most biomolecules, and the interior composition is regulated through specialised membrane transport machinery. However, many cellular processes are not delimited by a membrane, yet the molecules central to these processes need to colocalise to achieve effective reaction kinetics. Mechanistic and structural molecular biology has focussed on the identification, visualisation and characterisation of highly specific stoichiometric interactions of the proteins governing these processes, forming complexes at the 1–10 nm length scale. These macromolecular complexes further organise in compartments with linear dimensions ranging from 100 nm to several micrometers. These compartments, not delimited by membranes, go by many names including membraneless organelles, factories, hubs, clusters, foci, bodies, and perhaps most commonly, biomolecular condensates. These condensates typically contain tens to hundreds of different protein species, usually with multiple copies per protein. The first examplee of a biomolecular condensates was observed almost two hundred years ago, the nucleolus, with the observation of Cajal bodies in the early 20th century[69]. Over subsequent decades, the list of biomolecular condensates has further expanded[69]. Today, biomolecular condensates are associated with a diverse range of cellular processes, including ligand signalling, DNA repair, DNA replication, ribosome assembly and transcription[69,70]. Because the dysregulation of these fundamental processes are frequently linked to malignancies[70], the past decade has seen considerable interest in the physicochemical drivers of the condensate dynamics and their protein-concentrating abilities. However, the mechanisms by which macromolecular complexes interact to form these mesoscale biomolecular condensates remain poorly understood and challenging to model. Drawing on concepts from polymer physics and chemistry[71], biomolecular liquid–liquid phase-separation (LLPS) has emerged as an explanation for the biogenesis of these condensates. IDRs could drive LLPS by weak, multivalent and dynamic interactions between the protein components[69], with the classic stochiometric interactions given a secondary role. Nuclear receptors have also been associated with LLPS[72–74], and more notice has been given to the role of biomolecular condensates in transcriptional regulation[75]. With the rise of condensates as an orchestrator of spatiotemporal cellular processes, so did new models to describe the interactions between different components within these condensates, including new frameworks based on circuit topology[76,77]. Biomolecular condensates could offer an explanation for the differences in kinetic behaviour between our single-molecule observations (where transcription factors follow a bi-exponential model) and the observations from single-molecule tracking in cells, with power-law models fitting the data best[24,30]. However, although substantial work has led to important new findings[78–83], many questions remain. Observations of the LLPS of proteins invariably

happened with one or two protein components *in vitro*, while cellular condensates likely contain many different proteins. Super-resolution microscopy analysis of the dense fibrillar component, a region in the nucleus where ribosome biogenesis occurs, demonstrated a substructure considerably more complex[84] than was predicted by *in vitro* LLPS experiments[85]. This shows that LLPS observations *in vitro* are not generally predictive for what happens *in situ*. Furthermore, to improve signal to noise ratios, cell-based observations of biomolecular condensates often occur with the overexpression of proteins, which is no longer a physiological situation. The assumptions made in studies of LLPS and biomolecular condensates are one of the reasons for the lack of consensus regarding their biogenesis[86]. This does not imply that they are erroneous but rather underlines the substantial challenges ahead. The right interdisciplinary combination of new theoretical models, technical advancements and experimental approaches will likely enhance the understanding (and potential for therapeutic modulation) of biological processes.

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