Targeting DNA repeat sequences with Py-Im polyamides

Thesis by

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In Partial Fulfillment of the Requirements

for the degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

2015

(Defended March 27, 2015)

Dedicated to my family

Acknowledgements

I learned a great deal in graduate school, and a good measure of it is from the people I have interacted with over the years. Many people on- and off- campus that have positively influenced my years at Caltech. I would like to acknowledge at least some of them here.

I would like to thank my advisor *Prof. Peter Dervan* for his scientific guidance during my graduate studies. The intellectual freedom that he promotes in the lab, balanced by his advice on risk, has taught me to explore different areas of research in a considered manner. He has been supportive of, and appreciated the joy of, pursuing self-generated ideas even as I kept going "off the ranch". I expect the scientific judgment and understanding of new technologies that I have gained during my graduate education will prove essential to my future career. I would also like to thank my graduating committee chaired by *Prof. Bob Grubbs* and joined by *Prof. Jim Heath* and *Prof. Linda Hsieh-Wilson*. Their advice and support has been indispensible to my development in graduate school. Beyond science, the genuine interest of professors in the welfare of students makes Caltech an exceptional place.

My education was made much richer by my interactions with my labmates in the Dervan lab. In my earlier years, I learned a great deal from *Katy Muzikar*, *Jim Puckett*, and *Jordan Meier* who took the time to transfer their knowledge of some very time-intensive techniques. With all my colleagues over the years, I have appreciated the discussions of science, academia, culture, and our futures held over drinks, meals, and

late nights in the lab. My fellow graduate students: *Alexis Kurmis*, *Ben Li*, *Thomas Martinez*, *Dave Montgomery*, *Jerzy Szabowski*, *Fei Yang*; and post-doctoral colleagues: *Alissa Hare*, *Amanda Hargrove*, *Jevgenij Raskatov*, *Dan Gubler*, *Amanda Silberstein*, *Jamie Wang*, *Sam Weisbrod*, and *Tim Welch* were wonderful labmates. We are all so different, yet the work of this lab brought us all together for a short while— there is something special in that, and I look forward to when we might cross paths again.

My work would not have progressed without the help of *Igor Antoshechkin* in the Sequencing facility; and *Shelley Diamond* and *Diana Perez* in the Flow cytometry facility. They provided not only scientific help, but also moral support in the face of difficulties. Similarly, *Vicky Brennan*, *Joe Drew*, and *Agnes Tong* fix everything.

Thank you to all my friends, but in particular those in chemistry and the Korean (sometimes-) Tennis club. You have injected fun and relief into these years; my time spent with you defines a great deal of my Caltech experience. Some of our memories— grillin'n'chillin, "stop it, Myles", "Leonard", poker nights, "yeah, we're three people", nyan cat, tennis, "definitely a halibut"— I hope to build on over a lifetime of friendship.

Lastly, my family—*Mom*, *Dad*, and *Alex*, and *my extended family*… without you guys, I probably never would have started and I certainly couldn't have finished. It is with your love and support that I have the confidence to try more things than I fail at. There is nothing I can say to fully express my gratitude, so, I'll just say I love you.

Abstract

Hairpin pyrrole-imdazole polyamides are cell-permeable, sequence-programmable oligomers that bind in the minor groove of DNA. This thesis describes studies of Py-Im polyamides targeted to biologically important DNA repeat sequences for the purpose of modulating disease states. Design of a hairpin polyamide that binds the CG dyad, a site of DNA methylation that can become dysregulated in cancer, is described. We report the synthesis of a DNA methylation antagonist, its sequence specificity and affinity informed by Bind-n-Seq and iteratively designed, which improves inhibitory activity in a cell-free assay by 1000-fold to low nanomolar IC50. Additionally, a hairpin polyamide targeted to the telomeric sequence is found to trigger a slow necrotic-type cell death with the release of inflammatory molecules in a model of B cell lymphoma. The effects of the polyamide are unique in this class of oligomers; its effects are characterized and a functional assay of phagocytosis by macrophages is described. Additionally, hairpin polyamides targeted to pathologically expanded CTG•CAG triplet repeat DNA sequences, the molecular cause of myotonic dystrophy type 1, are synthesized and assessed for toxicity. Lastly, ChIP-seq of Hypoxia-Inducible Factor is performed under hypoxia-induced conditions. The study results show that ChIP-seq can be employed to study the genome-wide perturbation of Hypoxia-Inducible Factor occupancy by a Py-Im polyamide.

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Chapter 1

Introduction

1.1 Complexity coded in DNA

The blueprint for living organisms encoded in deoxyribose nucleic acid (DNA) determines every possible transcriptomic and proteomic state for all the cells in an organism. According to the central dogma of molecular biology put forth in 1958, DNA is the foundational molecule from which ribonucleic acid (RNA) is transcribed, and protein is translated from the RNA transcript (Figure 1.1A).[1] The coordination and control of these molecules are critical to the spatiotemporal organization of cell activity and tissue maintenance in organismal health. In humans, the 3 billion letter genome is organized in 23 chromosomes and encodes over 20,000 protein-coding genes.[2] In addition, over 15,000 long non-coding RNA transcripts have been identified and are thought to play a regulatory role.[3] The structural organization of DNA and its gene expression profile is specific to each cell-type and is accomplished by layers of exquisitely controlled processes. The etiology of many diseases can be found in misregulated DNA. Modulation by chemical means of DNA regulation may yield strategies for therapeutic benefit.

1.2 Epigenetic Organization of DNA

Eukaryotic DNA is condensed and regulated as chromatin to expose appropriate genes for transcription and maintain genome integrity in the face of a genotoxic environment (Figure 1.1A). The fundamental unit of organization is the nucleosome core particle, a histone octamer protein assembly wrapped by 147 base pairs of DNA (Figure 1.1B).[4] The wrapped DNA can be compacted densely into heterochromatin, which is less
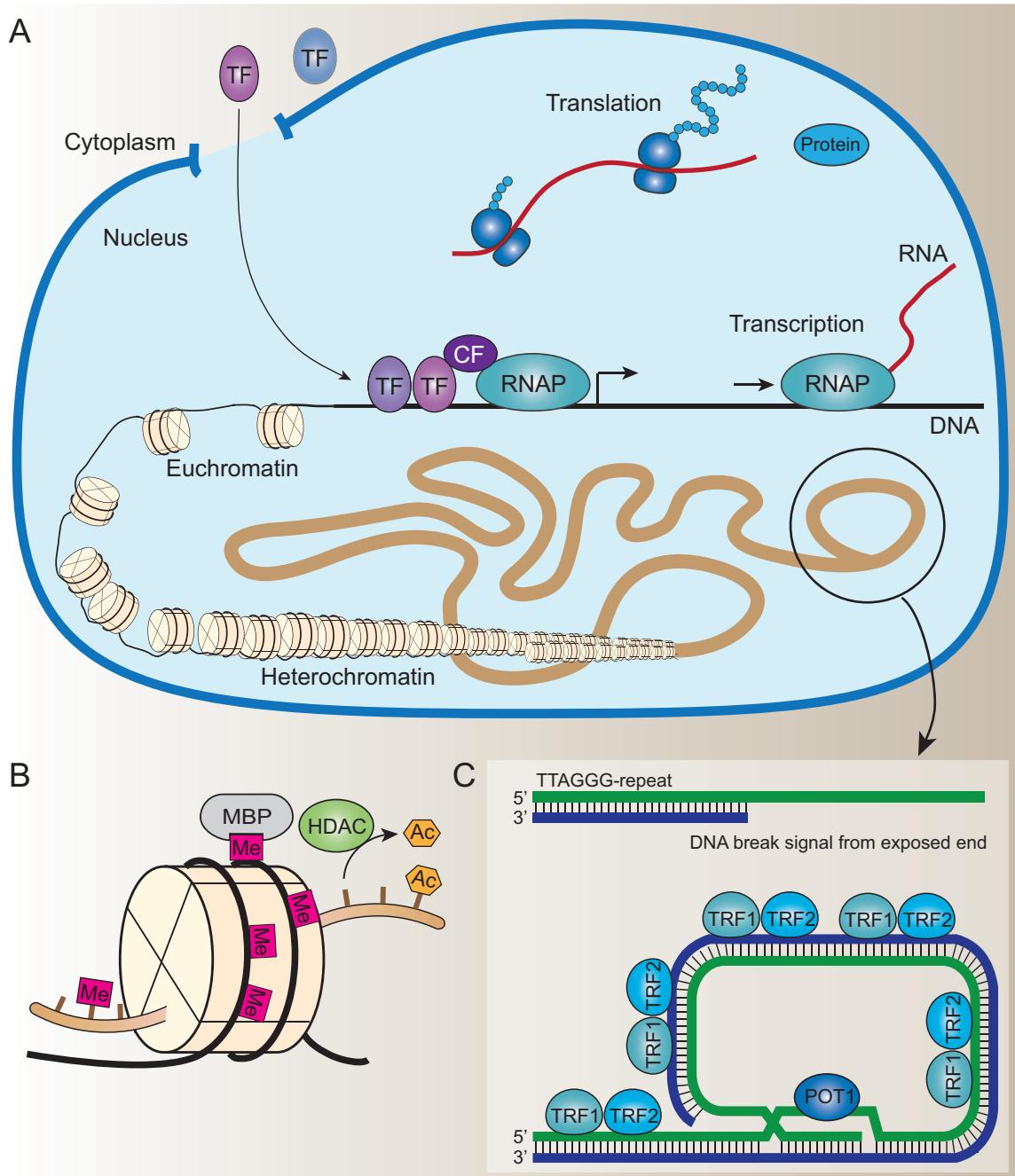
Figure 1.1 Structural organization of DNA in the cell. A) DNA (in black) is wrapped around the nucleosome core particle and organized as compact heterochromatin or transcriptionally active euchromatin. Transcription factors (TF) bind DNA and recruit cofactors (CF) and RNA polymerase II (RNAP) to transcribe mRNA from DNA. mRNA is translated into protein by ribosomes. B) The nucleosome core particle is comprised 147 base pairs of DNA wrapped around a histone octamer. Epigenetic modifications, including methylation (Me), acetylation (Ac), ubiquitiniylation, and SUMO-lation on the tails of histones govern chromatin structure. Methylated DNA is recognized by methyl-binding proteins (MBP) which recruit histone deacetylases (HDAC) to modify chromatin into a transcriptionally inactive state. C) Chromosomes end in the telomeric 5'-TTAGGG-3' hexamer repeat sequence, which can trigger a DNA damage response due to its similarity to a DNA double-stranded break. Telomeres form a

loop structure with invasion by the 5' strand to conceal the DNA end. TRF1 and TRF2 bind the telomere sequence and POT1 stabilizes the single-stranded DNA.

accessible for transcription and the requisite transcription machinery. Alternatively it can be packed more sparsely into euchromatin, which is a more transcriptionally active structure. The regulation of chromatin structure occurs by chemical annotation of the histones and DNA at the associated loci.[5] Histones can be methylated, acetylated, ubiquitinylated, phosphorylated, and SUMO-lated to modulate transcription or to respond to genotoxic stress. Proteins bound to enhancers modify, or recruit other enzymes that modify, the proximal histones that determine the transcriptional landscape of the cells. These epigenetic modifications are heritable and strongly correlate with the transcriptional programs that determine cell fate.[6] The key element of the epigenetic code on mammalian DNA is the methylation of the 5' carbon of cytosines in the palindromic CG dyad. This two base pair motif is concentrated in sequences called CpG islands, often associated with promoter sites.[7] The methylation of these CpG islands is transcriptionally repressive; methylated cytosines are bound by methyl binding proteins which recruit histone modifying enzymes to annotate the proximal histones into transcriptionally repressive heterochromatin (Figure 1.1B).[8] In this manner, DNA is purposefully structured and regulated for function.

In this organization of DNA, chromosome ends present a danger of being detected as DNA double-stranded breaks. Histones are sensitive to DNA damage and become phosphorylated upon detection of double stranded breaks, signaling towards repair or cell death.[9] To resolve the "end-protection problem", nature provides a structural solution.[10] The telomere is comprised of 6- base pair repeats of 5'-TTAGGG-3' spanning 5-30

kilobases with a 5' single strand overhang.[10] The repeating overhang is looped back to invade the double-stranded repeat region (Figure 1.1C).[11] The sequence repetition ensures complementarity and the end is hidden in the loop. Proteins including TRF1 and TRF2 of the shelterin complex, as well as POT1, which binds the single stranded portion, clamp this structure together.[11] The maintenance of this structure is important to maintain genome integrity, as its disruption is known to cause genotoxic stress signals and deleterious recombination.[12]

1.3 Transcriptional Regulation

In the context of this regulated DNA structure, transcription is enabled when transcription factor proteins bind enhancer regions coded in the DNA sequence. Transcription factors are proteins that bind DNA in a sequence-specific manner and function to either activate or repress transcription.[13] The enhancers of genes under a transcription factor's control contain the conserved cognate binding sequence for the protein. Transcription factors achieve specificity through their DNA binding domains (DBD). There are several conserved structural motifs for DNA binding and they include basic-helix-loop-helix, zinc finger, and high mobility group box DBDs (Figure 1.2).[14-16] These DBDs are linked to protein domains that transactivate transcription.

In mammals, these transcription factors do not bind in isolation but bind cooperatively to recruit cofactors and the RNA Polymerase II machinery to the transcription start site. The assemblage of multiple factors integrates various signaling

Myc/Max   Glucocorticoid Receptor   Lef-1
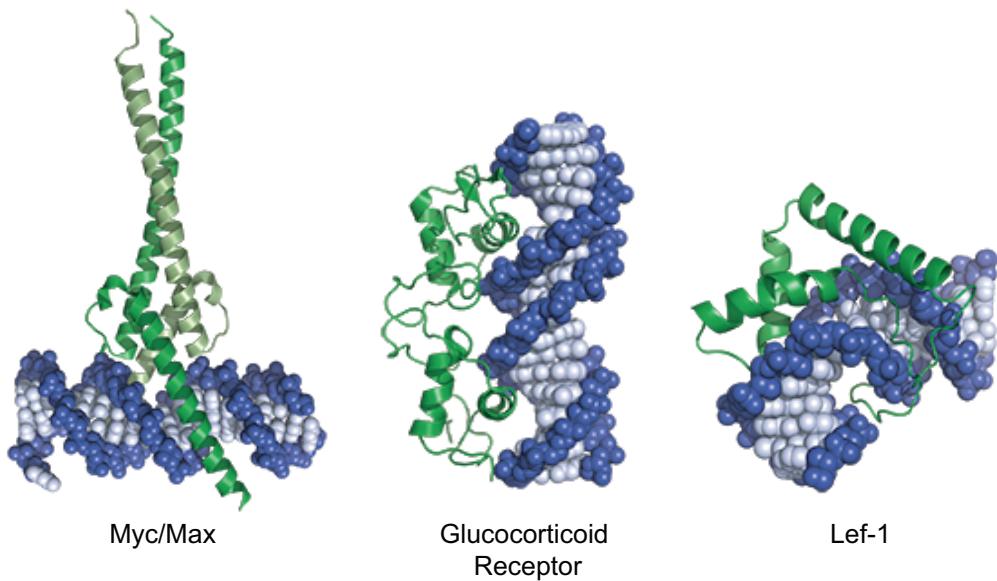
Figure 1.2 Crystal structures of transcription factors bound to DNA. DNA binding domains of transcription factors fall into several families, including basic-helix-loop-helix (Myc/Max, PDB 1NKP), zinc-finger (glucocorticoid receptor, PDB 1R40), and high mobility group (Lef-1, PDB 2LEF).[55]

cascades for precise detection of environmental cues.[13] Further, the expanded footprint of the bound proteins provides gene specificity. The interferon β (IFNB) gene enhancer is an example of the coordinate binding of multiple transcription factors for transcriptional activation. The DNA serves as a sequence-encoded scaffold for the binding of ATF-2, c-Jun, IRF-3A, IRF-7B, IRF-3C, IRF-7D, p50, and RelA at the enhancer of IFNB (Figure 1.3).[13] These factors in turn recruit the coactivators CBP/p300 in a multivalent fashion. The composite enhanceosome modifies chromatin and recruits RNA polymerase II to transcribe the IFNB gene into messenger RNA. Combining all these elements encoded in both the sequence and structure of DNA achieves exquisite control of the transcriptomic program.
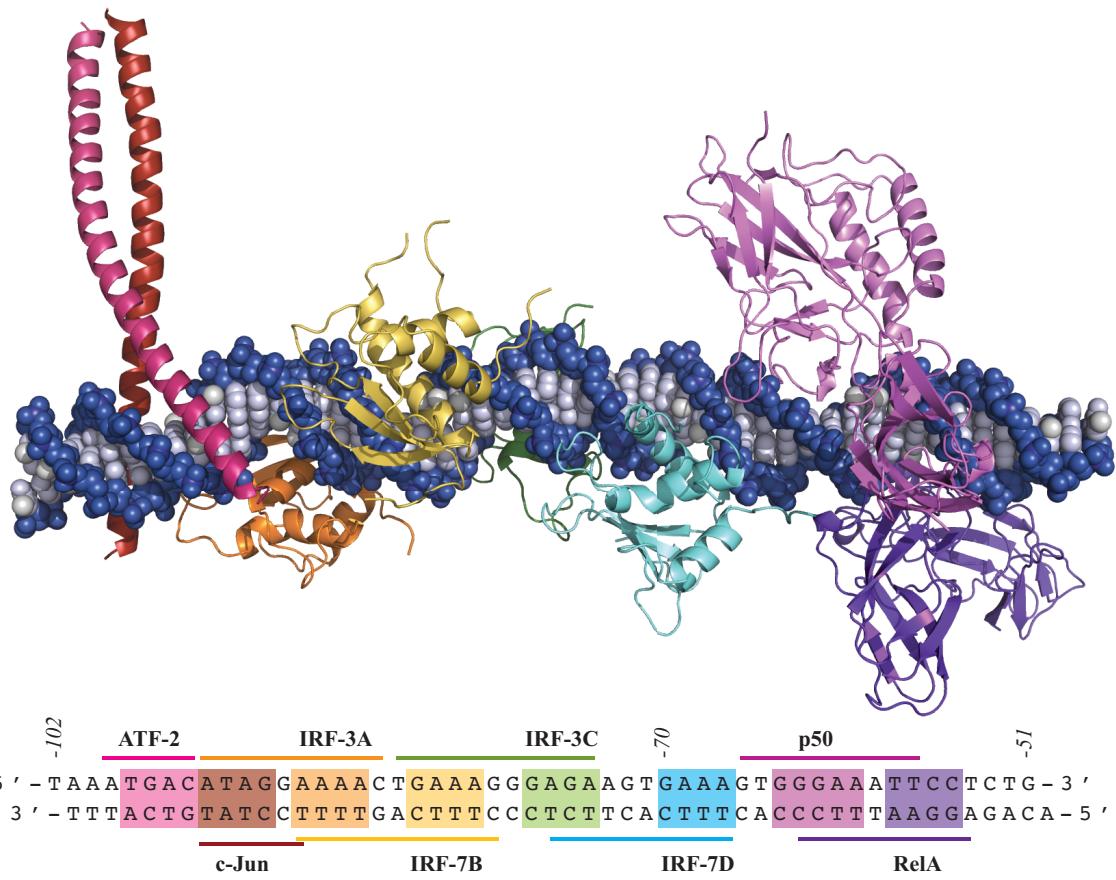
Figure 1.3 Model of the interferon β enhanceosome. [17]

1.4 Molecular recognition of DNA

The sequence-specific molecular recognition of DNA is key to this regulatory system. DNA is comprised of four nucleotides linked by anti-parallel phosphodiester backbones in a double helix (Figure 1.4). In the B-form DNA typically found in nature, the four nucleotides hydrogen-bond by the Watson-Crick pairings: adenosine (A) pairs with thymine (T) through two hydrogen bond interactions; cytosine (C) pairs with
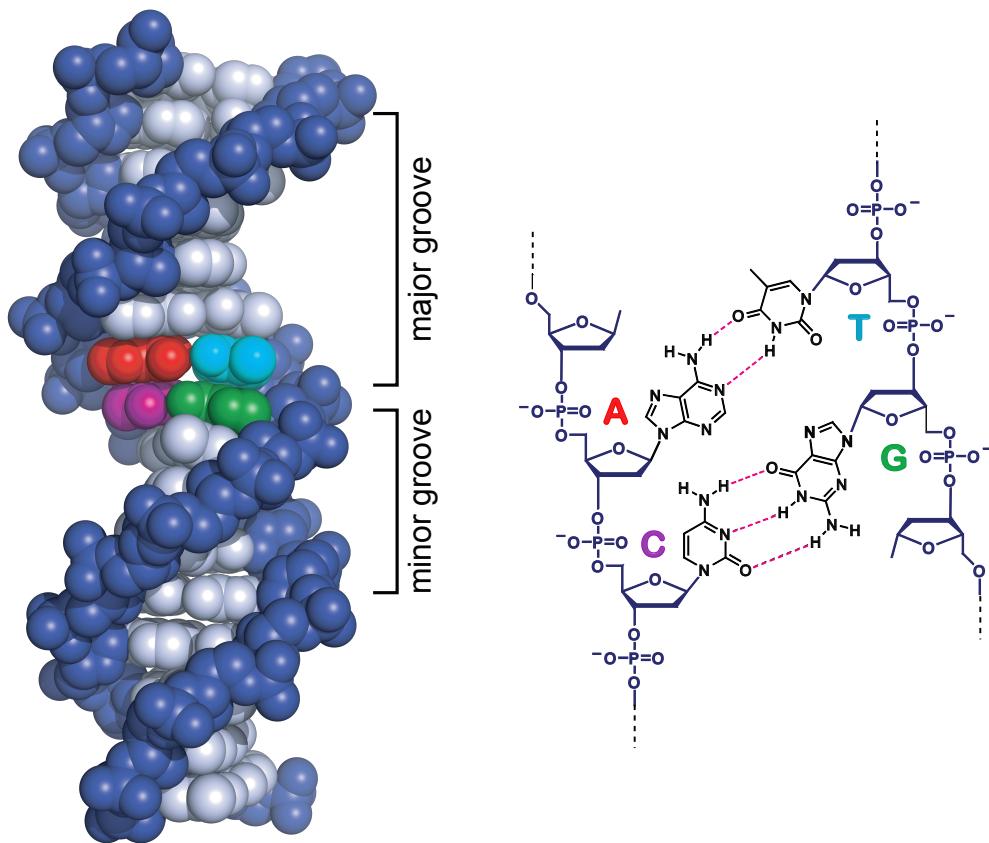
Figure 1.4 Structure of deoxyribose nucleic acid. DNA comprised of four nucleic acid bases linked by a phophodiester backbone in a double helix (PDB 1BNA) is shown.[59]

guanosine (G) through 3 hydrogen bonding interactions.[18] This specific pairing pattern provides for heritability of the genetic code as identical daughter genomes are synthesized during replication following the base complementarity. The exposed edges of the base pair form a wide major groove and a narrow minor groove on opposite sides of the double helix (Figure 1.4). Each of the four base pairs exposes a unique stereo-electronic edge in the grooves (Figure 1.5A). These sequence-specific stereo-electronics are recognized by DNA binding proteins. In addition, they are recognized by small molecule ligands which may be used to modulate the binding activity of the proteins.
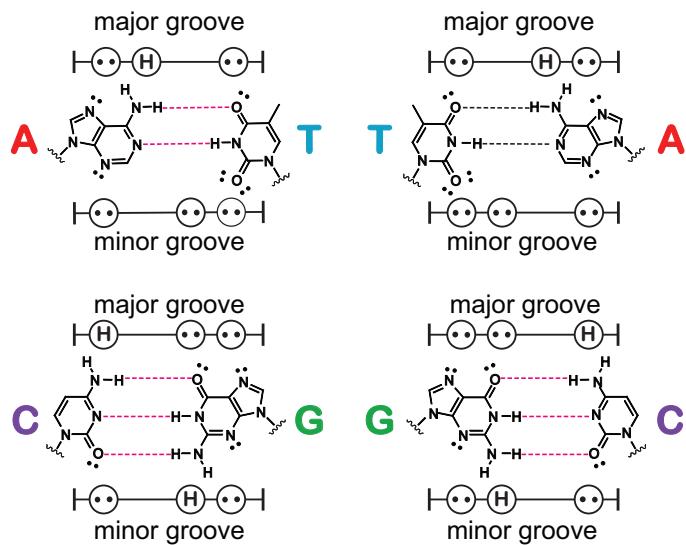
Figure 1.5 Each base pair has a unique stereo-electronic edge in both the major and minor groove.[55] Hydrogen-bonding patterns are shown for both the major and minor groove for each of the base pairs. Circles with two dots represent lone pairs and circles with an H represent a hydrogen on the exocyclic amine of guanine.

There are DNA-binding natural products with known sequence specificity that have been successfully employed as therapeutic drugs and scientific tools. Actinomycin D is a DNA-intercalator with a sequence preference for 5'-GC-3' that was one of the earliest chemotherapeutics for cancer (Figure 1.6).[19] Echinomycin is a bis-intercalating minor groove binding ligand that preferentially binds 5'-(A/T)CGT-3' and was found to downregulate hypoxia inducible factor transcriptional activity (Figure 1.6).[20-22] Fluorescent DNA-binding ligands such as DAPI have seen wide use as nuclear stains in microscopy (Figure 1.6).[23,24] Among DNA minor groove-binding ligands, distamycin and netropsin lend themselves to rational design and chemical modification due to their modular structure (Figure 1.6).[25]
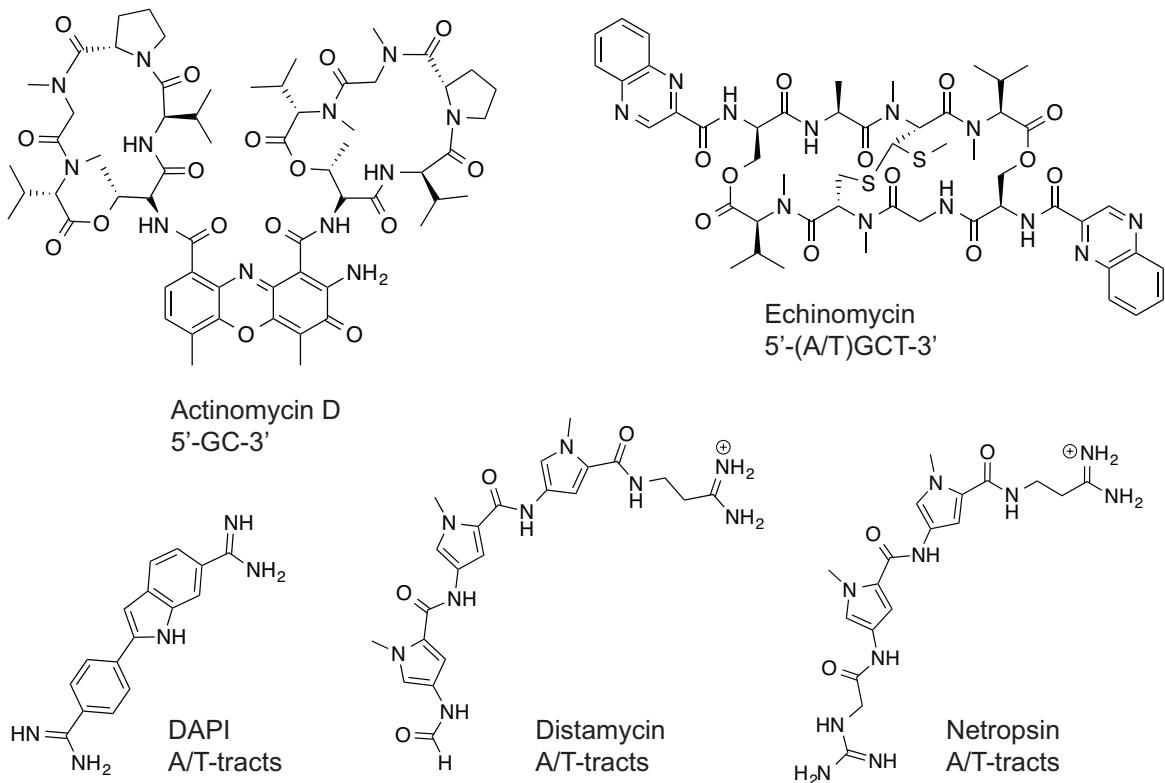
Figure 1.6 Structures of DNA binding small molecule ligands and their preferred binding sequences.

The minor groove surface of DNA presents a binding target for effecting modulation of dysfunctional biology in disease states. Distamycin preferentially binds AT-tracts, as there is steric hindrance from the exocyclic amine of guanosines in C•G/G•C on the minor groove floor (Figure 1.7A).[26] The introduction of a N-methyl imidazole ring (Im) to replace a N-methyl pyrrole ring (Py) provides a "hole" for the "bump" of the guanine residue.[27,28] In particular, the 2:1 binding of distamycin in the minor groove of DNA[29] (Figure 1.7B) suggested that linked Py-Im polyamides can target sequences through cofacial arrangement of the aromatic ring pairs to distinguish the edges of the four Watson-Crick base pairs.[30] Pairing rules for programmable specificity have been determined: Im/Py specifies a G•C base pair, Hp/Py codes for T•A base pairs
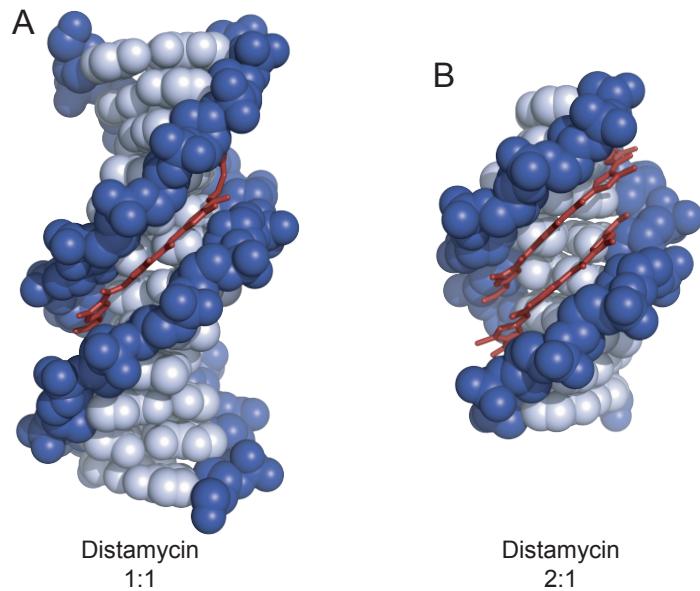
Figure 1.7 Crystal structures of distamycin bound in the minor groove of DNA. A) Distamycin bound 1:1 (PDB 2DND) and B) 2:1 in an antiparallel orientation (PDB 378D).[59]

and Py/Py binds both T•A/A•T (Figure 1.8).[27,28,31-34] This pairing register of the antiparallel polyamide strands can be covalently enforced with a γ-amino butyric acid linker to form a hairpin architecture.[35] However, Py-Im polyamide strands, particularly those containing many imidazoles, were found to be over-curved compared to natural DNA.[36] β-alanine residues were introduced to relieve the curvature in such cases, which allowed targeting longer sequences and sequences with more C•G/G•C residues.[37-41] The development of this molecular recognition technology has enabled sequence-specific targeting of DNA with affinities similar to that of transcription factors.[42]
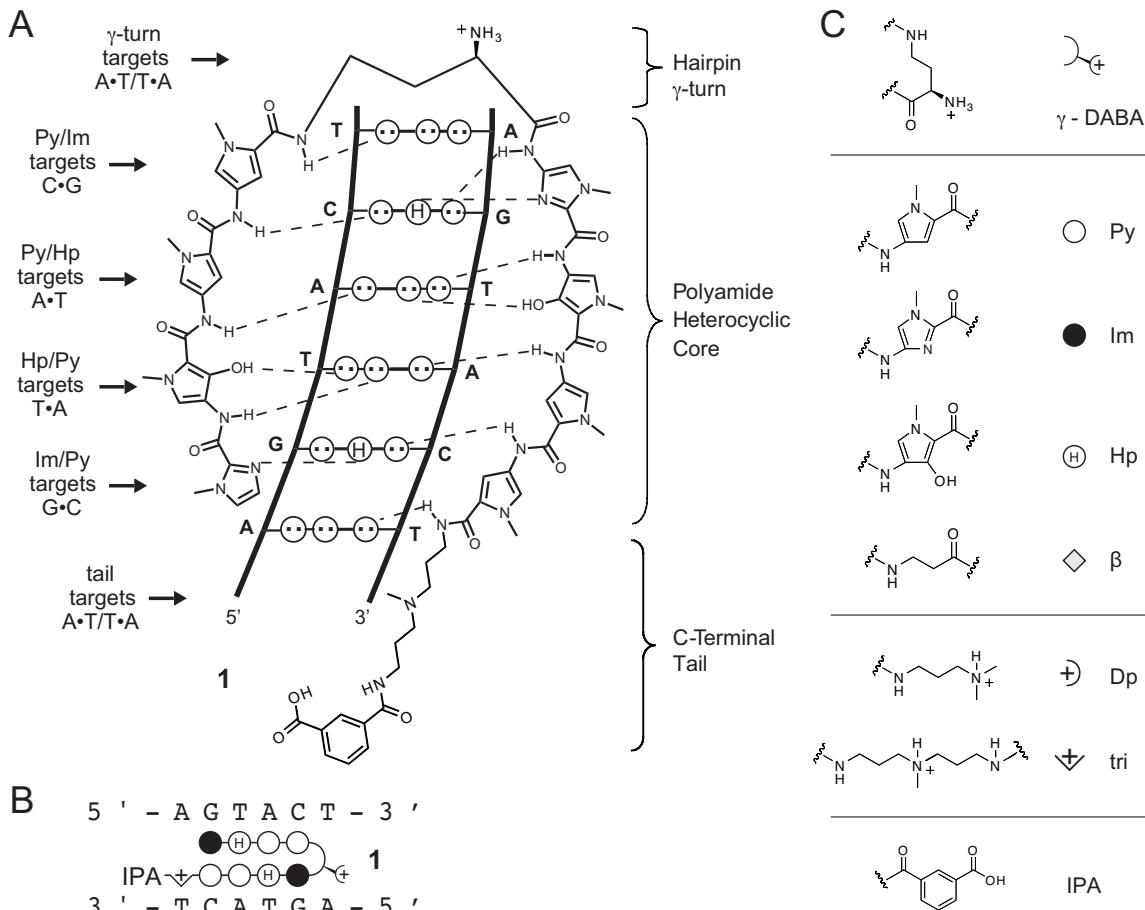
Figure 1.8 Pairing rules for Py-Im polyamide DNA sequence-specificity. A) Hydrogen-bonding pattern of the minor groove is depicted as described above. Model for the binding of a ImHpPyPy-γ-DABA-ImHpPyPy-tri-IPA polyamide bound to 5'-AGTACT-3'. Hydrogen bonds shown with dashed lines. B) Ball and stick notation for polyamides, with legend in C).

1.5 Biological modulation with Py-Im polyamides

Studies support Py-Im polyamides effecting biological modulation through its DNA binding capacity. The accessibility of DNA in the context of chromatin for Py-Im polyamide binding was demonstrated in a crystal structure of a polyamide bound to the nucleosome core particle (Figure 1.9A). In live cell culture experiments, the cell-permeability and nuclear localization of dye-conjugated polyamides were observed
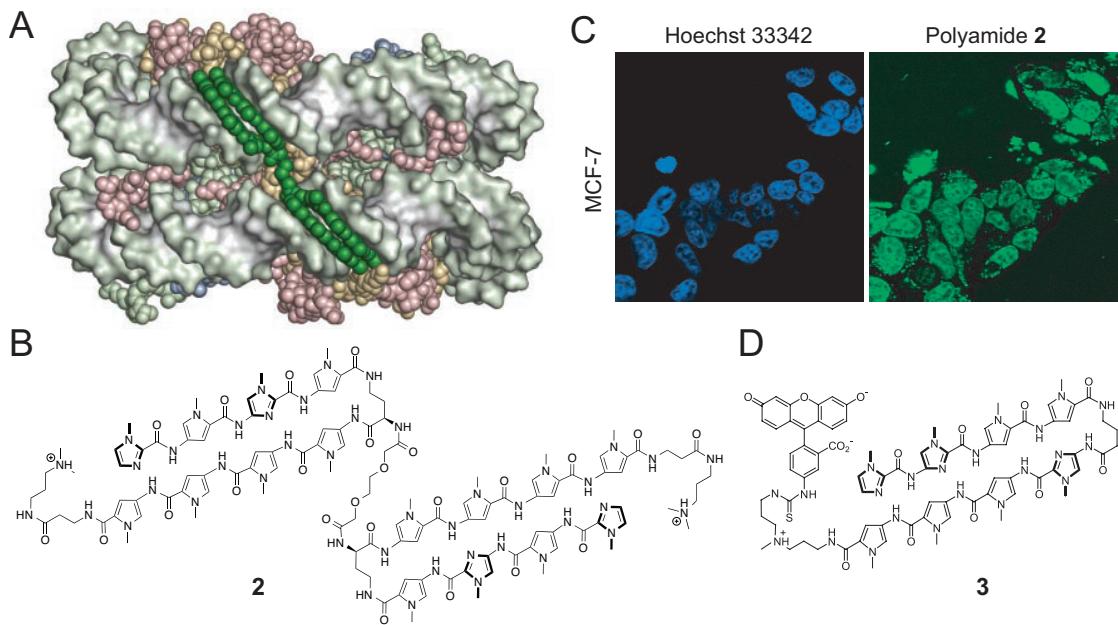
Figure 1.9 Py-Im polyamides access chromatin. A) Crystal structure of a nucleosome core particle with B) polyamide dimer **1** bound in the adjacent minor grooves.[60] C) Confocal microscopy of MCF-7 breast cancer cell line treated with nuclear-stain Hoechst or D) dye-conjugate polyamide **2**.[61] Images show co-localization to the nucleus.

through confocal microscopy in a wide range of cell lines (Figure 1.9C).[43-44] This demonstrated that access to chromatin with no external transfection was possible with these oligomers. Transcriptional modulation was demonstrated in cell culture by Py-Im polyamides targeted to the respective consensus binding sequences of the transcription factors hypoxia-inducible factor, androgen receptor, glucocorticoid receptor, and NF-κB.[46-50] An X-ray crystal structure of a cyclic polyamide bound to DNA provided a rationale for the exclusion of major-groove binding proteins upon minor groove binding by the polyamide.[51] The crystal structure revealed that polyamide binding caused an expansion of the minor groove and compression of the major groove (Figure 1.10). Py-Im polyamides were also found to disrupt processive DNA enzymes. For example, a high
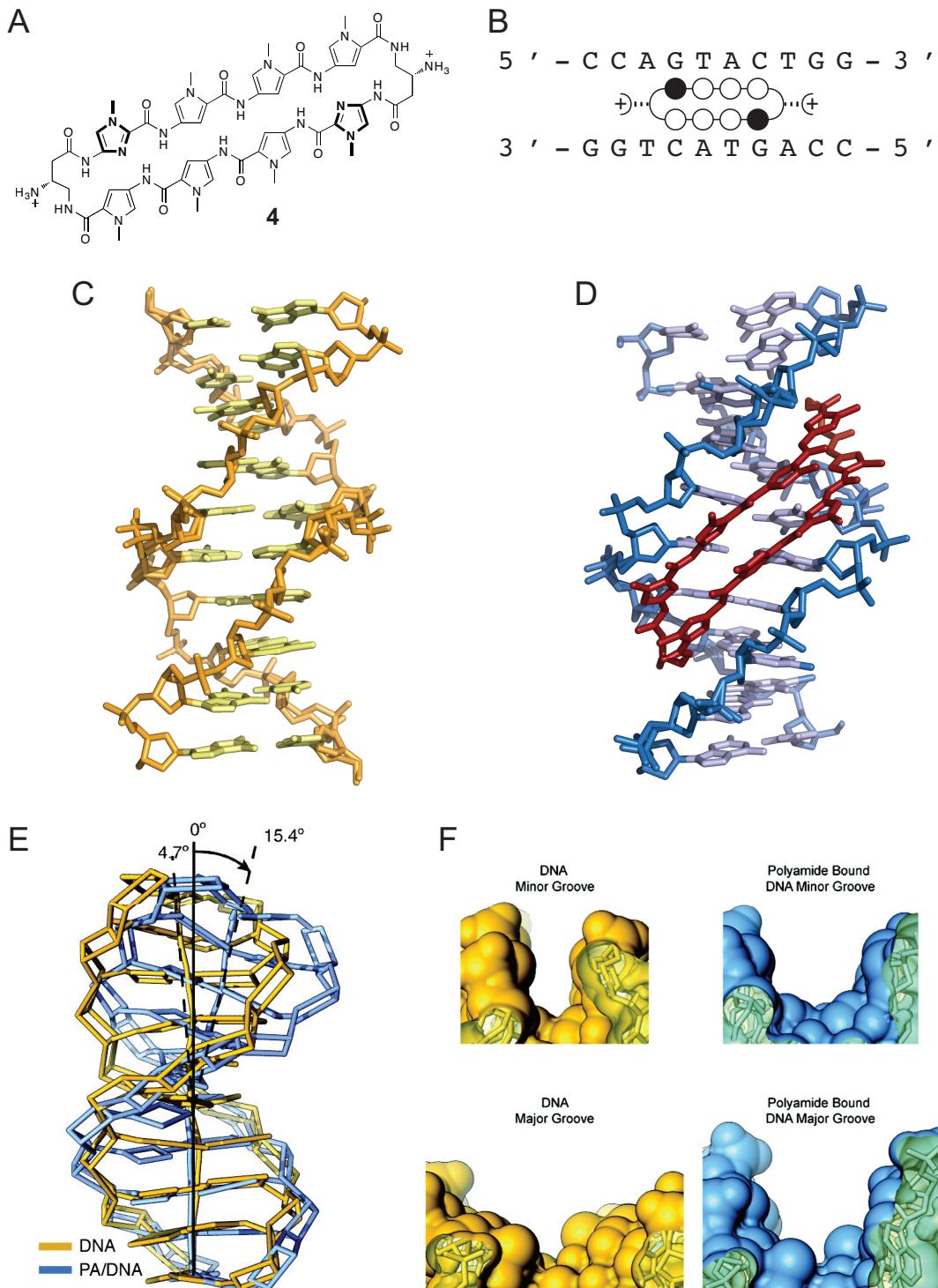
Figure 1.10 Allosteric disruption of major groove by minor groove binding cyclic polyamide.[51] A) Structure of cyclic polyamide **4**. B) Ball-and-stick notation for polyamide **4** shown with the sequence of the DNA oligonucleotide. C) Crystal structure of native B-form DNA (PDB 1D8G). D) Crystal structure of cyclic polyamide **4** bound in the minor groove of DNA (PDB 3OMJ). E) Binding of polyamide **4** causes a distortion to the native DNA structure. F) The binding of polyamide 4 causes a widening of the minor

groove (top), and a compression of the major groove (bottom). Native structure shown in yellow, polyamide-bound structure shown in blue.

affinity hairpin targeted to the androgen response element also disrupted RNA polymerase II elongation, causing cytotoxicity and p53 activation without DNA damage.[52] Another study showed that high doses of this polyamide can disrupt helicase activity and can cause replicative stress.[53] These results indicate DNA minor groove binding Py-Im polyamides can cause a variety of alterations to biological activity.

Studies in mouse models have shown that many of these effects observed in cell culture translate to the mouse with systemic treatment of the polyamide. Pharmacokinetics studies have shown that Py-Im polyamides are bioavailable and have reasonable exposure.[54] Cyclic architecture and acetylation of the turn of the same core aromatic oligomer sequence have each been explored and shown to have different pharmacokinetic and toxicity profiles.[55,56] Gene expression has been measured in the tumor of mouse xenograft models and shown to be modulated by polyamide treatment similarly to the experiments done in cell culture.[57] In a xenograft model of prostate cancer, the high-affinity Py-Im polyamide targeted to the androgen receptor response element reduced tumor size.[52] Indeed, a C-14 labeled analog of the molecule showed preferential localization to the tumor xenograft.[58] In sum, Py-Im polyamides are a class of molecules well-suited for the study of biological perturbation through DNA binding.

1.6 Scope of Work

This thesis describes studies of Py-Im polyamides targeted to biologically important DNA repeat sequences for the purpose of modulating disease states. In chapter

2, a Py-Im polyamide is designed to bind a sequence that contains two CG dyads and is tested as a DNA methylation antagonist. Chapter 3 explores the immunogenic signaling triggered by a polyamide that is targeted to the telomere repeat sequence. Chapter 4 describes work in assessing the animal toxicity of hairpin polyamides targeted to the CAG/CTG triplet repeat expansion to disrupt the transcription of the associated RNA, which is the molecular cause of myotonic dystrophy type 1. Chapter 5 describes a ChIP-seq experiment that explores the genome-wide perturbation of hypoxia-inducible factor occupancy induced under hypoxia by a Py-Im polyamide targeted to the consensus sequence of the hypoxia response element.

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