

PARALLEL, QUANTITATIVE ANALYSIS OF TRANSCRIPTION FACTORS

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ABSTRACT

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Cellular and tissue homeostasis is a result of complex processes that respond to the cellular microenvironment. To understand these processes and the signaling that initiates them, it is important to measure the levels of many cellular components. Continuing technology development, especially in high-throughput and parallel techniques, will provide new assays for such measurements. If designed well, these techniques can also be applied in the clinical setting, with the potential to improve human health through improved disease detection and diagnosis. Currently, the majority of these techniques measure the results of cellular signaling, e.g., changes in mRNA levels. It would provide complementary information to measure the levels and activities of transcription factors, the upstream mediators of cell signaling.

Transcription factors (TFs) are proteins that alter the expression of target genes in response to stimuli. They bind specific sites on chromosomal DNA, resulting in activation or repression of nearby genes. Many TFs respond to a variety of signals, and multiple TF levels can be altered by a single stimulus. Moreover, TF levels are dynamic, in general, changing with time in concert with changes in the cell phenotype and the microenvironment. As there are ~2000 TFs in humans and their levels change dynamically, measuring TF levels and activities in parallel is a challenging task.

To address this challenge, techniques have been developed to measure TF levels in parallel. While these assays have provided valuable information on cellular

processes, each has limitations. The aim of this dissertation was to develop a parallel, quantitative TF measurement method that leverages established DNA analytical technologies and would complement existing analytical approaches. This work focused on technology development and the application of the assay to poorly-characterized biological processes.

The initial focus was on assay development. The assay was based on magnetic bead separation of TF-bound DNA probes. We measured purified TFs, p50 (NF- κ B family) and c-Jun (AP-1 family), in parallel with ~10-fold improved sensitivity over existing approaches. TF levels were successfully measured in nuclear extracts from breast cancer cells. The results agreed with the previously published data, showing that the assay achieved successful parallel and quantitative detection of TFs.

To further demonstrate the applicability of the approach, temporal measurements of TF levels were performed in different cell types and in response to different stimuli. TNF- α treated HepG2 cells and breast cancer cells were selected as model systems. Levels of TFs, NF- κ B, Stat3, CREB, GR and TBP, were dynamically measured in these nuclear extracts. Furthermore, the same set of TF levels were measured in untreated and palmitic acid treated HepG2 cells. The observed changes in these TF levels have furthered our understanding of the molecular mechanisms associated with cellular exposure to saturated fatty acids such as palmitic acid. The results suggested that our developed assay can be generalized to multiple cell types and is useful for characterizing parallel, dynamic TF responses to multiple stimuli.

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KEY TO ABBREVIATIONS

PCR - Polymerase Chain Reaction

SELEX - Systematic Evolution of Ligands by EXponential enrichment

ELISA - enzyme-linked immunosorbent assay

ATP - Adenosine triphosphate

RT - reverse transcription

TF – Transcription factor

HTH – helix turn helix

HLH – helix loop helix

EMSA - electrophoretic mobility shift assay

FBS - fetal bovine serum

TSA - trichostatin A

PMA - phorbol 12-myristate 13-acetate

SA-PE - streptavidin-phycoerythrin

OATFA - oligonucleotide array-based transcription factor assay

SPA-OATFA - single-primer amplification technique

ChIP - chromatin immunoprecipitation

TNF- α – Tumor necrosis factor alpha

NF- κ B - nuclear factor kappa-light-chain-enhancer of activated B cells

Stat3 - Signal transducer and activator of transcription 3

CREB - cAMP response element-binding protein

GR - glucocorticoid receptor

TBP - TATA binding protein

PA – palmitic acid

NAFLD - non-alcoholic fatty liver disease

FFA - free fatty acids

CHAPTER 1 INTRODUCTION

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1.1 SIGNIFICANCE

Proper cell function is a result of many different biomolecular interactions within and between cells. Central among these biomolecules are nucleic acids, lipids and proteins, resulting in extraordinary molecular and functional diversity (e.g., roughly 20,000 genes leading to the expression of ~ 1,000,000 unique protein products). Characterization of the levels of these biomolecules and their interactions with each other has led to many different areas of research including the “omics” disciplines of genomics and proteomics. Functional proteomics provides information about proteins’ structure and function; expression proteomics gives information on the levels of these proteins in a given sample.

Questions regarding gene expression responses to stimuli have led to research on transcription factors (TFs), proteins that regulate gene expression. Profiling of transcription factors in parallel in response to intracellular and extracellular stimuli provides information on the activation of signal transduction pathways. Currently, there is considerable work being undertaken in all of these areas, leading to improved understanding of the molecular mechanisms resulting in biological phenotypes. Continued advancement in all of these fields depends on continued evolution of the tools for analysis of biological systems.

1.2 TRANSCRIPTION FACTORS: STRUCTURE AND SIGNALING

Transcription factors (TFs) are cell regulatory proteins that facilitate proper cell function by controlling gene expression in response to intracellular and extracellular stimuli (Orphanides et al., 2002). TFs have two domains; i) the DNA binding domain that binds specific sites of chromosomal DNA, thereby directing which gene(s) will be controlled and ii) the trans-activating domain that interacts with other proteins and co-factors initiating the required changes in gene expression. TFs regulate proper cell function across the range of cell process from development to differentiation.

1.2.1 Structure

Both the DNA sequence and TF structure plays important role in specific interaction of DNA and TF (Latchman, 1997). Binding of the TF to the DNA changes the structure of the DNA and altering the accessibility of the region to RNA polymerase, leading to gene activation or repression. Structural studies of TFs have shown that there are a number of common, TF structural motifs, including helix-turn helix, helix-loop-helix, zinc finger, and leucine zipper (Figure 1). Common among all of these motifs is the placement of an α -helix of the TF into the major groove of the DNA structure, with additional affinity provided by hydrogen bonding and Van der Waals interactions between the amino-acids and the nucleotide bases.

Helix-turn-helix (HTH) (Figure 1.1) was the first transcription factor motif discovered. As the name indicates, this structure has an α -helix, a turn that stabilizes the protein structure, and a second α -helix. HTH motifs are not separate, stable domains; rather they are always part of larger DNA binding proteins. This motif usually binds as dimers and occurs in many DNA binding proteins such as Lac repressor (Kaptein et al., 1985), 434 repressor (Anderson et al., 1987), or Trp repressor (Schevitz et al., 1985). Although the primary DNA-protein interactions occur between residues in the protein's "recognition helix" and bases in the DNA major groove, it has been shown that other parts of the protein also have significant roles in recognition of the target (Steitz et al., 1982).

Helix-loop-helix motifs (HLH) of transcription factors have two α -helices that mediate dimerization and a basic region that interact with DNA (Figure 1.1) (Murre et al., 1994). These two α -helices are connected by a loop and one helix is bigger than the other helix. Bigger helix is typically the one that has basic region to bind consensus sequence called E-box. Sequence of E-box is characterized as CANNTG (Chaudhary et al., 1999). HLH is primarily found in developmental genes such as MyoD (Ma et al., 1994) and HIF-1 (Wang et al., 1995).

Zinc finger motifs contain an antiparallel β -sheet and α -helix (Figure 1.1) (Pavletich et al., 1991). This family of proteins usually contains the repeat of sequence pattern Cys-

X_{20r4} -Cys- X_{12} -His- X_{3-5} -His. Zinc ions are chelated by the two cysteines in the β -sheet region and two histidines in the α -helix region of protein (Pavletich et al., 1991).

Leucine zipper TFs contain heptad repeats of leucines in a 30-40 amino-acid sequence (Figure 1.1). This type of protein usually has two domains, the leucine zipper domain and a basic region. The leucine zipper domains form two α -helices and stabilize the protein with hydrophobic interactions, while the basic region contains amino acids that bind to DNA (Pabo et al., 1992).

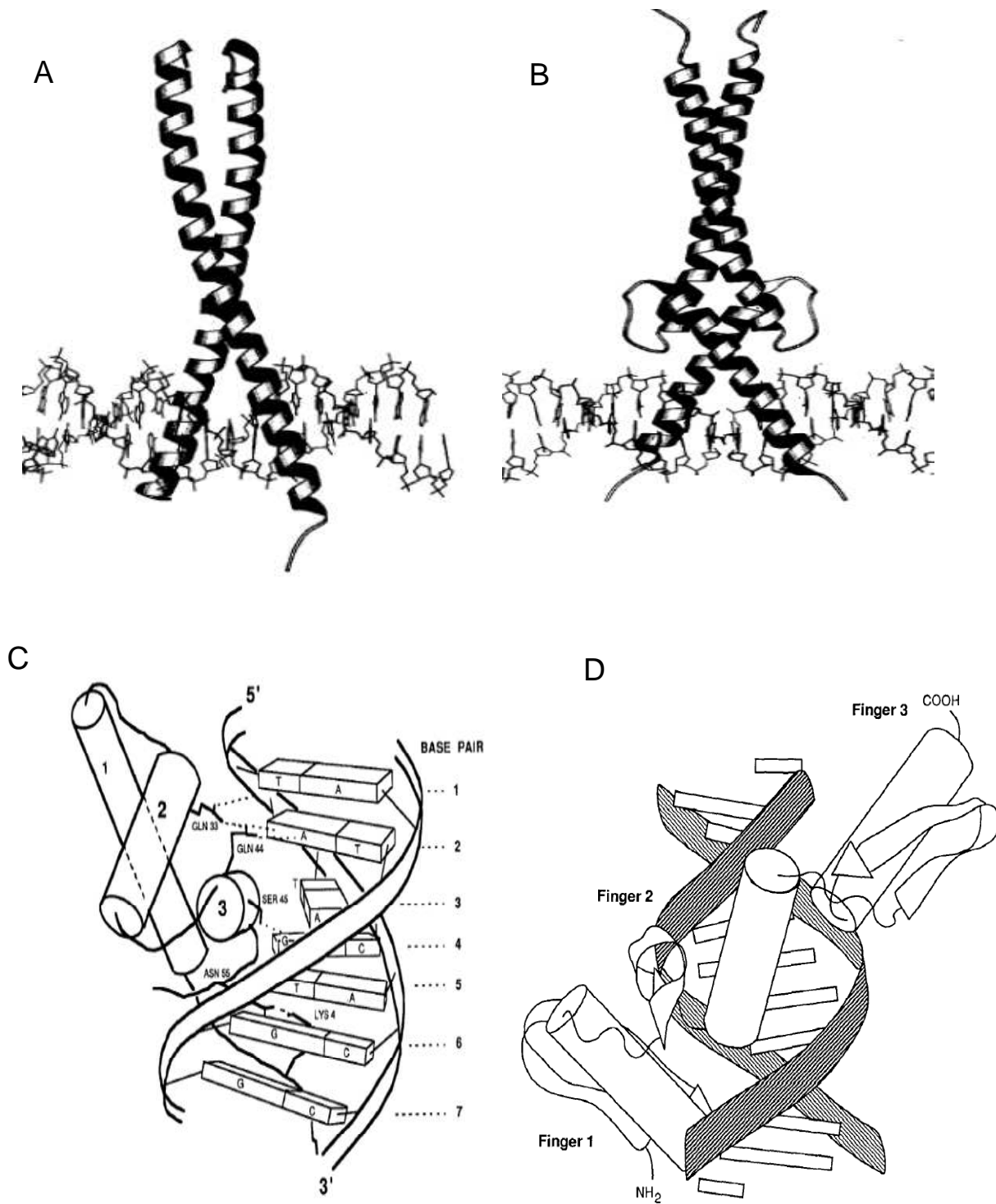


Figure 1.1: Structures of transcription factor motifs (a) leucine zipper (Ellenberg, 1994) (b) helix-loop-helix (Ellenberg, 1994) (c) Helix-turn-helix (Steitz et al., 1982) and (d) zinc finger motifs (Pavletich et al., 1991) respectively. α -helices of proteins are oriented to lie into major groove of DNA where their atoms form H-bond and Van der Waals interactions

1.2.2 Signaling

The activities of TFs are controlled by extracellular and intracellular signaling pathways resulting in the activation of transcription factors, either by transcription and translation of new TFs or by post-translational modification of Latent TFs, and their internalization into cellular nucleus. Latent TFs in the cytoplasm can be activated, for example, by tyrosine (e.g Stat) or serine (e.g c-Jun) phosphorylation (Darnell, 2002). The phosphorylated proteins are then bound by importin, which translocates the TFs to the nucleus (e.g NF- κ B). Nuclear TFs can be activated by phosphorylation following translocation of kinases from the cytoplasm to nucleus (e.g ETS) (Darnell, 2002). Additionally, most TFs require binding to other TFs or cofactors for gene expression to occur. For example, in Wnt signaling pathway, TCF/LEF transcription factors need b-catenin to activate the transcription. TCF/LEF transcription factors lack the transactivating domains, thus, require another factor to start the recruitment of RNA polymerase (MacDonald et al., 2009).

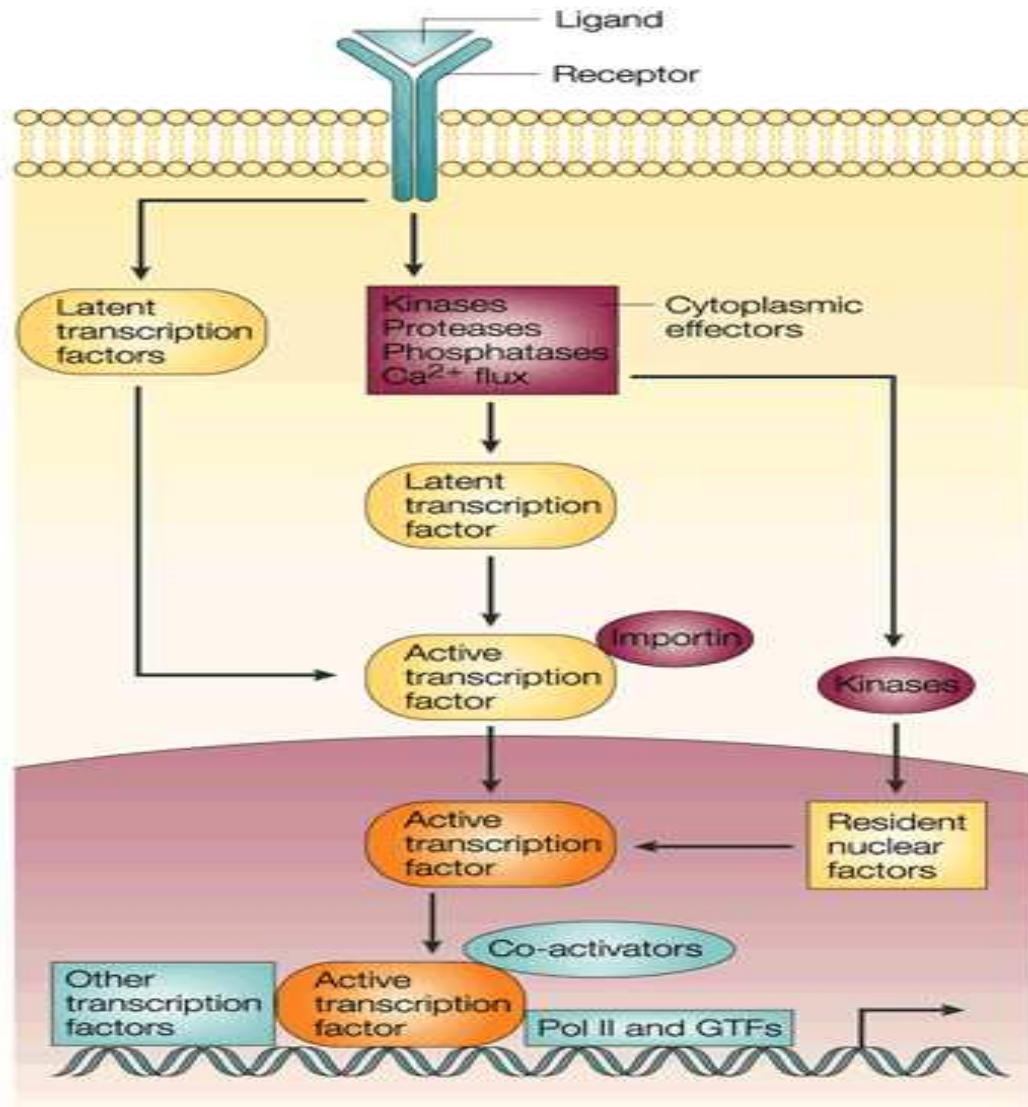


Figure 1.2: Generalized signaling pathways of TFs. Signaling pathways of TFs and their translocation to the nucleus. Latent TFs in cytoplasm can be activated by kinases or importin binding and internalize to nucleus (Darnell, 2002).

TF signaling networks are extraordinarily complex. Any one TF can respond to a variety of stimuli; additionally, multiple TFs are typically activated by any one stimulus. Thus, the profile of active TFs is complex and constantly changing (Babu, 2010; Emerson 2002). Aberrant TF activity results in improper cell function and can lead to disease

(e.g., cancer) (Barnes, 2006; Latchman, 1996; McCulley et al., 2012; Mees et al., 2009; Nebert, 2002). Therefore, monitoring of TF function is valuable for understanding biological processes and can support medical diagnoses and the development of novel therapeutics.

1.3 TECHNOLOGIES FOR TRANSCRIPTION FACTOR PROFILING

TF profiling studies can be divided into two categories; a) studies to identify target consensus sequences and target genes of a single TF and b) studies to profile TF levels in response to stimuli. Research in the first area is mainly based on protein microarrays, Chromatin immunoprecipitation and high-throughput SELEX (Berger et al., 2009; Johnson et al., 2007; Jolma et al., 2010; Park, 2009). With these tools, many TF factor binding sites have been determined, and large TF regulatory networks have been constructed (ref). While this work continues, more technology development is currently focused on tools to measure TF levels in parallel from biological samples.

The unique physical and chemical properties of TFs, e.g., pI, size, hydrophilicity, make identifying a single method for measuring them in parallel challenging. Individual TF levels can be measured with many different techniques such as Western blotting, chromatography, immunoassays, and electrophoresis (Moxley, 2005; Shen et al., 2002). However, extending these single TF methods to parallel analyses of TF levels is

often a considerable challenge. Likewise, these techniques depend on the detection of the TF proteins directly, which is a more complex challenge than detecting other cellular molecules, in particular nucleic acids.

1.3.1 Electrophoretic mobility shift assay (EMSA)

An alternative to direct readout of TF detection is leveraging the DNA binding character of TFs to convert detection of protein molecules (TFs) into detection of their cognate DNAs. This is done with one common method for detecting TFs, electrophoretic mobility shift assay (EMSA) (Ruscher et al, 2000). This technique is based on mobility differences of free DNA and TF-bound DNA. Typically, labeled DNA probes including the consensus sequence for a TF are mixed with TFs from a cellular extract. Samples are then separated in polyacrylamide gels, and the presence of TFs is detected based on the appearance of a shifted DNA band in the gel. While simple and easy to implement, EMSAs are limited by the loading capacity of gels, which in turn limits the number of TFs that can be detected. Moreover, the number of different TFs that can be detected is limited by the number of unique labels that can be attached to the DNA probes.

1.3.2 Reporter based assays

Another approach to reading out TF levels is through assays based upon the activities in initiating gene expression. Multiplex techniques for TF activities are often based on

the readout of reporter genes (Li et al., 2006). In these methods, an easily assayable reporter construct is placed behind the recognition sequence for the TF of interest, and the expression of the reporter is monitored. Thus, activation of the TF should lead to transcription of the natural targets as well as the construct. This approach was applied recently to the simultaneous measurement of 43 TF activities (Romanov et al., 2008). In a particularly creative approach, TF activation resulted in the expression of a set of transcripts, all of which contained the same, unique restriction enzyme site. After RNA isolation and RT-PCR, the resulting cDNAs were cleaved with the restriction enzyme resulting in a set of DNAs, each with a unique length specific for a given TF. The products were then assayed by capillary electrophoresis to determine the relative quantities of each length DNA product and, correspondingly, the relative activity of the corresponding TF. Using this approach, TF activities of HepG2 cells before and after induction with biologically active compounds were compared (Romanov et al., 2008). The value of the parallel TF measurements was further demonstrated by the measurement of unique TF activity profiles from cancer cell lines related to HepG2s, thus suggesting a potential value for TF profiling in the classification of tumors.

A commercial approach also relies on reporter gene constructs for multiplex TF measurements (Panomics/Affymetrix, Fremont, CA)(Figure 1.3). This approach also begins by transfection of cells with reporter plasmids. Reporter RNA is then isolated, reverse transcribed, and assayed by DNA microarray. This technique was applied to analyze trichostatin A (TSA) induction on cardiac myocytes *in vivo* (Davis et al., 2005). They monitored 24 TFs simultaneously and determined the interactions among them.

The results showed that the activity of the early growth response gene, EGR-1, increased with TSA induction. While reporter gene-based techniques provide valuable information for *in vitro* systems, they require manipulation of the cells prior to analysis, precluding their use for analysis of samples, for instance, from a clinical setting. Also, transfection of the reporter plasmids may alter the natural cellular signaling and function, resulting in an altered response to the stimulus being studied.

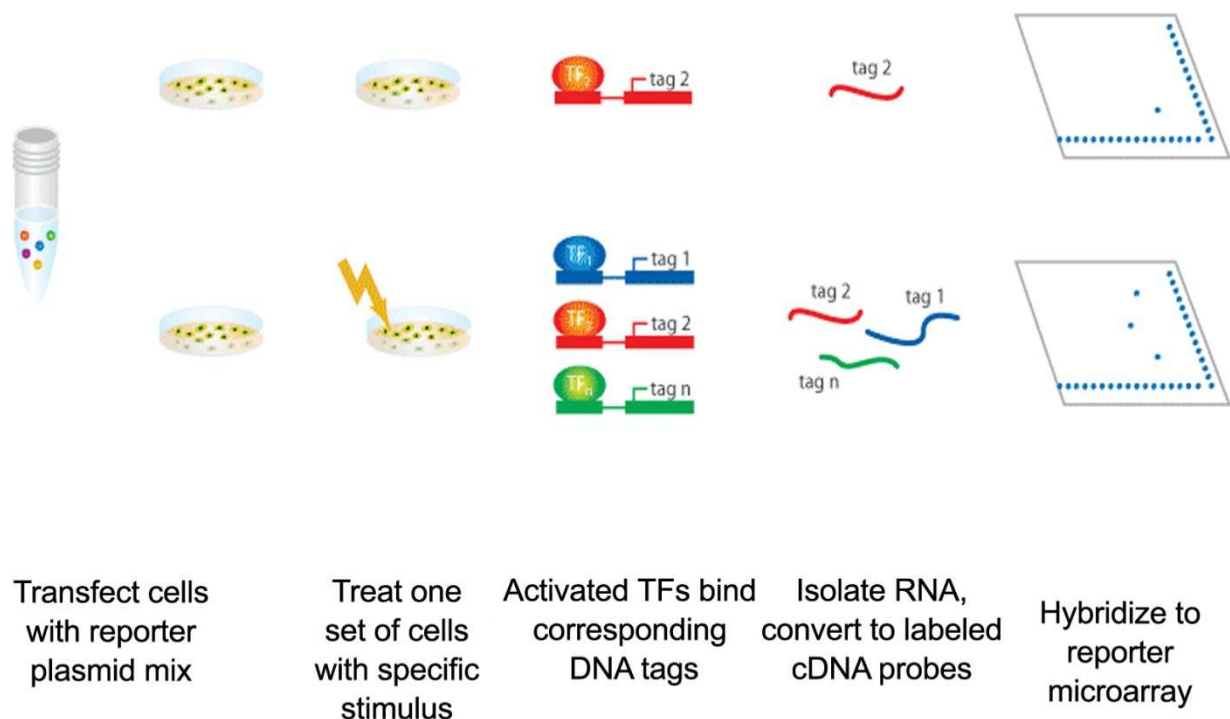


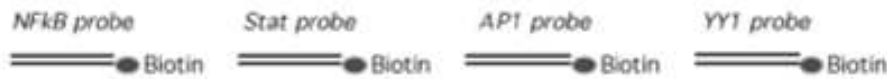
Figure 1.3: Reporter gene assay to profile TFs in parallel. After cells are transfected with reporter vector composed of TF binding sequence and reporter which has specific tag adjacent to TF consensus sequence. When TF binds to sequence, RNA tag is synthesized and its cDNA is analyzed with microarray.

1.3.3 Luminex assays

Similar commercial approaches that can be applied to isolated protein samples also leverage the Luminex cytometric platform. In these techniques (Panomics/Affymetrix and Marligen), cellular extracts are incubated with double stranded, biotinylated DNA probes containing TF recognition sites. After removal of unbound probes, bound probes are hybridized with complementary sequences attached to fluorescent beads.

Fluorescently-labeled streptavidin is then added to the mixture. In this way, the signals from the streptavidin fluorophore and the bead indicate the presence of a probe and its identity, respectively. These assays require only ~5 µg of cell extract for accurate TF measurements (Panomics/Affymetrix and Marligen). This technique was used to assess TF activity in untreated HeLa cells and those exposed to phorbol 12-myristate 13-acetate (PMA) and tumor necrosis factor α (TNF- α). Based on similarities in their TF activation profiles of NF- κ B, Ap1, Sp1, Ap2, CREB, PPAR, Stat5, E2F, NF-E2, p53 and Stat6, it was proposed that their signaling pathways share some common mechanisms (Yaoi et al., 2006). These methods have been used to detect up to 50 TFs in parallel but, again, the number of TFs that can be measured is limited by the number of unique bead spectra.

1. Mixture of pre-labeled TF probes



2. Incubate with nuclear extract



Spin column

Free probes

3. TF/probe complex



4. Recovery of TF-bound probes



5. Hybridization and detection by Luminex 100

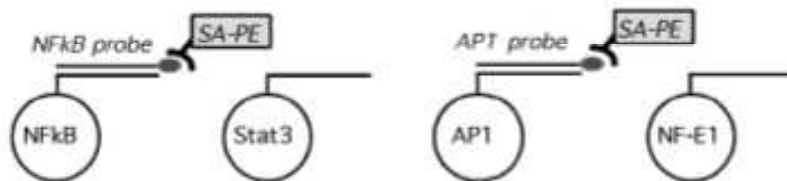


Figure 1.4: Microsphere based TF profiling assay. (1) nuclear extract is mixed with the pool of biotin labeled consensus DNA probes, (2) bound TF:DNA complexes are separated from free DNA, (3) TF:DNA complexes are mixed with the complementary DNA attached microspheres, (4) hybridized DNA are recovered, (5) following staining with streptavidin-phycoerythrin (SA-PE), detected with Luminex.

1.3.4 Array based assays

An array-based method to profile TF activities was developed by Panomics (Panomics/Affymetrix). In this method, after incubation of biotin-labeled TF probes with nuclear extracts, TF-bound probes are separated from free probes with spin columns. TF bound DNA complexes are then denatured, and free probes are hybridized to an array of sequences complementary to the TF recognition sequences. With the array, quantitative and qualitative data can be obtained. This method can profile up to 345 TF in parallel. This technique has been applied to the analysis of TF activities in breast cancer lines (Jiang et al., 2006), in analyzing the effects of the extra-cellular matrix on cancer cell phenotypes (Dozmorov et al, 2008), and in colon tumor cells (Li et al., 2007).

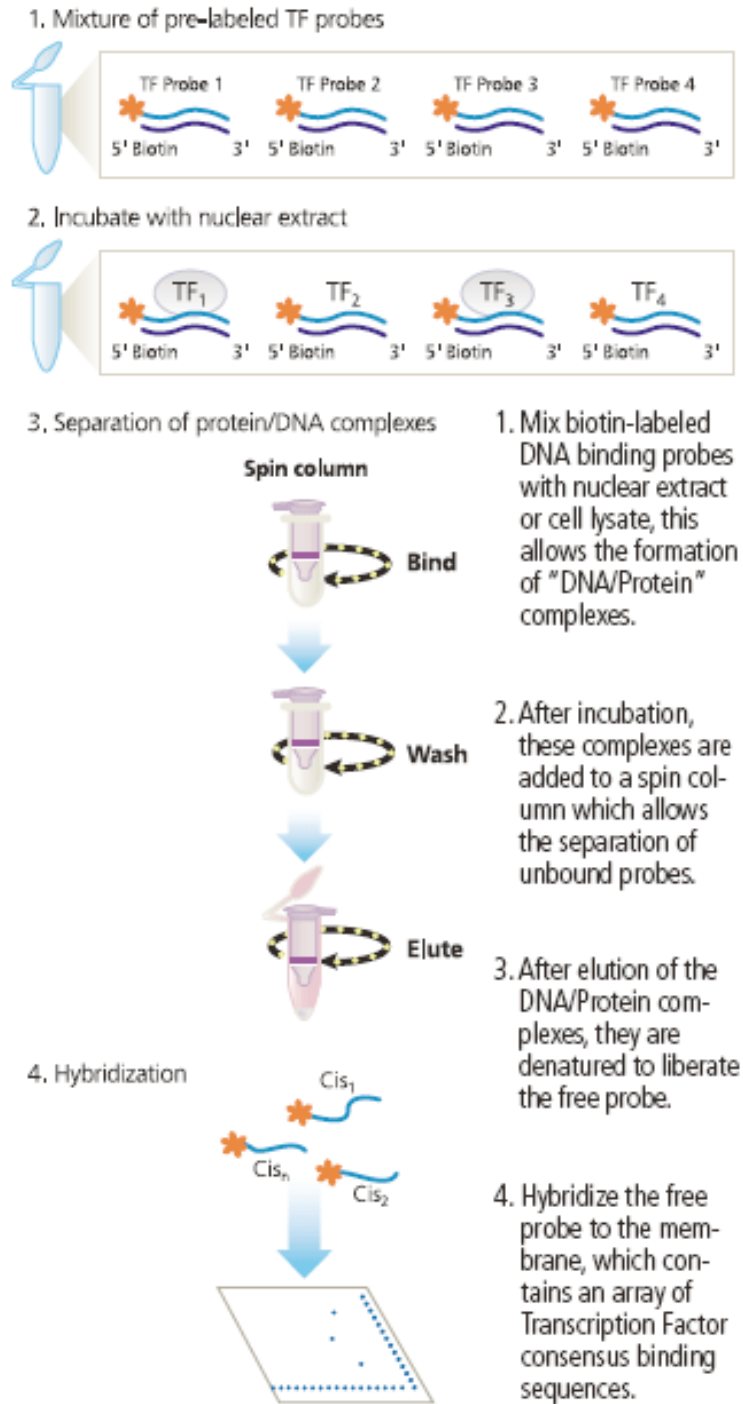


Figure 1.5: Array-based TF profiling assay. After incubation of biotin labeled consensus DNA sequences with nuclear extracts, DNA:TF complexes are separated from free DNA probe with spin columns. Recovered DNA probes are analyzed with microarray.

Researchers have also combined techniques to develop parallel analyses. One example is the oligonucleotide array-based transcription factor assay (OATFA) (Shoa et al., 2005). By this technique, multiple TFs were successfully analyzed in parallel both from mixtures of purified TFs and from cell extracts. They further improved the assay by combining OATFA method with single-primer amplification technique (SPA-OATFA). Using SPA-OATFA, 240 human TFs were simultaneously profiled in response to TPA and TNF- α (Qiao et al., 2008). While successful, this method is limited by the labor-intensive work of purification of DNA by gel electrophoresis. Likewise, this process is likely to lose considerable DNA, limiting the sensitivity of the assay.

All these current techniques to measure TFs in parallel have provided valuable information to understand cellular processes, however there is still need for a complementary assay to overcome the limitations of these assays. This ideal assay should at least met five characteristics; no manipulation of cells before the assay, lower detection limit, small sample size (10^6 cells or lower), parallel measurements (up to thousands of TFs), and quantitative.

1.4 APPROACHES AND SPECIFIC AIMS

We seek to develop a complementary technique to measure transcription factor levels in parallel leveraging high-throughput DNA readouts. We have approached the design

and development of this approach based on our goals for the ideal approach to parallel TF level analyses. With the ease of magnetic bead separation, increased sensitivity and scalable nature, our assay will be a complementary tool for TF analysis and will support studies to enhance our understanding of biological processes.

The specific Aims of the project are to:

1. Develop a quantitative, parallel transcription factor analysis technique

We have developed an assay based on magnetic bead separation of TF-bound DNA probes. To validate the assay, two transcription factors, NF- κ B and Ap1, were tested alone and also in nuclear extracts. Parallel measurements were also conducted in nuclear extract samples. Better sensitivity over current techniques was achieved.

2. Apply Transcription Factor Profiling to the analysis of a model system, palmitic acid treated HepG2 cells

To demonstrate that our technique can achieve parallel TF level measurements, transcription factor levels of palmitic acid-treated HepG2 cells were analyzed dynamically. First, TF levels in TNF- α treated HepG2 cells were measured to prove our assay can be expanded and used for different cell types. Further, TF levels in palmitic acid treated HepG2 cells were tested with time, yielding information about TF regulation and the effect of fatty acids on hepatocytic cells.

REFERENCES

REFERENCES

- Anderson JE, Ptashne M, Harrison SC (1987) Structure of the repressor-operator complex of bacteriophage 434. *Nature* 326:846-52
- Babu MM (2010) Structure, evolution and dynamics of transcriptional regulatory networks. *Biochemical Society Transactions* 38: 1155-1178
- Barnes PJ (2006) Transcription factors in airway diseases. *Laboratory Investigation* 86 (9): 867-872
- Berger MF, Bulyk ML (2009) Universal protein-binding microarrays for the comprehensive characterization of the DNA-binding specificities of transcription factors. *Nature Protocols* 4 (3): 393-411
- Chaudhary J, Skinner MK (1999). Basic helix-loop-helix proteins can act at the E-box within the serum response element of the c-fos promoter to influence hormone-induced promoter activation in Sertoli cells. *Mol. Endocrinol.* 13 (5): 774–86
- Darnell JE (2002) Transcription factors as target for cancer therapy. *Nature Reviews* 2:740-49
- Davis FJ, Pillai JB, Gupta M, Gupta MP (2005) Concurrent opposite effects of trichostatin A, an inhibitor of histone deacetylases, on expression of alpha-MHC and cardiac tubulins: implication for gain in cardiac muscle contractility. *Am. J. Physiol. Heart Circ. Physiol.* 288(3):H1477–1490
- Dozmorov MG, Kyker KD, Hauser PJ (2008) From microarray to biology: an integrated experimental, statistical and in silico analysis of how the extracellular matrix modulates the phenotype of cancer cells. *BMC Bioinformatics* 9(Suppl 9):S4
- Ellenberger T (1994) Getting a grip on DNA recognition: structures of the basic region leucine zipper and the basic region helix-loop-helix DNA binding domains. *Current opinions in structural biology*, 4:12-21
- Emerson BM (2002) Specificity of gene regulation. *Cell* 109 (3): 267-270
- Jiang X, Roth L, Lai C, Li X (2008) Profiling activities of transcription factors in breast cancer cell lines. *Assay Drug Dev Technol.* 4(3):293–305
- Johnson DS, Mortazavi A, Myers RM, Wold B (2007) Genome-wide mapping of in vivo protein-DNA interactions. *Science* 316 (5830): 1497-1502

- Jolma A, Kivioja T, Cheng L, Wei GH, Enge M, Taipale M, Vaquerizas JM, Yan J, Sillanpaa MJ (2010) Multiplexed massively parallel SELEX for characterization of human transcription factor binding specificities. *Genome Research* 20 (6): 861-873
- Kaptein R, Zuiderweg ERP, Sheek RM, Boelens R, van Gunsteren WF (1985) A protein structure from nuclear magnetic resonance data. *J. Mol. Biol.* 182:179-82
- Latchman DS (1996) Mechanisms of disease - Transcription-factor mutations and disease. *New England Journal of Medicine* 334 (1): 28-33
- Latchman DS (1997) Transcription factors: an overview. *Int. J. Biochem. Cell Biol.* 29: 1305-12
- Li Q, Dashwood WM, Zhong X, Nakagama H, Dashwood RH (2007) Bcl-2 overexpression in PhIP induced colon tumors: cloning of the rat Bcl-2 promoter and characterization of a pathway involving beta-catenin, c-Myc and E2F1. *Oncogene* 26(42):6194-6202
- Li XQ, Jiang X, Yaoi T (2006) High throughput assays for analyzing transcription factors. *Assay and Drug Development Technologies* 4 (3): 333-341
- Ma PC, Rould MA, Weintraub H, Pabo CO (1994) Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. *Cell* 77 (3): 451-9
- McCulley DJ, Black BL (2012) Transcription factor pathways and congenital heart disease. *Heart Development* 100: 253-277
- MacDonald BT, Tamai K, He X (2009) Wnt/ β -Catenin Signaling: Components, Mechanisms, and Diseases. *Developmental Cell* 17 (1): 9-26
- Mees C, Nemunaitis J, Senzer N (2009) Transcription factors: their potential as targets for an individualized therapeutic approach to cancer. *Cancer Gene Therapy* 16 (2): 103-112
- Moxley RA, Jarrett HW (2005) Oligonucleotide trapping method for transcription factor purification systematic optimization using electrophoretic mobility shift assay. *Journal of Chromatography A* 1070:23-34
- Murre C, Bain G, van Dijk MA, Engel I, Furnari BA, Massari ME, Matthews JR, Quong MW, Rivera RR, Stuver MH (1994) Structure and function of helix-loop-helix proteins. *Biochim. Biophys. Acta* 1218 (2): 129-35
- Nebert DW (2002) Transcription factors and cancer: an overview. *Toxicology* 181: 131-141

Orphanides, G, Reinberg, D (2002) A unified theory of gene expression. *Cell* 108 (4): 439-451

Pabo CO, Sauer RT (1992) Transcription factors; Structural Families and Principles of DNA recognition. *Annu. Rev. Biochem.*, 61:1053-95

Park PJ (2009) ChIP-seq: advantages and challenges of a maturing technology. *Nat Rev Genet* 10: 669–680

Pavletich NP, Pabo CO (1991) Zinc Finger- DNA recognition, *Science*, 252:809-17
Qiao J, Shao W, Wei HJ, Sun YM, Zhao YC, Xing WL, Zhang L, Mitchelson K, Cheng J (2008) Novel high-throughput profiling of human transcription factors and its use for systematic pathway mapping. *Journal of Proteome Research* 7 (7), 2769-2779

Romanov, S, Medvedev A, Gambarian M, Poltoratskaya N, Moeser M, Medvedeva L, Diatchenko L, Makarov S (2008) Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. *Nature Methods* 5 (3): 253-260

Ruscher K, Reuter M, Kupper D, Trendelenburg G, Dirnagl U, Meisel A (2000) A fluorescence based non-radioactive electrophoretic mobility shift assay. *Journal of Biotechnology* 78 (2): 163-170

Shao W, Wei HJ, Qiao JY, Zhao YC, Sun YM, Zhou YX, Cheng J (2005) Parallel profiling of active transcription factors using an oligonucleotide array-based transcription factor assay (OATFA). *Journal of Proteome Research* 4 (4), 1451-1456

Schevitz RW, Otwinowski Z, Joachimiak A, Lawson CL, Sigler PB (1985) The three dimensional structure of trp repressor. *Nature* 317:782-86

Shen Z, Peedikayil J, Olson GK, Siebert PD, Fang Y (2002) Multiple transcription factor profiling by enzyme-linked immunoassay. *Biotechniques* 32 (5): 1168-+

Steitz TA, Ohlendorf DH, McKay DB, Anderson WF, Matthews BW (1982) Structural similarity in the DNA-binding domains of catabolite gene activator and cro repressor proteins. *Proc. Natl. Acad. Sci.* 79:3097-100

Wang GL, Jiang BH, Rue EA, Semenza GL (1995) Hypoxia-inducible factor 1 is a basic helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *PNAS* 92(12): 5510-5514

Yaoi T, Jiang X, Li X (2006) Development of a fluorescent microsphere-based multiplexed highthroughput assay system for profiling of transcription factor activation. *Assay Drug Dev Technol.* 4(3):285–292

CHAPTER 2 QUANTITATIVE, SOLUTION-PHASE PROFILING OF MULTIPLE TRANSCRIPTION FACTORS IN PARALLEL

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2.1 ABSTRACT

Transcription factors are regulatory proteins that bind to specific sites of chromosomal DNA to enact responses to intracellular and extracellular stimuli. Transcription factor signaling networks are branched and interconnected so that any single transcription factor can activate many different genes and one gene can be activated by a combination of different transcription factors. Thus, trying to characterize a cellular response to a stimulus by measuring the level of only one transcription factor potentially ignores important simultaneous events that contribute to the response. Hence, parallel measurements of transcription factors are necessary to capture the breadth of valuable information about cellular responses that would not be obtained by measuring only a single transcription factor. We have sought to develop a new, scalable, flexible, and sensitive approach to analysis of transcription factor levels that complements existing parallel approaches. Here, we describe proof-of-principle analyses of purified human transcription factors and breast cancer nuclear extracts. Our assay can successfully quantify transcription factors in parallel with ~10-fold better sensitivity than current techniques. Sensitivity of the assay can be further increased by 200-fold through the use of PCR for signal amplification.

2.2 INTRODUCTION

Transcription factors (TFs) are cell regulatory proteins that facilitate proper cell function by controlling gene expression in response to intracellular and extracellular stimuli (Orphanides et al., 2002). TFs act through binding to specific sites of chromosomal DNA, thereby directing which gene(s) will be expressed. Any one TF can respond to a variety of stimuli; additionally, multiple TFs are typically activated by any one stimulus. Thus, the profile of active TFs is complex and constantly changing (Babu 2010; Emerson 2002). Aberrant TF activity results in improper cell function and can lead to disease (e.g., cancer) (Mees et al, 2009; Nebert 2002; Latchman 1996; McCulley et al., 2012; Barnes 2006). Therefore, monitoring of TF function is valuable for understanding biological processes and can support medical diagnoses and the development of novel therapeutics.

In studying TFs, there are two principal areas of research, i) identification of the consensus sequences and target genes of a single transcription factor and ii) measuring the levels of active TFs in response to a stimulus. Considerable work has been done in the first area using a variety of tools, including protein-binding microarrays and chromatin immunoprecipitation (ChIP) based approaches (Johnson et al., 2007; Berger et al., 2009). It is through the use of these tools that some TF regulatory networks have been constructed (Nishiyama et al, 2009; Matys et al., 2003; Matys et al., 2006).

Identifying the consensus target sequence for a given TF also provides an approach to quantify the level of the TF in response to stimuli. These techniques can be performed *in vitro* (e.g., electrophoretic mobility shift assays (EMSAs)) or in cells (e.g., reporter gene assays) (Ruscher et al., 2000; Bronstein et al., 1994; Benotmane et al., 1997). Each of these approaches can be applied for the analysis of single or multiple TFs, depending on the readout strategy (Shen et al., 2002; Li et al., 2006; Romanov et al., 2008). Cytometry based assays have been developed for measuring TFs in parallel based on fluorescent beads tagged with the TF consensus sequences (Yaoi et al., 2006). Additionally, an approach termed OATFA combined electrophoresis with an oligo microarray and showed success in analyzing multiple TFs in parallel both in purified TFs and in cell extracts (Qiao et al., 2008; Shao et al., 2005).

While all of these current techniques to measure TFs in parallel have provided valuable information to improve the understanding of cellular processes (Jiang et al., 2006), we are seeking to develop a complementary assay that approaches our perception of the ideal assay for parallel TF measurements. This ideal assay would meet the following characteristics: i) low detection limits (10^8 TF molecules or fewer), ii) small sample sizes (10^6 cells or fewer), iii) parallel measurements up to hundreds of TFs simultaneously, and iv) quantitative, rather than relative, measurements of TF levels. Moreover, we would want to avoid labor-intensive techniques like electrophoresis (as applied in EMSA and OATFA), manipulation of the cells prior to analysis (as in reporter gene assays), and expensive/proprietary technologies (as in cytometry-based approaches).

In this study, we have devised an approach to measure TF levels in parallel, based on streptavidin magnetic bead separation that begins to approach some of the ideal properties described above. For proof-of-concept, we have successfully analyzed purified transcription factors, p50 (NF- κ B family) and c-Jun (AP-1 family), in parallel with ~10-fold improved sensitivity over existing approaches. Also, nuclear extracts of breast cancer cells untreated and treated with TNF- α and IKK inhibitor were successfully analyzed with our method. Going forward, we envision straightforward coupling of our approach with modern technologies for DNA analysis (e.g., parallel sequencing) to expand the number of TFs that can be assayed in parallel using the approach.

2.3 MATERIALS AND METHODS

2.3.1 Magnetic beads assay strategy (Figure 2.1)

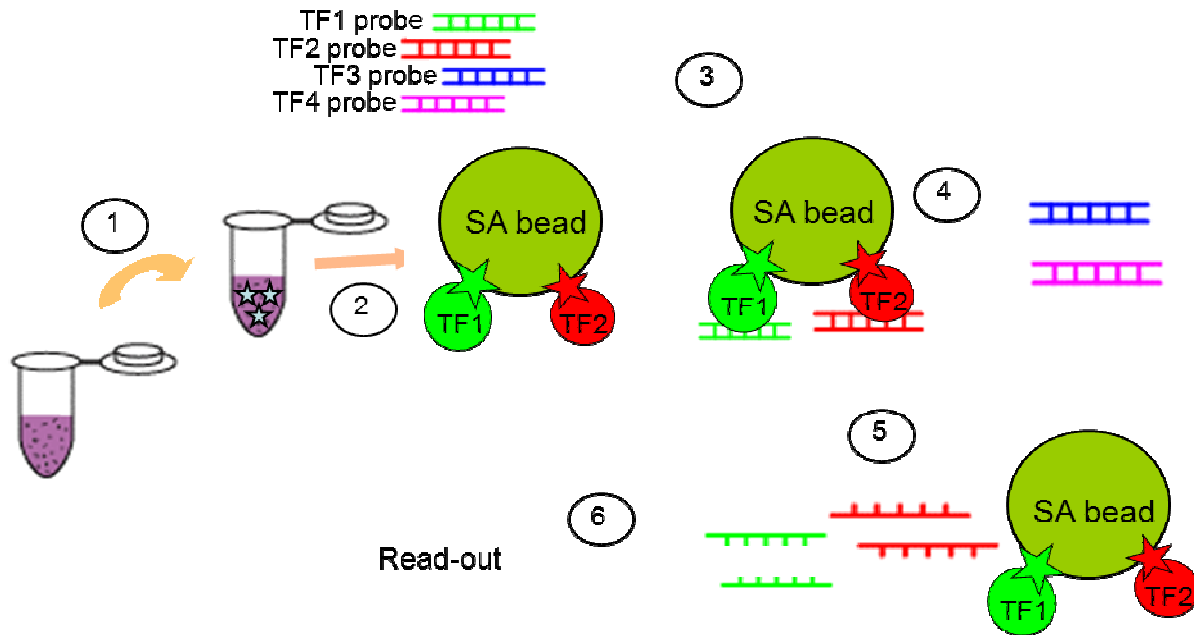


Figure 2.1: Schematic representation of proposed method. 1. Chemically biotinylate TFs in nuclear extracts; 2. Immobilize biotinylated TFs on streptavidin-coated magnetic beads (SA beads); Remove unbound proteins in the supernatant and wash the beads three times; 3. Mix TF-loaded beads with a library of DNA probes in binding buffer for 20 min at room temperature; 4. Apply magnet and remove unbound probes in the supernatant; Wash the beads three times to remove non-specifically bound probes; 5. Elute and denature retained DNA by incubating at 95°C for 15 min; 6. Apply magnet, recover eluted DNA in supernatant and analyze recovered DNA, using PCR amplification if necessary.

Biotinylated transcription factors were immobilized on streptavidin-coated magnetic beads (Dynal/Invitrogen, Oslo, Norway) by incubation at room temperature for 20 min in 1x PBS. A magnet was then applied, supernatant removed, and TF-bound beads recovered. Beads were washed three times with 50 μ l wash buffer I (1x PBS plus 0.1%BSA). Recovered beads were mixed with DNA probes in binding buffer (10 mM

Tris-HCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 1 mM CaCl₂, 0.2 mM KCl, 10 μM ZnCl₂, 4% glycerol, 20 mM acetic acid, 0.025μg/μL poly (dI-dC)) for 20 min at room temperature. Binding conditions were optimized by pH analysis (Figure S1), with pH = 6.5 selected to balance retention of specific signal with removal on non-specific signal. The magnet was applied again and the supernatant collected. The beads were then washed with wash buffer II (0.02% Tween 20 in water), with the supernatants collected after each wash step.

Scintillation Counting. The beads were resuspended with 50 μL of water and mixed with 10ml Safety Solve High Flash Point Scintillation Cocktail (Research Products International Corporation, Mount Prospect, IL). Signals for each fraction were quantified with scintillation counter and the percentage of signal in each fraction was calculated. An example of the measured signal in each fraction is shown in Table 2.1.

PCR Readout. The beads were resuspended in 25 μL of 1x TBE buffer and incubated at 95°C for 15 min to elute the retained DNA. This disrupted protein-DNA complexes without affecting biotin-streptavidin binding. With the magnet applied, eluted DNA molecules were recovered in the supernatant for PCR readouts.

Table 2.1: An example of the cpm measured for each step of pure protein detection. In this case, 3 nM NF- κ B was used.

Before	After	Wash1	Wash2	Wash3	Elution
1.68×10^5	1.33×10^5	1.43×10^3	554	250	3.24×10^4

2.3.2 PCR and parallel analysis.

For parallel analyses, two different primer sets were designed resulting in different length PCR products for recovered NF- κ B and Ap1 probes. Primer sequences are listed in Table 2.2. Eluted DNA probes (1 μ l of 25 μ l) were mixed with Ap1 and NF- κ B primer sets at 500 nM each and amplified for 20 cycles with Taq DNA Polymerase (New England Biolabs, Ipswich, MA) in 50 μ l reactions. The PCR program was: 95°C for 30 s (melting), 61°C for 30 s (annealing), and 72°C for 10 s (extension). 12 μ l of PCR product was mixed with 4 μ l of gel loading buffer, and 14 μ l was loaded onto native 4-20% TBE gels. Gels were run at 300 V for 20 min on ice, stained with SYBR Gold (Invitrogen, Carlsbad, CA), and visualized with UV light in a ChemiDoc XRS System (Bio-Rad, Hercules, CA).

Table 2.2: Sequences of DNA probes and PCR primers. TF binding consensus sequences are in bold. Complementary sequences of primers to DNA probes are underlined.

Transcription Factor	Probe name	Probe sequence
NF- κ B	NF- κ B oligo	5'-AGTTGAG GGGACTTTCCC CAGGA-3'
	NF- κ B complement	5'-TCCTG GGAAAGTCCC CTCAACT-3'
Ap1	Ap1 oligo	5'-CGCTTGAT GAGTCAG CCGGA-3'
	Ap1 complement	5'-TCCGGCT GACTCAT CAAGCG-3'
TBP	TBP oligo	5'-CGCCTACCTCATTT TATAT GCTCTGC-3'
	TBP complement	5'-GCAGAGCAT TATAAA ATGAGGTAGGCG-3'
Negative control	NC oligo	5'-TATTTAGGAGGAGTTCACCCACATAG-3'
	NC complement	5'-CTATGTGGTGAACCTCCTCCTAAATA-3'
NF- κ B primer	NF- κ B forward primer	5'-GTTTCTTCGACTTCGCGGCCT <u>TCCTGGGAAAG</u> - 3'
	NF- κ B reverse primer	5'-GTTTCTTCCTGCGGCGACCG <u>AGTTGAGGGGA</u> - 3'
Ap1 primer	Ap1 forward primer	5'- GCTGCCTGCCC <u>GCTTGATGA</u> -3'
	Ap1 reverse primer	5'- CTGCACGTCTG <u>TCCGGCTGAC</u> -3'

2.3.3 TF biotinylation

Pure transcription factors (p50 and c-Jun) were purchased from Active Motif (Carlsbad, CA). Transcription factors in pure protein solutions or nuclear extracts were biotin labeled chemically by EZ-Link-Iodoacetyl-PEG₂-biotin (Pierce, Rockford, IL), according

to the manufacturer's instructions. Briefly, pure proteins/nuclear extracts were mixed with EZ-Link-Iodoacetyl-PEG₂-biotin in reaction buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.0) at room temperature for 90 min. Unincorporated biotin molecules were removed with G-50 Sephadex columns (Roche Applied Science, Indianapolis, IN). Sephadex columns were washed three times with PBS prior to use.

2.3.4 DNA probe preparation and radiolabeling

All ssDNA probes were purchased from Integrated DNA Technology (Coralville, IA), and their sequences are listed in Table 2.2. Probes were hybridized by mixing the same amounts of complementary sequences in 1x STE buffer (10 mM Tris, 100 mM NaCl, and 1 mM EDTA), heating to 95°C for 5 min, followed by incubation at room temperature for 1 hour. These dsDNA probes were 5'-radiolabeled with 10 pmoles of [γ -³³P] ATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA); free radioactive ATP molecules were removed by G-25 Sephadex columns (Roche Applied Science, Indianapolis, IN).

2.3.5 Cell culture

The human breast cancer cell line, MDA-MB-231, was obtained from Dr. Kathleen Gallo in Michigan State University. The cells were cultured in Dulbecco's Modified Eagle Medium (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum, 2 mM glutamine, 100 μ g/mL streptomycin and 100 U/mL penicillin. Cells were maintained at 37°C and

10% CO₂ as described in (Wu et al., 2011). IKK inhibitor VII (EMD Millipore, Billerica, MA) and recombinant human TNF- α (R&D system, Minneapolis, MN) were used at the concentrations of 100 nM and 30 ng/ml, respectively. The cells were treated with IKK inhibitor VII or TNF- α for 2hr.

2.3.6 Nuclear extraction

Nuclear extraction was performed according to a protocol described by Lee (Lee et al., 1988). Briefly, cells were washed with PBS, then suspended and allowed to swell in buffer A (10 mM HEPES (pH=8.0), 1.5 mM MgCl₂, 10 mM KCl, protease inhibitor) on ice for 15 min. The cells were then lysed with a 25-gauge, 5/8 inch needle, and the nuclear pellets were collected by centrifugation. Nuclear pellets were re-suspended and incubated in buffer B (20 mM HEPES (pH=8.0), 1.5 mM MgCl₂, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA (pH=8.0), protease inhibitor) on ice for 30 min. After incubation, nuclear extracts (supernatants) were obtained by centrifugation at 12,000 g for 5 minutes.

2.3.7 Western blotting

The protein concentration of nuclear extracts was determined by Bradford assay (Bio-Rad, Hercules, CA) as described by Zhang (Zhang et al., 2011). Immunoblot was performed according to Liu (Liu et al., 2012). Briefly, 30 μ g protein samples were loaded and separated by 10% Tris-HCl gel, and transferred to nitrocellulose membrane.

Membranes were blocked with 5% BSA in 0.05% Tween 20-TBS (Tris buffered saline) (USB corporation, Fremont, CA) for 1 h. at room temperature. Primary antibodies, p50 (Cell signaling, Danvers, MA; diluted 1:500 in 5% BSA/0.05% Tween 20-TBS), c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:500 in 5% BSA/0.05% Tween 20-TBS), or TBP (Sigma, St. Louis, MO; diluted 1:1000 in 5% BSA/0.05% Tween 20-TBS), were incubated at 4°C overnight. Anti-mouse or anti -rabbit HRP-conjugated secondary antibody (Thermo Scientific, Asheville, NC; diluted 1:1000 times in 5% non-fat milk/0.05% Tween 20-TBS), was then added for 1 h at room temperature. After washing with Tween 20-TBS, blots were visualized by SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific, Logan, UT), according to the manufacturer's instructions.

2.4 RESULTS AND DISCUSSION

In conceiving our experimental approach, we sought a method that would be sensitive, quantitative, flexible, and scalable. In all cases, the goal was to use the presence of a TF's consensus DNA as proxy readout for the presence of the TF, given the far greater ease of parallel readouts of nucleic acids relative to proteins. Moreover, we wanted the assay to operate in the solution phase, as solution phase approaches are more flexible in how they are expanded and provide more opportunities when considering how to perform separations, a critical part of these types of experimental tools, especially considering the wide range of sizes and pI values among TFs.

With these considerations in mind, we developed an assay that relies on bead-based immobilization of all of the proteins in the sample to be analyzed, allowing solution phase recovery of bound DNAs for subsequent analyses. In our approach, we first biotinylate all of the proteins in the sample (Figure 2.1) followed by immobilization on streptavidin-coated magnetic beads. Followed by washes, bound DNA is then eluted for analysis. In this way, we control the sensitivity and the rate of false positives through the stringency of our wash steps. Using the DNA as the readout also allows for PCR amplification to enhance the detection limit of the approach. The fidelity of the assay depends on efficient and uniform biotinylation of all TFs in a sample. While many approaches exist for *post hoc* biotinylation of proteins (Bayer et al., 1990), we chose to target cysteines for the TFs we studied here, NF- κ B and Ap1. In the event that cysteines are involved in DNA recognition for other TFs, in particular zinc finger TFs (Pavletich et al., 1991), alternative biotinylation methods could be examined.

2.4.1 Recombinant TF measurements

To validate the assay, we first attempted to detect pure proteins, NF- κ B (p50) and Ap1 (c-Jun), in buffered solution. Biotinylation and immobilization were analyzed by western blot and Bradford assay, showing that the vast majority of proteins (~90% of total proteins based on Bradford and Western) were biotinylated and bound to the beads (Figure 2.2).

Table 2.3: Analysis of protein amount immobilized on the beads by Bradford assay (to detect all proteins) The Bradford results confirm that more than 83% of proteins from the nuclear extracts were retained by the beads during immobilization. This suggests that all or nearly all of the proteins in the original extract were biotinylated.

Bradford results

	Before immobilization	After immobilization	percent immobilized
TNF α	0.494	0.013	97.3
Ctrl	0.467	0.053	88.5
IKK inh.	0.514	0.083	83.8

Western results

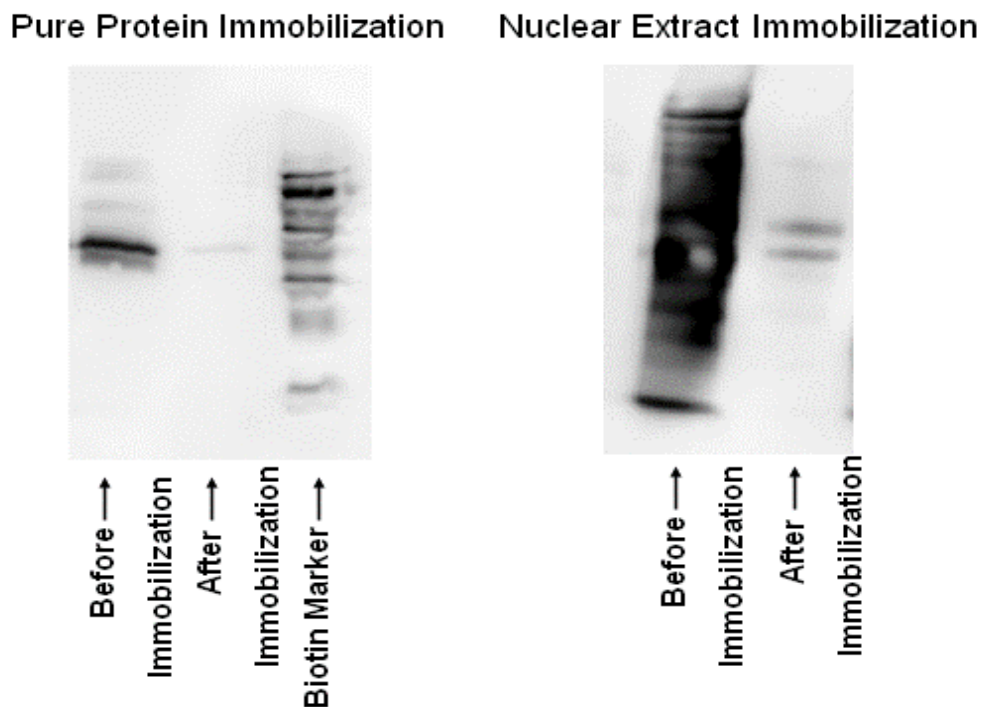


Figure 2.2: Analysis of protein amount immobilized on the beads by Western blot (with biotin antibody to detect biotinylated proteins). Protein immobilization was further supported by the Western blots showing that essentially all of the biotinylated protein in the sample was retained on the beads during the immobilization process.

The DNA binding properties of the biotinylated proteins were tested by EMSA and compared to unbiotinylated proteins, showing no difference between the biotinylated and native TFs (Figure 2.3).

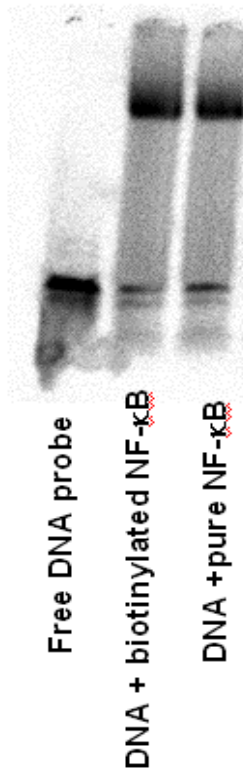


Figure 2.3: Binding analysis of biotinylated protein. The binding of the DNA probe to biotin labeled NF- κ B and un-labeled NF- κ B was analyzed by EMSA. As shown in the gel, the binding properties were not altered by biotinylation.

DNA probes (containing the consensus sequences for NF- κ B, Ap1, and TFIID, as well as a scrambled negative control (NC); sequences available in Table 2.2) were mixed with TF coated magnetic beads. Either radiolabeled NF- κ B probe was mixed with unlabeled Ap1, TFIID, and negative control probes (Figure 2.4A), or radiolabeled Ap1 probe was mixed with unlabeled NF- κ B, TFIID and negative control probes (Figure

2.4B). Our results showed the expected increasing signal with increasing protein concentration for both proteins. The detection limits for NF- κ B and Ap1 were 1.5 nM and 40 nM, respectively, reflecting the lower affinity of Ap1 for its consensus sequence (~300 nM) relative to NF- κ B (~8 nM).

A:

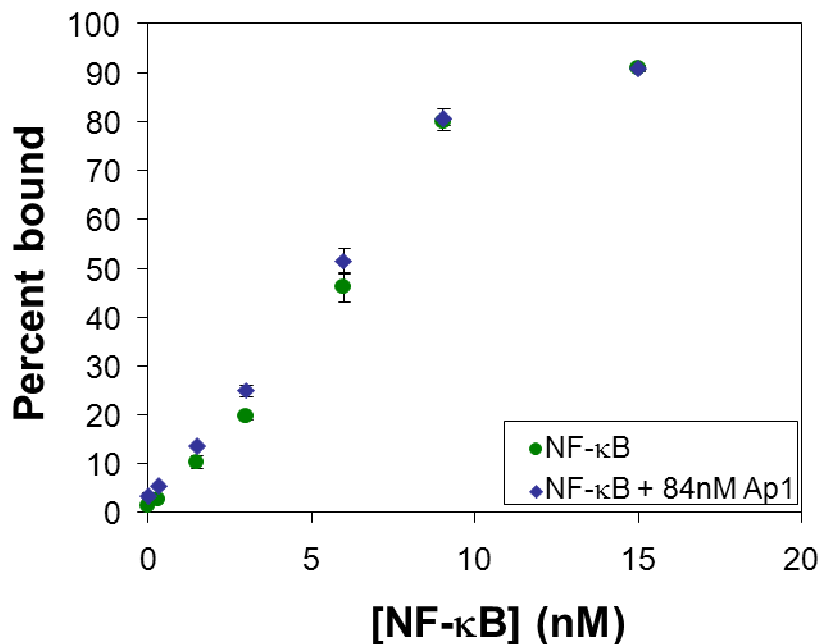
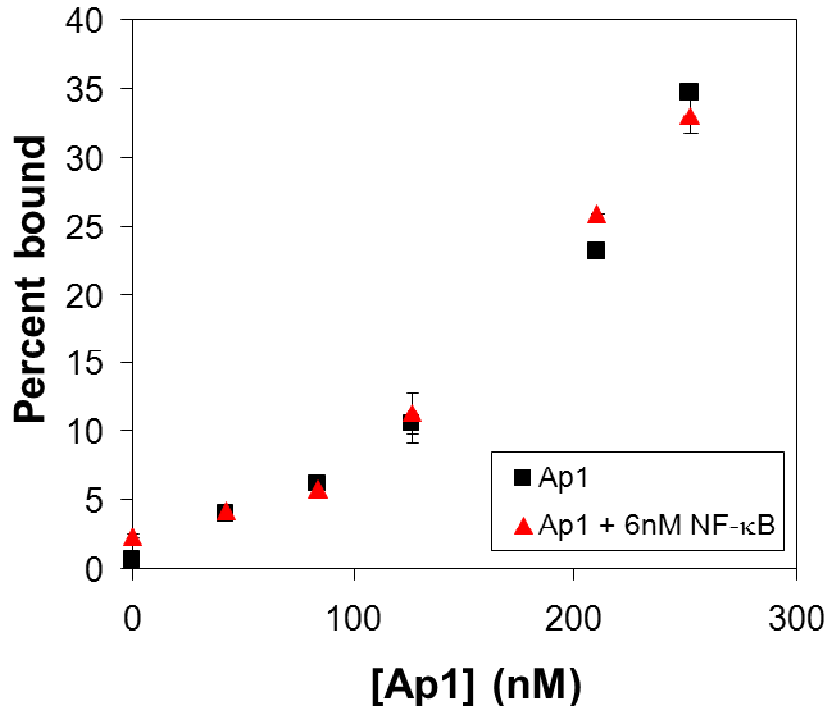


Figure 2.4 Detection of pure proteins alone and in the presence of a non-specific competitor. (A) Detection of NF- κ B:DNA complex with increasing amounts of NF- κ B protein in the absence (green circles) or presence (blue diamonds) of Ap1. NF- κ B protein was biotinylated, immobilized on the beads, and a mixture of DNA probes (radiolabeled NF- κ B probe mixed with unlabeled Ap1, TFIID, and negative control probes) were mixed with TF-coated magnetic beads. The percentage of radiolabeled NF- κ B probe remaining on the beads (relative to signal that did not bind or was washed from the beads) was plotted with respect to protein concentration. (B) Detection of Ap1:DNA complex with increasing amounts of Ap1 protein in the absence (black squares) or presence (red triangles) of NF- κ B. Ap1 protein was biotinylated, immobilized on the beads, and a mixture of DNA probes (radiolabeled Ap1 probe mixed with unlabeled NF- κ B, TFIID, and negative control probes) were mixed with TF-coated magnetic beads. The percentage of radiolabeled Ap1 probe remaining on the beads (relative to signal that did not bind or was washed from the beads) was plotted with respect to protein concentration. If not visible, error bars are within the plot symbol.

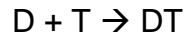
Figure 2.4 (cont'd).

B:



In our affinity-based detection scheme, the detection sensitivity, which is at best the concentration of DNA:TF complexes formed, depends on the concentrations of DNA probe and TF and their binding affinity (K_D). For our approach, a balance must be struck between sensitivity and ease of separation. Maximal sensitivity is best achieved using DNA probe concentrations above the K_D and greatly above the anticipated TF concentrations (see derivation below).

For single site DNA (D) binding to a transcription factor (T), the binding reaction can be written as;



where:

$$K_D = \frac{[D] * [T]}{[DT]}$$

[D], [T] and [DT] represent the concentration of DNA, transcription factor and DNA:transcription factor complex at equilibrium, respectively.

Using [D]₀ and [T]₀ for the initial concentrations of DNA and transcription factor, respectively, the equation becomes:

$$K_D = \frac{([D]_0 - [DT]) * ([T]_0 - [DT])}{[DT]}$$

At [D]₀ >> [T]₀, the equation can be simplified to:

$$K_D = \frac{[D]_0 * ([T]_0 - [DT])}{[DT]}$$

Then:

$$\frac{[DT]}{[T]_0} = f = \frac{[D]_0}{K_D + [D]_0}$$

If $[D]_0 \gg K_D$, then $[DT] = [T]_0$, meaning that all the transcription factor in the sample is bound to DNA. This condition gives maximal sensitivity. However, separation of complexes from the free DNA needs to be perfect, given the huge excess of free DNA relative to TF.

On the other hand, if protein is in excess ($[T]_0 \gg [D]_0$), the equation can be simplified to:

$$K_D = \frac{([D]_0 - [DT]) * [T]_0}{[DT]}$$

Then:

$$\frac{[DT]}{[D]_0} = f = \frac{[T]_0}{K_D + [T]_0}$$

While limited in sensitivity by the relative magnitude of $[T]_0$ and K_D , this case allows more straightforward separation as the labeled molecule, the DNA, is limiting.

However, as the DNA concentrations increase, the fraction of DNA probes bound by TF decreases, resulting in a more challenging separation problem; in other words, the number of free DNA probes approaches 100% of the total DNA. For our proof-of-principle experiments, we chose to operate in the regime where the TF was in excess,

minimizing false positives and simplifying the separation process, but potentially limiting sensitivity.

Nonetheless, consideration of the differences in affinity for various DNA:TF pairs is important when trying to design the approach for maximal sensitivity for all TFs in a parallel implementation. The flexibility of a solution-phase assay, as opposed to an array-based approach, would allow us to adjust the concentrations of our DNA probes to maximize the sensitivity for any TF. Specifically, we can increase, if needed, the concentration of DNA probes for TFs with lower affinity for their consensus sequences, thereby increasing the number of DNAs bound by these TFs and improving our sensitivity for them. The K_D values for TF-DNA complexes can vary but are typically in the nanomolar range. We have measured the K_D values of NF- κ B and Ap1 to be 8 nM and 300 nM, respectively (data not shown). Additionally, we could include multiple copies of a given consensus sequence within a probe as an alternative means of increasing the effective concentration of the probe. With that said, our assay in its current form can detect NF- κ B, the TF for which the majority of data exists, with sensitivity ~3-10 times greater than current approaches (Yaoi et al., 2006; Shao et al., 2005). We anticipate being able to achieve similar sensitivity gains for all TFs being measured.

In addition to sensitivity, it is critical that parallel measurements can be made with high fidelity and little to no cross-reactivity, where a TF binds to a DNA or DNAs other than

the probe with its unique consensus sequence. We wanted to establish the fidelity of our assay through comparison of the recovered DNA for the single TF measurements versus measurement of one TF in the presence of the other (Figure 2.4). The close correlation of the signals from these two studies establishes that our approach, at least for this pair of proteins, shows good fidelity for each TF. Clearly, this does not establish the fidelity for measurements examining even just tens of TFs, but it does give us confidence that the interactions we are measuring are specific and that the potential for expansion to broader parallel analyses exists.

2.4.2 Measuring TFs in nuclear extracts

While measurements of pure proteins, whether alone or in parallel, are useful for establishing the feasibility of the approach, it is critical to test the approach in the realistic context of nuclear extracts. We chose to examine nuclear extracts predicated on the assumption that TFs present in the nucleus are active and, ultimately, tell us more about cell function than whole cell or cytoplasmic levels of TFs. We first analyzed NF- κ B and Ap1 levels in nuclear extracts from breast cancer cells in culture. For both proteins, the measured protein quantity scaled with increasing quantity of nuclear extract (Figure 2.5). It is important to note that our assay detected NF- κ B levels in only 500 ng nuclear extracts, or what would be obtained from approximately 10^5 cells. For comparison, commercial assays typically require 5 μ g of nuclear extracts; thus, our assay is 10 fold more sensitive relative to those assays (Yaoi et al., 2006, Qiao et al., 2008).

A:

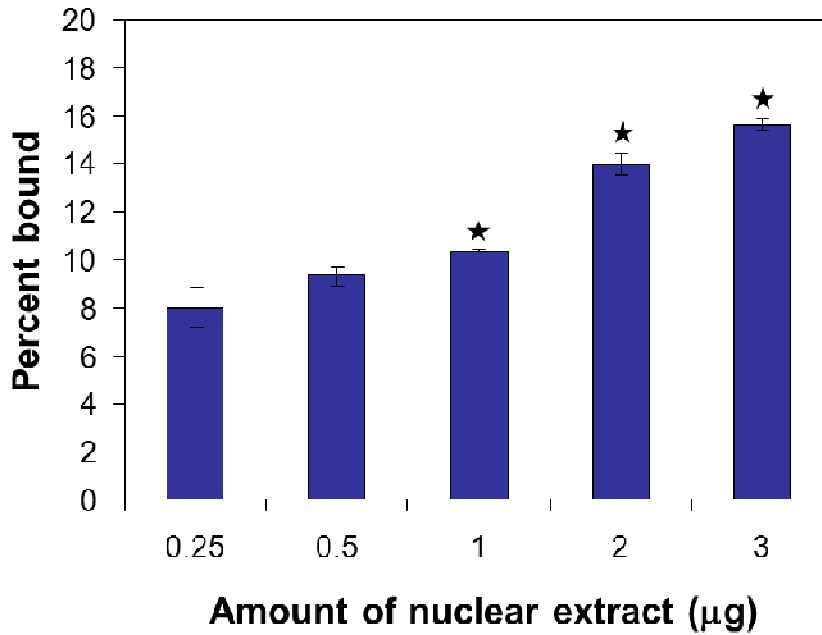
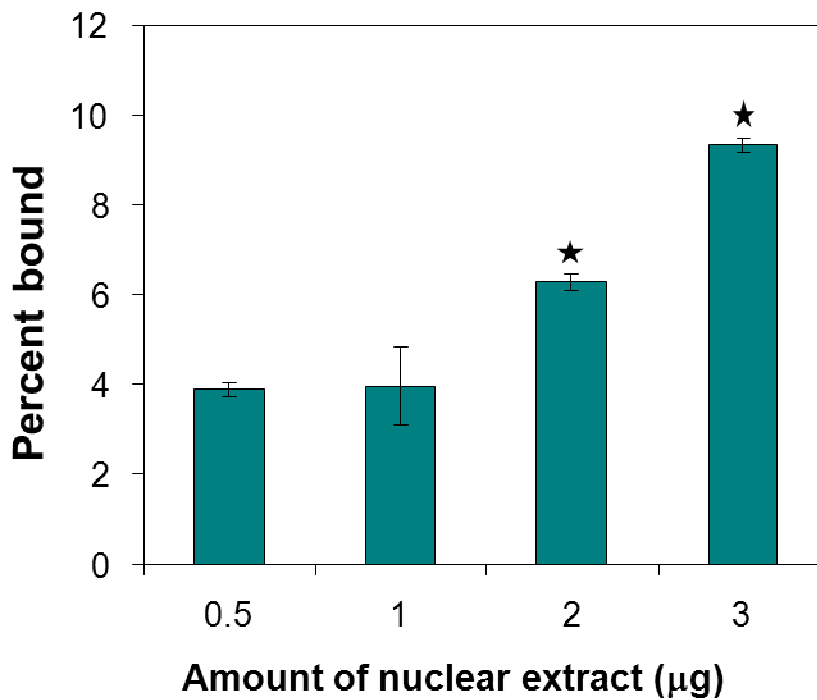


Figure 2.5: Detection of single TFs in nuclear extracts. Detection of NF- κ B:DNA complex (A) or Ap1:DNA complex (B) with increasing amounts of nuclear extracts. TFs in nuclear extract were biotinylated, immobilized on the beads, and a mixture of DNA probes (radiolabeled NF- κ B (A) or radiolabeled Ap1 (B) probe mixed with unlabeled TFIID, negative control, and Ap1 (A) or NF- κ B (B) probes) were mixed with TF-coated magnetic beads. The percentage of radiolabeled NF- κ B (A) or radiolabeled Ap1 (B) probe remaining on the beads (relative to signal that did not bind or was washed from the beads) was plotted with respect to the initial amount of nuclear extract analyzed. Background signal obtained from experiments using 0 μ g of nuclear extract has been subtracted from all points. (n = 3; * indicates p < 0.07 for NF- κ B and p < 0.1 for Ap1)

Figure 2.5 (cont'd).

B:



Given our success in analyzing a dose responsive signal for the proteins we measured, we wanted to test our approach against a stimulus where the impact on the TFs would be due to a biological response. For these studies, the same breast cancer cell line was treated with either TNF- α at 30 ng/ml for 2 hours or IKK inhibitor VII at 100 nM for 2 hours. We anticipated that these would increase and decrease levels of NF- κ B in the nuclear extracts, respectively (Kim et al., 2006; van Horssen et al., 2006). NF- κ B, Ap1, and TBP (as a control TF) levels in these treated cells and control samples were analyzed both with western blot and our assay. By both western blot and our technique, NF- κ B levels were found to be roughly two fold higher in TNF- α treated extracts and two fold lower in IKK inhibited extracts, relative to control extracts (Figure 2.6). Ap1 levels

were 1.5 times higher in TNF- α treated extracts and two fold lower in IKK inhibited extracts, relative to control extracts. As expected, TBP levels were unchanged in the extracts, again as measured by both our technique and western.

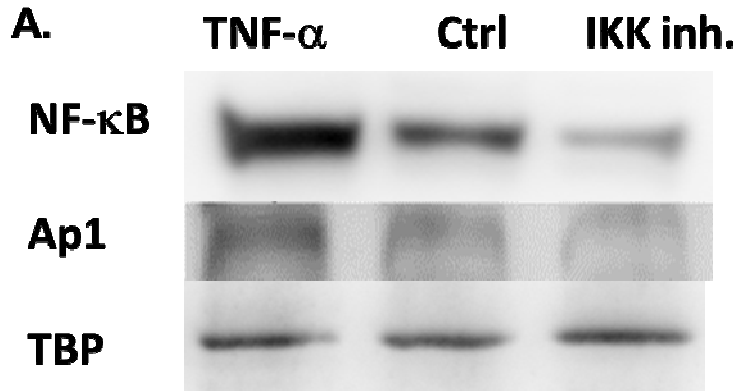
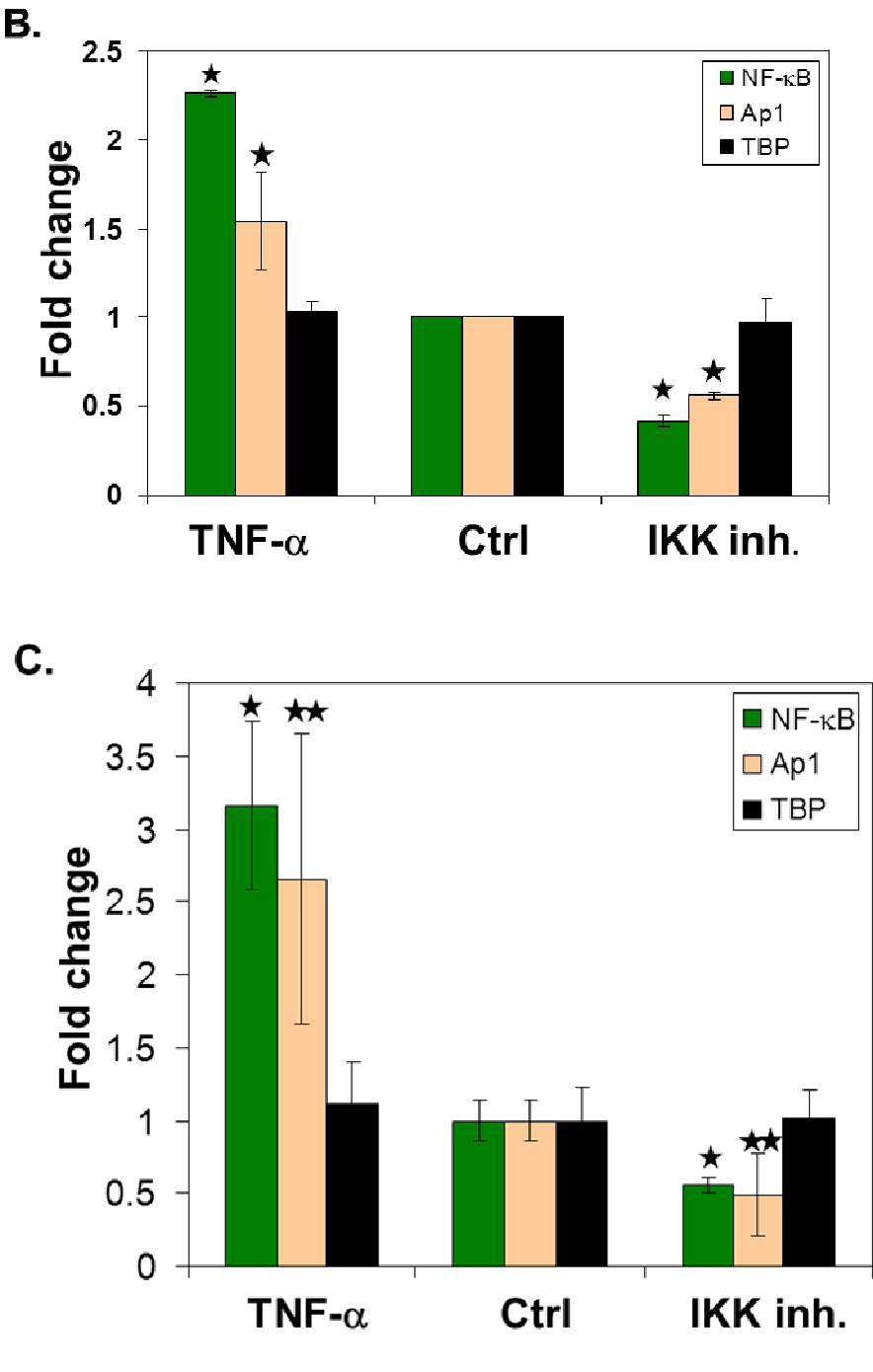


Figure 2.6: Quantification of TF levels in nuclear extracts after cell stimulation. (A) Representative western blot showing detection of NF- κ B, Ap1, and TBP in nuclear extracts after stimulation of the cultured cells with TNF- α (TNF- α), no treatment (ctrl), or inhibition of IKK (IKK inh.). The fold change in signal (ratio of sample to control) was plotted for each sample. (B) Quantification of the western blots for each protein. (n = 2) (C) TF levels measured by our technique. (n = 3) (* indicates p < 0.04 and ** indicates p < 0.15)

Figure 2.6 (cont'd).



In addition to the successful measurement of NF- κ B and Ap1 levels relative to control samples, our method allows us to estimate the absolute quantity of TF molecules in the sample. By comparison of the recovered DNA quantities of our samples relative to our standard curves, we estimate the number of NF- κ B and Ap1 molecules in 2 μ g nuclear extracts to be 10^{-13} moles and 4×10^{-12} moles, respectively. Based on the 10^5 cells used in our tests, this translates to 6×10^5 NF- κ B molecules/nucleus and 2.5×10^7 Ap1 molecules/nucleus.

2.4.3 Parallel TF measurements in nuclear extracts

To test the feasibility of parallel TF measurements, we wanted to use a PCR-based readout that would mirror potential future applications. In this case, we designed different length primers that would yield PCR products of unique length following amplification of the recovered DNA for each TF. The PCR products were then visualized and quantified after simultaneous separation by gel electrophoresis. We limited our PCRs to 20 cycles and confirmed that the products were not saturated (data not shown). As such, quantitative comparisons of the relative amounts of PCR product could be made using gel quantification. While we recognize that this type of PCR readout cannot be used for parallel measurements of many TFs, it is sufficient for our parallel measurements of two molecules. As before, we first confirmed the viability of this readout strategy with pure proteins (Figure 2.7). In our two tests, one protein was held constant while the other protein concentration was varied. In each test (Figures

2.7A and 2.7C), the PCR product obtained for each protein was proportional to the initial quantity of protein.

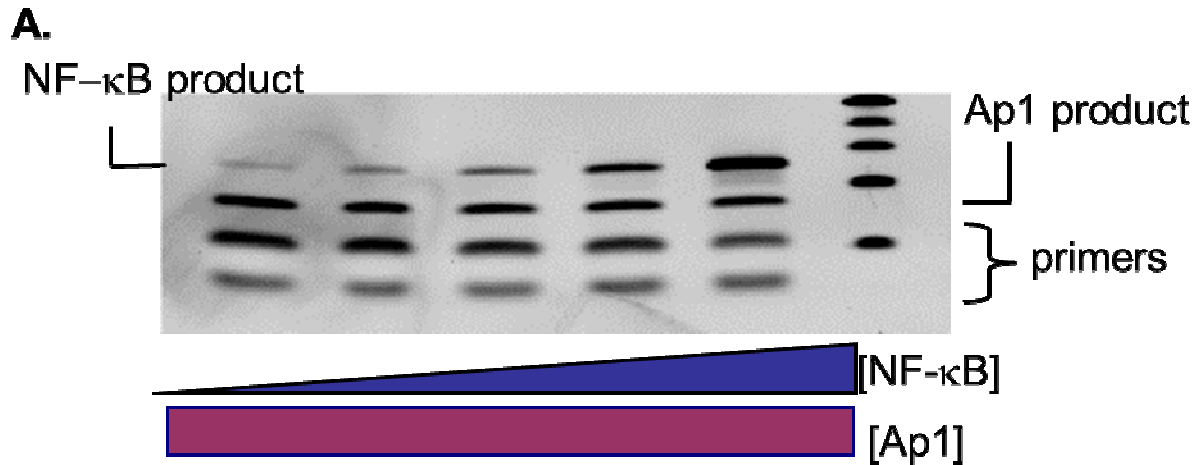
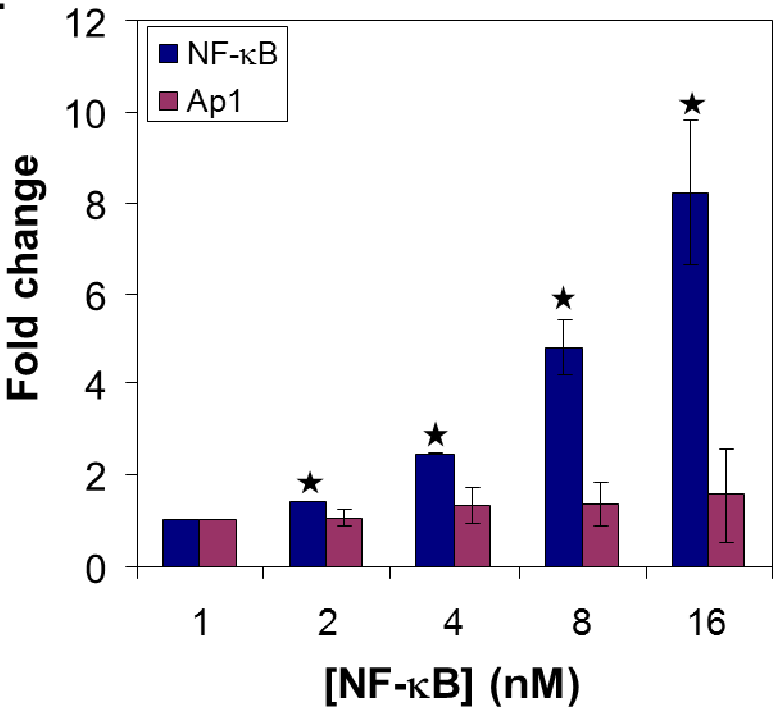


Figure 2.7: Parallel TF readout by PCR readout. NF- κ B and Ap1 PCR products of 62 bp and 40 bp, respectively, were analyzed by gel electrophoresis. Representative images are shown. 25 bp marker was also loaded for reference. (A) The amount of Ap1 was kept constant (120 nM) while the amount of NF- κ B was increased (1 – 16 nM). Eluted DNA from each sample was amplified with PCR and loaded into the gel. (B) Quantification of the gel images ($n = 3$). Band intensities were quantified with Quantity One software and the ratio of band intensity to intensity of DNA at the lowest NF- κ B concentration was plotted. (C) The amount of NF- κ B (8 nM) was kept constant while the amount of Ap1 was increased (30-240 nM). Eluted DNA from each sample was amplified with PCR and loaded into the gel (D) Quantification of the corresponding gel images ($n = 3$). Band intensities were quantified with Quantity One software and the ratio of band intensity to intensity of DNA at lowest Ap1 concentration was plotted. (* indicates $p < 0.015$)

Figure 2.7 (cont'd).

B.



C.

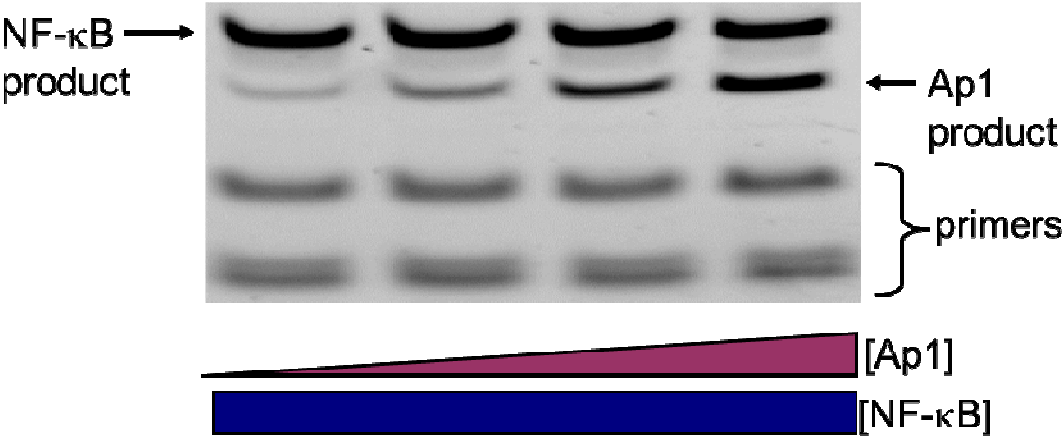
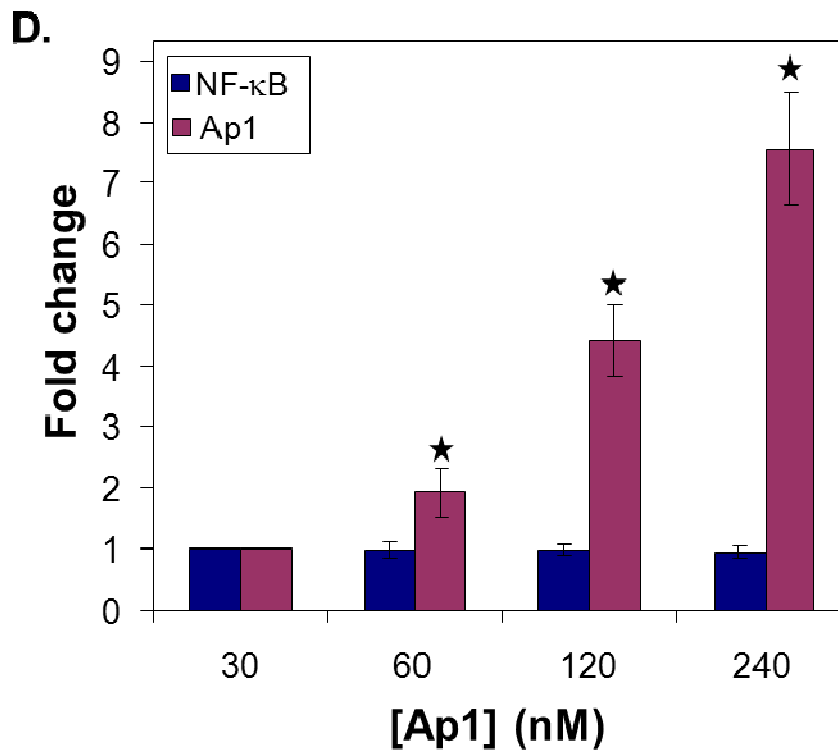


Figure 2.7 (cont'd).



We then applied our parallel approach to the IKK inhibited, control, and TNF- α stimulated nuclear extracts. The parallel analyses agree well with our single protein measurements (Figure 2.8). Amplification by PCR improves our detection limit by ~200 fold over currently available techniques. With the success of these experiments, we have demonstrated proof-of-concept for our approach to TF measurements that meets our requirements for flexibility, scalability, and sensitivity.

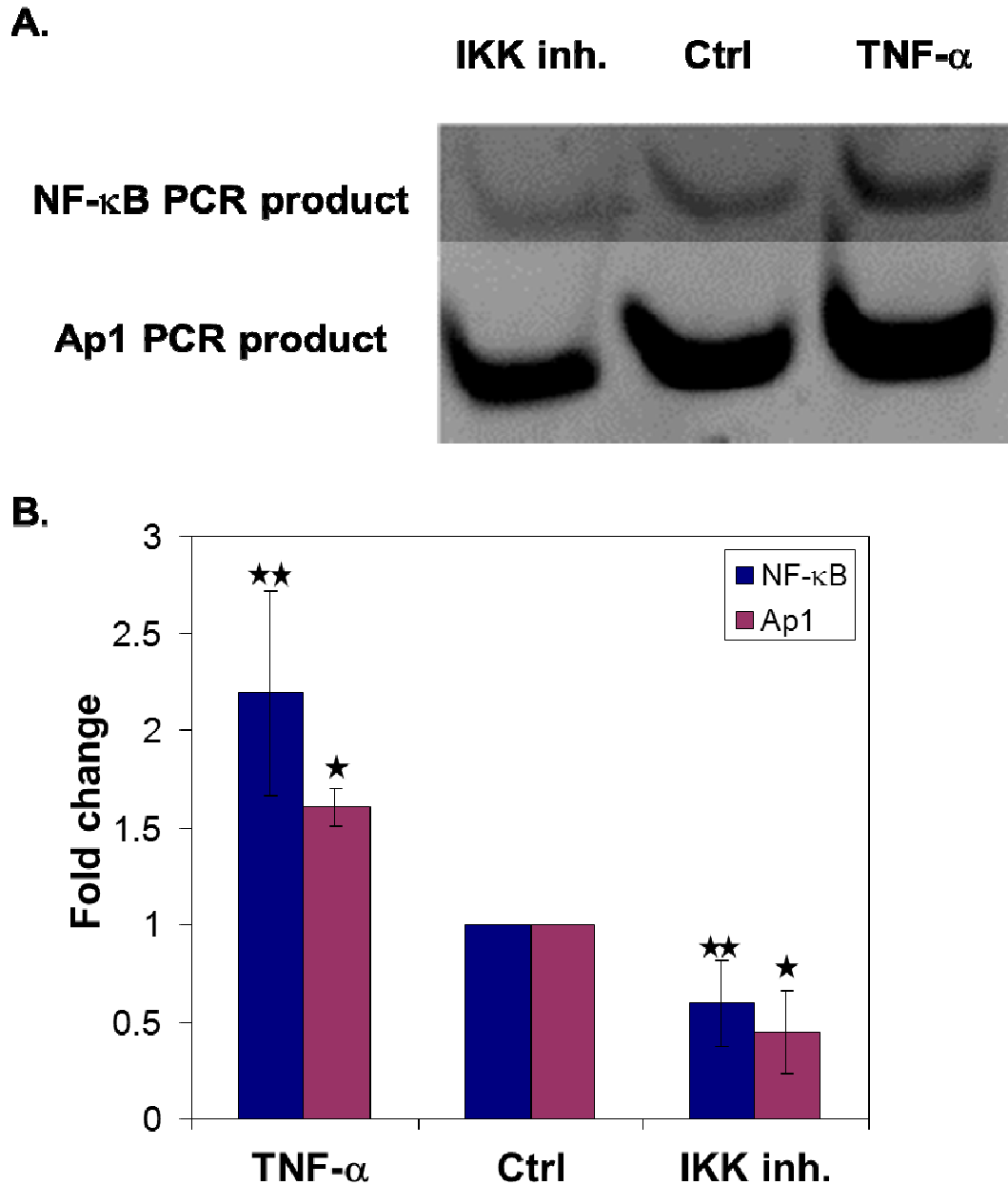


Figure 2.8: Parallel TF analysis in nuclear extracts after cell stimulation. After treatment of cells with an IKK inhibitor (IKK inh.) or TNF- α , nuclear extracts were analyzed for NF- κ B and Ap1 levels. A. PCR analysis by electrophoresis, representative gel. Contrast was adjusted for NF- κ B part of the gel for better visualization. B. Quantification of the corresponding gel images ($n = 3$). Band intensities were quantified with Quantity One software and ratio of sample to control was plotted. (* indicates $p < 0.07$ and ** indicates $p < 0.13$)

Nonetheless, our approach is still in development. In the near term, we are in the process of expanding the number of TFs we can measure in parallel. In this way, we will be able to investigate new biological phenomena where we will not necessarily have a clear expectation of the effect on all the TFs being measured. As we increase the number of TFs being measured, we will have to increase the total concentration of the DNA probes added to each sample. This has the potential to increase the frequency of non-specific interactions between the proteins and DNAs as well as among the various DNA sequences. We will continue to evaluate the stringency of our wash steps to maximize the accuracy of our approach at higher parallelism.

That said, perhaps the most critical factor in determining the feasibility of our assay in a parallel format is the fidelity of the interaction between a protein and its consensus sequence. In particular, many TFs are grouped in families that have highly similar consensus sequences (Kielbasa et al., 2005). For these, we will interpret data for a given consensus sequence as being indicative of higher levels of protein for the family of TFs. It would then be necessary to come back with a secondary approach (e.g., an antibody-based method) to identify the TFs with greater specificity.

2.5 CONCLUSIONS

We have developed a scalable, flexible, and sensitive approach for the analysis of TF levels. We have successfully analyzed NF- κ B and Ap1 in purified samples and nuclear

extracts, alone and in parallel, with improved sensitivity over existing approaches. Going forward, we anticipate that our method when further developed will provide an additional tool to enable scientists to understand cellular processes in response to stimuli, leading to improved disease diagnoses and accelerating therapeutic development.

REFERENCES

REFERENCES

- Babu MM (2010) Structure, evolution and dynamics of transcriptional regulatory networks. *Biochemical Society Transactions* 38:1155-1178
- Barnes PJ (2006) Transcription factors in airway diseases. *Laboratory Investigation* 86 (9):867-872
- Bayer EA, Wilchek M (1990) Protein biotinylation. *Methods in Enzymology* 184:138-160
- Benotmane AM, Hoylaerts MF, Collen D, Belayew A (1997) Nonisotopic quantitative analysis of protein-DNA interactions at equilibrium. *Analytical Biochemistry* 250 (2):181-185
- Berger MF, Bulyk ML (2009) Universal protein-binding microarrays for the comprehensive characterization of the DNA-binding specificities of transcription factors. *Nature Protocols* 4 (3):393-411
- Bronstein I, Fortin J, Stanley PE, Stewart G, Kricka LJ (1994) Chemiluminescent and bioluminescent reporter gene assays. *Analytical Biochemistry* 219 (2):169-181
- Emerson BM (2002) Specificity of gene regulation. *Cell* 109 (3):267-270
- Jiang X, Roth L, Lai CF, Li XQ (2006) Profiling activities of transcription factors in breast cancer cell lines. *Assay and Drug Development Technologies* 4 (3):293-305
- Johnson DS, Mortazavi A, Myers RM, Wold B (2007) Genome-wide mapping of in vivo protein-DNA interactions. *Science* 316 (5830):1497-1502
- Kielbasa SM, Gonze D, Herzog H (2005) Measuring similarities between transcription factor binding sites. *Bmc Bioinformatics* 6:11
- Kim HJ, Hawke N, Baldwin AS (2006) NF-kappa B and IKK as therapeutic targets in cancer. *Cell Death and Differentiation* 13 (5):738-747
- Latchman DS (1996) Mechanisms of disease - Transcription-factor mutations and disease. *New England Journal of Medicine* 334(1):28-33
- Lee KAW, Bindereif A, Green MR (1988) A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-messenger RNA splicing. *Gene Analysis Techniques* 5 (2):22-31.
- Li XQ, Jiang X, Yao T (2006) High throughput assays for analyzing transcription factors. *Assay and Drug Development Technologies* 4 (3):333-341

Liu L, Martin R, Chan C (2012) Palmitate-activated astrocytes via serine palmitoyltransferase increase BACE1 in primary neurons by sphingomyelinases. *Neurobiol Aging* Doi:10.1016/j.neurobiolaging.2012.05.017

Matys V, Fricke E, Geffers R, Gossling E, Haubrock M, Hehl R, Hornischer K, Karas D, Kel AE, Kel-Margoulis OV, Kloos DU, Land S, Lewicki-Potapov B, Michael H, Munch R, Reuter I, Rotert S, Saxel H, Scheer M, Thiele S, Wingender E (2003) TRANSFAC (R): transcriptional regulation, from patterns to profiles. *Nucleic Acids Research* 31 (1):374-378

Matys V, Kel-Margoulis OV, Fricke E, Liebich I, Land S, Barre-Dirrie A, Reuter I, Chekmenev D, Krull M, Hornischer K, Voss N, Stegmaier P, Lewicki-Potapov B, Saxel H, Kel AE, Wingender E (2006) TRANSFAC (R) and its module TRANSCOMP (R): transcriptional gene regulation in eukaryotes. *Nucleic Acids Research* 34:D108-D110.

McCulley DJ, Black BL (2012) Transcription factor pathways and congenital heart disease. *Current Topics in Developmental Biology* 100:253-277

Mees C, Nemunaitis J, Senzer N (2009) Transcription factors: their potential as targets for an individualized therapeutic approach to cancer. *Cancer Gene Therapy* 16 (2):103-112

Nebert DW (2002) Transcription factors and cancer: an overview. *Toxicology* 181:131-141

Nishiyama A, Xin L, Sharov AA, Thomas M, Mowrer G, Meyers E, Piao YL, Mehta S, Yee S, Nakatake Y, Stagg C, Sharova L, Correa-Cerro LS, Bassey U, Hoang H, Kim E, Tapnio R, Qian Y, Dudekula D, Zalzman M, Li MX, Falco G, Yang HT, Lee SL, Monti M, Stanghellini I, Islam MN, Nagaraja R, Goldberg I, Wang WD, Longo DL, Schlessinger D, Ko MSH (2009) Uncovering Early Response of Gene Regulatory Networks in ESCs by Systematic Induction of Transcription Factors. *Cell Stem Cell* 5 (4):420-433

Orphanides G, Reinberg D (2002) A unified theory of gene expression. *Cell* 108 (4):439-451

Pavletich NP, Pabo CO (1991) Zinc finger DNA recognition –crystal- structure of a ZIF268-DNA complex at 2.1-Å. *Science* 252 (5007):809-817

Qiao JY, Shao W, Wei HJ, Sun YM, Zhao YC, Xing WL, Zhang L, Mitchelson K, Cheng J (2008) Novel high-throughput profiling of human transcription factors and its use for systematic pathway mapping. *Journal of Proteome Research* 7 (7):2769-2779

Romanov S, Medvedev A, Gambarian M, Poltoratskaya N, Moeser M, Medvedeva L, Diatchenko L, Makarov S (2008) Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. *Nature Methods* 5 (3):253-260

Ruscher K, Reuter M, Kupper D, Trendelenburg G, Dirnagl U, Meisel A (2000) A fluorescence based non-radioactive electrophoretic mobility shift assay. *Journal of Biotechnology* 78 (2):163-170

Shao W, Wei HJ, Qiao JY, Zhao YC, Sun YM, Zhou YX, Cheng J (2005) Parallel profiling of active transcription factors using an oligonucleotide array-based transcription factor assay (OATFA). *Journal of Proteome Research* 4 (4):1451-1456

Shen Z, Peedikayil J, Olson GK, Siebert PD, Fang Y (2002) Multiple transcription factor profiling by enzyme-linked immunoassay. *Biotechniques* 32 (5):1168-+

van Horssen R, ten Hagen TLM, Eggermont AMM (2006) TNF-alpha in cancer treatment: Molecular insights, antitumor effects, and clinical utility. *Oncologist* 11 (4):397-408

Wu M, Liu L, Chan C (2011) Identification of novel targets for breast cancer by exploring gene switches on a genome scale. *Bmc Genomics* 12:19

Yaoi T, Jiang X, Li XQ (2006) Development of a fluorescent micro sphere-based multiplexed high-throughput assay system for profiling of transcription factor activation. *Assay and Drug Development Technologies* 4 (3):285-292

Zhang LX, Seitz LC, Abramczyk AM, Liu L, Chan C (2011) cAMP initiates early phase neuron-like morphology changes and late phase neural differentiation in mesenchymal stem cells. *Cellular and Molecular Life Sciences* 68 (5):863-876

CHAPTER 3 KINETICS OF THE EARLY TRANSCRIPTION RESPONSE TO CYTOKINE AND FATTY ACID STIMULATION

3.1 ABSTRACT

Transcription factors (TF) are key effectors of cell signaling pathways that regulate gene expression. TF networks are highly interconnected so that one signal can lead to changes in many TF levels, and one TF level can be changed by many different signals. TF regulation is central to normal cell function, with altered TF function being implicated in many disease conditions. Thus, measuring TF levels in parallel, and over time, is crucial for understanding the impact of stimuli on regulatory networks and on disease conditions. Here, we report the parallel analysis of temporal TF level changes in different cell types with multiple stimuli. We have analyzed short-term dynamic changes in the levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), Signal transducer and activator of transcription 3 (Stat3), cAMP response element-binding protein (CREB), glucocorticoid receptor (GR), and TATA binding protein (TBP), in breast cancer cells and HepG2 cells after TNF- α and palmitic acid exposure. In response to both stimuli, NF- κ B and CREB levels were increased, Stat3 decreased, and TBP was constant. Our temporal analysis showed that the changes in TF levels peak around 0.5 hour or 1 hour after stimulation. Based on our analysis, GR levels were unchanged in response to TNF- α stimulation and increased in response to palmitic acid treatment, indicating that it might be involved in palmitic acid signaling pathway but not TNF- α signaling pathway. Our results further illuminate the dynamics of TF responses to cytokine and fatty acid exposure, while further demonstrating the utility of parallel TF measurement approaches in the analysis of biological phenomena.

3.2 INTRODUCTION

Proper cellular responses to their microenvironments are crucial for cellular and tissue homeostasis. Abnormal cellular signaling can lead to diseases such as cancer and diabetes (Al-Quobaili et al., 2008; Courtois et al., 2006; Clevenger, 2004; Elsir et al., 2012; Maestro et al., 2007). Improved understanding of cellular signaling has led to improvements in disease diagnosis, the understanding of developmental processes, and the engineering of artificial tissues (Balaskas et al., 2012; Chen et al., 2008; Davidson et al., 2008). In fact, recent advances in cell signaling pathway analysis have already led to the advent of a new field of study, systems biology (Ideker et al., 2001). In general, systems biology relies on the vast amounts of data that can be generated from the various “omics” techniques. These data represent the results of cell signal transduction and would be complemented by incorporating parallel measurements of the levels of transcription factors, the upstream mediators of cellular signaling.

Transcription factors (TFs) are responsible for altering the cell state in response to stimuli by changing the transcription rates of their targeted genes. They interact with specific sites on genomic DNA, often recruiting other co-factors to the location, resulting in activation or repression of the corresponding genes. Analysis of cell signaling responses to stimuli is complicated by the fact that TFs generally control several genes, most genes are controlled by multiple TFs, and any given stimulus can result in the activation of multiple TFs (Awad et al., 2014; Babu et al., 2004; Karlebach et al., 2008). Moreover, TF levels must change in response to a stimulus but, in most cases, must

then return to baseline levels to avoid long-term perturbation of cellular function (Babu, 2010; Hao et al., 2012). For rapid response genes, their TFs are often present in the cytoplasm in an inactive state, are translocated to nucleus after a signaling cascade results in their activation, and then destroyed or trafficked out of the nucleus to halt their function (Calkhoven et al., 1996; Levy et al., 2002; Shaywitz et al., 1999, Whiteside et al., 1993). Thus, tracking TF activities requires analysis across many dimensions, including activation status, subcellular location, and time. With an estimated 2000 TFs in humans, profiling the levels of active TFs is a dynamic and complex task.

To address this challenge, we and others are developing quantitative, sensitive, parallel techniques for measurement of TF levels (Jiang et al., 2008; Li et al., 2006; Qiao 2008; Shen et al., 2002). We have previously demonstrated parallel measurements of TFs in breast cancer cells' extracts using our magnetic bead-based assay (Bilgin et al., 2013). In this study, we have expanded the number of TFs analyzed and applied our method to the kinetic analysis of cellular responses to multiple stimuli, specifically to cytokines (TNF- α) and saturated fatty acids (palmitic acid).

3.3 MATERIALS AND METHODS

3.3.1 DNA probe design and radiolabeling

Detailed design information and probe sequences are listed in Table 3.1. ssDNAs were purchased from Integrated DNA Technologies (Coralville, IA). Probes were generated

by hybridization of equimolar amounts of complementary ssDNAs in 1x STE buffer (10 mM Tris, 100 mM NaCl, and 1 mM EDTA), heating to 95°C for 5 min, followed by incubation at room temperature for 1 hour. After hybridization, dsDNA probes were 5'-radiolabeled with 10 pmoles of [γ -³³P] ATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA). Radiolabeled DNA probes were purified from unincorporated label with G-25 Sephadex columns (Roche Applied Science; Indianapolis, IN).

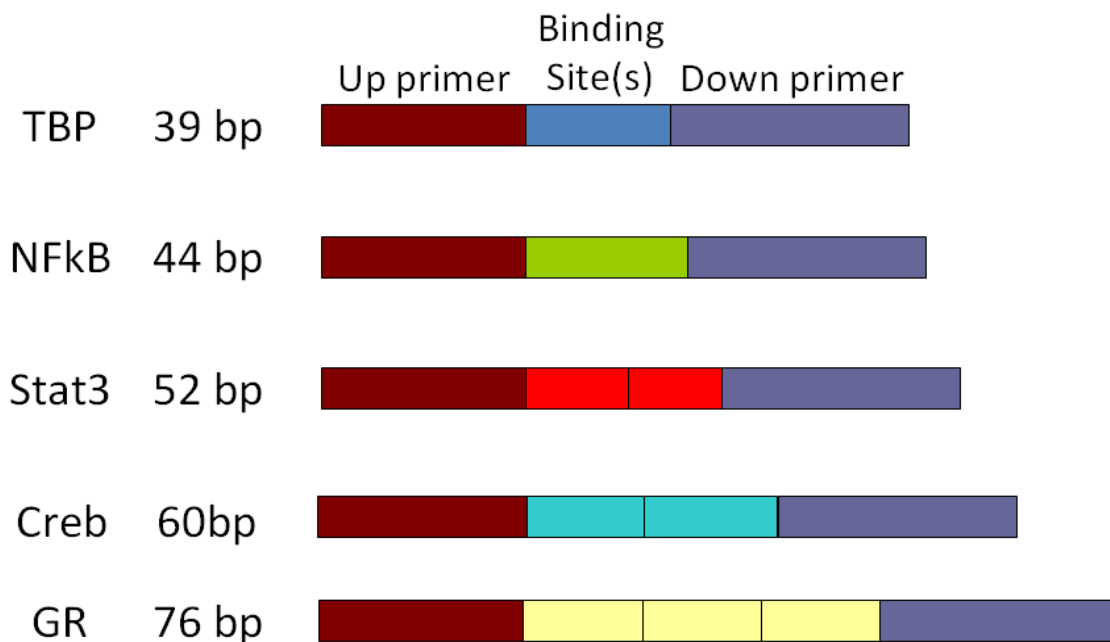


Figure 3.1: Designs of DNA probes and PCR primers. Repeats of TF binding sites are shown in design with the repeat of the same color.

Table 3.1: Sequences of DNA probes and PCR primers.

Transcription Factor	Probe name	Probe sequence
NF-κB	NF-κB oligo	5'- GATGTCCACGAGGTCTCTGGAAAGTCCCTACGCTGCAGGTCGAC-3'
	NF-κB complement	5'-GTCGACCTGCAGCGTAGGGACTTTCAGAGACCTCGTGGACATC-3'
CREB	CREB oligo	5'- GATGTCCACGAGGTCTCTGACGTCAGCGAGTGACGTCAGCGAGCTAC GCTGCAGGTCGAC-3'
	CREB complement	5'- GTCGACCTGCAGCGTAGCTCGCTGACGTCAGCTCGCTGACGTCAGAGA CCTCGTGGACATC-3'
TBP	TBP oligo	5'-GATGTCCACGAGGTCTCTTATAATACGCTGCAGGTCGAC-3'
	TBP complement	5'-GTCGACCTGCAGCGTATTATAAGAGACCTCGTGGACATC-3'
GR	GR oligo	5'- GATGTCCACGAGGTCTCTGTACACTGTGTTCTGTACACTGTGTTCTGT ACACTGTGTTCTTACGCTGCAGGTCGAC-3'
	GR complement	5'- GTCGACCTGCAGCGTAAGAACACAGTGTACAGAACACAGTGTACAG AACACAGTGTACAGAGACCTCGTGGACATC-3'
Stat3	Stat3 oligo	5'- GATGTCCACGAGGTCTCTTCCGGGAATTCGGGAATACGCTGCAGG TCGAC-3'

Table 3.1 (cont'd).

	Stat3 complement	5'- GTCGACCTGCAGCGTATCCCGGAATTCCCGGAAAGAGACCTCGTGG ACATC-3'
Internal Standard	Forward primer	5'-GATGTCCACGAGGTCTCTTACGCTGCAGGTCGAC-3'
	Reverse primer	5'-GTCGACCTGCAGCGTAAGAGACCTCGTGGACATC-3'
Universal Primers	Forward	5'-GATGTCCACGAGGTCTCT-3'
	Reverse	5'-GTCGACCTGCAGCGTA3'

3.3.2 Cell culture

Our experiments were conducted with two different cell lines, HepG2 (human hepatocellular carcinoma) and MDA-MB-231 (human breast adenocarcinoma). HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY), 100 µg/mL streptomycin (Life Technologies, Grand Island, NY) and 100 U/mL penicillin (Life Technologies, Grand Island, NY) in humidified incubator at 37°C and 5% CO₂. For tumor necrosis factor alpha (TNF-α) treatments, cells were grown to 90% confluence in 6-well plates and then treated with 50 ng/ml recombinant human TNF-α (R&D Systems; Minneapolis, MN) for periods of 0.5, 1, 2, 4 and 24 hours.

Control cells received fresh media without TNF- α . For palmitic acid treatments, cells were grown to 90% confluence in 6-well plates and then treated with media containing 0.7 mM palmitic acid (Sigma; St. Louis, MO) complexed with 2% (w/v) fatty acid free BSA (US Biologicals; Salem, MA) for 0.5, 1, 2, 4, 16, and 24 h. Control cells were grown in media containing 2% BSA for the same time periods.

MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (Life Technologies, Grand Island, NY) with 10% fetal bovine serum, 2 mM glutamine (Life Technologies, Grand Island, NY), 100 μ g/mL streptomycin, and 100 U/mL penicillin. Cells were maintained at 37°C and 10% CO₂ as described (Wu et al., 2011). For TNF- α treatments, cells were grown to 90% confluence in 6-well plates and then treated with 50 ng/ml recombinant human TNF- α for periods of 0.5, 1, 2, 4 and 24 hours. Control cells received fresh media without TNF- α .

3.3.3 Nuclear extraction

For HepG2 cells, nuclear extraction was performed as described (Saliobu et al., 1998). Briefly, after washing the cells with PBS, cells were resuspended and allowed to swell in five times the packed cell volume (PCV) of ice cold buffer (10 mM HEPES (pH = 7.9), 10 mM KCl, 0.1 mM EDTA, freshly added protease inhibitors (complete mini EDTA free cocktail tablets from Roche), and phosphatase inhibitors (phosphatase inhibitor cocktail from Sigma)) for 15 min. After adding 10% NP-40 solution to final concentration of 0.5%

(v/v), cells were vortexed for 20 seconds, and nuclear pellets were collected by centrifugation at 13,000 g for 1 min at 4°C. Nuclear pellets were washed three times with buffer (same as above) and resuspended in 1xPCV of ice cold buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, freshly added protease inhibitors (complete mini EDTA free cocktail tablets from Roche), and phosphatase inhibitors (phosphatase inhibitor cocktail from Sigma)). Pellets were shaken for 15 min, and nuclear extracts were obtained by centrifugation at 13,000 g for 15 min at 4°C. The total protein concentration in all extracts was measured by BCA Protein Assay kit (Thermo Scientific Pierce; Rockford, IL).

For MDA-MB-231 cells, nuclear extraction was performed according as described (Lee et al., 1988). Briefly, after washing the cells with PBS, the cells were trypsinized and allowed to swell in buffer (10 mM HEPES (pH = 8.0), 1.5 mM MgCl₂, 10 mM KCl, protease inhibitor (complete mini EDTA free cocktail tablets from Roche)) on ice for 15 min. The cells were then lysed with 15 strokes of a 25-gauge, 5/8 inch needle, and the nuclear pellets were collected by centrifugation at 12,000 g for 15 min. Nuclear pellets were resuspended and incubated in buffer B (20 mM HEPES (pH=8.0), 1.5 mM MgCl₂, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA (pH=8.0), protease inhibitor) on ice for 30 min. After incubation, nuclear extracts (supernatants) were obtained by centrifugation at 12,000 g for 5 min.

It should be noted that complete separation of nuclear and cytoplasmic extracts is

crucial for accurate TF analyses. High fidelity separations preclude latent TFs in the cytoplasmic fraction from giving a false positive signal for the nuclear extracts (Liu et al., 2011).

3.3.4 TF biotinylation

Nuclear extracts were chemically biotinylated by EZ-Link-Iodoacetyl-PEG₂-biotin (Thermo Scientific Pierce; Rockford, IL), according to the manufacturer's instructions. Briefly, nuclear extracts were mixed with EZ-Link-Iodoacetyl-PEG₂-biotin in reaction buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.0) at RT for 90 min. Biotinylated TFs were purified with G-50 Sephadex columns (Roche Applied Science, Indianapolis, IN). Sephadex columns were washed three times with PBS prior to use.

3.3.5 Magnetic bead-based TF quantification

TFs were measured according to our established technique (Bilgin et al., 2013). Biotin labeled TFs were immobilized on streptavidin-coated magnetic beads (Dynal/Invitrogen, Oslo, Norway) by incubation at room temperature for 20 min in 1x PBS. After applying a magnet, the supernatant was removed and the TF-bound beads recovered. Following three washes, the TF-loaded beads were mixed with dsDNA probes in binding buffer for 20 min at RT. The supernatant was collected by applying the magnet again, and the beads were washed with washing buffer (0.02% Tween 20 in water) the supernatants collected after each wash step.

Scintillation Counting. For single TF measurements, the technique was performed using one radiolabeled dsDNA probe while all other probes were unlabeled. After binding to the TF-loaded beads and washes to remove non-specifically bound probes, the beads were resuspended in 50 μ l of water and then mixed with 10 ml Safety Solve High Flash Point Scintillation Cocktail (Research Products International Corporation; Mount Prospect, IL). The signal from all fractions (supernatant, wash 1, wash 2, and beads) was measured, and the percentage of the signal retained on the beads was calculated.

Electrophoresis Readout. For parallel TF measurements, dsDNA probes were bound to the TF-loaded beads. After washes, the beads were resuspended in 1xTBE and heated at 95°C for 15 min to elute the retained DNA. A magnet was applied and the supernatant collected. Eluted DNA probes (1 μ l of the 25 μ l) were mixed with universal primers (300 nM) and amplified for 20 cycles with Taq DNA Polymerase (New England Biolabs; Ipswich, MA) in 50 μ l reactions. The PCR program was: 95°C for 30 s (melting), 61°C for 30 s (annealing), and 72°C for 10 s (extension). 12 μ l of PCR product was mixed with 4 μ l of gel loading buffer, and 14 μ l was loaded onto native 4-12% TBE gels. Gels were run at 300 V for 30 min on ice, stained with SYBR Gold (Invitrogen; Carlsbad, CA), and visualized in a ChemiDoc XRS System (Bio-Rad; Hercules, CA). Band intensities were quantified by QuantityOne software. For normalization, each signal was normalized to an internal standard included in each PCR reaction. The internal standard included the universal primer sites but did not contain a TF binding site. This accounted

for systematic errors from pipetting, electrophoresis, or quantification. After signals were normalized to the internal standard, the ratio of treated to control was calculated.

3.3.6 TF measurements by EMSA

Biotinylated TFs were mixed with dsDNA probes (one probe radiolabeled, the rest unlabeled) in a 20 μ l reaction volume of binding buffer (10 mM Tris-HCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 1 mM CaCl₂, 0.2 mM KCl, 10 mM ZnCl₂, 4% glycerol, 20 mM acetic acid, 0.025 μ g/ μ L poly (dI-dC)) for 30 min at RT. 15 μ l of the binding reaction were mixed with 5 μ l of gel loading buffer, and 18 μ l was loaded onto native 4-12% TBE gels. Gels were run at 300 V for 30 min on ice, dried, and detected by phosphorimaging using the Storm 860 (GE Healthcare; Pittsburgh, PA). Band intensities were quantified by QuantityOne software.

3.3.7 Statistical analyses

All experiments were performed at least 3 times. For gel images, representative results are shown. All error bars show the mean \pm SD value of experiments performed. Two-way student t-test was used to evaluate statistical significance of values compared to control samples. A 95% confidence limit was used to test significance relative to normalized controls.

3.4 RESULTS AND DISCUSSION

Having previously demonstrated applicability of our approach to parallel measurement of TFs (Bilgin et al., 2013), we sought to apply the technique to furthering our understanding of biological signaling kinetics while also demonstrating use of the technique for measuring a broader array of TFs in parallel. We chose to examine cytokine stimulation and fatty-acid exposure of cells as stimuli and designed our panel of TFs according to our expectations about which pathways could be activated by these stimuli. In addition, we tested our technique using extracts from two unique cell types to demonstrate the applicability of the method to different cell systems and to examine the commonalities and differences in response among the systems and stimuli.

3.4.1 TF measurements in MDA-MB-231 Breast Cancer cells stimulated with TNF- α

We first examined the changes in nuclear TF levels associated with TNF- α stimulation of MDA-MB-231 breast cancer cells. These experiments were intended to serve as an initial validation of our larger set of TF measurements. For these experiments, the levels of four different TFs were measured in parallel with respect to treatment time (Figure 3.2). These TFs were nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), signal transducer and activator of transcription 3 (Stat3), glucocorticoid receptor (GR), and TATA binding protein (TBP). For measurements of individual TFs (in which we only tested NF- κ B, Stat3, and TBP), our results showed an increase in

nuclear NF- κ B levels after TNF- α stimulation, with levels peaking at 1 h after stimulation (Figure 3.2A). NF- κ B levels returned to baseline after approximately 4 h, remaining at or near baseline levels for the remainder of the experiment. On the other hand, Stat3 levels decreased after 0.5 h, returning to baseline levels after 2 h. Our expected control TF, TBP, showed no significant change over the course of the experiment. To confirm our results, we performed electrophoretic mobility shift assays (EMSAs) on each of the samples. Strong agreement was seen between the EMSA (Figure 3.2B) and measurements by our method (Figure 3.2C).

Our approach aims to simplify measuring TFs in parallel, by detecting TF-bound dsDNA, rather than the TF itself. In this way, a challenging task, parallel protein detection, is reformulated into a relatively straightforward task, parallel nucleic acid detection. Our objective is to expand the technique to detection of hundreds of TFs in parallel with a high throughput technique such as parallel sequencing. For our current scale of 4 TFs in parallel, a PCR-based readout was applied as a proxy for such readouts. Each dsDNA probe was designed to include universal sense and antisense primer sequences and to yield a PCR product of unique length following amplification. To accomplish this, we included different numbers of binding sequence repeats for some TFs (Table 3.1). In our experiments, eluted DNAs from magnetic beads were PCR amplified with universal primers for 20 cycles, to achieve a semi-quantitative PCR readout. For all TFs, our single protein measurements with EMSA and scintillation counting correlated well with the levels measured in parallel (Figure 3.2D and 3.2E). In all detection techniques, NF- κ B levels were increased within 0.5 h and returned to

baseline after 4 h. In contrast, Stat3 levels decreased and recovered over the same time period. GR levels were unchanged with TNF- α treatment. This was our expectation based upon prior work in muscle cells (Dekalbab et al., 2007). The changes in NF- κ B and CREB levels agree with previously published reports examining the responses of these TFs to TNF- α stimulation (Nikolaidou-Neokosmidou et al., 2006; Regueira et al., 2009), though these were measured at a single timepoint. Our kinetic data demonstrate that TF levels in the nucleus can change rapidly both in the initiation of the response and in the return to baseline levels.

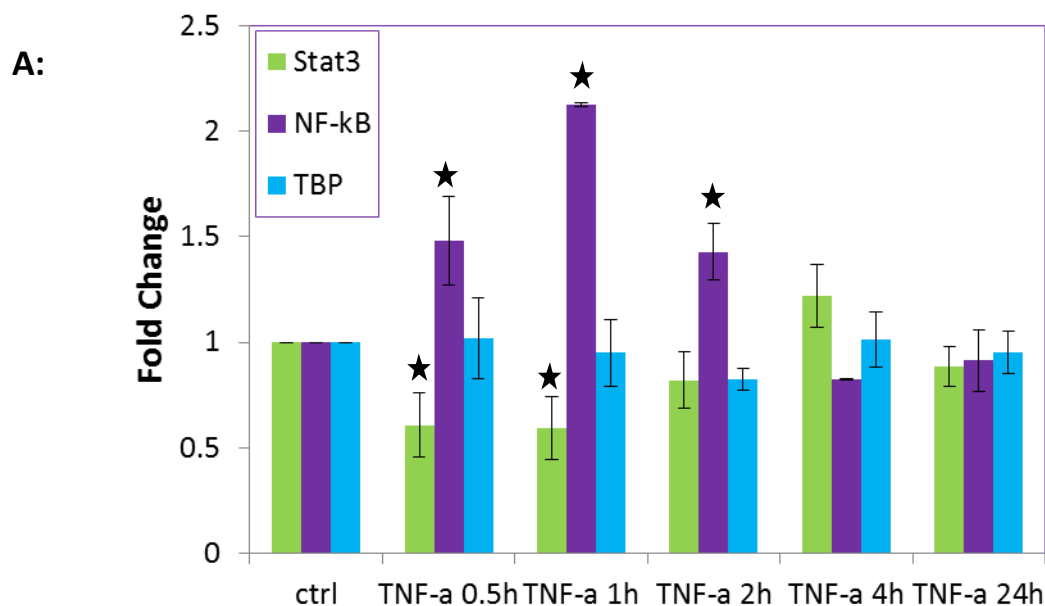
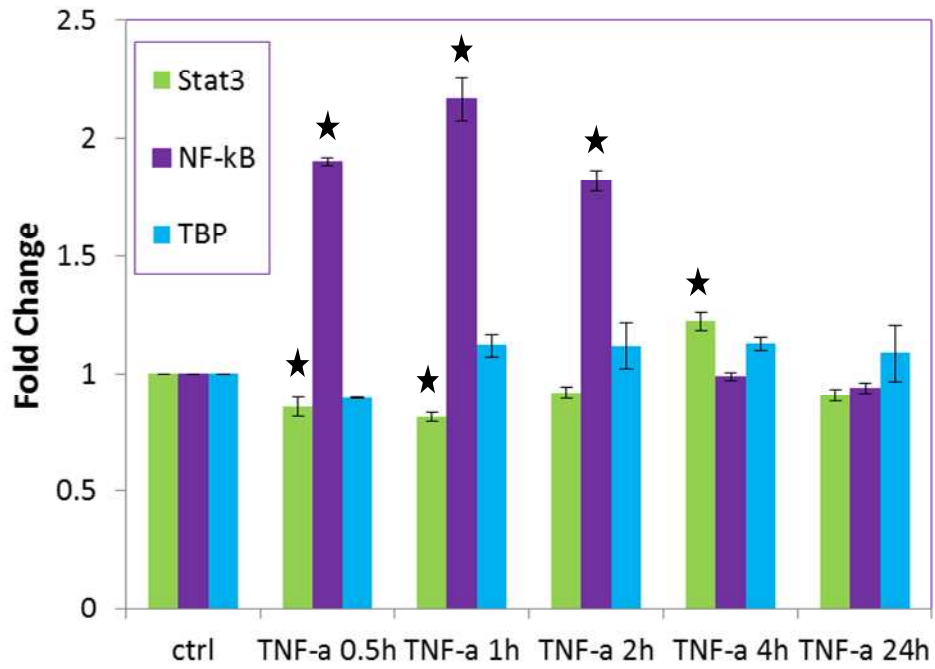


Figure 3.2: Quantification of TF levels in MDA-MB-231 Breast Cancer cells' nuclear extracts after TNF- α stimulation. (A) Single TF detection by bead assay. The percentage of radiolabeled TF probe remaining on the beads (relative to signal that did not bind or was washed from the beads) was calculated. Fold changes relative to control are shown. (n = 3, * indicates p < 0.05). (B) Single TF detection by EMSA. The fractions of bound and unbound DNA probe were quantified and the fraction of signal in the bound was calculated relative to the total signal from the lane. Fold changes relative to control are shown. (n = 3, * indicates p < 0.05). (C) Correlation between detection by EMSA and scintillation. All data points obtained with EMSA and scintillation methods were graphed and correlation analysis was done. (D) Parallel TF detection by bead assay. Signals were normalized with respect to an internal standard and then the ratio with respect to control was calculated. (n = 3, * indicates p < 0.05). (E) Correlation between detection by parallel PCR and scintillation. All data points obtained with two different methods were graphed and correlation analysis was done.

Figure 3.2 (cont'd).

B:



C:

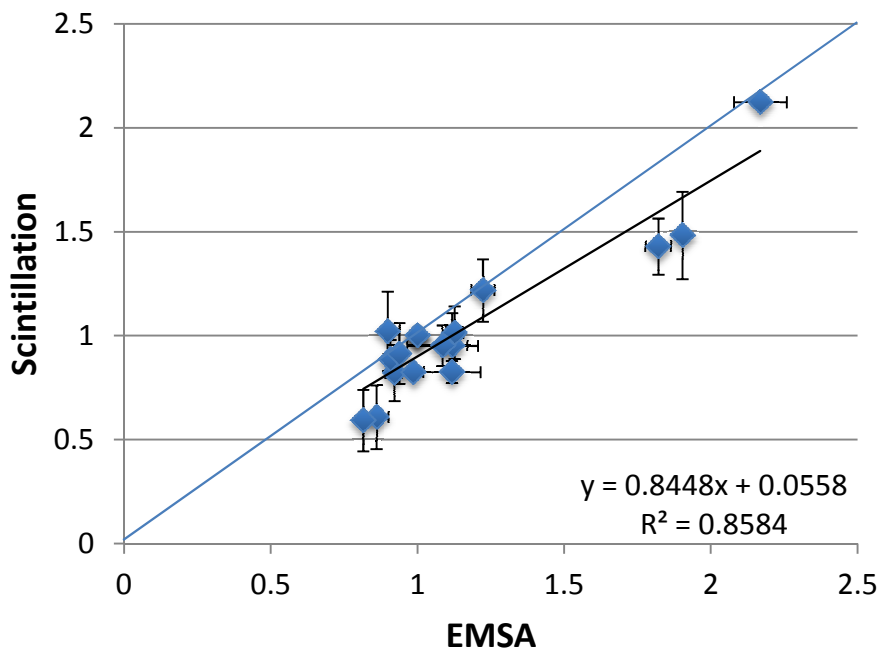
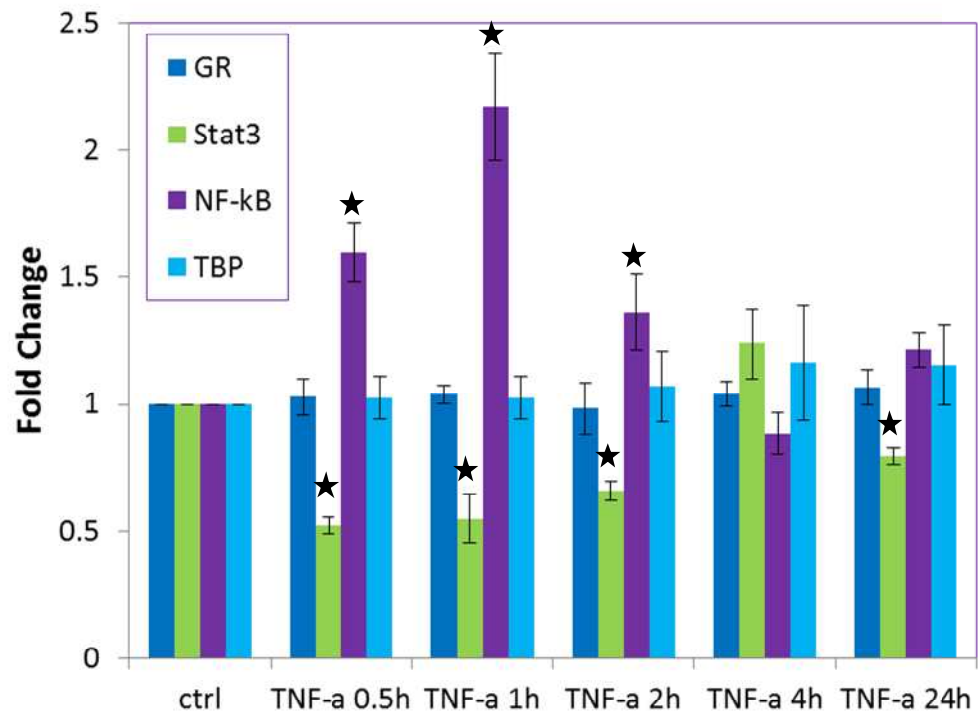
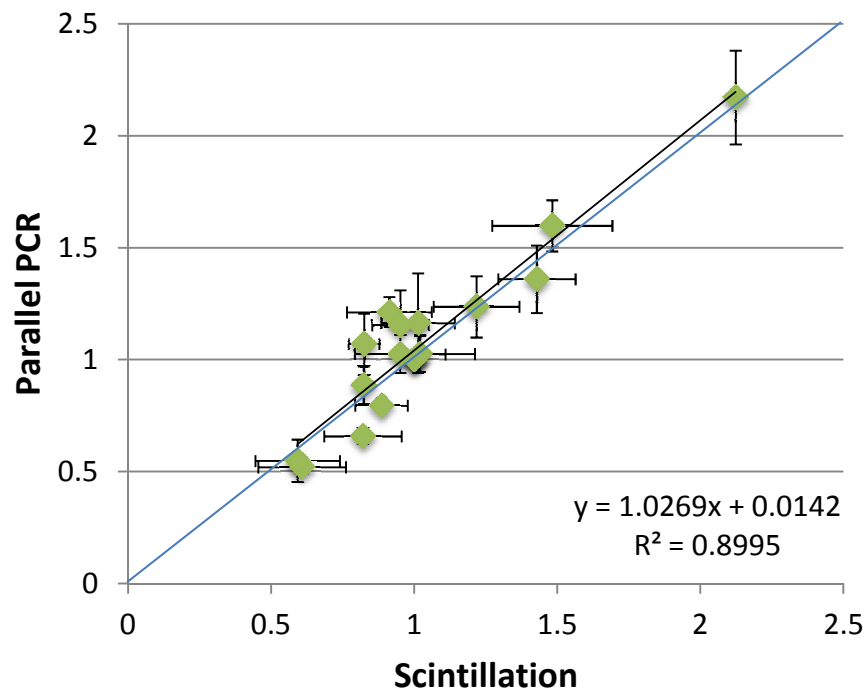


Figure 3.2 (cont'd).

D:



E:



3.4.2 TF measurements in HepG2 cells stimulated with TNF- α

To test the feasibility of our assay with different cell types, we measured TF levels in HepG2 cells. cAMP response element-binding protein (CREB) was included in this set of measurements. We and other have previously studied the effects of TNF- α on these TFs (Schwabe et al., 2006; Wullaert et al., 2006). As with the breast cancer cell experiments, individual TF levels were detected with scintillation counting and EMSA (Figure 3.3A, 3.3B), while multiple TFs were detected by our parallel PCR readout (Figure 3.3D). Results from the three different techniques correlated well, showing NF- κ B and CREB levels increasing, with CREB peaking and returning to baseline later than NF- κ B (Figure 3.3C and 3.3E). Over a similar timeframe, Stat3 levels decreased and also returned to baseline. After 4 h, all measured TF levels returned to control levels. TBP and GR levels were unchanged during the measurement period. These results align with those from the breast cancer cell experiments (Figure 3.2).

A:

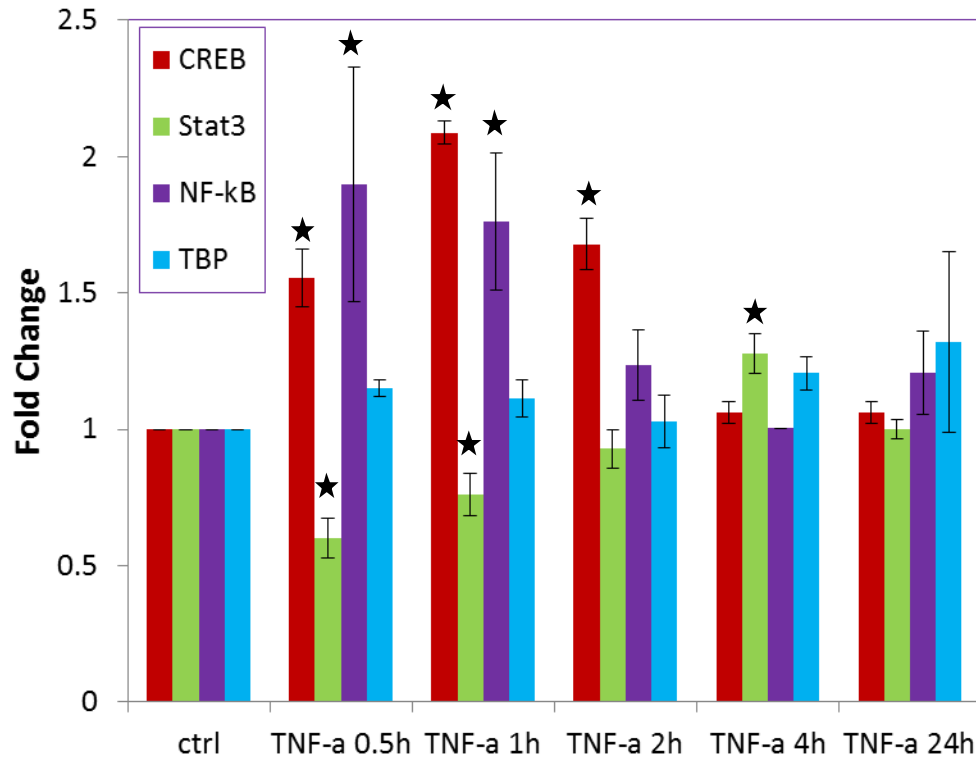
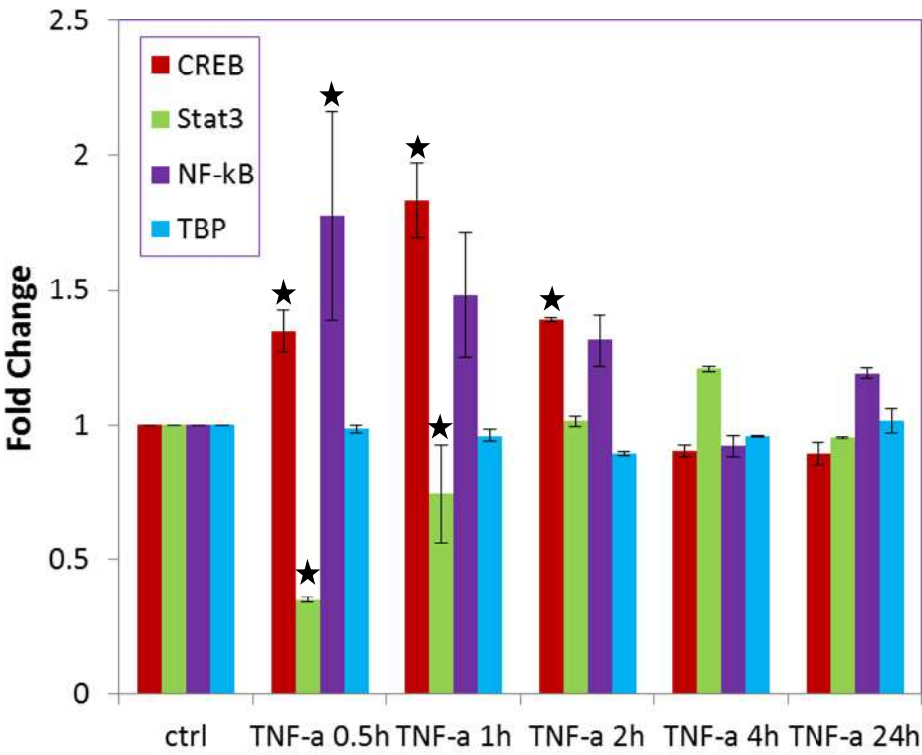


Figure 3.3: Quantification of TF levels in HepG2 nuclear extracts after TNF- α stimulation.

(A) Single TF detection by bead assay. The percentage of radiolabeled TF probe remaining on the beads (relative to signal that did not bind or was washed from the beads) was calculated. Fold changes relative to control are shown. (n = 3, * indicates p < 0.05). (B) Single TF detection by EMSA. The fractions of bound and unbound DNA probe were quantified and the fraction of signal in the bound was calculated relative to the total signal from the lane. Fold changes relative to control are shown. (n = 3, * indicates p < 0.05). (C) Correlation between detection by EMSA and scintillation. All data points obtained with EMSA and scintillation methods were graphed and correlation analysis was done. (D) Parallel TF detection by bead assay. Signals were normalized with respect to an internal standard and then the ratio with respect to control was calculated. (n = 3, * indicates p < 0.05). (E) Correlation between detection by parallel PCR and scintillation. All data points obtained with two different methods were graphed and correlation analysis was done.

Figure 3.3 (cont'd).

B:



C:

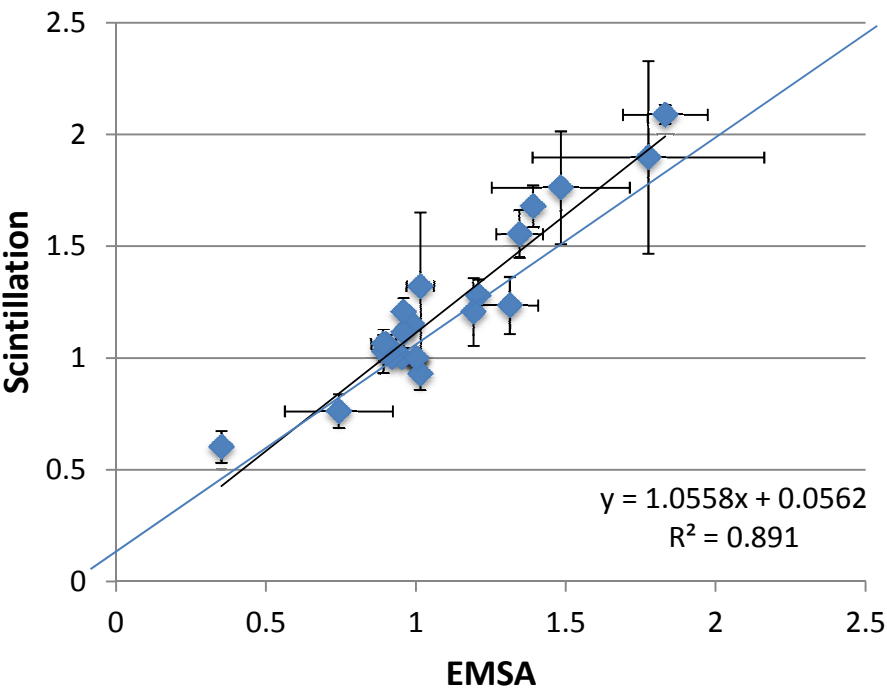
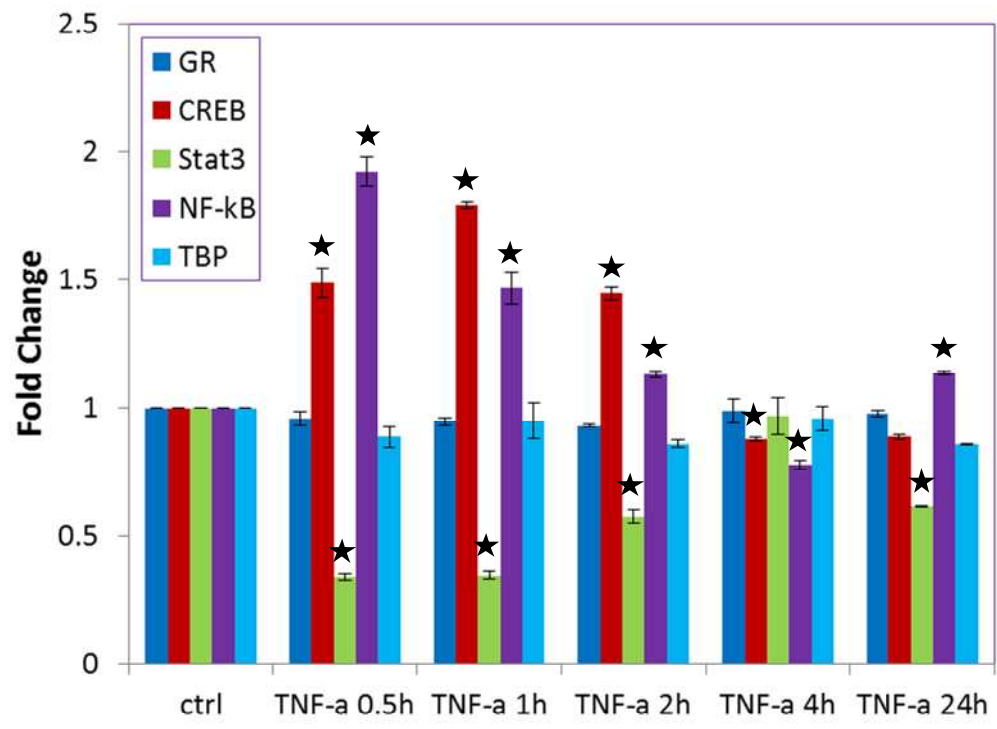
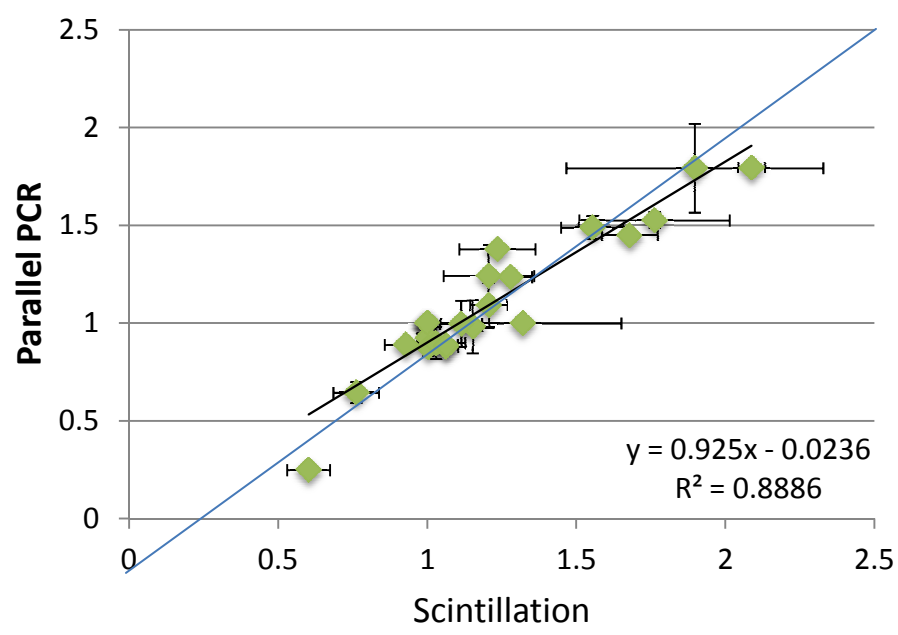


Figure 3.3 (cont'd).

D:



E:



In hepatocytes, TNF- α has been shown to initiate several different responses, including cytotoxicity (Ding et al., 2004). However, hepatocytes are capable of resisting the cytotoxic effects of TNF- α by activating NF- κ B (Tilg et al., 2000). Additionally, recent studies have suggested that TNF- α signaling can activate the IKK/JNK pathway (Wullaert et al., 2006; Zhan et al., 2011), which, in turn, enhances CREB activity (Johnson et al., 2002; Clarke et al., 2010). Both NF- κ B and CREB activate transcription of anti-apoptotic and cell proliferation genes (Park et al., 2005; Li et al., 2002). In line with this possible defensive response, we found rapid activation of both TFs by TNF- α .

The reduced nuclear levels of Stat3 may suggest a rapid export/degradation of nuclear Stat3 or a reduction in the rate of translocation from the cytoplasm to the nucleus. It is known that Stat3 localized to the cytoplasm may interact with protein kinase R (PKR) and inhibit its phosphorylation activity (Shen et al., 2012). PKR inhibits translation initiation and induces apoptosis via the FADD-dependent Caspase 8 pathway (Gil et al., 2000). Thus, lower levels of Stat3 in the nucleus, if accompanied by concomitant increases in cytoplasmic Stat3, may suggest a possible anti-apoptotic effect mediated by PKR repression.

For additional validation of our measured responses, we also compared the TF measurements to western blotting of each TF, with qualitative but not quantitative agreement (Figure 3.4). Western blotting is a technique that measures the total quantity of TF protein, while our approach and EMSA specifically measure the quantity of active

TF (as defined by its ability to bind its dsDNA recognition sequence). Since western blotting consistently showed elevated signals relative to the other approaches, this suggests that either TFs are being inactivated during sample preparation, which seems unlikely given the activity of the remaining proteins, or that a large fraction (~50%) of the TF molecules that are present in the nucleus are inactive prior to destruction or trafficking out of the nucleus (Darnell 2002). Our data also demonstrate how important kinetic measurements of TF levels can be to understanding biological processes. For our TFs, measurements at 24 h alone would have shown no response, hiding the early events in response to the stimulus that likely contribute to downstream changes in cellular function.

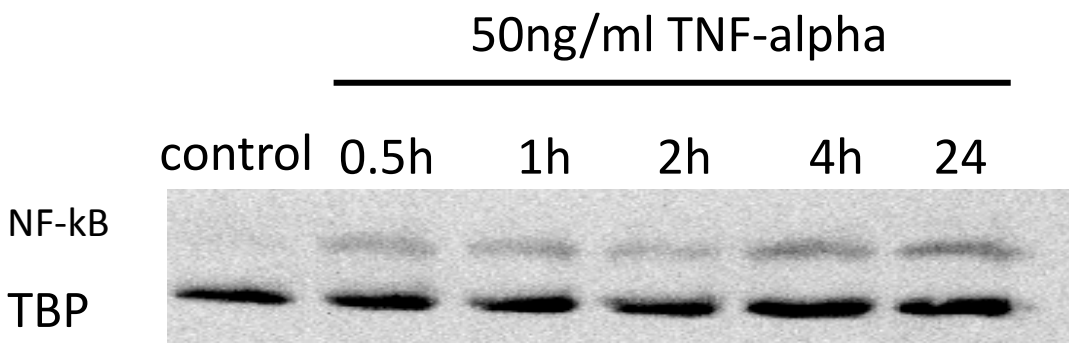
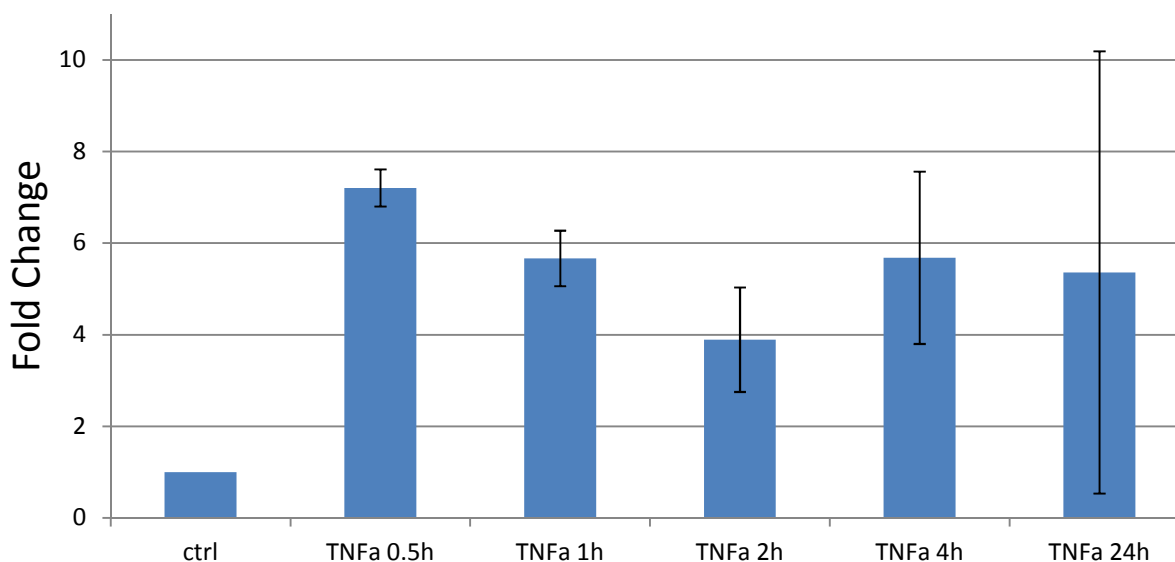


Figure 3.4: Western analysis of NF- κ B levels in TNF- α treated HepG2 cells. Representative of western blot image and quantification of NF- κ B levels from Western blot images.

Figure 3.4 (cont'd).



3.4.3 TF measurements in HepG2 cells stimulated with palmitic acid

In recent years, we and others have focused on the cytotoxic effects of FFAs on hepatocytes (Li et al., 2007; Wang et al., 2011). However, the signaling pathways associated with palmitic acid (PA) exposure are not well-established. Thus, we wanted to address which TFs of our analytical set are involved in the cellular response to PA exposure. Prior work had identified a role for NF- κ B and CREB (Cho et al., 2013). Our particular interest in GR is two-fold. First, GR was shown to increase activated CREB levels (Zhu et al., 2009), and CREB can act with GR to regulate genes where their binding sites are within 90 bp of each other (Imai et al., 1993). As above, TF expression levels were measured by scintillation counting or EMSA for individual TFs, or PCR for TFs in parallel (Figure 3.5). Our data consistently show that NF- κ B activity increased and reached a maximum at 30 min, then returned to baseline by 2 h, with a similar

pattern observed with CREB. As with our TNF- α studies, nuclear Stat3 levels decreased by 30 min and returned to basal levels by 2 h. Importantly, GR levels were increased by PA treatment, peaking at 1 h. TBP levels were unchanged as expected.

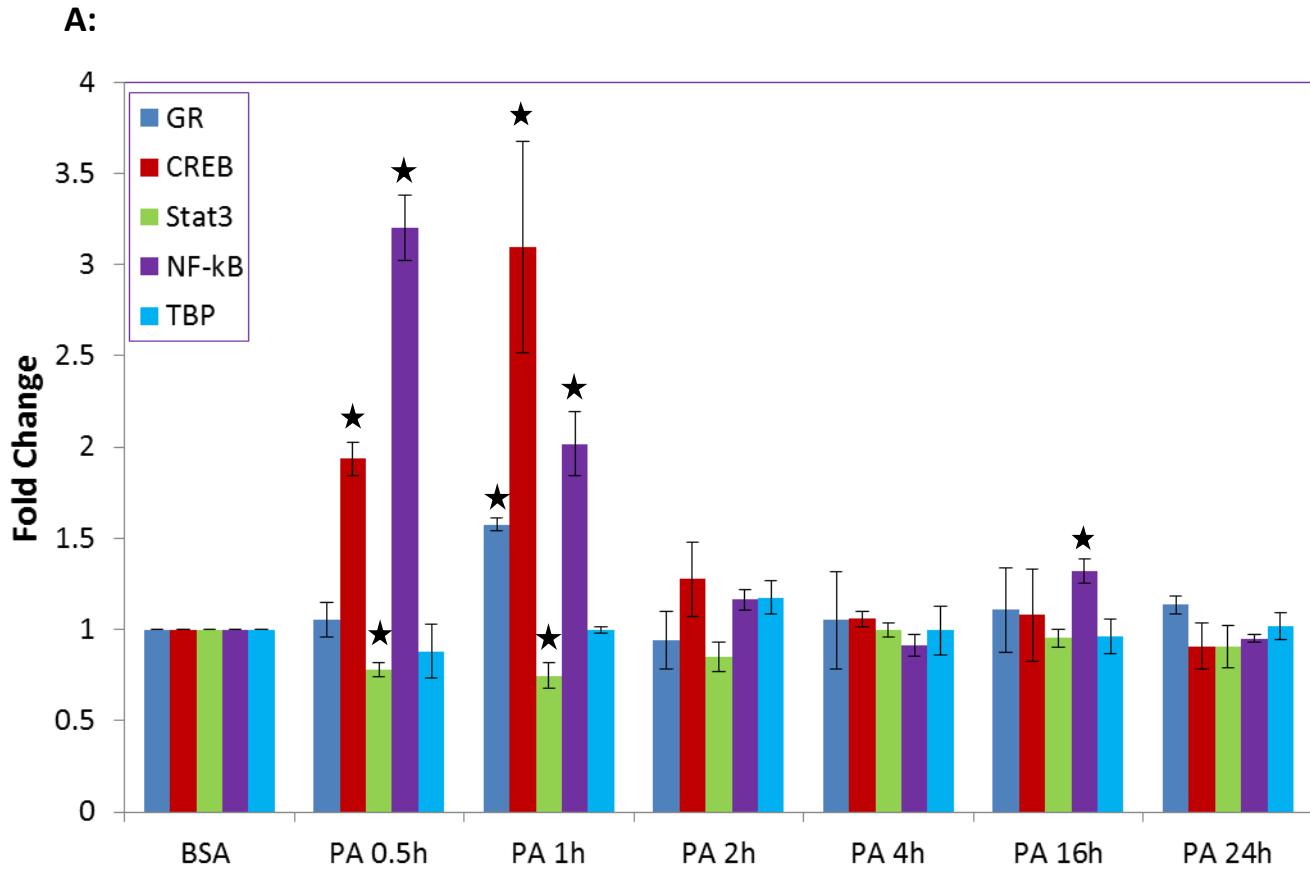


Figure 3.5: Quantification of TF levels in HepG2 cells' nuclear extracts after Palmitic Acid treatment. (A) Single TF detection by bead assay. The percentage of radiolabeled TF probe remaining on the beads (relative to signal that did not bind or was washed from the beads) was calculated. Fold changes relative to control are shown. (n = 3, * indicates p < 0.05). (B) Single TF detection by EMSA. The fractions of bound and unbound DNA probe were quantified and the fraction of signal in the bound was calculated relative to the total signal from the lane. Fold changes relative to control are shown. (n = 3, * indicates p < 0.05). (C) Correlation between detection by EMSA and scintillation. All data points obtained with EMSA and scintillation methods were graphed and correlation analysis was done. (D) Parallel TF detection by bead assay. Signals were normalized with respect to an internal standard and then the ratio with respect to control was calculated. (n = 3, * indicates p < 0.05). (E) Correlation between detection by parallel PCR and scintillation. All data points obtained with two different methods were graphed and correlation analysis was done.

Figure 3.5 (cont'd).

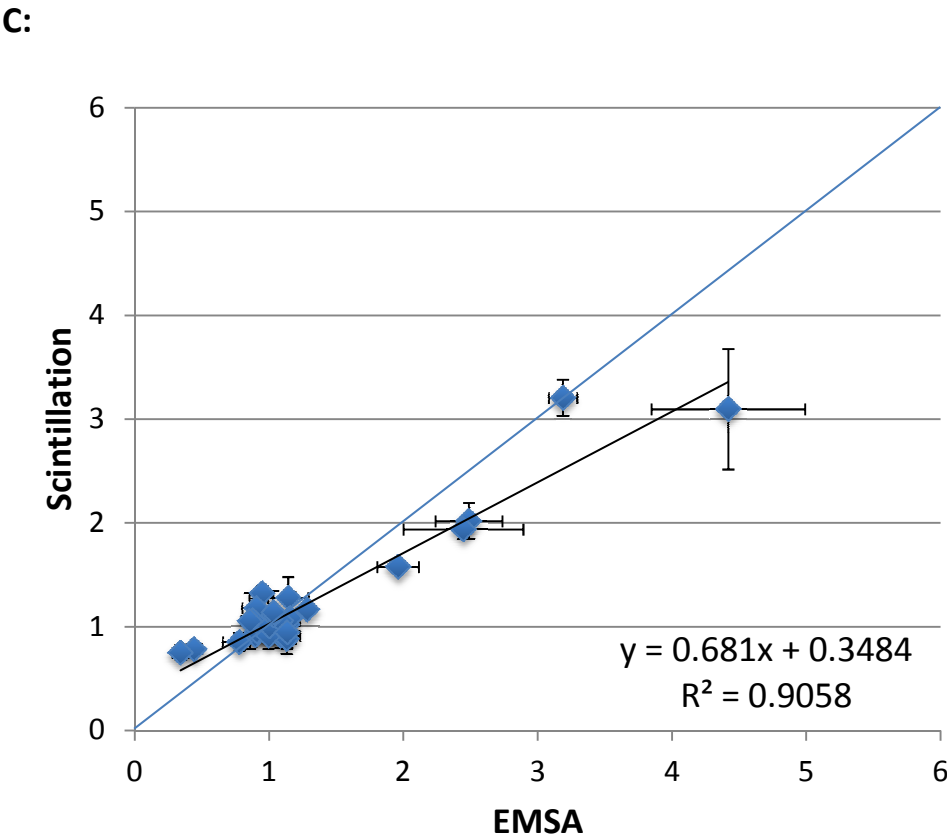
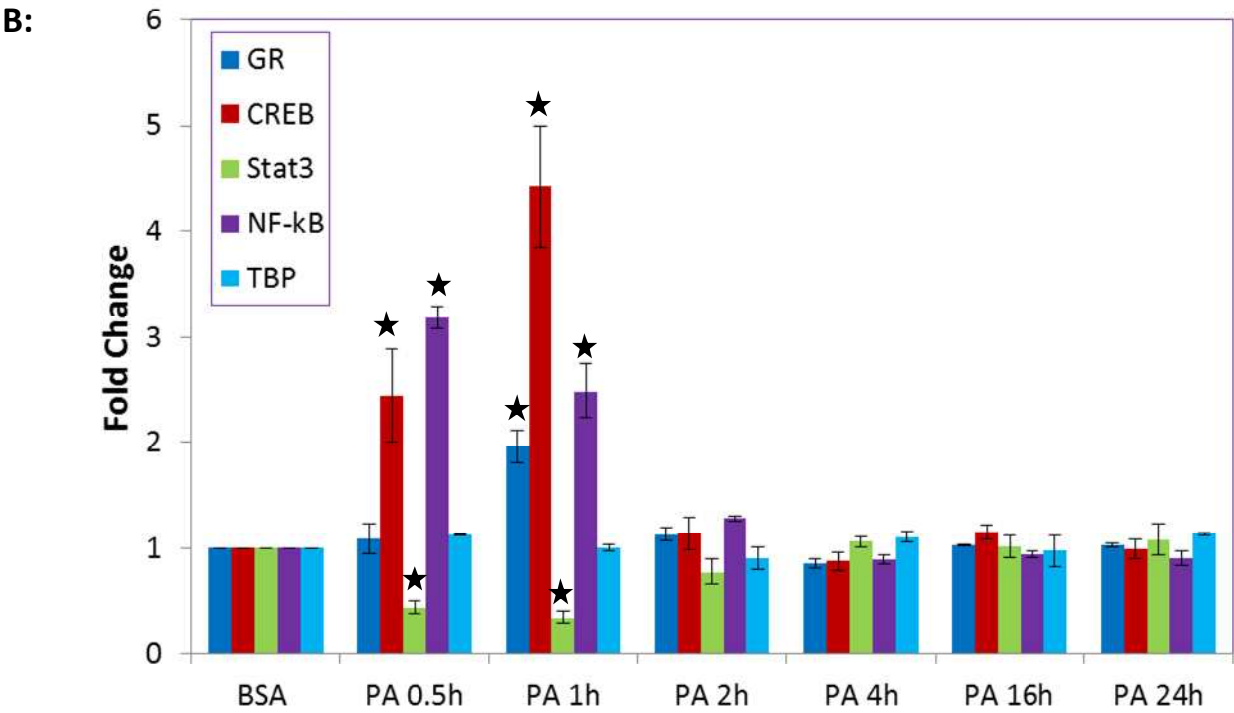
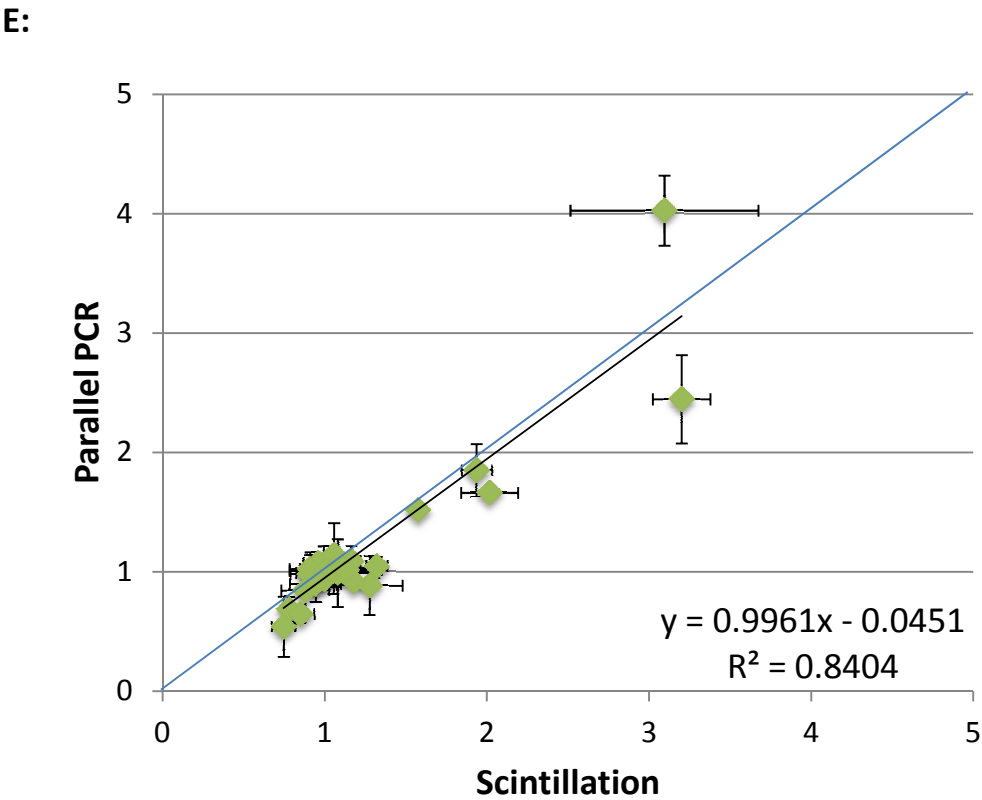
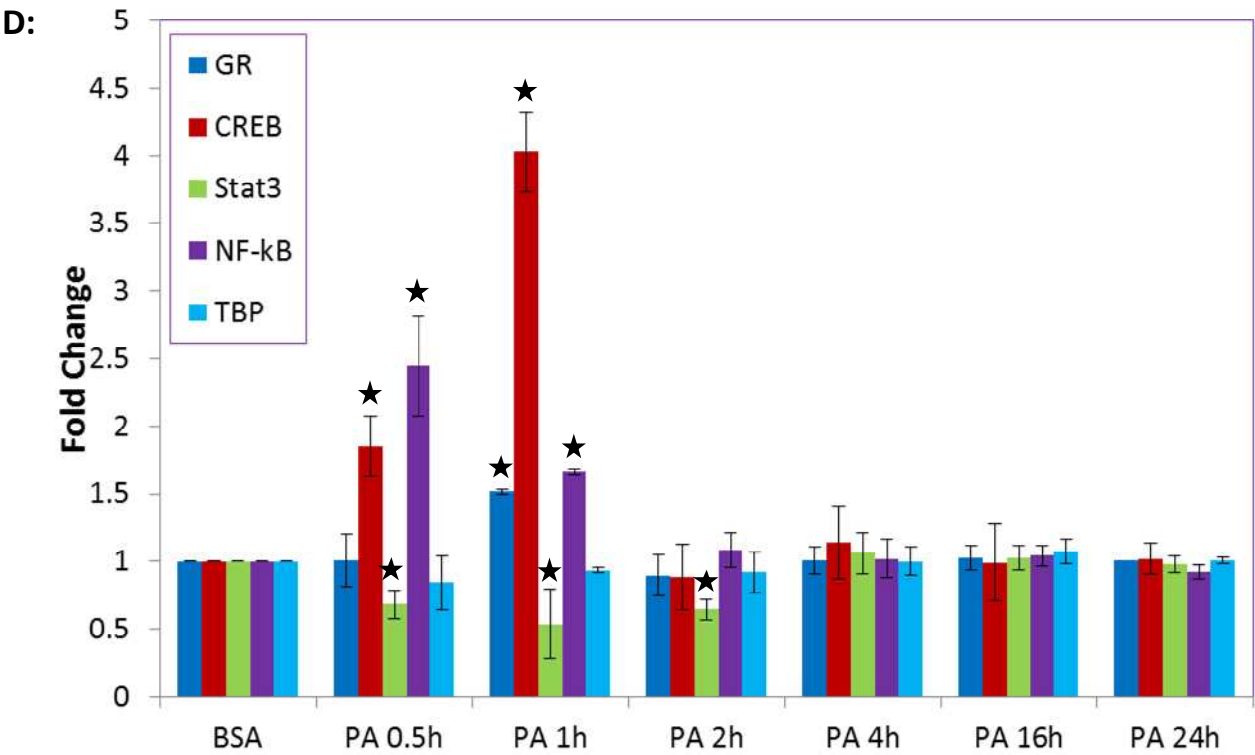


Figure 3.5 (cont'd).



These data show that HepG2 cells respond to PA treatment by dynamic changes in the trafficking of these TFs into or out of the nucleus. Of the TFs we measured, NF- κ B was most rapidly activated, followed soon after by CREB and GR. The increased activities of NF- κ B and CREB in response to elevated free fatty acids are in agreement with prior studies (Cho et al., 2013). Given that elevated levels of PA are cytotoxic to HepG2 cells (Unger et al., 2002), this response suggests that NF- κ B and CREB are activated to initiate an anti-apoptotic response to this stimulus, as in the TNF- α experiments.

The NF- κ B, CREB and Stat3 responses of HepG2 cells to palmitic acid and TNF- α are similar, indicating that similar pathways are activated by each stimulus. It has already been shown that circulating and liver levels of TNF- α are elevated in non-alcoholic fatty liver disease (NAFLD) (Bugianesi et al., 2007; Valenti et al., 2002). Additionally, free fatty acids have been shown to elevate TNF- α expression (Feldstein et al., 2004). It is unclear if TNF- α signaling is associated with the response to PA that we have measured, given the short time scales over which we detect significant changes in TF levels. It may be that the similarity in the responses is related to existing crosstalk between the two pathways.

Intriguingly, GR responds differently between the TNF- α and PA stimulated experiments. Currently, no data support a role of GR in mediating PA-induced cytotoxicity. However, it has been shown that elevated levels of glucocorticoids may be

associated with pathogenesis of NAFLD (Vegiopoulos et al., 2007). Also, in previous studies, GR has been shown to significantly enhance activated CREB levels (Cho et al., 2013), with both being increased in our results. GR and CREB are known to synergistically activate expression of certain genes, e.g., phosphoenolpyruvate carboxykinase (PEPCK), somatostatin, and even GR itself (Imai et al., 1993; Liu et al., 1994; Govindan et al., 2010). Importantly, activation of PEPCK by palmitic acid was observed with HepG2 cells previously (Gao et al., 2010). It has also been shown that there is crosstalk between GR and CREB in regulating neuronal genes (Focking et al., 2003). Taken together, our results and the literature suggest that genes regulated by GR (and perhaps particularly those co-regulated by CREB and GR) might be important in the cellular response to saturated fatty acid (palmitic acid) exposure.

In this work, we have shown that our assay can be applied to measure TF levels in parallel over time and is useful for the analysis of TFs involved in cytokine and fatty acid treatments of cultured cells. We believe that our assay can be used to profile other TF pathways and to study early cellular responses. We recognize that we only measured a limited number of TFs in parallel and only over a 24 h time period. Subsequent work and redesign of the technique will focus on inclusion of late-acting TFs, whose expression level changes would be expected after 8 h. This combination of early-acting and late-acting TFs would considerably enhance the information provided by the technique in analyzing biological systems. Combined with other parallel analytical techniques, our approach would contribute to a more complete picture of TF regulation and signaling in response to a variety of stimuli.

3.5 CONCLUSIONS

We have dynamically measured TF levels in response to cytokine and palmitic acid treatments by using our previously developed assay. With our assay, NF- κ B, CREB, Stat3, GR and TBP levels were successfully analyzed in a time-course. Our assay can be further improved to be used in profiling of larger sets of TFs, and it can be helpful to gain more insights on exploring regulation mechanisms, obtaining pathway mapping and modeling dynamic networks.

REFERENCES

REFERENCES

- Al-Quobaili F, Montenarh M (2008) Pancreatic duodenal homeobox factor-1 and diabetes mellitus type 2 (review). *Int. J. Mol. Med.* 21 (4): 399–404
- Awad S, Chen J (2014) Inferring transcription factor collaborations in gene regulatory networks. *BMC Systems Biology* 8(Suppl 1):S1
- Balaskas N, Ribeiro A, Panovska J, Dessaud E, Sasai N, Page KM, Briscoe J, Ribes V (2012) Gene regulatory logic for reading the Sonic Hedgehog signaling gradient in the vertebrate neural tube. *Cell* 148: 273–284
- Babu MM, Luscombe NM, Aravind L, Gerstein M, Teichmann SA (2004) Structure and evolution of transcriptional regulatory networks. *Curr Opin Struct Biol* 14:283-291.
- Babu MM (2010) Structure, evolution and dynamics of transcriptional regulatory networks. *Biochemical Society Transactions* 38: 1155-1178
- Bilgin B, Liu L, Chan C, Walton SP (2013) Quantitative, solution-phase profiling of multiple transcription factors in parallel. *Analytical and Bioanalytical Chemistry* 408(8): 2461-2468
- Bugianesi E (2007) Non-alcoholic steatophepatitis and cancer. *Clinics in Liver Disease* 11(1):191-207
- Calkhoven CF, Ab G (1996) Multiple steps in the regulation of transcription-factor level and activity. *Biochemical Journal* 312(2): 329-342
- Chen X, Xu H, Yuan P, Fang F, Huss M, Vega VB, Wong E, Orlov YL, Zhang W, Jiang J (2008) Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. *Cell* 133: 1106–1117
- Cho H, Wu M, Zhang L, Thompson R, Nath A, Chan C (2013) Signaling dynamics of palmitate-induced ER stress responses mediated by ATF4 in HepG2 Cells. *BMC Systems Biology* 7(9)
- Clarke DL, Clifford RL, Jindarat S, Proud D, Pang LH, Belvisi M, Knox AJ (2010) TNF alpha and IFN gamma synergistically enhance transcriptional activation of CXCL10 human airway smooth muscle cells via Stat-1, NF-kappa B and the transcriptional coactivator CREB-binding protein. *Journal of Biological Chemistry* 285(38): 29101-29110
- Clevenger CV (2004) Roles and Regulation of Stat Family Transcription Factors in Human Breast Cancer. *American Journal of Pathology* 165 (5): 1449-1460

- Courtois G, Gilmore TD (2006) Mutations in the NF-kappaB signaling pathway: implications for human disease. *Oncogene* 25: 6831–6843
- Darnell JE (2002) Transcription factors as target for cancer therapy. *Nature Reviews* 2: 740-49.
- Davidson EH, Levine MS (2008) Properties of developmental gene regulatory networks. *Proc. Natl. Acad. Sci.* 105: 20063–20066
- Dekelbab BH, Witchel SF, DeFranco DB (2007) TNF- α and glucocorticoid receptor interaction in L6 muscle cells: A cooperative downregulation of myosin heavy chain. *Steroids* 72: 705-712
- Ding WX, Yin XM (2004) Dissection of the multiple mechanisms of TNF-alpha-induced apoptosis in liver injury. *J Cell Mol Med.* 8: 445-54
- Elsir T, Smits A, Lindstrom MS (2012) Transcription factor PROX1: its role in development and cancer. *Cancer and Metastasis Reviews* 31 (3-4): 793-805
- Feldstein AE, Werneburg NW, Canbay A, Guicciardi ME et al. (2004) Free fatty acids promote hepatic lipotoxicity by stimulating TNF- α expression via a lysosomal pathway. *Hepatology* 40: 185–194
- Focking M, Holker I, Trapp T (2003) Chronic glucocorticoid receptor activation impairs CREB transcriptional activity in clonal neurons. *Biochemical and Biophysical Research Communications* 304: 720-723
- Gao D, Nong S, Huang X, Lu Y et al. (2010) The Effects of Palmitate on Hepatic Insulin Resistance Are Mediated by NADPH Oxidase 3-derived Reactive Oxygen Species through JNK and p38 MAPK Pathways. *J. Biol. Chem.* 285: 29965-29973
- Gil J, Esteban M (2000) Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): Mechanism of action. *Apoptosis* 5: 107-114
- Govindan MV (2010) Recruitment of cAMP-response element-binding protein and histone deacetylase has opposite effects on glucocorticoid receptor gene transcription. *J Biol Chem.* 285: 4489-510
- Hao N, O'Shea EK (2012) Signal dependent dynamics of transcription factor translocation controls gene expression. *Nature Structural and Molecular Biology* 19(1): 31-47
- Ideker T, Galitski T, Hood L (2001) A new approach to decoding life: Systems biology. *Annual Review of Genomics and Human Genetics* 2: 343-372

Imai E, Miner JN, Mitchell JA, Yamamoto KR et al.(1993) Glucocorticoid receptor-cAMP response element-binding protein interaction and the response of the phosphoenolpyruvate carboxykinase gene to glucocorticoids. *J. Biol. Chem.* 268: 5353–5356

Johnson GL (2002) Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and p38 Protein Kinases. *Science* 298(5600): 1911-1912

Jiang X, Roth L, Lai C, Li X (2008) Profiling activities of transcription factors in breast cancer cell lines. *Assay Drug Dev Technol.* 4(3):293–305

Karlebach G, Shamir R (2008) Modelling and analysis of gene regulatory networks. *Nat Rev Mol Cell Biol* 9:770-780

Lee KAW, Bindereif A, Green MR (1988) A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-messenger RNA splicing. *Gene Analysis Techniques* 5 (2):22-31

Levy D, Darnell JE Jr (2002) STATs: transcriptional control and biologic impact. *Nature Rev. Mol. Cell Biol.* 3, 651–662

Li XX, Stark GR (2002) NF kappa B-dependent signaling pathways. *Experimental Hematology* 30(4): 285-296

Li Z, Yang SX, Mittal S et al. (2007) A hierarchical approach employing metabolic and gene expression profiles to identify the pathways that confer cytotoxicity in HepG2 cells. *BMC Syst Biol.* 1

Liu JL, Papachristou DN, Patel YC (1994) Glucocorticoids activate somatostatin gene transcription through co-operative interaction with the cyclic AMP signalling pathway. *Biochem. J.* 301: 863–869

Liu X, Fagotto F (2011) A Method to Separate Nuclear, Cytosolic, and Membrane-Associated Signaling Molecules in Cultured Cells. *Science Signaling* 4 (203): p12
Maestro MA, Cardalda C, Boj SF, Luco RF, Servitja JM, Ferrer J (2007) Distinct roles of HNF1beta, HNF1alpha, and HNF4alpha in regulating pancreas development, beta-cell function and growth. *Endocr Dev* 12: 33–45

Nikolaidou-Neokosmidou V, Zannis VI, Kardassis D (2006) Inhibition of hepatocyte nuclear factor 4 transcriptional activity by the nuclear factor kappaB pathway. *Biochem J.* 398(3):439-50

Park JM, Greten FR, Wong A (2005) Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis- CREB and NF-kappa B as key regulators. *Immunity* 23(3): 319-329

Qiao J, Shao W, Wei HJ, Sun YM, Zhao YC, Xing WL, Zhang L, Mitchelson K, Cheng J (2008) Novel high-throughput profiling of human transcription factors and its use for systematic pathway mapping. *Journal of Proteome Research* 7 (7), 2769-2779

Regueira T, Lepper PM, Brandt S, Ochs M, Vuda M, Takala J, Jakob SM, Djafarzadeh S. (2009) Hypoxia inducible factor-1 α induction by tumour necrosis factor- α , but not by toll-like receptor agonists, modulates cellular respiration in cultured human hepatocytes. *Liver Int.* 29(10):1582-92.

Saliou C, Rihn B, Cillard J, Okamoto T, Packer L (1998) Selective inhibition of NF- κ B activation by the flavonoid hepatoprotector silymarin in HepG2: Evidence for different activating pathways. *FEBS letters* 440(1-2): 8-12

Schwabe RF, Brenner DA (2006) Selective inhibition of NF- κ B activation by the flavonoid hepatoprotector silymarin in HepG2: Evidence for different activating pathways. *Am. J. Physiol. Gastrointest. Liver Physiol.* 290(4):G583-9

Shaywitz AJ, Greenberg ME (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu. Rev. Biochem.* 68, 821–861

Shen Z, Peedikayil J, Olson GK, Siebert PD, Fang Y (2002) Multiple transcription factor profiling by enzyme-linked immunoassay. *Biotechniques* 32 (5): 1168-+

Shen S, Niso-Santano M, Adjemian S, Takehara T, Malik SA, Minoux H, Souquere S, Mariño G, Lachkar S, Senovilla L, Galluzzi L, Kepp O, Pierron G, Maiuri MC, Hikita H, Kroemer R, Kroemer G. (2012) Cytoplasmic STAT3 represses autophagy by inhibiting PKR activity. *Mol Cell.* 48(5):667-80

Tilg H, Diehl AM (2000) Cytokines in alcoholic and nonalcoholic steatohepatitis. *N Engl J Med.* 343: 1467-76.

Unger RH, Orci L (2002) Lipoapoptosis: its mechanism and its diseases. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids* 1585: 202–212

Valenti L et al. (2002) Tumor necrosis factor α promoter polymorphisms and insulin resistance in nonalcoholic fatty liver disease. *Gastroenterology* 122(2): 274-280

Vegiopoulos A, Herzig S (2007) Glucocorticoids, metabolism and metabolic diseases. *Molecular and Cellular Endocrinology* 275(1-2): 43-61

Wang X, Nath A, Yang X, Portis A et al. (2011) Synergy Analysis Reveals Association between Insulin Signaling and Desmoplakin Expression in Palmitate Treated HepG2 Cells. *PLoS ONE* 6: e28138

Whiteside ST, Goodbourn S (1993) Signal transduction and nuclear targeting: regulation of transcription factor activity by subcellular localisation. *Journal of Cell Science* 104 (4): 949–55

Wu M, Liu L, Chan C (2011) Identification of novel targets for breast cancer by exploring gene switches on a genome scale. *Bmc Genomics* 12:19

Wullaert A, Heyninck K, Beyaert R (2006) Mechanisms of crosstalk between TNF-induced NF-kappaB and JNK activation in hepatocytes. *Biochem. Pharmacol.* 72(9):1090-101

Zhan S, Rockey DC (2011) Tumor necrosis factor α stimulates endothelin-1 synthesis in rat hepatic stellate cells in hepatic wound healing through a novel IKK/JNK pathway. *Experimental Cell Research* 317(7): 1040-1048

Zhu XO, Yang Z, Guo CM, Ni XT et al. (2009) Paradoxical Stimulation of Cyclooxygenase2 Expression by Glucocorticoids via a Cyclic AMP Response Element in Human Amnion Fibroblasts. *Mol. Endocrinol.* 23: 1839-1849

CHAPTER 4 CONCLUSIONS AND FUTURE WORK

4.1 CONCLUSIONS

The work presented here was targeted at developing a new technology for parallel, quantitative analyses of transcription factors. The utility and scalability of the approach rely on changing the protein readout to a DNA readout and taking the advantage of easy high-throughput detection of DNA.

The biggest challenge, separation of TF-bound DNA from free DNA, was successfully solved by immobilizing TFs on magnetic beads. Previously separations based on size exclusion, gel extraction, and hydrophobicity were tried; however, due to the unique properties of each TF and the similar size of DNA probes to TFs, none of these techniques succeeded completely.

The sensitivity of the DNA readout was increased by PCR amplification. Thus, changing the protein signal to the DNA signal provided better sensitivity and ease of qualitative and quantitative analyses. Sensitivity of the current techniques was compared based on the number of cells used to detect the TFs in parallel. For clinical studies, the smaller the sample size, the more convenient it is to apply the analysis. Thus, sensitive detection in small sample sizes is essential; in our case, 10^5 cells were enough, a number easily obtained from *in vivo* samples.

Furthermore, the assay was applied to temporal detection of TF levels in different cell types and in response to different stimuli. It was proven that the technique can be successfully used for simultaneous, parallel detection of TFs, and it has the potential for further application in various types of cells and stimuli. Kinetic measurements of TF levels demonstrated that dynamic analysis of TF levels is essential to capture the breadth of valuable information about cellular processes. The TFs assayed in our system were early-response TFs, thus changes in their activity profiles were within hours, sometimes sooner. To analyze late-response TFs, longer treatment times than those studied here would need to be measured.

4.2 FUTURE WORK

4.2.1 Continued assay development

Further development of the assay will be necessary to increase the performance of the system. In particular, design of the DNA probes will have to be performed carefully when expanding the number of TFs being assayed to avoid cross reactivity and to obtain better sensitivity. Moreover, the total concentration of the DNA probes added to each sample will be increased, which also has the potential to increase the frequency of non-specific interactions among the various DNA sequences. With the simplicity of adjusting the DNA probe concentrations in our solution-phase assay, as opposed to an array-based assay, continued development of the optimal conditions for DNA probe design and quantity will improve the technique for better TF profiling.

The design of DNA probes can be based on the current and expanding data of TF binding sites, which can be obtained from JASPAR (Mathelier et al., 2014). JASPAR is the largest open access database of matrix-based nucleotide profiles. Binding preferences of TFs from multiple species are defined in the database, mainly derived from chromatin immunoprecipitation-seq experimental datasets in the literature. The fifth major release of the database in 2014 greatly expanded and updated the quantity of TF binding-site information available.

That said, perhaps the most critical factor when expanding the number of TFs measured in parallel is the fidelity of the interaction between the protein and its consensus binding sequence. It is especially important to differentiate among TFs in the same family that might have similar binding sequences and therefore have a higher likelihood of crossreacting. To avoid the misinterpretation of data, it will be important, as with any parallel technique, to confirm findings with a more specific secondary approach such as western blotting, ELISA, or other antibody based detection.

For the number of TFs quantified in this work, a parallel readout by electrophoresis was sufficient to demonstrate the potential of the technique to measure TFs in parallel; however, to achieve our desired scale, it will be essential to combine our technique with high-throughput DNA readout technologies, such as parallel sequencing or microarrays. Multiple high-throughput DNA analytical technologies are relatively mature at this point, with researchers having made a substantial focus on increasing sensitivity and quality

while decreasing costs of the assays (Pareek et al., 2011). Additional contributions from complementary technologies, like microfluidics, will likely further enhance the analytical capacity of these readouts (Guo et al., 2012). We expect the flexibility of our approach to make it feasible to apply any of these known and as yet unknown readout technologies to the analysis of our recovered TF probes.

Once our technology is optimized for broadly parallel analyses and designed to leverage high-throughput DNA technologies, it will be still more useful for dynamic profiling of TFs in different cell types and in response to different signals. Data generated by this new technology in various conditions will enable scientists to better understand cellular processes in response to stimuli, leading to disease diagnoses, development of novel therapeutics, and mapping of cellular process pathways. For example; identification and quantification of TFs in a complex disease like Alzheimer's may lead to understanding of disease progression. This will further contribute to early diagnosis of the disease as well as monitoring desired and adverse effects of the therapy (Jiang et al., 2013).

4.2.2 Alternative methods

In addition, other proteomics techniques can be applied to TF profiling studies. Currently, methods based on mass spectrometry are greatly expanding and accelerating the available proteomic information (Michalski et al., 2011; Choudhary et al., 2010; Nilsson et al., 2010). Combining MS with other instruments like LC has further

improved the quality of the data obtained. However, at this point, due to the relatively low concentrations of TFs, MS-based assays are not applicable to TF profiling research (Zhang et al., 2007). Once current challenges like increasing signal/noise ratio in identifying and sequencing peptides, detecting and quantifying specific peptides, and increasing throughput, are resolved, these techniques will be more directly applicable for TF measurements.

In vivo molecular imaging also has potential utility for TF profiling. TF binding probes that can be delivered in nanoparticles to the nuclei of target cells could be visualized by fluorescence, chemiluminescence, radioactivity, or other labeling methods. These methods will enable researchers to track the molecular events of TF function to better understand the dynamics of signals leading to disease progression as well as their cellular localization. This kind of application would require high-affinity/high-specificity reagents with appropriate imaging groups and high-resolution imaging tools. Such methods could be applied to TF monitoring studies; however, their application would be limited by the number of molecules that could be imaged at one time. Once high-throughput imaging techniques are well established, this type of method could be applied to dynamic, parallel, *in vivo* TF profiling.

The other area that will be improved in the future is computational and mathematical models for understanding TF regulation. The quantities of data obtained with all the experimental methods being developed will need to be turned into knowledge. For this,

technologies of computational sciences that integrate biosciences with bioinformatics are essential. Also, effectively modeling these data will enable prediction of unknown TF responses. Establishment of novel tools and algorithms will identify connections of TF profiles with different diseases, leading to personalized medicine applications. One example of these kinds of tools is discrete dynamic modeling (Wu et al., 2009). This model is constructed based on the relationship of regulators in a network and each component in the network is assigned with three potential states (0: lower than control, 1: control, 2: higher than control level). The network is simulated by regulatory relationships of the network and their transitions from one state to another. By this, potential TFs regulated by specific stimuli can be modeled. Application of this model on identification of active TFs in multiple disease types could give potential TFs profile and data obtained from the model combined with experimental data could give insights on disease mechanisms and pathways.

4.2.3 Alternative applications

In addition, this new technology which is capable of detecting protein-DNA interactions in parallel is suited not only to TF detection but also to measurements of all other DNA-binding proteins. Likewise, the analysis can be inverted to study the binding preferences of a given TF. By immobilizing the protein of interest followed by mixing with pool of DNA, binding partners of the protein can be identified and their affinities can be measured specifically. The sequences and quantities/frequencies of the bound DNAs could then be analyzed by parallel DNA sequencing technologies.

4.2.4 Remaining Questions

Our assay's application on TF pathway analysis of HepG2 cells stimulated with free fatty acids has identified and dynamically quantified some TFs that are important in free fatty acid signaling. However, there are still more TFs that wait to be analyzed with high-throughput technologies. Additionally, there are TFs that are regulated by multiple pathways like those associated with insulin resistance or obesity. These two factors are already indicated as being risk factors for liver cancer. Further in our studies, we would like to model all TFs that are potential regulators of these factors and experimentally evaluate them with our assay. We could then potentially construct a novel depiction of the mechanism of free fatty acid signaling and define suitable TF candidates for targeted therapy.

TFs are also key effectors in development. Developmental progression is controlled by specific TFs organized spatially and temporally, giving rise to appropriately patterned embryos. There is an increasing number of studies trying to understand how combinations of TFs regulate gene expression during development leading to correct patterns of gene expression. Application of our assay to developmental studies could provide complementary information to define which, where, and when TFs act. The data generated could provide baseline information useful for correcting developmental abnormalities.

Overall, the work presented here were not ideal, still needing to be optimized and incorporated with high-throughput readout technologies. With continued improvements and customization to particular TFs, the assay can mature to a flexible, quantitative, parallel TF measurement platform. The advantages of better sensitivity, no manipulation of cells prior to analysis, and high selectivity, make this technique a convenient tool for analysis of cellular processes. Data obtained with our technique and other assays will link between TF signaling and phenotypes and their dynamic changes in different cells and tissues in response to different stimuli. Understanding TF signaling pathways is key to understanding biological processes. That said, the data generated is complex, and analyzing it is challenging. Computational tools and models will be essential to understand complex disease prognosis and progression, drug design, drug screening, and biomarker identification. These applications will enable early diagnosis of diseases, disease monitoring, and, in turn, development of true personalized treatments.

REFERENCES

REFERENCES

- Choudhary C, Mann M (2010) Decoding signalling networks by mass spectrometry-based proteomics. *Nature reviews Molecular cell biology*, 11:427-439
- Guo MT, Rotem A, Heyman JA, Weitz DA (2012) Droplet microfluidics for high-throughput biological assays. ***Lab Chip***, 12: 2146-2155
- Jiang W, Zhang Y, Meng F, Lian B, Chen X, et al. (2013) Identification of active transcription factor and miRNA regulatory pathways in Alzheimer's disease. *Bioinformatics*, 29: 2596–2602
- Mathelier A, Zhao X, Zhang AW, Parcy F, Worsley-Hunt R, Arenillas DJ, Buchman S, Chen CY, Chou A, Ienasescu H, Lim J, Shyr C, Tan G, Zhou M, Lenhard B, Sandelin A, Wasserman WW (2014) Jasp 2014: an extensively expanded and updated open-access database of transcription factor binding profiles. *Nucleic Acids Res.*, 42 (D1):D142–D147
- Michalski A, Damoc E, Hauschild JP, Lange O, et al. (2011) Mass spectrometry-based proteomics using Q Exactive, a high-performance benchtop quadrupole Orbitrap mass spectrometer. *Molecular and Cellular Proteomics*, 10 (9):M111.011015
- Nilsson T, Mann M, Aebersold R, Yates JR, Bairoch A, Bergeron JJM (2010) Mass spectrometry in high-throughput proteomics: ready for the big time. *Nature Methods*, 7(9): 681-685
- Pareek CS, Smoczynski R, Tretyn A (2011) Sequencing technologies and genome sequencing. *Journal of Applied Genetics*, 52 (4): 413-435
- Wu M, Yang XR, Chan C (2009) A Dynamic Analysis of IRS-PKR Signaling in Liver Cells: A Discrete Modeling Approach. *PLoS One* 4(12):e8040
- Zhang L, Kasif S, Cantor AC (2007) Quantifying DNA-protein binding specificities by using oligonucleotide mass tags and mass spectroscopy. *Proc. Natl. Acad. Sci.* 104(9): 3061-3066