Chapter 4

Progress Towards Polyamide Inhibition of Myc-Activated Gene Expression by Antagonism of the E-box Fragment 5'-WCGWGW-3'

The work outlined in this chapter was performed in collaboration with Daniel A. Harki (California Institute of Technology).

Abstract

Deregulated expression of the Myc transcription factor is a potent and frequently encountered defect of human cancer, and a known contributor to cancer pathogenesis or disease progression. Inactivation of the *myc* oncogene or Myc protein represents a promising avenue for the development of novel anticancer agents. We describe here the synthesis and biochemical characterization of sequence-specific, DNA-binding polyamides as antagonists of Myc-activated gene expression. Our goal is to employ DNA-bound polyamides to sterically block Myc-activated gene expression in human cancer cells. While several of these polyamides showed promising cellular uptake properties in live cells, modulation of Myc-regulated gene expression was not observed in MCF-7 (breast cancer) or A549 (lung carcinoma) cell lines. We believe that a study of polyamide inhibition of Myc-regulated genes, and we anticipate future developments in polyamide design will allow for cellular uptake in other relevent cell lines which may display a different genetic response to polyamide treatment.

4.1 Introduction

The c-Myc (Myc) transcription factor, the protein product of the *c-myc* (*myc*) proto-oncogene, participates in an array of cellular processes, such as proliferation, growth, differentiation, and apoptosis (**Figure 4.1**).^{1,2} Unfortunately, deregulated Myc expression is a potent and frequently encountered defect of human cancer, and a known contributor to cancer pathogenesis or disease progression.^{1,3} Myc is deregulated and overexpressed in most cancer cells, and de-activation of *myc* in established, Myc-induced transgenic tumors triggers proliferative arrest and redifferentiation of tumor cells, resulting in rapid tumor regression.⁴



Figure 4.1 The Myc-Max transcription factor

The Myc-Max basic-helix-loop-helix leucine zipper heterodimer binds a target DNA site, known as the *E*-box, having a consensus sequence 5'-CACGTG-3'. c-Myc regulates downstream target genes resulting in activation of cell cycle regulation, apoptosis, or inhibition of cell adhesion. Examples of c-Myc target genes associated cellular functions are listed.

Structurally, Myc resembles most transcription factors, possessing a helix-loop-helix leucine zipper surface for protein dimerization and a basic region that forms contacts with DNA surfaces.^{3,5,6} The active Myc transcription factor exists as a heterodimer with Max (Myc associated protein X), which binds DNA and interacts with other distinct proteins.^{3,5}

Interestingly, the Myc-Max heterodimer targets different DNA sequences for gene activation and suppression. Myc-Max activates gene expression by binding E-box sequences in gene promoters (5'-CACGTG-3') and suppresses gene expression by targeting proximal promoter regions (not E-boxes) in other genes.^{1,3,7-13} Myc also associates with proteins involved in chromatin remodeling, transcriptional regulation, and the maintenance of Myc stability.¹³

Inactivation of the *myc* oncogene or Myc protein represents a promising avenue for the development of novel anticancer agents.^{3,14} Previous studies directed at the *myc* oncogene have utilized triplex-forming oligonucleotides (TFOs) that target the *myc* promoter. In particular, a TFO conjugated to daunomycin (DNA intercalator) was recently reported to reduce Myc promoter activity in prostate and breast cancer cells.¹⁵ Similarly, RNAi knockdown of *myc* expression¹⁶ and antisense oligodeoxynucleotides targeting *myc*¹⁷ in the MCF-7 breast cancer cell line have yielded promising results. Recently, small molecule inhibitors of Myc-Max heterodimerization have been identified from library screening¹⁸ and diversity-oriented synthesis,¹⁹ and shown to inhibit Myc-dependent proliferation and Myc-Max heterodimerization, respectively, in micromolar quantities.

However, in spite of these encouraging results, Myc is essential for cell growth, differentiation, proliferation and, critical for cancer patients, stem cell compartment maintenance of regenerative adult tissues such as the gastrointestinal tract, skin and bone marrow. Hence, blocking Myc function systemically might trigger devastating and irreversible side effects. As Myc functions in cells by both initiating and suppressing gene expression as the Myc-Max heterodimer, we hypothesize that cellular abatement of Myc will invariably contribute to additional gene deregulation.³ It has been estimated that Myc can bind ~25,000 sites in the human genome and regulate a significant number of genes.²⁰

may relinquish control of genes normally repressed by Myc. Furthermore, an autoregulatory pathway that monitors Myc protein levels has been proposed as the mechanism by which cells regulate Myc gene expression.²¹ Decreasing intracellular Myc may actually increase Myc expression. DNA-binding polyamides offer a new approach for controlling Myc overexpression that circumvents such problems—the blocking of Myc-Max heterodimer binding to E-box DNA sequences (5'-CACGTG-3') by a sequence-specific hairpin polyamide (**Figure 4.2**).



Figure 4.2 Polyamide inhibition of Myc-Max DNA binding

Simplified model of the inhibition of Myc-Max modulated gene expression by a sequence-specific DNA-binding polyamide. (a) E-box DNA sequence found in gene promoters (b) Myc-Max binds and activates gene expression (c) Polyamide bound to the E-box DNA sequence (d) Bound polyamide antagonizing Myc-Max binding and inhibiting gene expression.

We describe here the synthesis and biochemical characterization of sequence-specific, DNA-binding polyamides as antagonists of Myc-activated gene expression. Our goal is to employ DNA-bound polyamides to sterically block Myc-activated gene expression in human cancer cells. Accordingly, a small library of polyamides targeting the E-box (5'-CACGTG-3') sequence recognized by Myc in gene promoters has been synthesized and evaluated biochemically. By antagonizing Myc binding to E-box DNA sequences, as opposed to decreasing cellular levels of Myc protein or disrupting Myc-Max heterodimer formation, polyamides may counteract the effects of elevated Myc levels in cells without relinquishing control of genes normally repressed by Myc. Importantly, this strategy should be relatively specific for inhibiting only the Myc processes associated with gene activation, since only a minority of the known Myc-Max binding sites *in vivo* possess the consensus 5'-CACGTG-3' sequence targeted by our proposed polyamide library.²⁰

4.2 Experimental Design

I. Design and synthesis of a polyamide library to bind 5'-WCGTGW-3'

A small library of polyamides targeting the Myc E-box sequence (5'-CACGTG-3') was synthesized on solid-phase using protocols developed in the Dervan laboratory.²² Compounds **1-6** possess different modifications to the polyamide skeleton to optimize binding affinity and specificity (**Figure 4.3**). The molecular design of these compounds is based upon previous studies in the Dervan laboratory that examined the utility of placing a β -alanine unit targeting the G•C base pair proximal to the C•G in the terminal position of a polyamide²³ and immediately following the chiral turn.²⁴ This initial library consists of compounds containing beta-alanine-diaminopropylamine ("Dp") tails.



Figure 4.3 Initial polyamide library.

Compounds 1-6, synthesized by D. Harki and used in initial binding affinity screening studies

II. Assessment of binding affinities and specificities of initial polyamide library

Polyamides **1-6** were screened for favorable binding affinities and specificities by melting temperature analysis of duplex DNA²⁵ possessing the polyamide match DNA binding site (5'-CGTG-3') or single base pair mismatches. Our interest in determining the specificity of the N-terminal pyrrole cap unit led us to design this DNA binding site with a T in place of the C normally found in the biologically relevant E-box, thus removing the binding ambiguity that would be presented by the palindromic E-box consensus sequence. As shown in **Table 4.1**, polyamides **1** and **2** were found to have the highest DNA duplex stabilization. Modifications to polyamide **2**, such as introduction of terminal acetamide and formamide caps (compounds **3** and **4**, respectively), as well as elimination of the first β -alanine moiety (**5**), failed to yield a higher affinity binder. Based on these results, **1** and **2** were chosen for further biological studies. Eight-ring hairpin **6** failed to yield reliable melting temperature curves in this initial screen, and was not further utilized.

 Table 4.1 Assessment of binding affinities and specificities of polyamides 1-5

	X-	Y: C-G	G-C	T–A	A-T
	DNA only	56.9 ± 0.3	57.9 ± 0.3	55.2 ± 0.1	55.9 ± 0.5
1	÷ → → → → → + → → → → → → → → → → → → →	68.9 ± 0.1 [12.0]	65.1 ± 0.1 [7.2]	64.7 ± 0.2 [9.4]	61.3 ± 0.0 [5.4]
2	€€€€€€€	71.9 ± 0.2 [15.1]	68.2 ± 0.2 [10.3]	66.0 ± 0.1 [10.8]	61.8 ± 0.1 [5.9]
3		64.1 ± 0.3 [7.2]	60.1 ± 0.2 [2.2]	62.8 ± 0.4 [7.6]	58.5 ± 0.3 [2.6]
4		70.6 ± 0.4 [13.7]	67.1 ± 0.4 [9.2]	65.7 ± 0.2 [10.4]	63.2 ± 0.3 [7.3]
5	€ € € € € € € € € € € € € € € € € € €	69.8 ± 0.3 [12.9]	66.4 ± 0.1 [8.4]	65.0 ± 0.2 [9.8]	60.6 ± 0.5 [4.7]

Tm oligo: 5' - GGTAXGTGTGGG-3'3' - CCATYCACACCC-5'

Melting temperature studies of duplex DNA (2 nmoles/oligo) treated with polyamides (2.0-2.4 nmoles). Averages and S.D. were calculated from at least four analyses. Values in brackets represent Δ Tm versus untreated DNA duplex. These melting temperature studies were performed by D. Harki.

III. Design and synthesis of a polyamide library to disrupt Myc-Max DNA binding

Based on the results of the screening, a small library of polyamides derived from compounds **1** and **2** was designed to bind the 5'-WCGTGW-3' sequence of the E-box in gene promoters and block Myc-Max-activated gene expression. Over the course of experimentation, this library evolved to contain polyamides of several different scaffolds and tail substituents, including hairpins containing both diaminopropylamine ("Dp") as well as diamino-N-methyl-dipropyl amine ("triamine") tails, hairpins containing a β -alanine moiety in the tail region, hairpins lacking this β -alanine tail moiety, and even cyclic polyamides (**Figure 4.4**). Polyamides **7-11** contain an isophthalic acid tail modification, a modification that has been shown in previous studies by the Dervan laboratory to facilitate cellular uptake.²⁶



Figure 4.4 Library of compounds synthesized by K. Muzikar and D. Harki for biological studies

IV. Assessment of binding affinities and specificities of biological polyamide library

The binding affinities and specificities of polyamides **7** and **8** were rigorously characterized by quantitative DNase I footprint titrations as well as thermal melting analysis (**Figure 4.5**).²⁷ These polyamides are analogues of **1** and **2**, respectively, and contain the isophthalic acid tail modification often noted to encourage positive nuclear localization in cell culture. The 5'-terminal base pair was interrogated for specificity since this position is often deemed the hardest to obtain specificity. As shown in **Figure 4.5**, extremely high binding affinities for both polyamides were observed. Polyamide **7** bound its match DNA sequence with $K_a = 4.0 \times 10^9 \text{ M}^{-1}$ and polyamide **8** bound the same match site with $K_a = 1.3 \times 10^{10} \text{ M}^{-1}$. To our delight, high specificities against binding to the mismatch sites was observed for both compounds; with polyamide **8** exhibiting 10-fold preference for match site I versus mismatch sites II-IV. Based on these findings we concluded that polyamides **7** and **8** are more than sufficient to proceed to biological studies.



Figure 4.5 Binding studies of polyamides 7 and 8

A) Sequence of the pDHKM1 plasmid insert B) Storage phosphor autoradiograms from quantitative DNase I footprint titrations of polyamides 7 (left) and 8 (right). Lane 1, intact DNA, lane 2 G reaction, lane 3 A reaction, lane 4, DNase control. lanes 5-15: DNase I digestion products in the presence of 1pM, 3pM, 10pM, 30pM, 100pM, 300pM, 1nM, 3nM, 10nM, 30nM, 100nM polyamide, respectively. C) Summary of binding affinities as measured by DNase footprinting and by melting temperature studies of duplex DNA (2 nmoles/ oligo) treated with polyamides (2.0-2.4 nmoles). Averages and S.D. were calculated from at least four analyses. Values in brackets represent ΔTm versus untreated DNA duplex. Values in parenthesis represent K_a relative to K_a for match site. Melting temperature experiments were performed by D. Harki.

V. Evaluation of polyamide uptake and localization in cell culture

In order to assess the ability of the polyamides to traffic across the cell membrane and localize to the nucleus of cells, fluorescein isothiocyanate (FITC) conjugates **13-16** were synthesized (**Figure 4.6**) so that uptake could be evaluated by confocal microscopy.



Figure 4.6 Fluorescein isothiocyanate (FITC) conjugate polyamides synthesized by K. Muzikar and D. Harki

Polyamides **13** and **14** were added at two micromolar concentration to A549 (human lung carcinoma), MCF-7 (human breast cancer), LNCaP (human prostate cancer) and P493 (human lymphocytes) cells. For A549, MCF-7, and LnCaP cell lines (**Figure 4.7**) significant amounts of fluorescence was observed in the nucleus when dosed both alone and when co-administered with the calcium channel blocker verapamil (25μ M), suggesting that both compounds access the nucleus and accumulate to reasonable concentrations in these cell lines. In P493 cells, however, uptake is negligible for both compounds, with only small amounts of **13** and **14** localized to the nucleus when co-administered with verapamil (25μ M).



Figure 4.7 Cellular uptake of polyamides 13 and 14

Confocal laser scanning microscopy images of polyamide-fluorescein conjugates 13 and 14 in several different cancer cell lines. For each group of pictures, the left images show 2uM polyamide treatment with 14 hour incubation; the right images show 2μ M polyamide and 25μ M verapamil treatment with 14 hour incubation; top images show fluorescence signal from polyamide; bottom images show fluorescence signal overlaid on visible light image.



Figure 4.8 Cellular uptake of polyamides 13-16

Confocal laser scanning microscopy images of polyamide-fluorescein conjugates **13-16** in NCI H82, H60 cell lines. For each group of pictures, the left images show 2uM polyamide treatment with 14 hour incubation; the right images show 2μ M polyamide and 25μ M verapamil treatment with 14 hour incubation; top images show flourescence signal from polyamide; bottom images show flourescence signal overlaid on visible light image.

The favorable uptake of **13** and **14** in the aforementioned three cell lines suggests both polyamides have cell permeability properties sufficient for further *in vivo* studies, while studies in P493 cells were not pursued. Additionally, polyamides **13-16** were added at 2 μ M concentration to NCI-H60 (non-small-cell lung cancer) and NCI-H82 (small-cell lung cancer) cells (**Figure 4.8**), which are known to have elevated levels of c-myc gene.²⁸ Uptake of all four compounds in these cell lines appears favorable both with and without verapamil co-administration, although less so in the NCI-H60 cells. **Table 4.2** summarizes the uptake properties of compounds **13-14** in the cell lines described above.

	25mM verapamil	P493	MCF-7	A549	LnCap	H82	H60
	-	-	++	++	++	++	+
13	+	++	++	++	++	++	+
FITC-t-		-	++	++	++	++	+
14	+	++	++	++	++	++	+
	-	n.d.	n.d.	n.d.	n.d.	+	++
нис- 	+	n.d.	n.d.	n.d.	n.d.	+	++
		n.d.	n.d.	n.d.	n.d.	++	-
16	+	n.d.	n.d.	n.d.	n.d.	++	++

 Table 4.2 Cellular localization of polyamide-dye conjugates in cultured cells

++, Nuclear staining exceeds that of the medium; +,nuclear staining less than or equal to that of the medium, but still prominent; -, very little nuclear staining, with the most fluorescence seen in the cytoplasm and/or medium; --, no nuclear staining. n.d. = not determined.

While it is estimated that ~25,000 genes are regulated in some way by Myc, there is a surprising dearth of known primary gene targets of Myc. Identification of Myc-regulated genes has generally relied on experimental activation of Myc followed by monitoring of changes in mRNA levels.^{29,30} More than 10 investigative works reported the use of high-throughput screening based on cDNA microarrays or the SAGE assay, significantly expanding the list of genes that are up- or downregulated by Myc. Based on an updated online compilation,³¹ this list now includes over 1500 genes. It remains unclear, however, how many of these genes are direct targets of Myc. In many studies a large fraction of Myc-target genes respond weakly, or even fail to respond, to Myc activation, depending on the cell type or experimental conditions used. Additionally, lists of genes identified in high-throughput screens such as RNA microarrays,³² ChIP-screening,^{33,34} or ChIP-PET³⁵ are only partially overlapping, especially between cell lines, and many genes were identified only once. Thus, we still possess a fragmentary picture of the loci that are directly targeted by Myc, and no accurate estimate of their numbers.

Table 4.3 E-box and surrounding DNA sequences of several known direct Myc target ge	enes
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gene	E-box sequence	gene	E-box sequence
RCC1	GCCGGCCC CACGTG AAGCCCGGAGG	ECA39	CTGAGCGC C<u>ACGTG</u> T CACTGCACAG
RCC1	ACTTCGAC CACGTG TGACTTGTGTG	cdc25A	GGGCCTGC CACGTG CACCCCGCCCG
ODC	TGTGCGGC CACGTG TCGCGAGGCCC	cdc25A	ACTACAGA CACGTG CCACCACACCC
ODC	GCAGGGGA CACGTG GTCGCCGAGCG	РТ	GCAACGAG CACGTG GCCTGGGGCGC
eIF4e	CCATCGGC CACGTG ACCAGTCCTTT	P53	TCCCCTCC CACGTG CTCACCCTGGC
eIF4e	ATATCCGT CACGTG GCCAGAAGCTG	MrDb	TGACTCAC CACGTG CATACCTATGG

Shown in bold is the six base pair E-box site. Underlined are 5'-WCGWGW-3' polyamide match sites. Sites without an underline present a GC base pair mismatch under the turn moiety.

Despite this complexity, we have identified a small list of genes that are known direct targets of Myc, and whose promoter E-boxes have been rigorously identified (**Table 4.3**). We anticipate that polyamides that bind and interfere with the expression of these genes would be useful in then mining the genome for other, perhaps previously unidentified, direct targets of Myc. Of the genes identified in **Table 4.3**, the gene that has by far the most relevance to cancer biology is the cap-binding protein eukaryotic translation initiation factor 4E (eIF4E). A functional promoter region of eIF4E has been identified 400 nucleotides upstream of the transcription initiation site with two essential E-box motifs in the immediate promoter region. ³⁸ Of these two E-boxes, only one has a full match site (5'-ACGTGA-3') for the polyamide, while the other (5'-ACGTGG-3') presents a G•C base pair under the turn moiety (**Figure 4.9**)



Figure 4.9 Schematic representation of E-box locations relative to transcription start site of eIF4E

During initiation of transcription, eIF4E binds the 7-methylguanosine cap of mRNA and recruits a transcript to the translational machinery. Increased cap-dependent mRNA translation rates are frequently observed in human cancers. Mechanistically, many human tumors often overexpress the cap binding protein eukaryotic translation initiation factor 4E (eIF4E), leading to enhanced translation of numerous tumor-promoting genes.

Shown are the sequences of the two E-boxes with a representative polyamide shown bound. Note that while there is a full polyamide match binding site in E-box 1, there is a severe mistmatch under the turn moiety of the polyamide, it is likely this polyamide has a poor binding affinity for this site.

eIF4E-specific antisense oligonucleotides have been shown to repress expression of eIF4E-regulated proteins (e.g., VEGF, cyclin D1, survivin, c-myc) in human cells (HeLa, A549, MCF-736) as well as induce apoptosis of the carcinogenic cells. Intravenous administration of eIF4E-specific antisense oligonucleotides has been shown to reduce eIF4E expression in human tumor xenografts, as well as significantly suppress tumor growth.³⁷

VII. Assessment of binding affinities of polyamides towards eIF4E E-box

The initial thermal melting temperature studies were performed on 12-mer oligos that were designed to have an unambiguous polyamide binding site (5'-TACGTGT-3') in order to probe the specificity of the polyamide in the 5' position avoiding the palindromic sequence 5'-CACGTG-3'. However, it is the second sequence that is relevant within the context of the eIF4E E-box, hence we decided to perform a T_m analysis on a 12-mer oligo containing the sequence 5'CACGTG-3'. **Table 4.4** shows that polyamides 7 and 11 demonstrate essentially the same thermal stabilization energy ($\Delta T_m \sim 4 \circ C$) as each other for the eIF4E duplex. While comparisons cannot be drawn relating ΔT_m s across different DNA oliomers, this is the same general magnitude of stabilization previously seen on the 5'-TACGTGT-3' sequence.

	Tm oligo:	5 ' - GGC <mark>CACGTG</mark> ACC-3 ' 3 ' - CCG <mark>GTGCAC</mark> TGG-5 '	5 ' - TGGG <mark>TACGTG</mark> TGGG- 3 ' 3 ' - ACCC <mark>ATGCAC</mark> ACCC- 5 '
	DNA only	62.4 ± 0.6	63.7 ± 0.3
7		65.6 ± 0.5 [3.2]	70.3 ± 0.2 [6.5]
11		66 .4 ± 0.5 [4.0]	n.d.

Table 4.4 Thermal melting temperature studies of polyamides 7 and 11 on eIF4e E-box 1

Melting temperature studies of duplex DNA (2 nmoles/oligo) treated with polyamides (2.0-2.4 nmoles). Averages and S.D. were calculated from at least four analyses. Values in brackets represent ΔTm versus untreated DNA duplex. n.d. = not determined.

VIII. Selection of inducible and endogenously Myc-overexpressed cell lines

Unlike previous transcription factors that have been inhibited by polyamide-DNA binding, the Myc-Max transcription factor does not function in response to an antagonist; that is, this is not an endogenously inducible system. This gives an added level of complexity to the assessment of gene regulatory activity, hence for our initial studies of these polyamides in cell culture we intended to use an artificial system that would allow us to control cellular Myc levels. Two such cell lines were available from the literature, and we requested and received these cell lines.

P-493 cells:

The P493-6 cell line is a model for cell cycle activation by *myc* in Burkitt lymphoma cells. P493-6 is a human EBV-EBNA1 positive B-cell line in which *myc* is expressed under the control of a tetracycline-regulated promoter. ³⁹ These cells were sent as a gift from Professor Dirk Eick (Institute of Clinical Molecular Biology and Tumor Genetics, Munich, Germany). Unfortunately, as can been seen in **Figure 4.7**, cellular localization of compounds **13** and **14** was negligible in this cell line and further studies were not pursued.

MCF-7cl35 cells:

The Shiu lab at University of Manitoba has developed a clonal MCF-7 human breast cancer cell line (MCF-7cl35) harboring a stably-transfected human c-myc gene, whose expression is controlled by the bacterial reverse tetracycline transcription activator protein.⁴⁰ Expression of endogenous genomic c-myc gene in MCF-7 cells is abolished by the potent pure estrogen antagonist, ICI 182,780. These cells were sent as a gift from Professor Robert P.C. Shiu (University of Manitoba, Winnipeg, Manitoba, Canada) and were cultured as instructed by the Shiu lab, which is as reported in their papers: in DMEM with sodium pyruvate, glutamine (4mM), penicillin-streptomycin (4mM), and 5% FBS.⁴



Figure 4.10 Doxycycline-induced Myc and eIF4E expression in MCF7-cl35 cells

Myc induction by doxycycline (a water-soluble version of tetracycline) as well as repression of endogenous *myc* expression by ICI was reproduced when the cell line was first received. However, as the cells were passaged over time, the intensity of the *myc* signal induction decreased from 5-fold (passage number three) to less than 2-fold (passage number seven). Additionally, while Myc expression was induced, no induced expression of the target gene eIF4E was seen in most instances, and what induction is seen for both genes is wildly variable. **Figure 4.10** shows a compilation of data that represents the irreproducible nature of data garnered from this cell line. The large error bars seen in **Figure 4.10** were not

A) Myc expression is induced 5-Fold by 12-hour incubation with doxycycline and and repressed by ICI inhibitor compound in accordance with literature precident (cell passage number 3). B) Doxycycline time course indicates Myc is induced maximally 5-fold at 3 hours doxycyline treatment, while eIF4E is induced over 15-fold at this time point (cell passage number 4). C) Doxycycline treatment fails to induce Myc or eIF4E over a 1-8 hour range (cell passage number 7).

a one-time occurrence, but were seen in every experiment performed (over 10 independent experiments). We hypothesize that the plasmid containing the inducible tet-Myc promoter may not be as stably integrated as we expected. There is no additive in the culture medium as described by the Shiu group to keep a selective pressure on the cells to maintain the plasmid, and it may be that the plasmid does not remain in the cells after several passages. While working with an inducible Myc cell line was desirable, we decided to move our efforts into an endogenous system.

NCI-H82 cells:

NCI-H82 is a small-cell lung cancer cell line in which c-myc DNA sequences are amplified about 25-fold, with a resulting 24-fold increase in c-myc RNA relative to normal cells.⁴¹ This line grows as aggregates of cells in suspension, and is available for purchase from the American Type Culture Collection (ATCC). In order to establish a baseline for comparison for gene repression, Validated Stealth siRNA was purchased from Invitrogen and optimized for use with the NCI-H82 cell line. Figure 4.11 shows a representative qRT-PCR experiment using the siRNA against Myc in NCI-H82 cells.



Figure 4.11 siRNA against Myc in NCI-H82 cells

IX. qRT-PCR analysis of Myc-inducible gene expression following polyamide treatment

NCI-H82 cells were dosed with compounds 7-10 at 10 μ M concentration for 48 hours, after which time their mRNA was harvested, reverse transcribed, and analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) for the expression levels of Myc and eIF4E along with several other genes from Table 4.3 (ODC, RCC1). Unfortunately, no appreciable effect on eIF4E gene expression (or ODC or RCC1) is seen following 1, 5, or 10 μ M concentration treatment of compounds 7-10 (data not shown). Figure 4.12 shows representative results of three independent biological trials, while error bars indicate the SD of four technical biological replicates of the given trial. While the levels of the actual Myc gene are slightly reduced, no appreciable effect is seen on eIF4E expression in excess of that which may be caused by the slight Myc repression. siRNA treatment concurrent with these biological trials indicates that reduced levels of expression would have likely been detectable if present.



Figure 4.12 qRT-PCR results in NCI-H82 cells

Representative qRT-PCR results after 48 hours treatment of siRNA or 10 μ *M concentrations of polyamides 7-12 in NCI-H82 cells. Error bars represent SD of at least three technical replicates.*

X. Enzyme-linked immunosorbent assay (ELISA)

In collaboration with Summer Undergraduate Research Fellow (SURF) Nickolaus Krall, an ELISA assay was developed that would allow us to measure the protein concentration of the eIF4e and Myc proteins. In the first step an ELISA^{42,43} was developed to measure eIF4E levels in crude cell lysate. Starting from a design suggested previously⁴⁴ the optimal concentrations of antibodies, buffer compositions, and appropriate incubation conditions were determined. To test the hypothesis that the polyamides only affect Myc binding to E-box DNA and hence expression levels of target genes but not Myc protein expression itself an ELISA for the measurement of Myc protein levels in crude cell lysate was also developed. Due to lack of gene response to polyamide treatment, these assays were never used to measure change of protein levels in response to polyamide, but the detailed experimental protocol developed can be found in the Materials and Methods section of this chapter (Section **4.4**, **ix**).

4.3 Closing Remarks

We have described the development of cell-permeable sequence-specific polyamides that bind to the Myc-Max E-box DNA sequence. By antagonizing the discrete binding event between Myc-Max and its cognate DNA sequence we hope to regain control of deregulated gene expression facilitated by Myc overexpression. A library of matched and mismatched polyamides was prepared by solid- and solution-phase synthesis, and their binding affinities and specificities for E-box (5'-CACGTG-3') DNA was determined by quantitative DNase I footprinting titration and thermal melting temperature analysis. The favorable cellular permeability and intracellular localization properties of the FITC conjugates of the compounds was observed by laser-scanning confocal microscopy. However, the ability of the polyamides to inhibit Myc-Max binding in vivo was not demonstrated in the cell lines and conditions described here. It is possible that improvements to the uptake of these or other E-box-targeted polyamides into alternate cell lines would demonstrate different biological effects in those cell lines. We believe that a study of polyamide inhibition of Myc-regulated genes has the potential to uncover a novel mechanism for the regulation of MYC-overexpressed genes, and we anticipate future developments in polyamide design may give this project new direction.

4.4 Materials and Methods

i. Polyamide synthesis

Chemicals not otherwise specified are from Sigma-Aldrich.

Synthesis of polyamides 1-6:

Polyamides **1-6** were synthesized by Dan Harki using pre-loaded Boc- β -Ala-PAM resin (50 mg, 0.81 meq/g, Peptides International) according to published manual solid-phase synthesis protocols.^{22,45} The resin was cleaved with neat 3-dimethylamino-1-propylamine (1 mL) at 37 °C for 16 h. Products were purified by preparatory reverse-phase HPLC on a Beckman Gold system using either a Waters Delta-Pak 25°—100 mm, 15 µm 300 Å C18 PrepPak Cartridge reverse-phase column or a Varian Dynamax 21.4°—250 mm Microsorb 8 µm 300 Å C8 reverse-phase column in 0.1% (w/v) TFA with acetonitrile as the eluent. The appropriate fractions were lyophilized after characterization by analytical HPLC, UV-visible spectroscopy, and MALDI-TOF or ESI mass spectrometry.

1: (MALDI-TOF) $[M+H]^+$ calcd for $C_{51}H_{70}N_{21}O_{10}^+$ 1136.6, observed 1136.6 2: (MALDI-TOF) $[M+H]^+$ calcd for $C_{54}H_{71}N_{22}O_{10}^+$ 1187.6, observed 1187.6 3: (MALDI-TOF) $[M+H]^+$ calcd for $C_{56}H_{74}N_{23}O_{11}^+$ 1244.6, observed 1244.6 4: (MALDI-TOF) $[M+H]^+$ calcd for $C_{55}H_{72}N_{23}O_{11}^+$ 1230.6, observed 1230.7 5: (MALDI-TOF) $[M+H]^+$ calcd for $C_{51}H_{66}N_{21}O_{9}^+$ 1116.5, observed 1116.5 6: (MALDI-TOF) $[M+H]^+$ calcd for $C_{57}H_{72}N_{23}O_{10}^+$ 1238.6, observed 1238.6

Synthesis of polyamides 7-10:

Polyamides **7-10** were synthesized by Katy Muzikar and Dan Harki using Boc-β-Ala-PAM resin according to published manual solid-phase synthesis protocols

as referenced above. The protected FmocHN- γ -turn amine was deprotected with 20% piperidine in DMF and reprotected as the Boc derivative with a solution of Boc₂O (Fluka) and DIEA in DMF. The Boc-protected resin was cleaved with 1 mL of 3,3'-diamino-N-methyldipropylamine (triamine) at 37 °C for 16 h. Products were purified by preparatory reverse-phase HPLC and the appropriate fractions were lyophilized after characterization by analytical HPLC, UV-visible spectroscopy, and MALDI-TOF or ESI mass spectrometry. Conjugates were formed by pre-activating isophthalic acid (3.0 equiv) with PyBOP (2.9 equiv, Novabiochem) in a solution of DIEA (20 equiv) and DMF at 37 °C for 30 min, followed by reaction of the activated solution with the polyamide for 1 h at room temperature. Conjugates were deprotected with neat TFA for 10 min at room temperature before purification by preparatory reverse-phase HPLC. Lyophilization of the appropriate fractions yielded the polyamide conjugates 7-10, which were characterized as described above. Extinction coefficients were calculated according to standard protocols.²⁷ (i.e., estimating 69,500 cm⁻¹ M⁻¹ for standard 8-ring polyamides leads to an approximation of 8,690 cm⁻¹ M⁻¹ per ring for polyamides containing a β -alanine moiety in place of a ring. Calculated extinction coefficient was rounded to the nearest hundred, thus the extinction coefficient for a 7-ring polyamide is calculated to be 60,800 cm⁻¹ M⁻¹).

7: (MALDI-TOF) $[M+H]^+$ calcd for $C_{51}H_{70}N_{21}O_{10}^+$ 1136.6, observed 1136.6 8: (MALDI-TOF) $[M+H]^+$ calcd for $C_{54}H_{71}N_{22}O_{10}^+$ 1187.6, observed 1187.6 9: (MALDI-TOF) $[M+H]^+$ calcd for $C_{56}H_{74}N_{23}O_{11}^+$ 1244.6, observed 1244.6 10: (MALDI-TOF) $[M+H]^+$ calcd for $C_{63}H_{80}N_{22}O_{14}^+$ 1369.6, observed 1369.9

Synthesis of polyamide 11:

Polyamide 11 was synthesized by a different route than the rest of the compounds owing to the fact that imidazole monomer does not stably load onto oxime resin, and synthesis on Boc- β -Ala-Pam resin installs the undesired β -alanine moiety.



Scheme 4.1 Synthesis of polyamide 11.

As shown in **Scheme 4.1**, this necessitated several off-resin synthetic steps. Because a β -alanine moiety was desired in the second position from the resin, the core of **11** was synthesized according to standard protocols as described above on Boc- β -Ala-Pam resin. The core **11a** was saponified off the resin via incubation with 0.5M lithium hydroxide (LiOH) to provide the carboxylic acid **11b**, which was purified by preparatory reverse-phase HPLC. This was subsequently coupled with 4-[(tert-Butoxycarbonyl)amino]-1-methylimidazole-2-carboxylic acid (NH₂-Im-OEt) following established protocols,⁵³ yielding compound **11c**, which was purified by preparatory reverse-phase HPLC. **11c** was saponified in 0.5 M sodium hydroxide (NaOH) to yield the carboxylic acid **11d** which was purified by preparatory reverse-phase HPLC. This was conjugated to 3,3'-diamino-N-methyldipropylamine via PyBOP activation, resulting in compound **11e**, which was purified by preparatory reverse-phase HPLC. **11e** was conjugated to IPA, deprotected, and purified by HPLC as described above.

11b: (MALDI-TOF) $[M+H]^+$ calcd for $C_{43}H_{56}N_{15}O_{11}^+$ 957.4, observed 958.5 **11c**: (MALDI-TOF) $[M+H]^+$ calcd for $C_{50}H_{65}N_{18}O_{12}^+$ 1109.5, observed 1109.6 **11d**: (MALDI-TOF) $[M+H]^+$ calcd for $C_{48}H_{61}N_{18}O_{12}^+$ 1081.4, observed 1037.7 ($[M-CO_2+H]^+$)

11e: (MALDI-TOF) $[M+H]^+$ calcd for $C_{55}H_{79}N_{21}O_{11}^{++}$ 1208.6, observed 1208.8 **11**: (MALDI-TOF) $[M+H]^+$ calcd for $C_{58}H_{74}N_{21}O_{12}^{-+}$ 1256.6, observed 1256.8

Synthesis of polyamide 12:

Cyclic polyamide **12** was synthesized following the methodology established for solution-phase cyclic polyamide synthesis.⁵⁴ Briefly, polyamide half-strands **12a** and **12b** (**Scheme 4.2**)were synthesized and were conjugated together by solution-phase synthetic methods.⁵³ After conjugation of the half-strands, **12c** was saponified to **12d**, which was converted to the pentafluorphenol (Pfp) ester **12e**. Each step of this synthesis up to this point proceeded in similar yields to those previously published and all intermediates were characterized by ¹HNMR and analyzed for purity by analytical scale reverse-phase HPLC. Each product was >90% pure as assessed by these methods. Pfp ester **12e** was deprotected and cyclized to form **12f**, an insoluble compound that was not characterized. The Cbz protecting groups present in **12f** were cleaved and the resulting solution purified by reverse-phase HPLC to yield compound **12** in trace amounts. This synthesis provided the 30 nmols necessary to perform qRT-PCR analysis of its effect in cell culture for one biological replicate. Due to the lack of biological activity of cyclic polyamide **12**, the detailed characterization of these 34 intermediates has been excluded from this thesis.

12: (MALDI-TOF)
$$[M+H]^+$$
 calcd for $C_{58}H_{74}N_{21}O_{12}^+$ 1256.6, observed 1256.8



Scheme 4.2 Synthesis of cyclic polyamide 12.

Synthesis of polyamides 13-16:

Polyamides **13-16** were synthesized by Katy Muzikar and Dan Harki using Boc- β -Ala-PAM resin according to published manual solid-phase synthesis protocols as referenced above. The protected FmocHN- γ -turn amine was deprotected with 20% piperidine in DMF and reprotected as the Boc derivative with a solution of Boc₂O (Fluka) and DIEA in DMF. The Boc-protected resin was cleaved with 1 mL of 3,3'-diamino-N-methyldipropylamine (triamine) at 37 °C for 16 h. Products were purified by preparatory reverse-phase HPLC and the appropriate fractions were lyophilized after characterization by analytical HPLC, UV-visible spectroscopy, and MALDI-TOF or ESI mass spectrometry.

Conjugates were formed by reacting fluorescein-5-isothiocyanate (FITC, Invitrogen) with the polyamide in a solution of DIEA (20 equiv) and DMF for 1 h at room temperature. Conjugates were deprotected with neat TFA for 10 min at room temperature before purification by preparatory reverse-phase HPLC. Lyophilization of the appropriate fractions yielded the polyamide conjugates **13-16**, which were characterized as described above. Extinction coefficients were calculated according to standard protocols.

13: (MALDI-TOF) $[M+H]^+$ calcd for $C_{74}H_{86}N_{23}O_{15}S^+$ 1568.6, observed 1569.7 14: (MALDI-TOF) $[M+H]^+$ calcd for $C_{77}H_{87}N_{24}O_{15}S^+$ 1619.7, observed 1620.8 15: (MALDI-TOF) $[M+H]^+$ calcd for $C_{76}H_{88}N_{23}O_{16}S^+$ 1610.7, observed 1611.6 16: (MALDI-TOF) $[M+H]^+$ calcd for $C_{79}H_{89}N_{24}O_{16}S^+$ 1661.7, observed 1662.6

ii. UV absorption spectrophotometry of of DNA thermal stabilization

Melting temperature analysis was performed on a Varian Cary 100 spectrophotometer equipped with a thermo-controlled cell holder possessing a cell path length of 1 cm. An aqueous solution of 10 mM sodium cacodylate, 10 mM KCl, 10 mM MgCl₂, and 5 mM CaCl₂ at pH 7.0 was used as analysis buffer. This buffer was degassed under vacuum and DNA duplexes and polyamides were added to a final concentration of 2 μ M DNA, 3 μ M polyamide for each experiment. Prior to analysis, samples were heated to 90 °C and cooled to a starting temperature of 23 °C with a heating rate of 5 °C/min for each ramp. Denaturation profiles were recorded at $\lambda = 260$ nm from 23 to 90 °C with a heating rate of 0.5 °C/min. Each sample was subjected to denaturation in technical duplicate, and the reported numbers are the average of at least three experimental replicates. The reported melting temperatures were defined as the maximum of the first derivative of the denaturation profile. Plasmid pDHKM1 was constructed following standard protocols. The following hybridized insert (Integrated DNA Technologies) was ligated into the BamHI/HindIII polycloning site in pUC19 Plasmid (Sigma) using a Rapid DNA Ligation Kit (Roche) according to the manufacturer's protocols:

5'-GATC AGTGG GTACGTGT GGGCTCAGTGG GTAGGTGT GGGCTCAGTGG GTATGTGT GGGCTCAGTGG GTAAGTGT GGGCT-3'

5'-AGCT AGCCC ACACTTAC CCACTGAGCCC ACACATAC CCACTGAGCCC ACACCTAC CCACTGAGCCC ACACGTAC CCACT-3'

The ligated plasmid was then transformed into JM109 subcompetent cells (Promega) by standard methods (30 minute incubation on ice followed by 45 second heat shock (42 °C) followed by 1 hour incubation at 37 °C). Colonies were selected for α -complementation on agar plates containing 50 mg/L ampicillin, 120 mg/L IPTG, and 40 mg/L X-gal after overnight growth at 37 °C. Cells were harvested after 16 h growth at 37 °C in LB medium containing 50 mg/L ampicillin. Plasmid was then purified by midi-prep kit (Promega). The presence of the desired inserts was determined by capillary electrophoresis dideoxy sequencing methods (Laragen).

iv. Preparation of 5'-labeled DNA for DNase I footprinting

Two primer oligonucleotides, 5'-AATTCGAGCTCGGTACCCGGG-3' (forward, corresponding to EcoRI restriction enzyme cut site) and 5'-CTGGCACGACAGGTTTCCCGA-3' (reverse, corresponding to the PvuII restriction enzyme cut site) were constructed for PCR amplification. The forward primer was radiolabeled using [α -³²P]-dATP (MP Biomedicals) and polynucleotide kinase (Roche), followed by purification using ProbeQuant G-50 spin columns (GE Healthcare). The desired PCR product was generated from the plasmid pDHKM1 using the primer pair and Expand High Fidelity PCR Core Kit (Roche) following the manufacturer's protocol. The labeled fragment was purified on a 7% nondenaturing preparatory polyacrylamide gel (5% cross-link) and visualized by autoradiography. The radiolabeled band was excised, crushed, and soaked overnight (14 hours) in 2 M NaCl. The gel pieces were removed by centrifugal filtration and the DNA was precipitated with 2-propanol (1.5 volumes). The pellet was washed with 75% ethanol, lyophilized to dryness, and then resuspended in 1 mL of RNase-free water. Chemical sequencing reactions were performed according to published protocols.^{46,47}

v. DNAse I footprint titrations

All reactions were carried out in a volume of 400 uL according to published procedures.²⁷ Footprinting was performed on 1 pM, 3 pM, 10 pM, 30 pM, 100 pM, 300 pM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM solutions of polyamides **7** and **8**, where polyamide solutions were quantitated at λ =310 nm using ε =52,100 M⁻¹ cm⁻¹ (compound **7**) and ε =60,800 M⁻¹ cm⁻¹ (compound **8**). Polyamides were equilibrated with the radiolabeled DNA for 14 h prior to DNase I cleavage at 23 °C. DNA was precipitated and subjected to gel electrophoresis as previously described. Quantitation by storage phosphor autoradiography and determination of equilibrium association constants were as previously described.²⁷ The human lung carcinoma cell line NCI-H82 (American Type Culture Collection HTB-175) was maintained in RPMI-1640 Medium (ATCC Catalog No. 30-2001) supplemented with L-glutamine (4 mM), penicillin/streptomycin (4 mM) and 10% fetal bovine serum (Irvine Scientific). Cell growth and morphology were monitored by phase-contrast microscopy.

The human lung carcinoma cell line A549 (ATCC CCL-185) was maintained in F-12K Medium, (ATCC Catalog No. 30-2004) supplemented with L-glutamine (4 mM), penicillin/streptomycin (4 mM) and 10% fetal bovine serum as recommended by the ATCC. Cell growth and morphology were monitored by phase-contrast microscopy.

The human breast adenocarcinoma cell line MCF-7 (American Type Culture Collection HTB-22) was maintained in Eagle's Minimum Essential Medium (ATCC Catalog No. 30-2003), supplemented with 0.01 mg/ml bovine insulin; 10% fetal bovine serum, as recommended by the ATCC. Cell growth and morphology were monitored by phase-contrast microscopy.

The human prostate adenocarcinoma cell line LnCaP (ATCC CRL-1740) was maintained in RPMI-1640 Medium (ATCC Catalog No. 30-2001), supplemented with 10% fetal bovine serum, as recommended by the ATCC. Cell growth and morphology were monitored by phase-contrast microscopy.

Cells were trypsinized for 5-10 min at 37°C, centrifuged for 5 min at 2,000 rpm and 5 °C in a Beckman-Coulter Allegra 6R centrifuge, and resuspended in fresh medium to a concentration of 1×10^6 cells per mL. Incubations were performed by adding 150 µL of cells into culture dishes equipped with glass bottoms for direct imaging (MatTek, Ashland, MA). The cells were grown in the glass-bottom culture dishes for 24 h. Then 5 µL of a 60 µM polyamide solution was added and the cells. For verapamil-treated cells, 5 µL 750 mM verapamil(±) was added in addition to polyamide. The plates were then incubated in a 5% CO2 atmosphere at 37 °C for 10-14 h. Imaging was performed with a 40x oil-immersion objective lens on a Zeiss LSM 5 Pascal inverted laser scanning microscope. Polyamide–fluorescein conjugate fluorescence and visible-light images were obtained using standard filter sets for fluorescein.^{46,47} 12-Bit images were analyzed using Zeiss LSM and ImageJ software.

viii. Determination of relative mRNA levels via qRT-PCR

siRNA:

Myc Validated Stealth DuoPak SKU# 12936-50, was purchased from Invitrogen. This included Duplex #1 and Duplex #2, of proprietary sequence and concentration. Stealth RNAiTM siRNA Negative Control Kit (Invitrogen, Cat. No. 12935-100) was purchased and the "Medium GC Duplex #1" was used. siRNA was transfected using LipofectamineRNAiMAX (Invitrogen, Cat. No. 13778-075) according to the manufacturer's instructions. The amounts of these reagents used in most assays was 1 μ L Lipofectamine RNAiMax and 1.4 μ L of supplied siRNA.

Note: at the time of the printing of this thesis, Invitrogen has discontinued the siRNA product described. Brief attempts to use the replacement product yielded poor results, but a thorough optimization of conditions was not performed.

RNA isolation:

Cells were plated in 24-well dishes at a density of 40 x 10^4 cells/mL in 0.5 mL of culture medium without antibiotic supplements (which are lethal in combination with siRNA treatment) and allowed to attach for 16-20 hours. Polyamides or siRNA were added (according to instructions above) and the cells were incubated for 48 hours. The medium was removed, cells were washed with ice-cold PBS and immediately lysed with RLT buffer from an RNeasy kit (Qiagen). Further RNA isolation was carried out with the RNeasy kit as described in the manufacturer's manual. The isolated total RNA was quantified. The yields were 12-15 µg per well.

Reverse transcription:

A 2.5 μg sample of total RNA was used to reverse-transcribe cDNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Random hexamers were used as primers. The total volume for each RT reaction was 20 μl.

Real-time quantitative RT-PCR:

Analysis was performed using the Myc and eIF4E gene primers described below, purchased from Integrated DNA Technologies. Quantitative real-time RTPCR was performed using Applied Biosystems SYBR Green RT-PCR master mix according to the manufacturer's instructions. Temperature cycling and detection of the SYBR Green emission were performed with an ABI 7300 real-time instrument using Applied Biosystems Sequence Detection System version 1.2. Statistical analysis was performed on three independent experiments. To amplify the 88-bp fragment from the 3'-translated region of Myc:

Forward primer: 5'-CAGCGACTCTGAGGAGGAAC-3' Reverse primer: 5'-CTCTGACCTTTTGCCAGGAG -3'

To amplify the 118-bp fragment from the 3'-translated region of eIF4E: Forward primer: 5'-TTTTGGGCTCTGTACAACCA-3' Reverse primer: 5'-CTCCCCGTTTGTTTTTCTCA -3'

RNA was standardized by quantification of the β -glucuronidase gene as an endogenous control,⁴⁸ Using the following primers:

Forward primer: 5'-CTCATT TGGAATTTTGCCGATT -3' Reverse primer: 5'- CCGAGTGAAGATCCCCTTTTTA -3'

ix. Determination of relative protein levels via Enzyme-Linked Immunosorbent Assay (ELISA)

In collaboration with Nickolaus Krall, Summer Undergraduate Research Fellow (SURF) student, summer 2008:

Cells were incubated at 37 °C, 5% CO₂. Cells were induced by the addition of 2 μ l of 1mg/ml doxycyclin (in H₂O) to each well at the indicated time point for each experiment. At the indicated harvest time point for each experiment the cells were detached from the plates by the addition of trypsin. Cells were counted via hemocytometer and subsequently pelleted by centrifugation at 130 RCF for 10 minutes. The supernatant was removed and the pellet was rinsed twice with PBS and repelleted before the addition of RIPA buffer supplemented with protease inhibitor cocktail (PIC) and PMSF. Pellets were agitated by

pipetting and left on ice for 20-30 minutes before cell debris was pelleted by centrifugation at 1000 RCF for 5 minutes. The supernatant was transferred to a fresh tube and frozen at -80°C in an isopropanol slow-freeze bath.

Normalization

Frozen cell lysate was thawed on ice and adjusted to a constant protein or DNA concentration prior to determining eIF4E concentrations. Total protein concentration was determined with a Bradford assay.¹⁹ One ml of Bradford reagent (BioRAD) was added to 10 μ L of crude lysate and 10 μ l of water contained in a 1 ml plastic cuvette and mixed vigorously. A ready-to-go BSA standard set (BioRAD) was used as a reference. One ml of Bradford reagent was added to 10 μ l of BSA standard solution and 10 μ l of RIPA buffer contained in a 1 ml plastic cuvette. The absorbance was measured at 595 nm (A₅₉₅) with a UV/Vis Spectrometer (Agilent) and a linear standard curve constructed from the BSA reference dilutions. Using this standard curve total protein concentrations in samples were calculated from the A₅₉₅. All measurements were carried out in duplicate and the average value used to adjust all samples to the same total protein concentration by dilution with appropriate amounts of RIPA buffer. For normalization to constant total DNA, DNA was measured with the built in function of a Nano Drop UV/Vis spectrophotometer and samples adjusted to the same DNA content with RIPA buffer.

eIF4E ELISA:

96-well plates (Greiner BioOne Microlon 600) were coated overnight with donkey anti-mouse IgG (Jackson, 50 μ l of 5 μ g/ml antibody in PBS with 0.05% NaN₃ per well, 2-8°C). Plates were subsequently washed (3x 200 μ l of PBS with 0.05% Tween20 per well), then blocked (200 μ l of 5% dry milk powder in PBS per well) for 1 h at room temperature. Plates were washed again, then incubated with mouse anti-eIF4E IgG (BD

Signal Transduction Laboratories, 50 µl of a 1:450 dilution in antibody buffer [i.e., PBS, 0.25% BSA and 0.05% Tween20] per well) for 1 h at room temperature. After another wash they were incubated with samples (50 µl per well) and standards (Globozymes recombinant eIF4E; 50µl of 200, 100, 50, 25, 15, 10, 5, 1 and 0.1 ng/ml in antibody buffer per well) for 1 h at room temperature. Plates were then washed again and incubated with rabbit anti eIF4E (Cell Signaling, 50µl of a 1:1000 dilution in antibody buffer) for 1 h at room temperature, followed by another wash step. Plates were subsequently incubated with goat anti-rabbit IgG HRP conjugate (Jackson, 50µl of a 1:80,000 dilution in antibody buffer per well for 1 h at room temperature), washed, and finally developed with TMB substrate (Sigma, 50 µl per well) After 30 minutes the reaction was stopped with an equal volume of 1 M HCl. The output signal was read as the absorbance at 450 nm (A450) with a Perkin Elmer multiwell plate reader. The absorbance of eIF4E standards was fit to a 4-parameter logistic curve^{17,18} using Kaleida Graph and used to calculate unknown eIF4E concentrations in samples from A450 values.

Myc ELISA:

The Myc ELISA closely followed the eIF4E ELISA protocol above but differed in choice and concentration of antibodies. Coating with donkey anti-IgG was carried out as described above. The secondary coating antibody was a monoclonal mouse anti-c-Myc IgG (Sigma, clone 9E10, 50 µl of a 1:300 dilution in antibody buffer per well). Incubation with samples was as above. The primary detection antibody was a polyclonal antibody rabbit anti c-Myc IgG raised against the 262 N-terminal amino acids (Santa Cruz, 50 µl of a 1:250 dilution in antibody buffer per well). The secondary detection antibody was a HRP conjugated goat anti-rabbit IgG (Jackson, 50 µl of a 1:20,000 dilution in antibody buffer).

4.5 References

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