UNIVERSITY OF CALIFORNIA, SAN DIEGO

Cancer, Cell Fate, and Transcription: Regulation of the p53 Transcriptional Response by Structurally Diverse Core Promoters

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biological Sciences

by

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2010

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The dissertation of José Manuel Morachis is approved, and it is acceptable in quality and form for publication of microfilm and electronically:

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2010

DEDICATION

“The eye cannot see what the mind cannot comprehend”

-Unknown

This dissertation is dedicated to my wonderful mother Maria Magdalena, family members, and to my better half, Lorem Samantha.

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ACKNOWLEDGEMENTS

I will start from the beginning by thanking my high school biology teacher Mr. Robert Peterson. Mr. Peterson was able to teach me the basics of biology in a way that allowed me to comprehend concepts (not simply memorizing facts). His take home message was to understand the details but never forget to view the whole picture. Next I would like to acknowledge Dr. Ruben Alarcon who gave me my first opportunity to do research. He was inspiring and provided important guidance that persuaded me to pursue a research career. I thank Dr. Ernest Martinez for allowing me to pursue exciting and challenging research during my last two years of undergrad. The training I received from Dr. Martinez was essential for my Ph.D. research.

I would like to thank my thesis adviser Dr. Beverly Emerson for giving me the opportunity to join her laboratory. I take great pride in being one of the top two students to ever work in her lab. Bev provided an enviroment that allowed me to mature scientifically and professionally. I would like to acknowledge my committee members Jim Kadonaga, Lorraine Pillus, Kathy Jones, and Bing Ren.

I thank Chris Murawsky for providing excellent technical advice and support throughout my entire Ph.D. training. Chris was like a big brother, teaching me great techniques in lab but more importantly teaching me about beers from around the world. I thank Ruo Huang for her excellent assistance and collaboration. I thank my great friends and colleagues that have passed through the Emerson lab (Dave Liston, Daniela Ishimaru, Buddy Weissman, Mathias Kaeser, and Mark Zentner) and for those currently there (Chris Murawsky, Fernando Lopez-Diaz, Michael Witcher, Sri Kripa Balakrishnan, Michal Krawczyk, and Ruo Huang). I thank the never-ending battles of foosball with everyone in the lab, how can I ever forget. I would also like to acknowledge members

from the Kathy Jones lab for providing advice, reagents, and great friendship. I especially want to acknowledge my good friend Jose Sierra, who passed away this year. I am grateful to Wolfgang Fischer and Jessica Read of the Salk Institute Mass Spectrometry Core Facility for their identification of NF-Y. I thank Michael Witcher and other members of the Emerson lab for reviewing my manuscript and providing significant technical advice and reagents. I thank Dr. Cheng-Ming Chiang and his wife Shwu-Yuan (Sue) Wu for taking me into their laboratory for two weeks in Dallas. They provided their generous gifts of purified TBP and TFIID. I thank Eric Scheeff and Gerard Manning for a bioinformatics analysis of core promoters.

I thank all my classmates and close friends that made my last five years fun and interesting. I especially thank my friends Nate Heintzman, Will Alaynick, and Sara Mednick for being great friends and advising me through my Ph.D. maze. Nate and Will were true scholars and provided a nexus to ideas and activities that strengthened my graduate experience. I thank Chihunt Wong and Julia Clagget for collaborating with me to create BioEASI. I thank Rebecca Sanders, Larry Bock, and others for collaborating with me to create MySDscience and the Science Festival.

I would like to thank my family for being patient and very supportive. There are too many family members to thank but I would like to thank my mother Maria and my sister Gisela for being there for me whenever I needed them. I thank my Aunt Xochilt for being supportive and always believing in me. I thank Jeomar, Stephanie, and Luis Mario for sometimes joining me at the lab during weekends and listening to my advice. I thank my Grandma Vicenta for great dinners and support.

And finally I would like to thank Lorem Que, my fiancé, for being highly supportive during my 5 years in graduate school. I couldn't have done it without you.

Collaborators subject to authorship deriving from this work include:

Beverly M. Emerson, Ph.D.

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Chris Murawsky, Ph.D.

Jose M. Morachis, Ruo Huang, Beverly M. Emerson. "Identification and characterization of three kinase inhibitors that block transcription initiation of *p21* and *Fas/APO1*." (In preparation). Chapters 5 and 6.

Jose M. Morachis, Chris M. Murawsky, Beverly M. Emerson. "Regulation of the p53 transcriptional response by structurally diverse core promoters." (Genes & Development, in press). Chapters 1-4, and 6.

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Jennifer H Dao, Robert J M Kurzeja, **Jose M Morachis**, Henrike Veith, Jeffery Lewis, Violeta Yu, Christopher M Tegley, Philip Tagari (2009). "Kinetic characterization and identification of a novel inhibitor of hypoxia-inducible factor prolyl hydroxylase 2 using a time-resolved fluorescence resonance energy transfer-based assay technology." Anal Biochem 384(2): 213-23.

Awards and Honors

1. H.A. and Mary K.Chapman Scholar, 2009, Salk Institute
2. HHMI, Med into Grad Fellowship at UCSD's Moore's Cancer Center, 2008
3. National Research Service Award (NRSA) NIH Pre-Doctoral Fellowship, 2006 - 2009
4. National Science Foundation AGEP Pre-Doctoral Fellowship, 2004 - 2005
5. Amgen Inc. Summer Graduate Fellowship, 2004
6. Minority Access to Research Careers (MARC) undergraduate research Fellowship, 2002- 2004, University of California, Riverside.
7. Nathaniel T. Coleman Scholarship, 2003, UC Riverside
8. Summer Undergraduate Research Fellowship (SURF). 2002, Rockefeller University, NY
9. California Alliance for Minority Participation (CAMP) summer fellowship sponsored by the NSF and the University of California, Riverside, 2001
10. Summer Youth Intern; Kaiser Permanente (Fontana, CA). 2000

ABSTRACT OF THE DISSERTATION

Cancer, Cell Fate, and Transcription: Regulation of the p53 Transcriptional Response by Structurally Diverse Core Promoters

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University of California, San Diego, 2010

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The aim of my dissertation research was to understand the mechanisms underlying the transcriptional response to stress by the tumor suppressor protein p53 and and their influence on cell fate decisions. This was accomplished by several complementary experimental approaches that compared two p53-mediated transcriptional programs, cell cycle arrest and apoptosis. Since p53 is central to the

cellular response to stress signals, it is important to define the events that are involved during p53 activation of cell cycle arrest and pro-apoptotic genes. p53 target promoters are structurally diverse and display pronounced differences in RNA polymerase II (RNAP II) occupancy even in unstressed cells, with higher levels observed on cell cycle arrest genes (*p21*) compared to apoptotic genes (*Fas/APO1*). This occupancy correlates well with their ability to undergo rapid or delayed stress-induction. To understand the basis for such distinct temporal assembly of transcription complexes, the role of core promoter structures in this process was examined. My studies revealed that the *p21* core promoter directs rapid, TATA box dependent assembly of RNAP II pre-initiation complexes (PIC), but permits few rounds of RNAP II re-initiation. By contrast, PIC formation at the *Fas/APO1* core promoter is very inefficient but supports multiple rounds of transcription. A downstream element within the *Fas/APO1* core promoter is essential for its activation and binds NF-Y. It is known that NF-Y acts as a bi-functional transcription factor that regulates the expression of *Fas/APO1 in vivo*. Thus, two critical parameters of the stress-induced p53 transcriptional response are the kinetics of gene induction and duration of expression through frequent re-initiation. These features are intrinsic, DNA-encoded properties of diverse core promoters and may be fundamental to anticipatory programming of p53 response genes upon stress.

Analysis of the *p21* and *Fas/APO1* promoters by *in vitro* transcription resulted in the identification of three pharmacologic agents that should aid in further dissecting transcriptional mechanisms employed by p53 target genes and other genes regulated by RNAP II. Three kinase inhibitors (hypericin, rottlerin, and Sp600125), previously not associated with inhibiting transcription, were discovered to block transcription initiation efficiently.

Chapter 1

Background and Introduction

Background:

Cells are constantly being attacked by internal and external stresses and therefore several pathways have evolved to keep them, and ultimately organisms, in homeostasis. Two important cellular events that respond to stress are cell cycle arrest and programmed cell death. Both of these processes are invaluable and help the cell deal with stressful events such as DNA damage. Upon stress, a cell must rapidly halt cell growth until homeostasis is achieved or the organism faces the possibility of acquiring dangerous mutations that could ultimately lead to tumouregenesis. If the damage is too severe, it must quickly shift its energies to shut itself down through programmed cell death (apoptosis). These processes must be poised for action but at the same time, be highly regulated such that aberrant cell cycle arrest or apoptosis does not occur. A strategy used by cells to manage this problem is via the multi-functional p53 protein.

p53, once termed the “Cellular Gatekeeper,” is responsible for the regulation of many cell cycle and pro-apoptotic genes and is mutated in over 50% of all cancers (1-3). Many factors influence cell fate “decisions” in response to stress, including: the severity and duration of certain stresses such as DNA damage, serum deprivation and hypoxia (4). The cell, through the action of proteins like p53, must somehow integrate signals from these various stresses to ultimately initiate appropriate transcriptional programs (Figure 1.1). How p53 is capable of surveying the cell and affecting its fate is only vaguely understood.

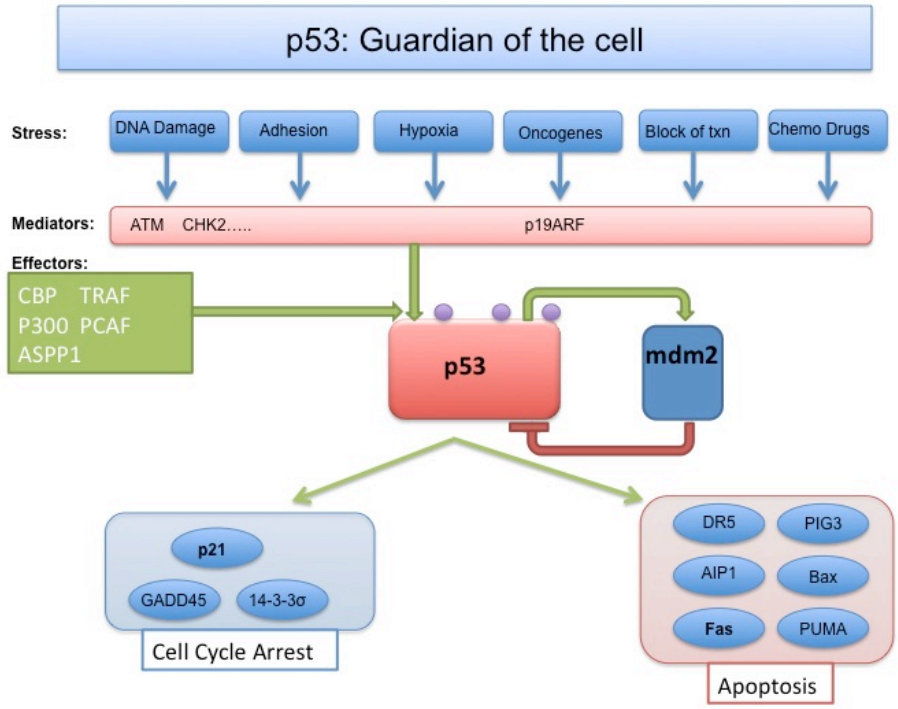

Figure 1.1 p53 summary diagram

A well-characterized p53 target gene involved in cell cycle arrest is *p21 (CIP1/WAF1/CDKN1A)* (5-6). In normal cells, *p21* functions by inhibiting cyclin-dependent kinases which prevent phosphorylation and subsequent inactivation of Rb. p53 activation of *p21* eventually arrests cell cycle progression at the G1/S boundary. It is believed that p53 uses p21 to arrest the cell cycle in response to stressful events to prevent the cell from duplicating any damaged DNA until it is repaired. Another well-studied p53 target gene is *Fas/APO1*; a member of the tumor necrosis factor receptor (TNFR) superfamily (7). *Fas/APO1* plays an important role in induction of apoptosis by chemotherapeutic agents and during immune surveillance (8)

Upon genotoxic stress p53 is post-translationally modified in a variety of ways that includes phosphorylation and acetylation. Under normal conditions, p53 is maintained at very low levels with the help of Mdm2 (an E3 ubiquitin ligase) that binds p53 and directs its degradation. Upon stress signaling, p53 is phosphorylated, released by Mdm2 and is no longer degraded by the 26S proteosome (9). Once p53 levels increase, a variety of transcriptional programs are initiated that depend on the severity and type of stress. The ability of p53 to function in numerous cellular events has been clearly demonstrated (10). However, the gene specific mechanisms utilized by p53 are still poorly understood. This is likely to be one of the reasons that the generation of therapeutic agents directed towards p53 and its target genes have been delayed. Only recently has progress been made to elucidate the biochemical interactions and properties of p53 at its target promoters in order to understand its role in influencing cell fate decisions in response to stress.

Introduction:

The ability of cells to undergo cell cycle arrest or apoptosis after acquiring malignant alterations is of fundamental importance to normal surveillance mechanisms that have evolved to prevent tumor progression. The tumor suppressor protein p53 is a critical component of this anti-tumor response by regulating diverse gene pathways that control cell cycle arrest, angiogenesis, DNA repair, senescence and apoptosis (11-13). Induction of cell cycle arrest (at G1-S) by p53 results mainly from transcriptional activation of *p21* (*CDKN1A*), leading to inhibition of cyclin-dependent kinase (CDK)-cyclin complexes and proliferating nuclear antigen (PCNA) (14-15). The molecular events that promote p53-dependent apoptosis are more complex and occur through activation of critical genes involved in the intrinsic mitochondrial apoptotic pathway

(*PUMA*, *APAF-1*, and *NOXA*) and the extrinsic death-receptor pathway (*Fas/APO1, KILLER/DR5*). The kinetics and magnitude of target gene expression after p53 activation varies considerably, with those involved in cell cycle control being expressed early while pro-apoptotic genes undergo delayed transcription (16).

A critical issue that remains to be elucidated is how p53 “chooses” which of its multiple target genes to activate or repress in response to a given stress. In this regard, an important source of p53 functional diversity that could contribute to selective gene regulation and cell fate choice resides within the core promoters of p53 target genes. The core promoter is defined as the DNA sequence required to direct accurate transcriptional initiation by the RNAP II complex. It contains the region around the initiation site and usually one or more conserved sequence motifs such as the TATA box, initiator (Inr), TFIIB recognition element (BRE), downstream promoter element (DPE), and downstream core element (DCE) which impose different regulatory requirements for transcription initiation (17,18) The series of regulatory events that direct the activity of p53 target promoters must ultimately relay through the basal RNAP II machinery. Thus, it is important to understand not only the relationship of p53 to the RNAP II complex but also how architectural diversity among its promoters affects this relationship and contributes to the overall stress-induced transcriptional program.

Previous studies have shown that different levels of RNA polymerase II (RNAP II) transcription pre-initiation complexes (PICs) are assembled on endogenous p53 target promoters even before stress-induction (19). These levels correlate with the timing of transcriptional activation during the stress response. The pro-cell cycle arrest gene *p21* contains high levels of RNAP II and other initiation components in unstressed cells and

is rapidly induced by DNA damaging agents. This is achieved by conversion of RNAP II to an elongating form through recruitment of elongation factors to distinct regions of the *p21* gene (19). By contrast, pro-apoptotic genes like *Fas/APO1* and *PUMA* contain very low levels of RNAP II and display delayed induction kinetics relative to *p21*. These genes are more likely to be controlled at the level of initiation. Interestingly, high levels of promoter-bound RNAP II complexes do not correlate with the duration of gene expression during the damage response since mRNA synthesis from pro-apoptotic genes can equal or exceed that of *p21*. Prolonged transcription after damage may depend, in part, on the efficiency of RNAP II re-initiation from specific promoters. In addition, p53 is required to assemble the RNAP II complex on the endogenous *p21* promoter before stress and to differentially recruit and retain specific initiation and elongation factors after distinct types of DNA damage *in vivo* (19,20)**.** However, since p53 has been shown to interact with both *p21* and pro-apoptotic genes before stress (19,21,22), the variation in promoter structure among these genes is likely to influence the composition and rate of assembly of promoter-specific RNAP II transcription complexes. This, in turn, affects whether activation of specific genes occurs with early or delayed kinetics and how long expression is sustained during the stress response.

In **Chapters 1-4**, I present my work investigating the role of core promoter architecture in directing the transcriptional kinetics of two functionally and structurally diverse p53 target genes, *p21* and *Fas/APO1* using biochemical and cell-based approaches. Remarkably, we find that differences in RNAP II affinity and re-initiation kinetics observed between the endogenous *p21* and *Fas/APO1* genes can be recapitulated *in vitro* using DNA templates in a manner dependent upon their respective core promoters. We find that the *p21* core promoter has a high intrinsic affinity for RNAP

II and rapidly assembles pre-initiation complexes *in vitro* whereas the opposite is observed for *Fas/APO1*. Although *Fas/APO1* has a low affinity for RNAP II, once a PIC is formed this promoter directs very efficient re-initiation of transcription in marked contrast to *p21,* which re-initiates poorly. Consequently, the affinity for RNAP II does not necessarily dictate prolonged gene expression through high re-initiation frequency and these two important aspects of transcription are independently regulated. Upon mutagenesis of the *p21* and *Fas/APO1* core promoters, we defined the TATA box in *p21* and a downstream element in *Fas* as key regulators of promoter-specific RNAP II activity. Additionally, we identified the bi-functional transcription factor NF-Y as a *Fas* downstream element-interacting factor and demonstrated that it plays a role in regulating basal expression of endogenous *Fas/APO1 in vivo*.

These studies support the notion that diverse core promoters among p53 target genes play a fundamental role in stress-induced cell fate decisions by regulating the kinetics of gene activation and the duration of expression through RNAP II re-initiation. Interestingly, these intrinsic features of distinct core promoter structures are “hard-wired” into their DNA and occur independently of chromatin, p53 or coactivators, which act to impose further levels of control. Thus, the programming of p53 target genes in unstressed cells to be “poised” by engaged RNAP II for rapid activation or “unpoised” for delayed, but sustained expression is, in part, genetically determined to anticipate how and when these genes will need to function in the stress response. This default, intrinsic programming can be modulated by epigenetic events and specific p53 and coactivator complexes that tailor gene activity and cell fate choices in a tissue- and stress-specific manner (23). Thus, diverse core promoter architecture provides another important

mechanistic level by which p53 coordinates a physiologically appropriate response to diverse stress conditions.

In **Chapter 5**, I present the results from my studies of small molecules that inhibit mRNA transcription with the aim to find new tools to study transcriptional regulation and potential therapeutics. Our current understanding of transcription has been influenced by the use of small molecule inhibitors and reagents that have allowed researchers to dissect and define the multiple steps and factors involved. In addition, these reagents have also been proven potentially beneficial as pharmacologic agents to treat various diseases such as cancer and HIV.

Inhibitors of kinases that act on the C-terminal domain (CTD) of RNAP II have provided important details about the factors involved in transitioning from transcription initiation to elongation, the capping enzymes that modifies the 5' end of mRNAs, polyadenylation factors that modify the 3' end, the Mediator complex that regulates transcription initiation, and histone modifying enzymes (24-27). In this way, the CTD couples transcription with histone modification, mRNA splicing and polyadenylation through a series of dynamic interactions that occur at different steps of transcription (25). The CTD consists of a simple heptapeptide sequence of Tyr-Ser-Pro-Thr-Ser-Pro-Ser that is repeated 27-52 times and does not exist in RNAP I or RNAP III. The CTD is highly phosphorylated *in vivo* at two sites that appear at distinct stages of the transcription cycle and function to regulate association with cofactors. Serine 5 is phosphorylated by the Cdk7 subunit of the initiation factor TFIIH when bound to the core promoter. This modification recruits capping enzymes and also converts RNAP II into an elongating form (28-30). Serine 2 is phosphorylated by the Cdk9 subunit of the Positive

Transcription Elongation Factor b (P-TEFb) and is implicated in recruitment of 3' RNA processing factors, efficient 3' cleavage and polyadenylation (31, 32). Inhibition of P-TEFb/Cdk9 by DRB or flavopiridol blocks transcription of most genes *in vivo* (33). CTD phosphorylation events are coordinated with the action of specific CTD phosphatases. Fcp1 preferentially dephosphorylates Ser 2 over Ser 5 *in vivo* (34) and a family of small CTD phosphatases (SCPs) and the Ssu72 phosphatase catalyze selective dephosphorylation of Ser 5 (35,36). In addition, the peptidyl prolyl isomerase Pin1 modulates RNAP II function by influencing CTD phosphorylation (37). Similar to the regulation of transcriptional initiation, it is likely that control of elongation occurs through the combinatorial use of elongation factors and that gene- and signaling-specific elongation factors exist. Identifying new reagents that inhibit novel factors/targets may yield more detailed information of the mRNA transcription process.

One of the most intriguing ways that the p53 response can be activated is by pharmacological agents that inhibit cellular mRNA synthesis, which is perceived as a form of stress. Several seminal studies have shown that blocking transcription by cyclin-dependent kinase inhibitors (Cdki) roscovitine (Seliciclib, CYC202), DRB (5,6-dichloro-1-b-D-ribofuranosylbenzimidazole), and H7 results in nuclear accumulation of p53, induction of p53 target genes, and apoptosis (38,39). These Cdk inhibitors act by competing for the ATP binding site on the kinase and have somewhat broad-spectrum substrate specificities, including Cdk2/cyclin E, Cdk7/cyclin H and Cdk9/cyclin T (40). Inhibition of Cdk7 and Cdk9 abolishes RNAP II phosphorylation within its carboxyl-terminal domain (CTD) and prevents transcriptional elongation. It has been proposed that the transcription machinery itself may be a pivotal stress sensor that directs cell fate decisions by gauging the severity of damage (41). On this basis, selective interference of

transcription has become an active area of pursuit for the development of potential anti-tumor therapeutics (42). Indeed, flavopiridol and UCN-01 were the first Cdk inhibitors tested in clinical trials and have shown promising results, particularly in the treatment of certain chronic leukemias (43-44). In addition, roscovitine (Seliciclib, CYC202) is currently in phase II clinical trials due to its ability to induce apoptosis of multiple myeloma cells by inhibiting RNAP II phosphorylation and down-regulating the antiapoptotic factor Mcl-1 (45). Clearly, a more detailed understanding of which specific kinases need to be inhibited by small-molecules to promote apoptosis through transcription interference is required to extend their therapeutic efficacy to certain cancers and other diseases. In chapter 5, I present the initial findings from a small molecule screen to identify new factors that inhibit transcription initiation.

References:

1. Harms, K., S. Nozell, and X. Chen, The common and distinct target genes of the p53 family transcription factors. Cell Mol Life Sci, 2004. 61(7-8): p. 822-42.

2. Vogelstein, B., D. Lane, and A.J. Levine, Surfing the p53 network. Nature, 2000. 408(6810): p. 307-10.

3. Levine, A.J., p53, the cellular gatekeeper for growth and division. Cell, 1997. 88(3): p. 323-31.

4. Vousden, K.H. and X. Lu, Live or let die: the cell's response to p53. Nat Rev Cancer, 2002. 2(8): p. 594-604.

5. el-Deiry, W.S., et al., WAF1, a potential Mediator of p53 tumor suppression. Cell, 1993. 75(4): p. 817-25.

6. Prives, C. and P.A. Hall, The p53 pathway. J Pathol, 1999. 187(1): p. 112-26.

7. Muller, M., et al., p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. J Exp Med, 1998. 188(11): p. 2033-45.

8. Kim, R., et al., The role of Fas ligand and transforming growth factor beta in tumor progression: molecular mechanisms of immune privilege via Fas-mediated apoptosis and potential targets for cancer therapy. Cancer, 2004. 100(11): p. 2281-91.

9. Grossman, S.R., et al., Polyubiquitination of p53 by a ubiquitin ligase activity of p300. Science, 2003. 300(5617): p. 342-4.

10. Lowe, S.W., E. Cepero, and G. Evan, Intrinsic tumour suppression. Nature, 2004. 432(7015): p. 307-15.

11. Vousden, K. H. (2009) Functions of p53 in metabolism and invasion, Biochem Soc Trans 37, 511-517.

12. Vousden, K. H., and Prives, C. (2009) Blinded by the Light: The Growing Complexity of p53, Cell 137, 413-431.

13. Murray-Zmijewski, F., Slee, E. A., and Lu, X. (2008) A complex barcode underlies the heterogeneous response of p53 to stress, Nat Rev Mol Cell Biol 9, 702-712.

14. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) WAF1, a potential Mediator of p53 tumor suppression, Cell 75, 817-825.

15. Abbas, T., and Dutta, A. (2009) p21 in cancer: intricate networks and multiple activities, Nat Rev Cancer 9, 400-414.

16. Zhao, R., Gish, K., Murphy, M., Yin, Y., Notterman, D., Hoffman, W. H., Tom, E., Mack, D. H., and Levine, A. J. (2000) Analysis of p53-regulated gene expression patterns using oligonucleotide arrays, Genes Dev 14, 981-993.

17. Sandelin, A., Carninci, P., Lenhard, B., Ponjavic, J., Hayashizaki, Y., and Hume, D. A. (2007) Mammalian RNA polymerase II core promoters: insights from genome-wide studies, Nat Rev Genet 8, 424-436.

18. Heintzman, N. D., and Ren, B. (2007) The gateway to transcription: identifying, characterizing and understanding promoters in the eukaryotic genome, Cell Mol Life Sci 64, 386-400.

19. Espinosa, J. M., Verdun, R. E., and Emerson, B. M. (2003) p53 functions through stress- and promoter-specific recruitment of transcription initiation components before and after DNA damage, Mol Cell 12, 1015-1027.

20. Gomes, N. P., Bjerke, G., Llorente, B., Szostek, S. A., Emerson, B. M., and Espinosa, J. M. (2006) Gene-specific requirement for P-TEFb activity and RNA polymerase II phosphorylation within the p53 transcriptional program, Genes Dev 20, 601-612.

21. Kaeser, M. D., and Iggo, R. D. (2002) Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity *in vivo*, Proc Natl Acad Sci U S A 99, 95-100.

22. Szak, S. T., Mays, D., and Pietenpol, J. A. (2001) Kinetics of p53 binding to promoter sites *in vivo*, Mol Cell Biol 21, 3375-3386.

23. Espinosa, J. M. (2008) Mechanisms of regulatory diversity within the p53 transcriptional network, Oncogene 27, 4013-4023.

24. Hirose, Y. and J.L. Manley, RNA polymerase II and the integration of nuclear events. Genes Dev, 2000. 14(12): p. 1415-29.

25. Bentley, D.L., Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors. Curr Opin Cell Biol, 2005. 17(3): p. 251-6.

26. Kizer, K.O., et al., A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. Mol Cell Biol, 2005. 25(8): p. 3305-16.

27. Xiao, T., et al., Histone H2B ubiquitylation is associated with elongating RNA polymerase II. Mol Cell Biol, 2005. 25(2): p. 637-51.

28. Kanin, E.I., et al., Chemical inhibition of the TFIIH-associated kinase Cdk7/Kin28 does not impair global mRNA synthesis. Proc Natl Acad Sci U S A, 2007. 104(14): p. 5812-7.

29. Trigon, S., et al., Characterization of the residues phosphorylated *in vitro* by different C-terminal domain kinases. J Biol Chem, 1998. 273(12): p. 6769-75.

30. Kobor, M.S. and J. Greenblatt, Regulation of transcription elongation by phosphorylation. Biochim Biophys Acta, 2002. 1577(2): p. 261-275.

31. Ahn, S.H., M. Kim, and S. Buratowski, Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing. Mol Cell, 2004. 13(1): p. 67-76.

32. Ni, Z., et al., Coordination of transcription, RNA processing, and surveillance by P-TEFb kinase on heat shock genes. Mol Cell, 2004. 13(1): p. 55-65.

33. Chao, S.H. and D.H. Price, Flavopiridol inactivates P-TEFb and blocks most RNA polymerase II transcription *in vivo*. J Biol Chem, 2001. 276(34): p. 31793-9.

34. Cho, E.J., et al., Opposing effects of Ctk1 kinase and Fcp1 phosphatase at Ser 2 of the RNA polymerase II C-terminal domain. Genes Dev, 2001. 15(24): p. 3319-29.

35. Yeo, M., et al., A novel RNA polymerase II C-terminal domain phosphatase that preferentially dephosphorylates serine 5. J Biol Chem, 2003. 278(28): p. 26078-85.

36. Krishnamurthy, S., et al., Ssu72 Is an RNA polymerase II CTD phosphatase. Mol Cell, 2004. 14(3): p. 387-94.

37. Xu, Y.X. and J.L. Manley, Pin1 modulates RNA polymerase II activity during the transcription cycle. Genes Dev, 2007. 21(22): p. 2950-62.

38. Ljungman, M., et al., Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene, 1999. 18(3): p. 583-92.

39. Ljungman, M. and M.T. Paulsen, The cyclin-dependent kinase inhibitor roscovitine inhibits RNA synthesis and triggers nuclear accumulation of p53 that is unmodified at Ser15 and Lys382. Mol Pharmacol, 2001. 60(4): p. 785-9.

40. Dai, Y. and S. Grant, Cyclin-dependent kinase inhibitors. Curr Opin Pharmacol, 2003. 3(4): p. 362-70.

41. Ljungman, M. and D.P. Lane, Transcription - guarding the genome by sensing DNA damage. Nat Rev Cancer, 2004. 4(9): p. 727-37.

42. Derheimer, F.A., C.W. Chang, and M. Ljungman, Transcription inhibition: a potential strategy for cancer therapeutics. Eur J Cancer, 2005. 41(16): p. 2569-76.

43. Sausville, E.A., Cell cycle regulatory kinase modulators: interim progress and issues. Curr Top Med Chem, 2005. 5(12): p. 1109-17.

44. Senderowicz, A.M., Inhibitors of cyclin-dependent kinase modulators for cancer therapy. Prog Drug Res, 2005. 63: p. 183-206.

45. MacCallum, D.E., et al., Seliciclib (CYC202, R-Roscovitine) induces cell death in multiple myeloma cells by inhibition of RNA polymerase II-dependent transcription and down-regulation of Mcl-1. Cancer Res, 2005. 65(12): p. 5399-407.

ACKNOWLEDGEMENTS:

Collaborators subject to authorship deriving from this work include:

Beverly M. Emerson

Chris M. Murawsky

Ruo Huang, M.S.

Jose M. Morachis, Ruo Huang, Beverly M. Emerson. "Identification and characterization of three kinase inhibitors that block transcription initiation of p21 and Fas/APO1." (In preparation)

Jose M. Morachis, Chris M. Murawsky, Beverly M. Emerson. "Regulation of the p53 transcriptional response by structurally diverse core promoters." (Genes & Development in press).

Chapter 2

Intrinsic features of diverse p53 core promoters regulate differences in RNAP II binding affinity and re-initiation kinetics

To understand the function of diverse core promoter structures that exist among p53 target genes, we examined which features may influence the differences in apparent RNAP II binding affinities and expression rates observed among p53 target genes using an *in vitro* transcription system. Initially, we assumed that p53 interaction with chromatin-assembled recombinant genes would be required to recapitulate these aspects of regulation. We began by simply measuring the relative amounts of transcription obtained from cloned natural *p21* and *Fas/APO1* promoter-reporter plasmids as naked DNA *in vitro* using protein extracts from unstressed Hela cells. These promoters have very dissimilar structures but each directed efficient RNA synthesis *in vitro* with *p21* being the stronger template (Fig. 2.1A, B). Although the *Fas/APO1* gene has been reported to have multiple start sites within a relatively close region of about 200 bp, only one major initiation site was utilized under *in vitro* transcription conditions. This mapped to approximately 80 bp upstream of the ATG start codon and within 4 bp of previously reported start site predictions and RACE analysis (*1, 2*).

To define the relative promoter strength and affinity of the transcriptional machinery, the *p21* and *Fas/APO1* DNA templates were transcribed after pre-initiation complex (PIC) formation in the presence of the detergent sarkosyl, which limits transcription to a single-round by inhibiting RNAP II re-initiation (diagrammed in Fig. 2.2) (*3-5*). By performing kinetic analyses of transcription in the presence and absence of sarkosyl, we could measure two important parameters: the rate of PIC formation and the number of rounds of re-initiation directed by each promoter.

To analyze the rate of PIC formation, PICs were allowed to assemble on each promoter between 0-2 hours with further PIC formation or RNAP II re-initiation blocked by the addition of sarkosyl to 0.04%. Immediately afterwards, transcription was initiated

by addition of nucleotide triphosphates (NTPs) (Fig. 2.3A). This revealed that the *p21* promoter was activated as early as 5 minutes after addition of NTPs, reaching a plateau at about 1 hour. By contrast, the *Fas/APO1* promoter was much less efficient than *p21* in assembling PICs, with negligible transcription detected under these single-round conditions even after 2 hours (Fig. 2.3A-B). We also observe similar kinetics on APAF-1 (Fig. 2.3C). These results, on naked DNA templates, were surprisingly consistent with the ChIPs analysis in unstressed cells showing dramatic differences in levels of RNAP II binding to the endogenous *p21* and *Fas/APO1* promoters (*6*). This suggests that variations in RNAP II binding affinities are directed by intrinsic features of p53 target promoters encoded in their DNA sequence since neither chromatin nor p53 were required for this level of basic regulation, although both obviously modulate transcriptional activity at more complex stages.

Next, we analyzed the RNAP II re-initiation frequency by measuring the approximate rounds of transcription directed from each promoter *in vitro*. PIC formation was allowed to occur on each template for 1 hour, followed by the addition of NTPs to initiate RNA synthesis. Once transcription was initiated, we conducted a time course of sarkosyl addition from 0-30 minutes to stop further re-initiation at specific time intervals (Fig. 2.4A). These results were quantified using a phosphoimager by comparing the transcriptional output at each time point with that of a single-round at time zero. Unexpectedly, this analysis revealed that, although the *p21* promoter can assemble a PIC far more rapidly than *Fas/APO1* (Fig. 2.3A), it is much less efficient in directing RNAP II re-initiation (4 rounds) than *Fas/APO1* (21 rounds). Previous *in vitro* assays using sarkosyl on more classical promoters have reported a maximal 2-6 rounds of transcription using mammalian or *Drosophila* protein extracts (*3, 4*). To confirm our

results we tested whether differences in the general sensitivity of sarkosyl towards transcription of *p21* and *Fas/APO1* existed, but found none (Fig. 2.4B). As a more rigorous verification of the large difference in re-initiation that we observed between p21 and *Fas/APO1*, we developed an assay to estimate rounds of transcription without using sarkosyl. First, the *p21* or *Fas/APO1* templates were immobilized on magnetic beads and incubated with Hela extracts to form a PIC on each promoter. Then, instead of adding sarkosyl to eliminate re-initiation, unbound RNAP II and other proteins were washed away from each template with buffer before starting transcription by the addition of NTPs (Fig. 2.4C). By this means, only engaged RNAP II complexes at the time of washing were capable of initiating single-round transcription whereas templates left with excess unbound RNAP II (unwashed) could reinitiate multiple rounds of RNA synthesis. The results of this experiment were quantified as before and closely matched that obtained under sarkosyl conditions, with *p21* undergoing 7 rounds of transcription and *Fas/APO1*, 18 rounds (Fig. 2.4D).

The preceding experiments were performed using full-length, natural *p21* and *Fas/APO1* recombinant promoters (Fig. 2.1A). We now asked whether the pronounced differences in RNAP II behavior observed between the two templates were regulated by DNA sequences within or outside of their respective core promoters. To this end, we generated templates containing core promoter sequences from -149 to +42 of *p21* and -50 to +78 of *Fas/APO1* (Fig. 2.5A). Both the full-length and core promoters of each gene generated approximately equal amounts of RNA synthesis (Fig. 2.5B), consistent with the general observation that *in vitro* transcription of DNA templates mainly reflects their core promoter activity. A time course of sarkosyl addition during PIC formation followed by NTP addition, similar to that shown in Figure 3A, demonstrated that the core

promoters of *p21 and Fas/APO1* direct PIC formation and re-initiation efficiency in an identical manner as observed with the full-length promoters (Fig. 2.5C). Taken together, these results support the notion that the differences in levels of RNAP II binding to p53 target genes in unstressed cells and the kinetics of gene expression upon stress-induction are strongly influenced by intrinsic features of structurally diverse core promoters at the level of DNA.

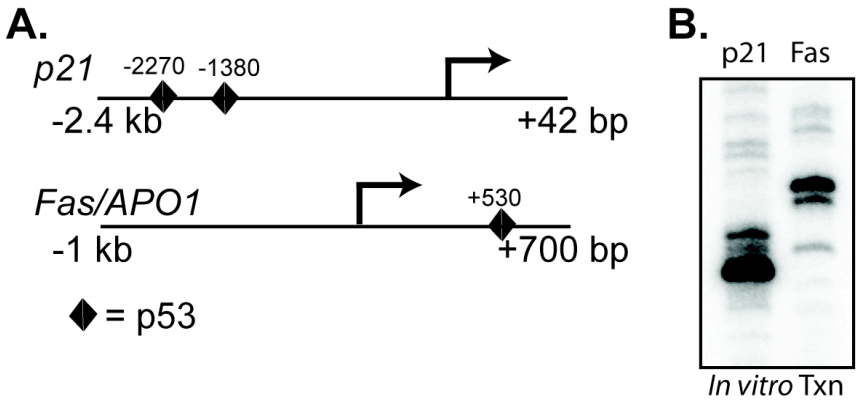

Figure 2.1 Promoter structure of p21 and Fas/APO1.

(A) Schematic diagram of p21 and Fas/APO1 promoters including their respective p53 binding sites. (B) In vitro transcription of p21 or Fas/APO1 plasmids was analyzed by primer extension.

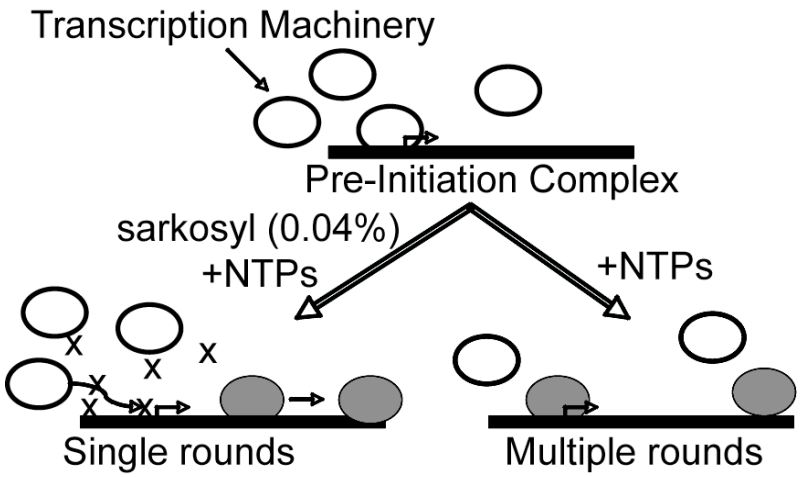

Figure 2.2 Sarkosyl assay.

Schematic diagram of single and multiple round-transcription in the presence of sarkosyl. Addition of sarkosyl to assembled or pre-formed Pre-initiation complexes (PICs) prevents new PIC formation. Templates without sarkosyl undergo multiple rounds of transcription.

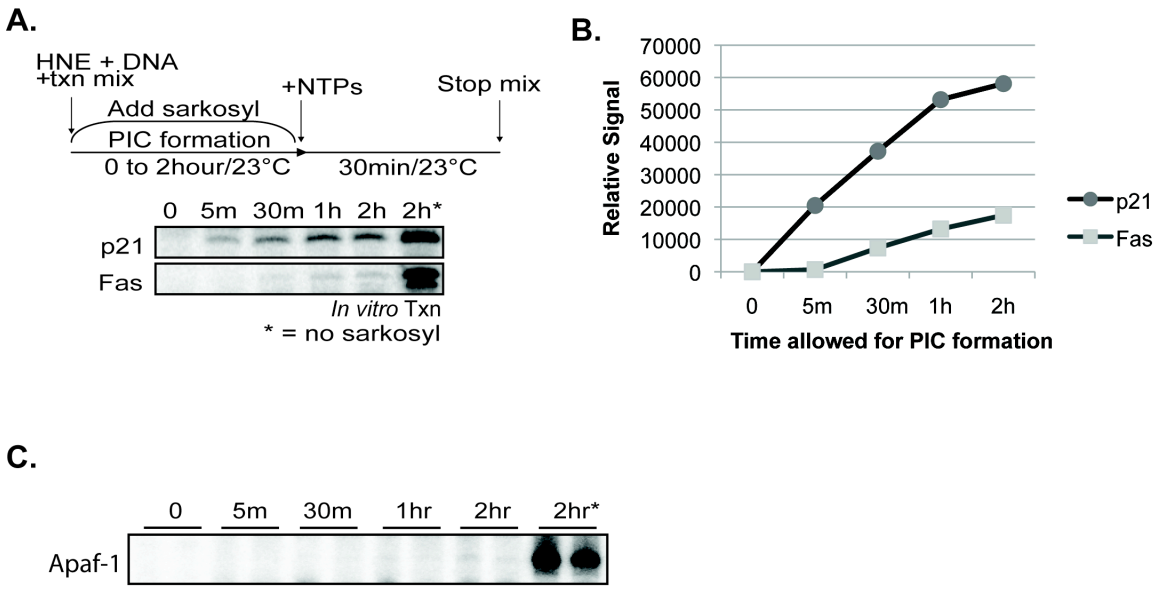

Figure 2.3 Functional characterization of the p21 and Fas/APO1 core promoters.

(A) Rates of PIC formation were analyzed by adding sarkosyl just before (time 0), or various times after combining DNA templates with Hela nuclear extracts. Transcription was initiated by adding NTPs. (B) Quantification and graphical representation of A. (C) PIC formation analysis (similar to "A") of the APAF-1 promoter.

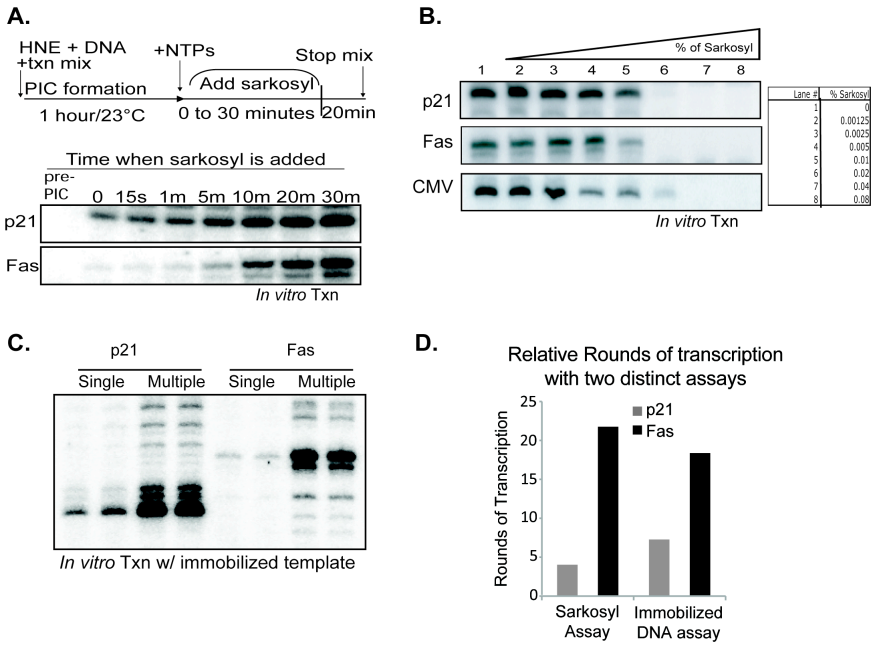

Figure 2.4 Rounds of transcription. *(A) Rounds of transcription were analyzed by allowing PICs to form for 1 hour before initiating transcription (time 0). The initiated reactions then underwent multiple rounds of transcription before sarkosyl addition at intervals from 15 seconds to 30 minutes. The first lane shows that sarkosyl addition to DNA templates before exposure to Hela extract prevents formation of functional PICs. (B) Sarkosyl sensitivity was analyzed by primer extension from in vitro transcription reactions using the p21, Fas/APO1 and CMV templates. The reactions were set up as described previously in the presence of increasing amounts of sarkosyl prior to PIC formation and initiation of transcription. (C) Rounds of transcription were also measured using DNA templates immobilized on magnetic beads. Extract and immobilized DNA were mixed for 1 hour to allow PIC formation similar to A. For single round transcription, the DNA template was washed with buffer followed by addition of NTPs to allow elongation whereas this wash step was not included for multiple rounds of transcription. (D) Rounds of transcription were quantified by dividing the signal from multiple rounds of transcription by the signal from single round transcription.*

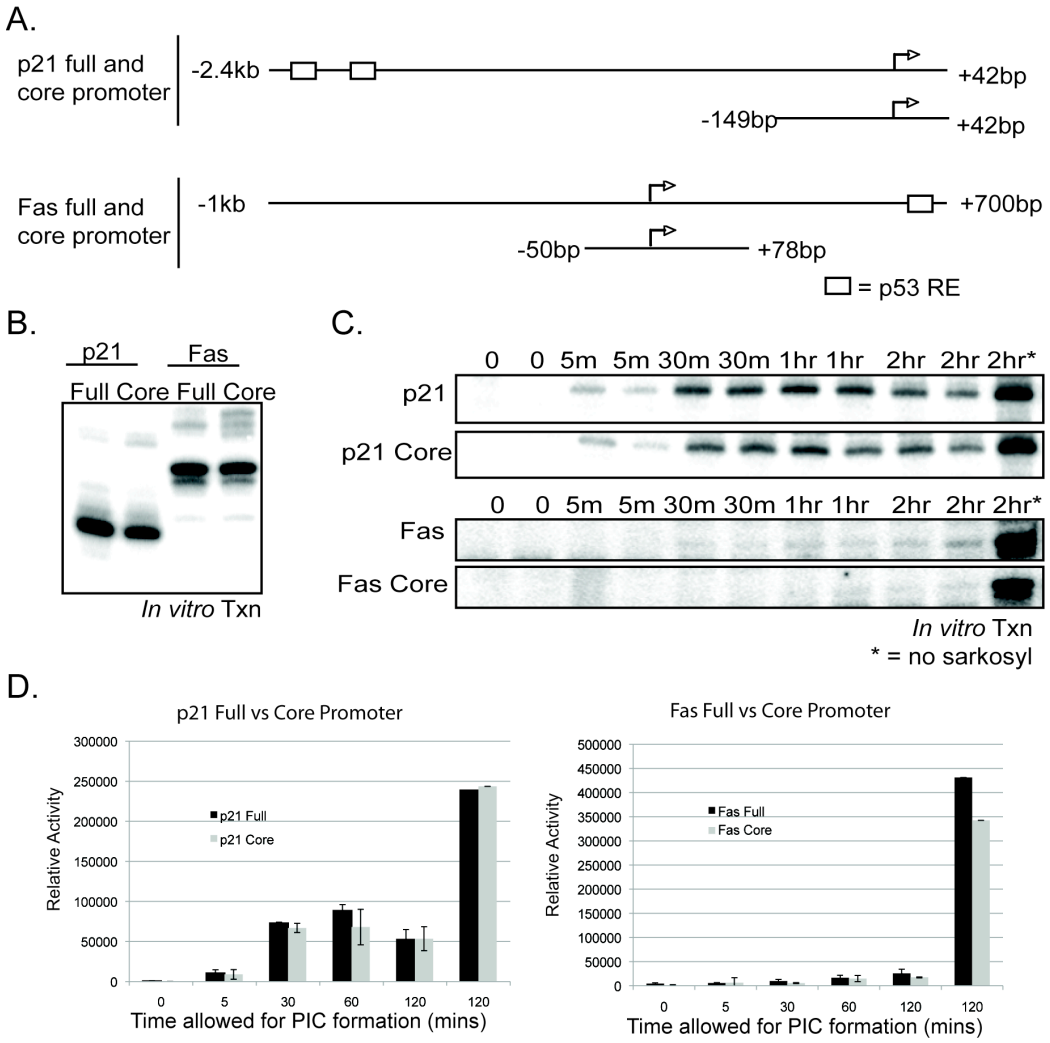

Figure 2.5 Analysis of PIC assembly kinetics on full-length or core p21 and Fas/APO1 promoters.

(A) Diagram of the full-length promoters containing p53 response elements and core promoters used. (B) In vitro transcription reactions using Hela extracts to compare promoter strength between full-length and core promoter templates. (C) Rates of PIC formation on p21 and Fas/APO1 full-length or core promoters were measured by in vitro transcription under conditions similar to Fig.3A. (D) Graphical representation of C.

REFERENCES:

1. Behrmann, I., Walczak, H., and Krammer, P. H. (1994) Structure of the human APO-1 gene, Eur J Immunol 24, 3057-3062.

2. Cheng, J., Liu, C., Koopman, W. J., and Mountz, J. D. (1995) Characterization of human Fas gene. Exon/intron organization and promoter region, J Immunol 154, 1239-1245.

3. Kadonaga, J. T. (1990) Assembly and disassembly of the Drosophila RNA polymerase II complex during transcription, J Biol Chem 265, 2624-2631.

4. Hawley, D. K., and Roeder, R. G. (1987) Functional steps in transcription initiation and reinitiation from the major late promoter in a Hela nuclear extract, J Biol Chem 262, 3452-3461.

5. Cai, H., and Luse, D. S. (1987) Transcription initiation by RNA polymerase II *in vitro*. Properties of preinitiation, initiation, and elongation complexes, J Biol Chem 262, 298-304.

6. Espinosa, J. M., Verdun, R. E., and Emerson, B. M. (2003) p53 functions through stress- and promoter-specific recruitment of transcription initiation components before and after DNA damage, Mol Cell 12, 1015-1027.

ACKNOWLEDGEMENTS:

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Chapter 3

Mapping critical elements within the p21 and Fas/APO1 core promoters

To extend our observation that the p21 and *Fas/APO1* core promoters were each sufficient to direct dissimilar RNAP II binding kinetics, we mapped the functional elements within each promoter by scanning mutagenesis of progressive 10 bp blocks to the transverse bases using a PCR-based strategy (Invitrogen) (Fig. 3.1A-B). All mutations were generated in the context of the full-length promoters and were assessed by *in vitro* transcription using Hela extracts. An examination of the *p21* promoter set revealed that the TATA box was the most critical sequence in the core region since mutation of this element decreased transcription to a negligible extent (Fig. 3.1C, Scan C). Mutation of other sequences had far less deleterious effects except to change the major transcriptional start site, such as Scan D (adjacent to the TATA box) and Scan F (impairs the Initiator); or create additional initiation sites like Scan B (disrupts an Sp1 site flanking the TATA box) and Scan E (disrupts sequence just upstream of the start site).

A similar analysis of the *Fas/APO1* promoter set revealed little change in transcriptional efficiency of templates containing scanning mutations of sequences from -52 to the Initiator region (Scans A-E) (Fig. 1B). Interestingly, mutation of sequences just 3' of the Initiator from +7 (Scans F-G) completely abolished transcription, whereas adjacent 3' sequences (Scans H-I) had no effect. To further define this essential region, transversion mutations in consecutive blocks of 5 bp were created within Scans F-G. After transcriptional analysis of these mutated templates, the essential sequences for *Fas/APO1* expression *in vitro* were localized to a 15 bp element (Scans F.2, G.1, and G.2) residing between +12 to +26 (Fig. 1E). The reactions were quantified and a graphical representation of the results is shown (Fig. 3.1F)

To verify that this region was also responsible for basal transcription *in vivo*, we created a luciferase reporter system using the core promoters of p21 and *Fas/APO1* and compared the wildtype activity to the mutated regions important for transcription (Fig. 3.2). Similar to previous studies, the TATA box mutation (p21 C) drastically reduced luciferase activity from the *p21* promoter. The core promoter mutations within the Fas Scan F and Scan G mutations also decreased the basal activity of *Fas/APO1* to varying degrees. Thus, our mutagenesis studies identified the most critical sequences for *p21* and *Fas/APO1* intrinsic core promoter function as the *p21* TATA box and a *Fas/APO1* downstream element.

Analysis of the TATA box and *Fas/APO1* downstream elements using chimeric *p21* and *Fas/APO1* promoters

Next, we examined whether the distinct characteristics of RNAP II binding and re-initiation observed on the *p21* and *Fas/APO1* core promoters were regulated by the *p21* TATA box or the *Fas/APO1* downstream element. To address this, a chimeric promoter called Fas-TATA was constructed in which the *p21* TATA sequence (ATATCAG) was inserted into the *Fas/APO1* promoter to replace the region from -29 to -23 (GAGGCCA) and generate the sequence, TATATCAGG beginning at -30 (Fig. 3.4A). A functional analysis of the Fas-TATA promoter-template revealed that it was much more efficiently transcribed *in vitro* than templates containing the wildtype *Fas/APO1* (Fas-wt) promoter (Fig. 3.3B). Moreover, a time course of sarkosyl addition under single-round transcription conditions, analogous to the experiment shown in Figure 2.3A, showed that the Fas-TATA promoter was capable of forming detectable functional PICs much faster, like *p21* but unlike *Fas/APO1* wildtype, and could still direct multiple rounds

of re-initiation (Fig. 3.3C). Thus, the chimeric promoter has the dual capacity of high-affinity PIC formation, conferred by the TATA box, and efficient rates of re-initiation, like *Fas/APO1*. Interestingly, mutation of the Fas downstream element (Scan F) within the Fas-TATA hybrid template ("F-TATA") resulted in a severe reduction of transcription (Fig. 3.3D). This indicates that the TATA box can synergize with the Fas downstream element within the context of the *Fas/APO1* full-length promoter to greatly stimulate expression but that the downstream element is an essential feature that cannot be functionally replaced by the TATA box. No sequences homologous to the Fas downstream element have been found within the *p21* core promoter and none of our scanning *p21* mutations significantly impaired TATA function, suggesting that the TFIID complex can act relatively independently in these *in vitro* assays (Fig. 3.1A).

We further analyzed the function of the Fas downstream element (Scans F-G) by placing that sequence (+7 to +26 bp) in the *p21* promoter at the corresponding location from +7 to +26 (Fig. 3.4A-B). We discovered that the Fas downstream element repressed p21 transcription and this was mediated specifically by the Scan F sequence +7 to +16 bp (compare lanes 2 and 3). When either the Scan F or G downstream element was inserted in *p21* promoters lacking a functional TATA box, only negligible transcription was obtained. This indicates that in the context of the *p21* promoter, unlike the *Fas/APO1* promoter, the Fas downstream element cannot synergize with the TATA box nor functionally replace it (lanes 6-8).

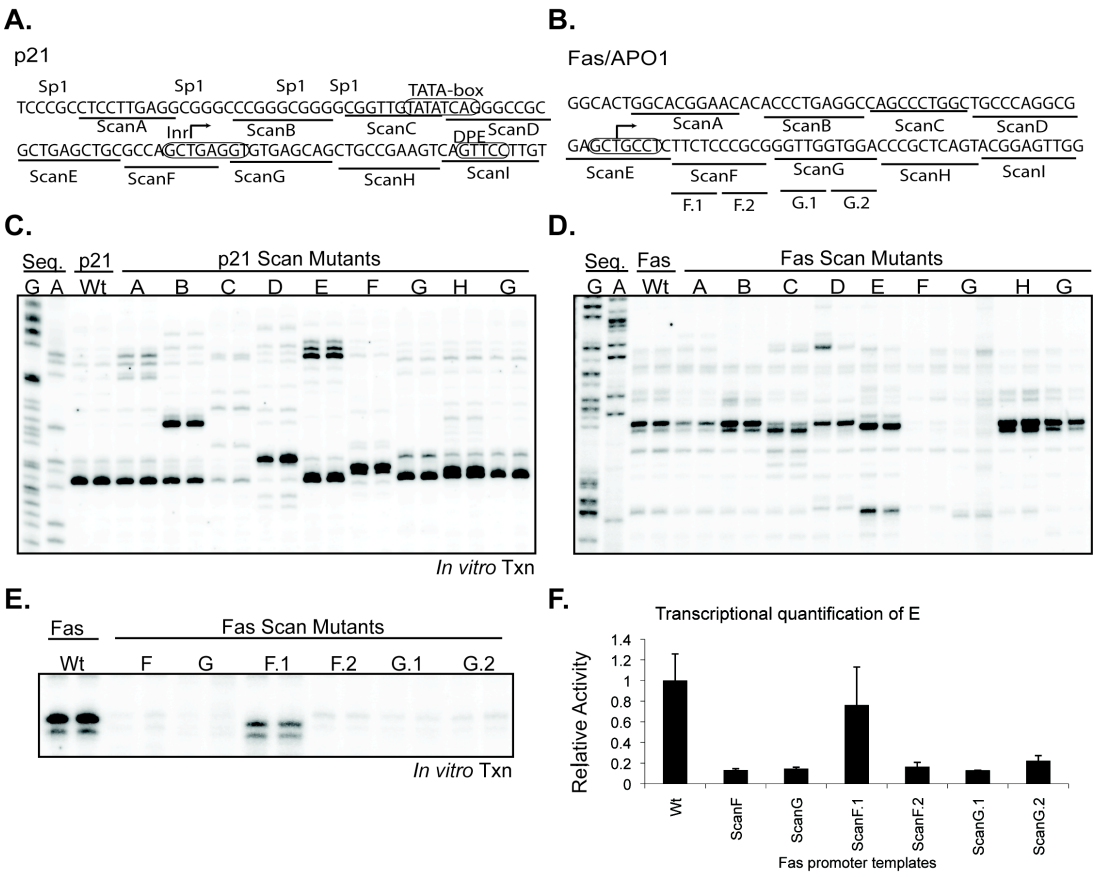


Figure 3.1 Functional core promoter elements mapped by scanning mutagenesis. *(A-B) Diagram of p21 (A) and Fas/APO1 (B) promoter sequences indicating the location of scanning mutations. Progressive 10 bp transversion mutations were generated in the context of the full-length promoters. (C) In vitro transcription reactions of p21 scanning mutations. The two left lanes labeled "G" and "A" contain DNA sequencing reactions used to map the transcription start site(s). (D) Same as (C) but using Fas/APO1 scanning mutations. (E) Transcriptional analysis of the scanning mutations "F" and "G" of Fas/APO1 that were further defined by creating four 5 bp block mutations between scans F and G to create Fas scan mutants F.1, F.2, G.1, and G.2. (F) Graphical representation from the reactions of E.*

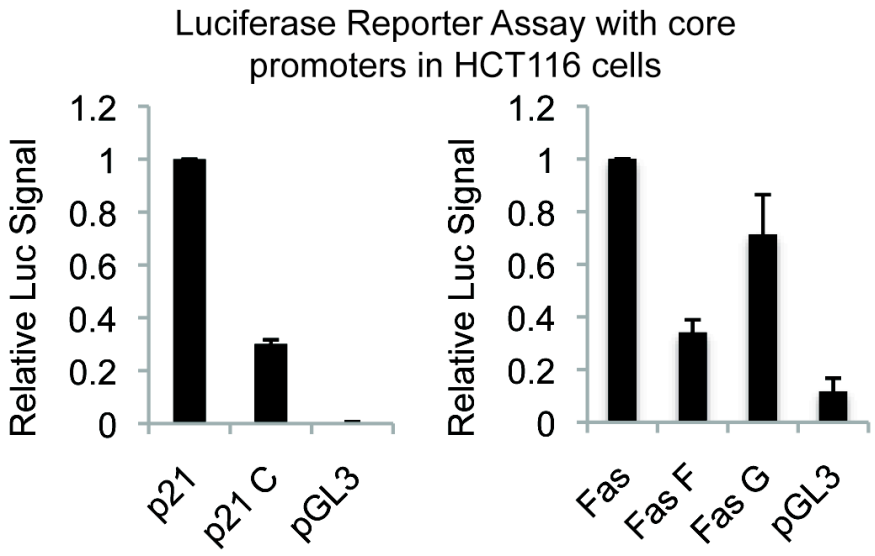


Figure 3.2 Mutant analysis using luciferase reporter assays

Luciferase activity in HCT116 cells using transiently transfected p21 or Fas/APO-1 core promoters within a pGL3 luciferase plasmid.

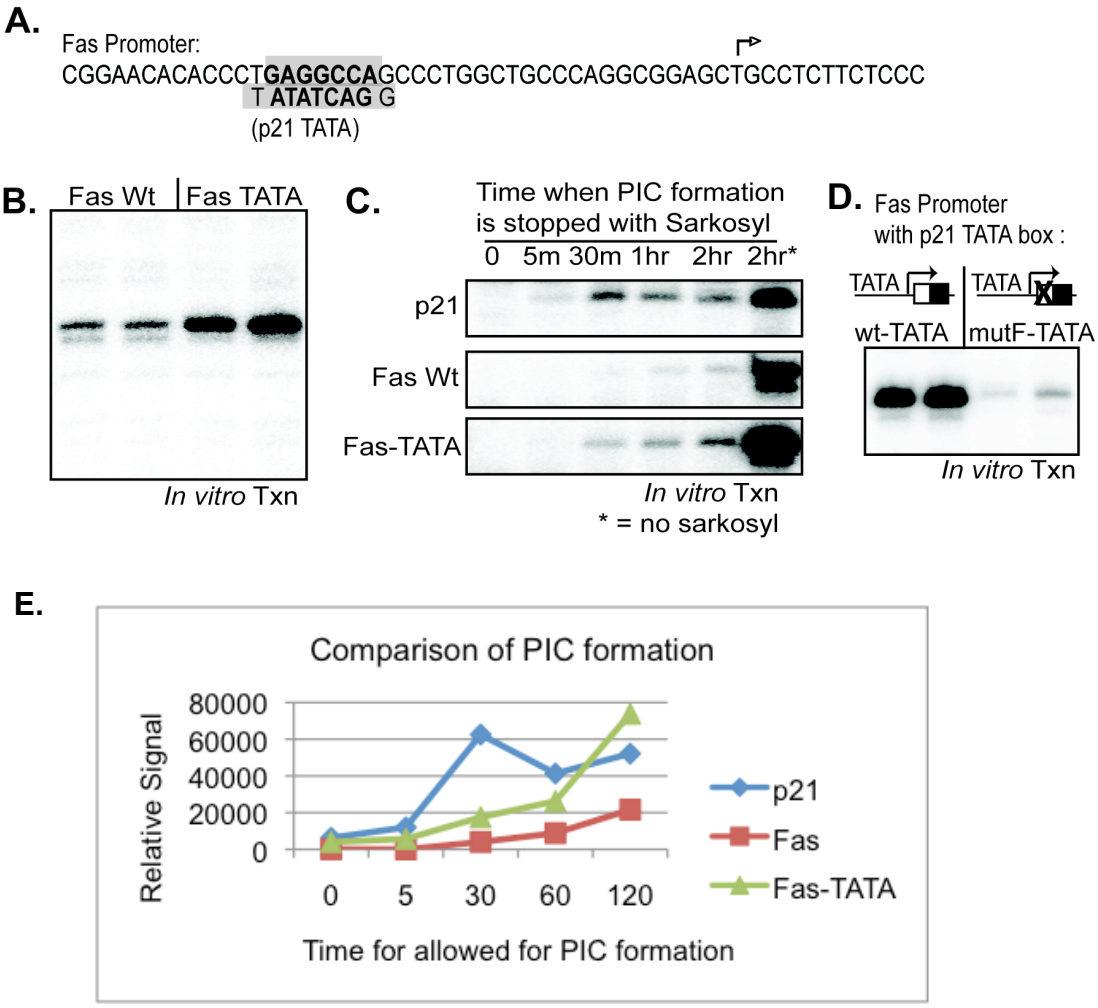
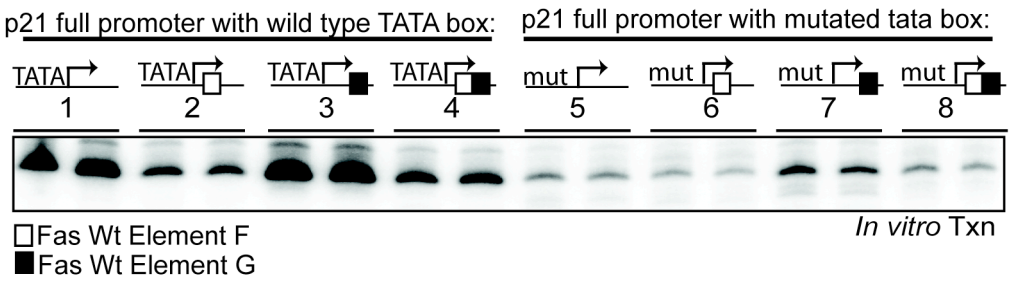


Figure 3.3 Analysis of critical core promoter elements using chimeric templates.

Diagram of the Fas/APO1 promoter sequence indicating the location in which the p21 ATATCAG sequence was inserted to replace -23 to -29 and create the Fas-TATA promoter. (B) In vitro transcription to examine the activity of Fas-TATA compared to the Fas/APO1 promoter. (C) In vitro transcription analyzing the rate of PIC formation of p21, Fas/APO1, and Fas-TATA. Hela *nuclear extract was allowed to bind to templates for 0-2 hrs before addition of sarkosyl similar to Fig. 2.3A. To generate multiple rounds of transcription, sarkosyl was not added to the last lane (2 hr*). (D) Transcriptional analysis of Fas-TATA compared to F-TATA (Fas-TATA with the scan F mutation). (E) Graphical representation of the PIC formation from C.*

A.



B.

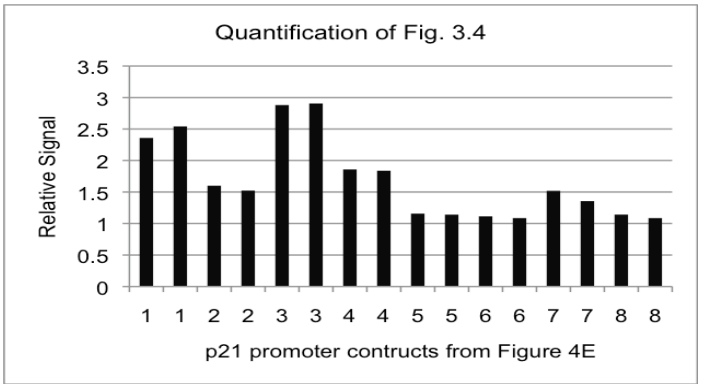


Figure 3.4 Analysis of the hybrid p21 promoter containing the Fas/APO1 downstream element.

(A) Analysis of the Fas downstream element within the p21 wildtype or mutated full-length promoter. (B) Graphical representation of the PIC formation from C.

REFERENCES:

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Chapter 4

Binding and regulation of the *Fas/APO1* core promoter downstream element by NF-Y

Having established the regions of the *p21* and *Fas/APO1* that are essential for *in vitro* transcription, I wished to identify which proteins bound to them. A DNase I protection analysis demonstrated that both native TFIID and recombinant TBP could interact with the *p21* TATA box, however no evidence of binding was observed on *Fas/APO1*. This substantiates the notion that significant differences in binding affinities for general transcriptional factors exist between these diverse core promoters, which may directly influence the assembly of compositionally distinct PICs (Figure 4.1).

To determine whether the downstream promoter element that is essential for *Fas/APO1* transcription specifically interacts with proteins present in our Hela transcription extracts, we performed an electrophoretic mobility shift assay (EMSA) using oligonucleotides that span -1 to +35 bp, which includes the sequences in Scans F-G (Fig. 5A). We observed specific protein complex formation using wildtype oligos that was not generated with oligos containing mutations in the 20 bp corresponding to elements F-G (Fig. 5B). Although a different shift was observed using the mutant probes, it likely results from an unrelated protein since the mutations removed all the functional sequences (middle 20 bp out of 36). Moreover, a 10-fold excess of mutant oligo failed to compete for binding of the specific protein complex to the wildtype oligo (Fig. 5C).

I next identified and characterized the protein(s) binding to this DNA region by oligonucleotide-affinity chromatography, similar to a strategy used to purify a corepressor of p53, *SnoN*, on the alpha-fetoprotein gene (*1*). In this approach, DNA sequences comprising the Fas activator element (Scans F-G), that are capable of protein binding by EMSA, were immobilized as multiple copies on streptavidin-coated magnetic beads. The multimers were produced using a self-primer PCR method and

immobilized through a 5'-biotin linkage (*2, 3*). The multimerized DNA molecules were generated with an average length of one thousand basepairs (Figure 4.3A). Protein-DNA binding reactions were conducted in the presence of the synthetic polymer dI-dC to reduce non-specific interactions and specific protein-DNA complexes were captured with a magnet to remove unbound material. We also incorporated a "pre-clearing" step in which Hela extracts were initially incubated with an immobilized, mutated Fas downstream element (which abolishes specific protein binding as determined by EMSA) to further minimize contaminating proteins in our subsequent analysis (Figure 4.3B). Proteins that "flowed-through" the pre-cleared chromatographic steps were enriched for factors that specifically interact with the Fas downstream element. The immobilized templates were then incubated with Hela extract containing the potential Fas downstream element interacting protein(s), washed, and step-eluted with 0.1, 0.25, 0.5, and 1M NaCl buffer (diagrammed in Fig. 4.3B). The fractions were analyzed by EMSA, which showed that the downstream element binding activity eluted mainly between 0.1-0.25M NaCl (Fig. 4.3C).

Fractions eluted from both the immobilized wildtype and mutated downstream element oligos, were then compared by SDS-PAGE. Analysis of silver-stained gels revealed a specific band between 49-64 kDa in the 0.1 and 0.25M NaCl elutions that was not bound to the mutated template (Figure 4.4A, asterisk). This protein band (along with the corresponding area of the mutant lane) was excised from the gel, digested into peptides, and analyzed by MALDI-TOF mass spectrometry. Using this technique, we were able to obtain a list of potential factors present in each sample. The factors with the highest scores that were not present in the non-specific mutated elutions were considered. From this list, NF-Y subunit C (gamma) had the highest score, and was

therefore chosen for further analysis. Immunoblotting for the NF-Y subunit C confirmed the mass spectrometry results (4.4B). NF-Y is a trimeric complex that is comprised of subunits A, B, and C (*4*). To verify that the complete trimeric complex actually binds to the downstream Fas promoter element we performed a supershift EMSA assay using antibodies against two of the three NF-Y subunits (anti-NF-YA and anti-NF-YC), using anti-YY1 and anti-Bmi as controls. The specific shift previously observed by EMSA was clearly supershifted using either anti-NF-YA or anti-NF-YC antibody whereas no supershift was observed with the control antibodies (Fig. 4.4C). The reactions in lanes 8 and 9 are similar to 5 and 7, respectively, except that Hela extract was incubated with the probe before addition of the antibody.

NF-Y binds to the *Fas/APO1* promoter *in vivo* and activates *Fas/APO1*, but not *p21*, transcription *in vitro*

NF-Y is a conserved transcription factor that binds with high specificity to CCAAT (or reverse: ATTGG) motifs in the promoter regions in a variety of genes. The C subunit forms a tight dimer with the B subunit, and is a prerequisite for association with subunit A. The resulting trimer binds to DNA with high affinity and is responsible for the transcriptional regulation of numerous promoters (*5-7*). The DNA sequence within the critical promoter region for *Fas/APO1* transcription contains "…GGG**TTGG**TGG…" and is very similar, although not identical, to the reverse ATTGG NF-Y consensus sequence.

To examine the physiological relevance of NF-Y to *Fas/APO1* gene regulation, we used the human breast cancer MCF7 cell line since previous studies have shown that *Fas/APO1* plays a role in apoptosis in these cells (*8, 9*). Treatment of MCF7 cells

with 5-Fluorouracil (5-FU) for 14 hours resulted in highly elevated *Fas/APO1* mRNA synthesis (Fig. 4.5A). A ChIP analysis was performed in these cells, in the presence or absence of 5-FU, to determine whether NF-Y was associated with the *Fas/APO1* promoter *in vivo*. For these experiments, we used an antibody to the NF-Y subunit A and mapped protein binding with primers near the transcriptional start site of *Fas/APO1* and a control region far downstream. The *p21* and *CCNB1* promoters were used as negative and positive controls, respectively, for NF-Y A binding. These studies revealed that NF-Y A is bound near the *Fas/APO1* initiator region (Inr) in unstressed MCF7 cells and this interaction increases upon 5-FU treatment. In contrast, negligible NF-Y A binding was observed on the p21 promoter, regardless of 5-FU treatment. A strong recruitment of NF-Y A was also detected at the known target promoter *CCNB1* (Fig. 4.5B). These ChIP results are consistent with our biochemical data showing specific binding of NF-Y to the Fas downstream core promoter element by EMSA and recruitment to immobilized *Fas/APO1* templates (Fig. 4.4) and thereby confirm the biological relevance of this protein-DNA interaction.

We next analyzed whether NF-Y influenced *Fas/APO1* transcription *in vivo*. To this end, the three subunits of NF-Y were each overexpressed to similar levels in MCF7 cells using CMV-driven cDNA constructs (Fig. 4.5C) and mRNA levels of the endogenous *Fas/APO1* gene were subsequently measured by qPCR. We found that overexpression of the NF-Y trimeric complex activated *Fas/APO1* mRNA nearly 2-fold whereas transcription of endogenous p21 and a control gene, SDHA, remained unchanged (Fig. 4.5D). This data suggests that NF-Y positively regulates *Fas/APO1* expression and can function selectively on p53 target genes, consistent with our biochemical studies. These results also support the hypothesis that the specialized roles

of NF-Y and other components of the basal transcription machinery in regulating the p53 response are directed by the diverse core promoter structures of p53 target genes.

Several studies have identified a relationship between p53 and NF-Y in which they interact directly to repress various cell cycle genes (10). Interestingly, NF-Y knockdown by siRNA causes apoptosis while activating many p53 target genes (11). We analyzed RNA levels in NF-Y knockdown cells and observed activation of *Fas/APO1*, *p21*, and *PUMA* genes, possibly by indirect induction (Fig. 4.6). This is consistent with previous studies, although *Fas/APO1* expression was not shown (11). The observation that p53 targets are among those affected by ablation of NF-Y is not entirely surprising since it has been shown that depletion of NF-Y from HCT116 cells results in mis-regulation of hundreds of genes (Benatti et al.). This study showed the down-regulation of 478 genes and up-regulation of 803 genes, indicating that NF-Y knockdown affects multiple genes in a global manner. Taken together, this data indicates that NF-Y is a critical, functionally diverse transcription factor that regulates genes in both a positive and negative fashion. Loss of NF-Y results in a crisis that culminates in apoptosis.

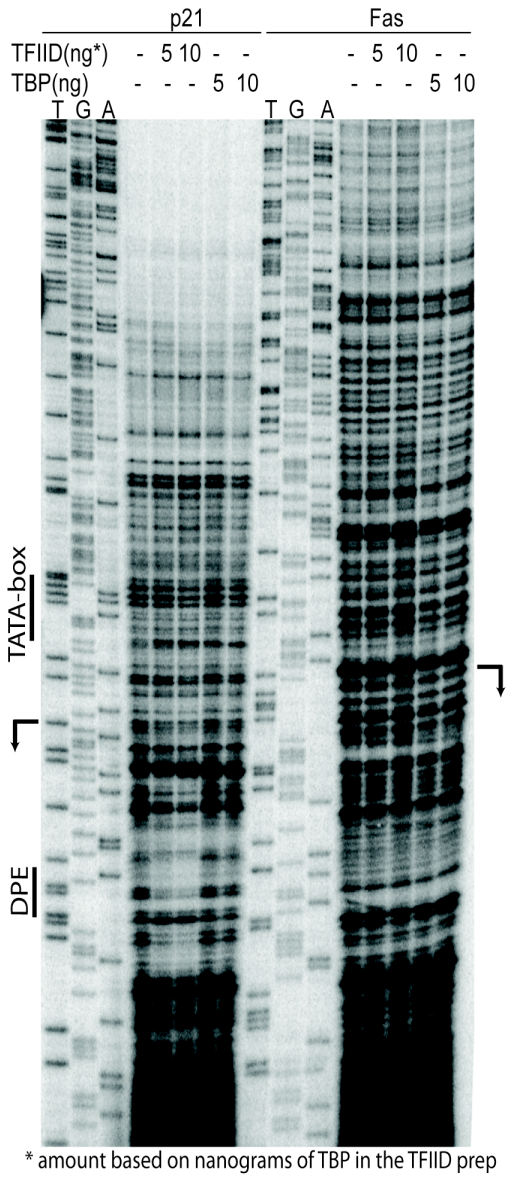

Figure 4.1 DNase I footprinting analysis of TBP and TFIID.

The DNase I footprinting assay was performed as before (Lee and Chiang 2009) using 5-10ng of purified TBP alone or as part of the multi-subunit TFIID complex. Both TFIID and TBP produced a DNase I hypersensitive site immediately adjacent to the TATA box on the p21 promoter. TFIID, but not TBP, protected a region corresponding to the initiator and DPE sequences. There were no significant footprints observed in the Fas/APO1 promoter using the same amounts of TBP/TFIID.

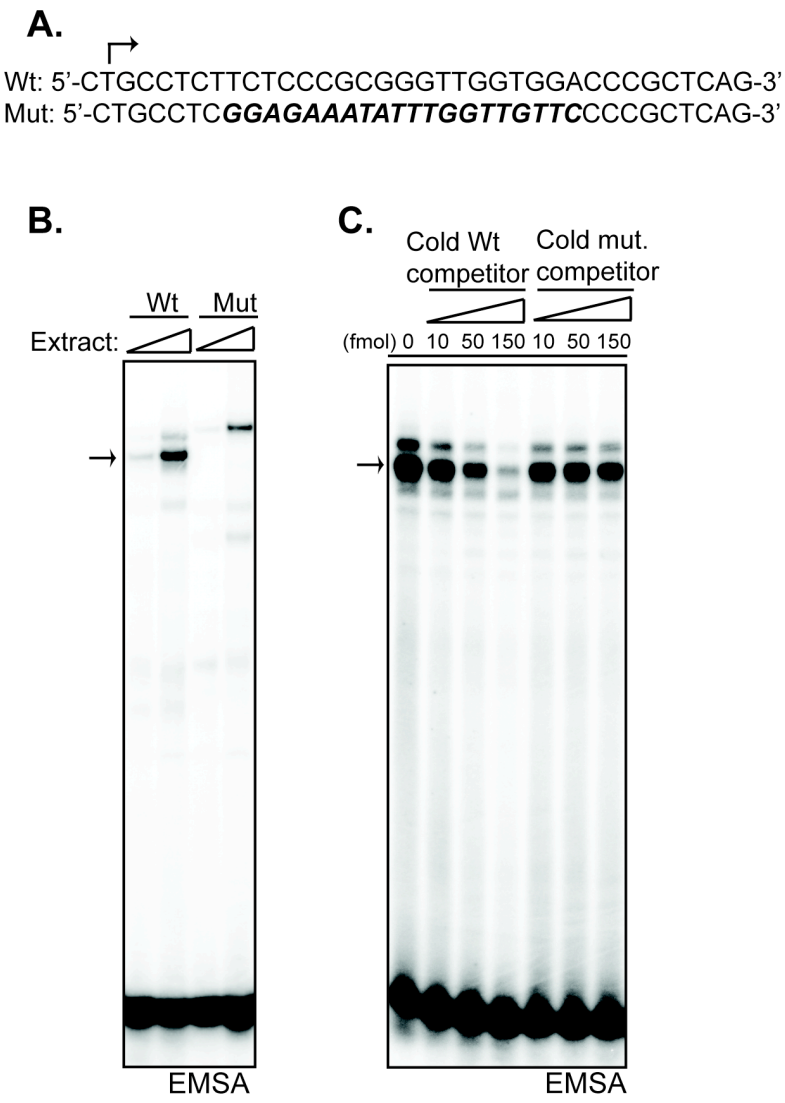

Figure 4.2 Identification of a specific activity that binds to the critical Fas downstream element.

(A) Sequences of the wildtype or mutant probes used for electrophoretic mobility shift assay (EMSA). (B) EMSA analysis of Fas downstream element (Fas "F-G") binding protein(s) from Hela nuclear extracts. (C) EMSA competition assays using cold wildtype or mutated competitor F-G oligonucleotides. Wildtype Fas "F-G" oligos were shifted with 4 μg of Hela nuclear extract in the absence (first lane) or presence of increasing concentrations of cold probes.

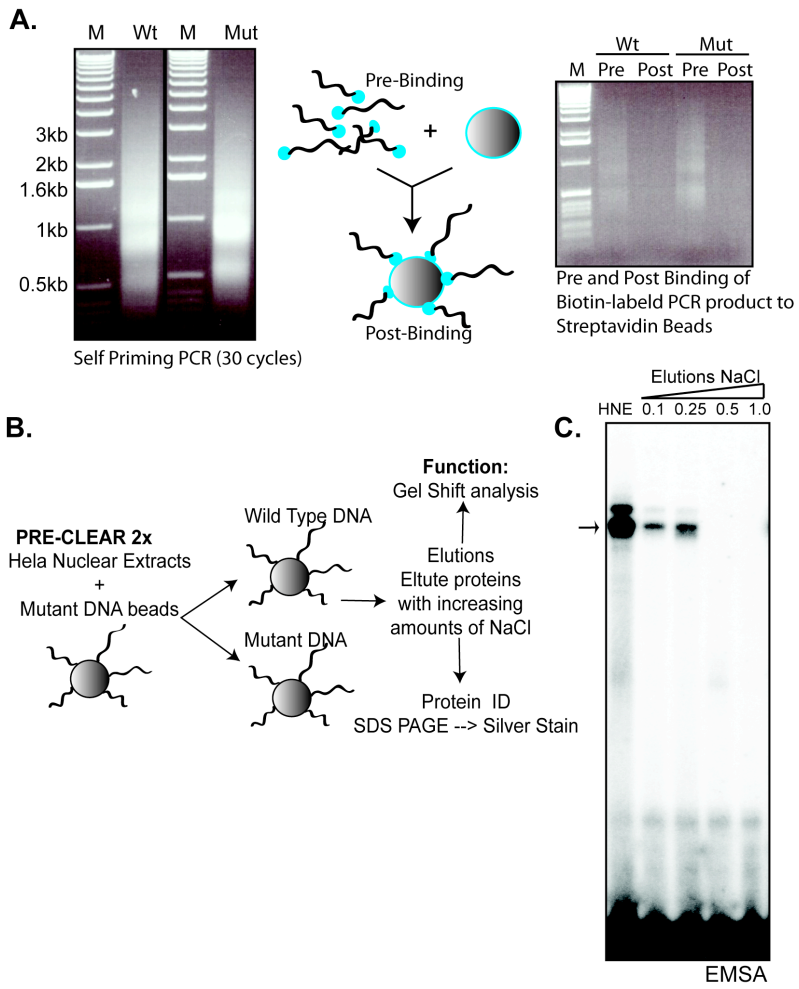

Figure 4.3 Experimental strategy to identify the Fas downstream element binding activity. *(A) Analysis of the self-priming DNA multimerization reactions of wildtype and mutated Fas F-G sequences. Immobilization was performed under high salt conditions according to Invitrogen's protocols and then evaluated for efficiency using agarose gel electrophoresis (pre-bound vs post-bound). (B) Schematic diagram describing the steps performed to capture and characterize the protein complex that binds to the Fas downstream element. The DNA affinity pulldown was performed using immobilized multimers of the DNA sequence used for EMSA. (E) Proteins that bound to the wildtype and mutated sequence were step eluted with buffer containing 0.1M, 0.25M, 0.5M or 1M NaCl and then tested for binding activity by EMSA.*

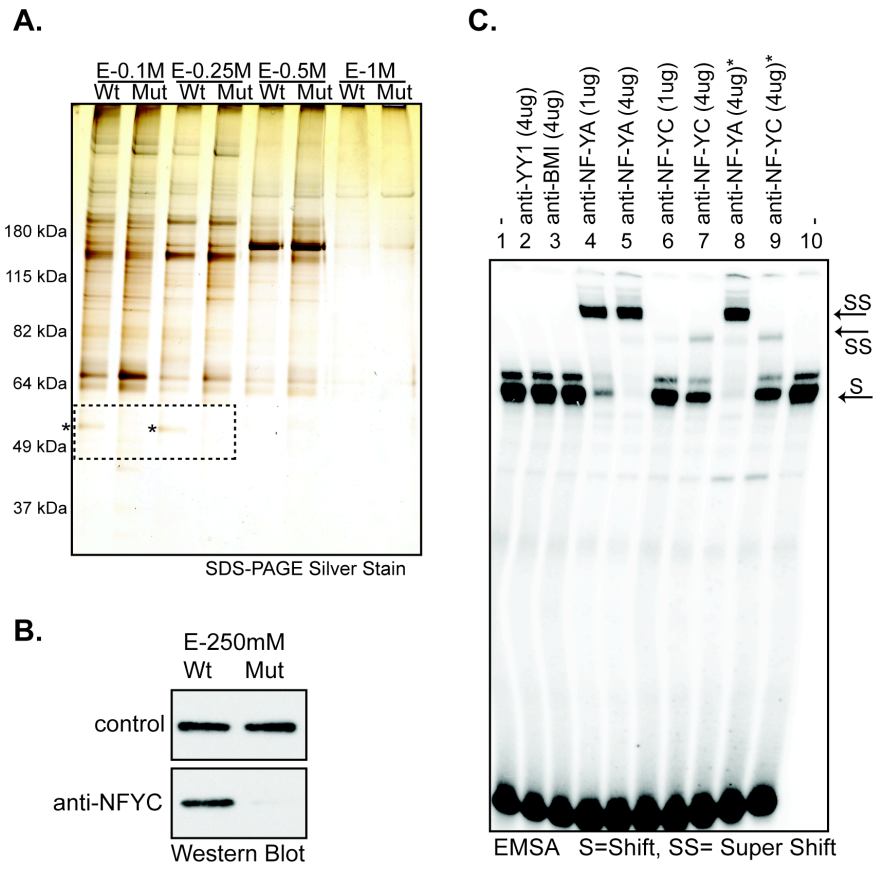

Figure 4.4 Fractionation and identification of NF-Y as an activity that binds to the critical Fas downstream element. *A. Elutions from the DNA affinity assays were separated by SDS-PAGE and stained with silver. The gel regions encompassing the unique polypeptides, noted with (*) were excised and subjected to Mass Spec. NF-Y, subunit C, was identified with a high score. (B) To verify the latter results, we used Western Blot analysis to compare 0.25M elutions from wildtype and mutated templates. Using anti-NF-YC antibody (SantaCruz), we detected NF-YC specifically in elutions from the wildtype sequence. (C) Supershift EMSA analysis to test the specificity of NF-Y binding. In lanes 1-7, binding reactions were incubated for 30 minutes followed by addition of the specified antibody and incubated for 15 minutes. For lanes 8 and 9, antibodies were incubated with Hela nuclear extract for 10 minutes before mixing with the DNA probe.*

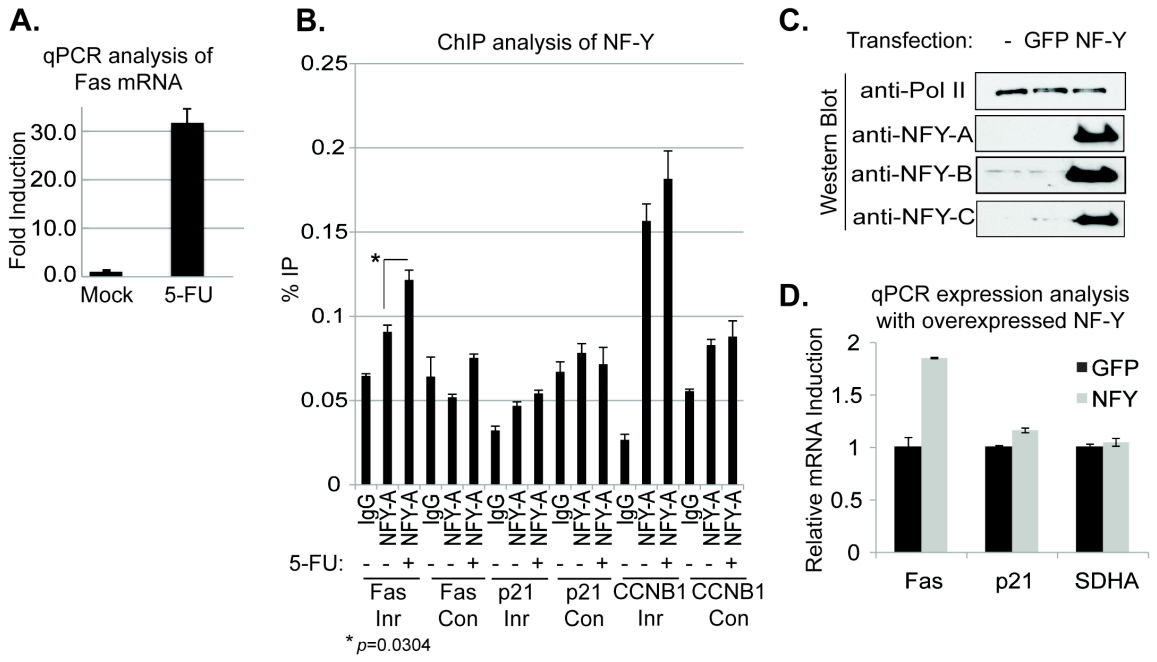

Figure 4.5 NF-Y binds to and regulates the FAS/APO-1 promoter in MCF7 cells.

(A) mRNA analysis of Fas/APO1 activation upon 5-FU (50ng/ml) treatment for 14 hours. (B) Chromatin immunoprecipitations of the FAS/APO-1, p21, and CCNB1 genes were assayed for the presence of NF-Y in MCF7 cells. (C) Western Blot analysis of NF-Y subunits overexpressed in MCF7 cells. CMV driven NF-YA, NF-YB, and NF-YC plasmids were used for transfection and assayed with subunit specific antibodies. (D) mRNA from MCF7 cells over-expressing NF-Y were analyzed by q-PCR.

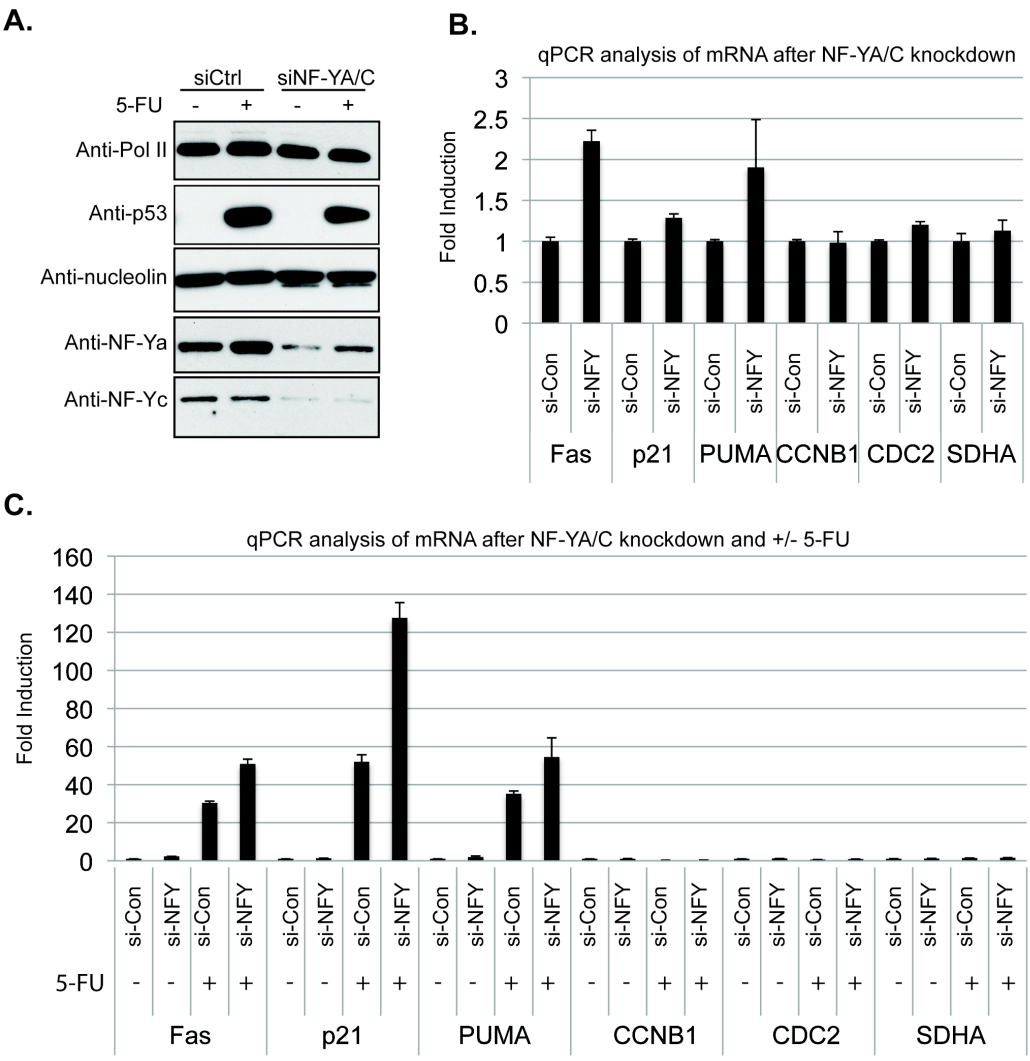

Figure 4.6 Depletion of NF-Y with si-RNA in MCF7 cells. *A. Cells were double transfected using Lipofectamine 2000 (Invitrogen) with control si-RNA or si-RNA targeting NF-YA and NF-YC (Santa Cruz). Cells were then treated or untreated with 50ng/ml 5-FU and proteins were harvested 8 hours later. Western Blot analysis was performed using antibodies against NF-YA, NF-YB, RNAP II, p53, and nucleolin. (B) mRNA analysis upon depletion of NF-Y. (C) mRNA analysis upon depletion of NF-Y and treatment with 5-FU (50ng/ml) for 8 hours.*

REFERENCES:

1. Wilkinson, D. S., Ogden, S. K., Stratton, S. A., Piechan, J. L., Nguyen, T. T., Smulian, G. A., and Barton, M. C. (2005) A direct intersection between p53 and transforming growth factor beta pathways targets chromatin modification and transcription repression of the alpha-fetoprotein gene, *Mol Cell Biol 25*, 1200-1212.

2. Hemat, F., and McEntee, K. (1994) A rapid and efficient PCR-based method for synthesizing high-molecular-weight multimers of oligonucleotides, *Biochem Biophys Res Commun 205*, 475-481.

3. Yaneva, M., and Tempst, P. (2006) Isolation and mass spectrometry of specific DNA binding proteins, *Methods Mol Biol 338*, 291-303.

4. Mantovani, R. (1999) The molecular biology of the CCAAT-binding factor NF-Y, *Gene 239*, 15-27.

5. McNabb, D. S., Xing, Y., and Guarente, L. (1995) Cloning of yeast HAP5: a novel subunit of a heterotrimeric complex required for CCAAT binding, *Genes Dev 9*, 47-58

6. Sinha, S., Maity, S. N., Lu, J., and de Crombrugghe, B. (1995) Recombinant rat CBF-C, the third subunit of CBF/NFY, allows formation of a protein-DNA complex with CBF-A and CBF-B and with yeast HAP2 and HAP3, *Proc Natl Acad Sci U S A 92*, 1624-1628.

7. Caretti, G., Salsi, V., Vecchi, C., Imbriano, C., and Mantovani, R. (2003) Dynamic recruitment of NF-Y and histone acetyltransferases on cell-cycle promoters, J Biol Chem 278, 30435-30440.

8. Ruiz-Ruiz, C., Robledo, G., Cano, E., Redondo, J. M., and Lopez-Rivas, A. (2003) Characterization of p53-mediated up-regulation of CD95 gene expression upon genotoxic treatment in human breast tumor cells, J Biol Chem 278, 31667-31675.

9. Hernandez-Vargas, H., Ballestar, E., Carmona-Saez, P., von Kobbe, C., Banon-Rodriguez, I., Esteller, M., Moreno-Bueno, G., and Palacios, J. (2006) Transcriptional profiling of MCF7 breast cancer cells in response to 5-Fluorouracil: relationship with cell cycle changes and apoptosis, and identification of novel targets of p53, Int J Cancer 119, 1164-1175.

10. Imbriano, C., Gurtner, A., Cocchiarella, F., Di Agostino, S., Basile, V., Gostissa, M., Dobbelstein, M., Del Sal, G., Piaggio, G., and Mantovani, R. (2005) Direct p53 transcriptional repression: *in vivo* analysis of CCAAT-containing G2/M promoters, *Mol Cell Biol 25*, 3737-3751.

11. Benatti, P., Basile, V., Merico, D., Fantoni, L. I., Tagliafico, E., and Imbriano, C. (2008) A balance between NF-Y and p53 governs the pro- and anti-apoptotic transcriptional response, *Nucleic Acids Res 36*, 1415-1428.

ACKNOWLEDGEMENTS:

Collaborators subject to authorship deriving from this work include:

Beverly M. Emerson

Chris M. Murawsky

Jose M. Morachis, Chris M. Murawsky, Beverly M. Emerson. "Regulation of the p53 transcriptional response by structurally diverse core promoters." (Genes & Development in press).

Chapter 5

Drug screen to identify inhibitors of transcription initiation

I have extended my analysis of the *p21* and *Fas/APO1* promoters by leveraging the *in vitro* transcription system to identify novel pharmacologic agents that may aid in further dissecting the transcription mechanisms of p53 target genes. An intriguing route for activating p53 transcriptional programs is through the use of pharmacological agents that inhibit mRNA synthesis. Pertinent examples of such drugs are a class of cyclin-dependent kinase inhibitors that affect the phosphorylation status of the C-terminal domain (CTD) of RNAP II. The transition from pre-initiation to initiation and finally elongation is believed to be influenced by modification of the CTD which then functions as a platform for the ordered assembly of different pre-mRNA processing machinery. I, and others (Gomes et al. 2005) have tested the affects of 5,6-di-chloro-1-b-D-ribofuranosyl-benzimidazole (DRB), a well known CDK9 kinase inhibitor, and found that it was able to activate specific subsets of p53 target genes in a cell type specific manner. DRB has a long history in the transcription field because it has allowed the dissection of multiple elongation steps and assisted in the identification of important transcription factors. Advanced analogs of DRB have also been developed and have shown promising therapeutic potential for several types of cancer. Interestingly, DRB and many drugs that affect basic transcriptional steps were initially intended or believed to inhibit kinases involved in cell cycle progression or other signaling pathways.

In an effort to extend these observations and identify new compounds, we decided to screen 80 commercial kinase inhibitors (BIOMOL International, see Table 1 below) for their activity towards early transcriptional steps on the *p21*, *Fas/APO1*, SCP1 (super core promoter 1) promoters (1). As our assay uses nuclear extracts and naked DNA templates (natural promoters), we felt comfortable excluding complications arising

from cell signaling or chromatin targeting. An additional advantage is that the reactions only measure transcription initiation and elongation of up to about 100bp. The *in vitro* transcription reactions were first tested for their sensitivity to DMSO since all drugs are dissolved in DMSO. We titrated increasing amounts of DMSO and found that our system can tolerate up to 4% DMSO (v/v) (Fig 5.1). In order to expedite screening, the 80-kinase inhibitors were first combined into 20 cocktail mixes containing four drugs each at a concentration of 1250μM. The 4-drug cocktails were added (50μM final concentration) to the transcription mix and Hela nuclear extract prior to initiating transcription with NTPs (Fig. 5.1A). The resulting transcriptional activities were measured by primer extension followed by gel separation and compared using a phosphoimager. Using this strategy, we were able to identify four cocktail mixes (C-7, C-8, C-14, and C-16) that significantly reduced transcriptional activity from all of the promoter templates (Fig. 5.1B).

The four cocktail mixes were further analyzed by testing each of the compounds independently. We observed that only a single compound from cocktails C-7, C-14, and C-16 significantly inhibited transcriptional activity. Interestingly, none of the compounds in cocktail 8 were able to inhibit transcription on their own and suggests a requirement for combinatorial inhibition. The three active compounds identified using this strategy were hypericin, rottlerin, and Sp600125. These three small molecules have not previously been implicated, to our knowledge, in blocking basal transcription initiation. Hypericin induces apoptosis in cancer cells and is a potent antiviral agent. The compound inhibits protein kinase C, irreversibly damages sarco/endoplasmic reticulum and other cellular membranes, decreases cellular pH, and inhibits mitochondrial function (2, 3). Rottlerin (mallotoxin) has been shown to be a strong inhibitor of MAPK-activated protein kinase 2, p38-regulated/activated kinase, protein kinase A, and glycogen

synthase kinase 3-beta (4). Sp600125 is novel and selective inhibitor of c-Jun N-terminal kinase (JNK) (5).

Known kinase inhibitors typically interfere with transcription by blocking the phosphorylation of the CTD of RNAP II. We tested the three compounds we identified for their ability to inhibit Ser2 phosphorylation in HCT116 cells. We treated cells with 50μM of each drug and analyzed the lysates by SDS-PAGE followed by immunoblotting using antibodies towards phospho-Ser2 of the CTD, total RNAP II, and p53 (Fig. 5.4). We included DRB as a positive control of phospo-Ser2 inhibition. In striking contrast to DRB, the three drugs do not appear to inhibit Ser2 phosphorylation. Experiments are underway evaluating their effect on Ser5 phosporylation since this marker is believed to be more critical during transcription initiation. Interestingly, only Sp600125 appears to activate p53 to levels comparable to DRB even though all drugs caused visible cellular toxicity (Fig. 5.4 and data not shown). In an effort to further characterize these compounds, we analyzed their half maximal inhibitory concentrations (IC50) to measure the effectiveness of the compounds in inhibiting transcription. Hypericin has an IC50 of approximately 5-10μM, comparable to other known transcription inhibitors (Fig. 5.5). Analyses of the IC50s for rottlerin and SP600125 are currently underway.

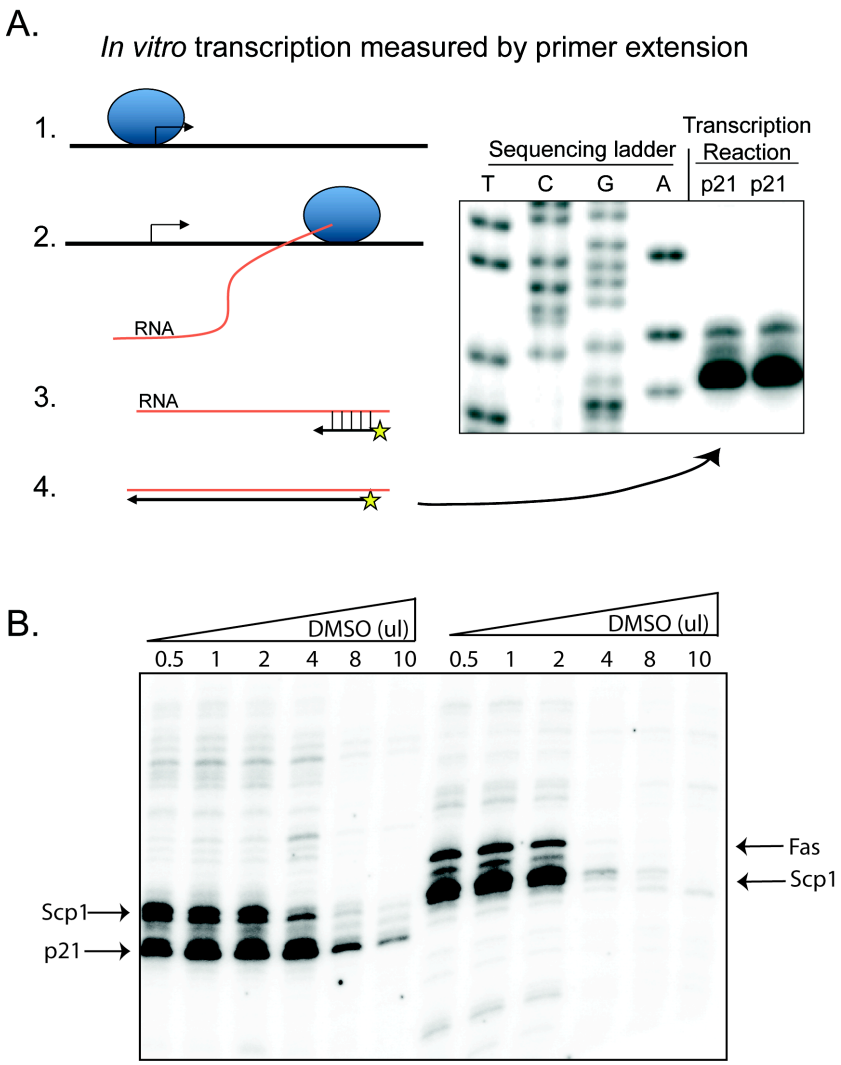

Figure 5.1 In vitro transcription assay used to screen compound library. *(A) Diagram of the in vitro transcription assay showing (1) PIC formation and initiation, (2) elongation and production of mRNA, (3) annealing of radioactive labeled primer, (4) primer extension and detection by polyacrilymide gel electrophoresis. (B) Transcriptional analysis of p21, Fas, and Scp1 as a function of increasing amounts of DMSO.*

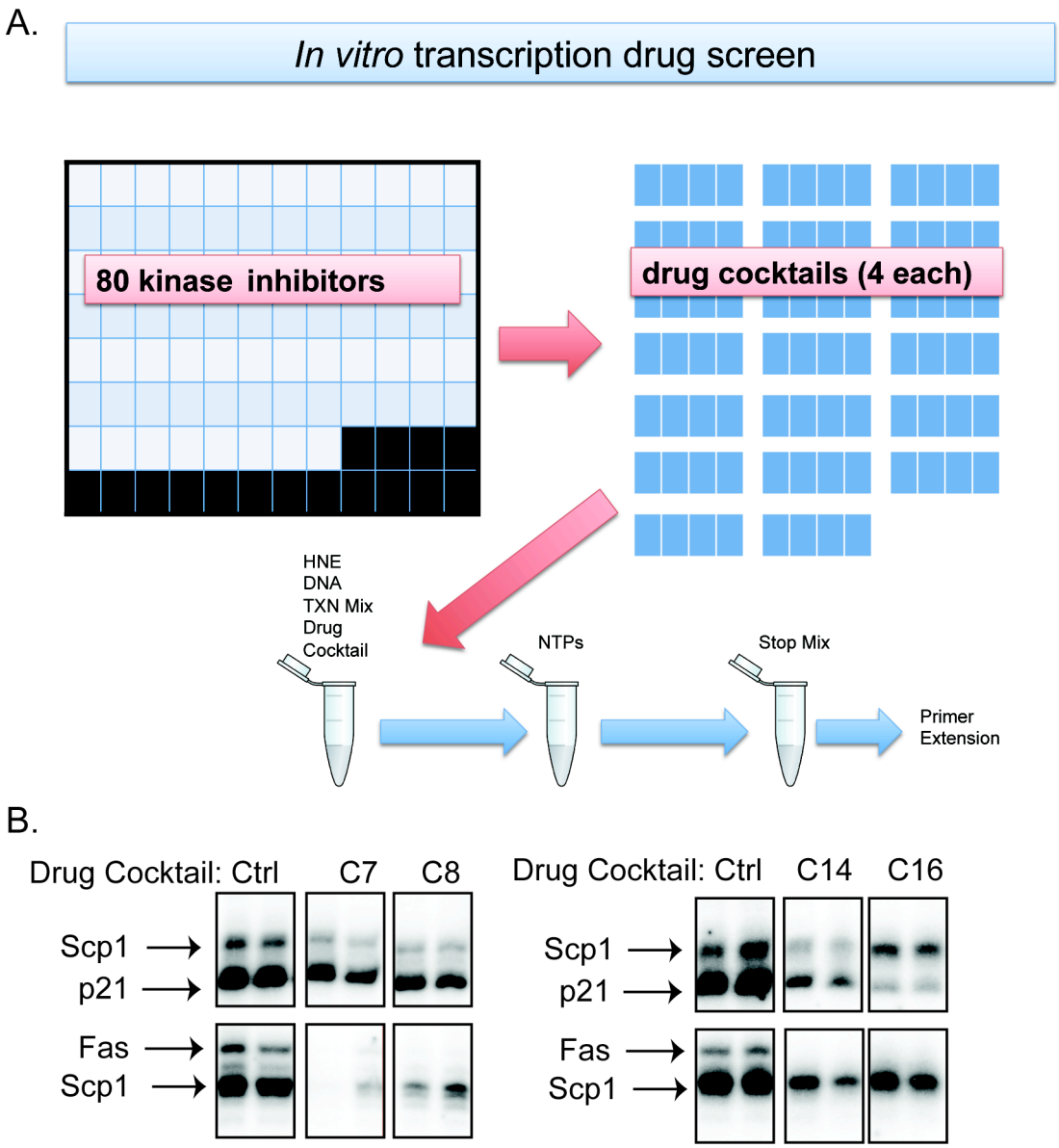

Figure 5.2 Kinase inhibitor screen identified 4 cocktails that block in vitro transcription. (A) *Diagram of the in vitro transcription drug screen using 80 kinase inhibitors from BIOMOL. The 80 compounds were mixed into 20 cocktails containing 4 drugs each at final concentrations of 1.25mM concentrations (50μM in final reactions). (B) Four of the drug cocktails yielded significant reduction in transcription compared to the controls.*

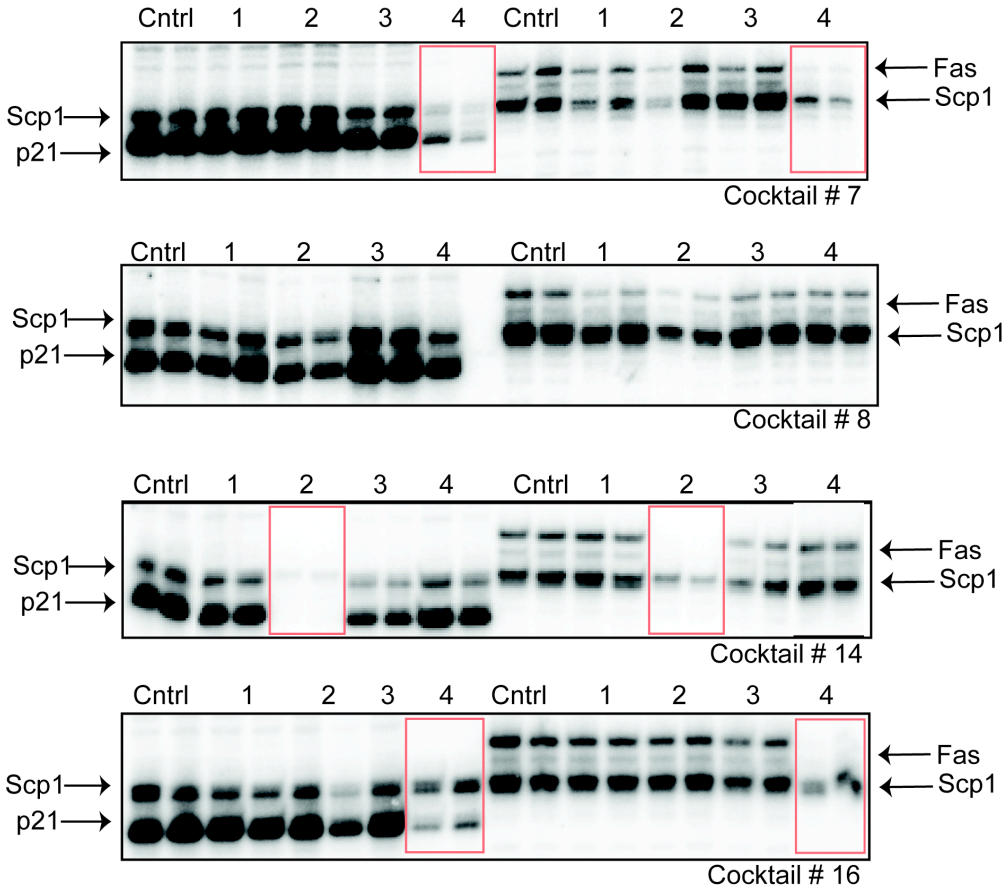

Figure 5.3 Identification of the active drugs within the four inhibitory cocktail mixes. *Three active compounds clearly inhibited transcription and are highlighted with red rectangles. In vitro transcription reactions were performed in duplicates. The compounds are hypericin (from cocktail #7), rottlerin (from cocktail #14), and Sp600125 (from cocktail #16).*

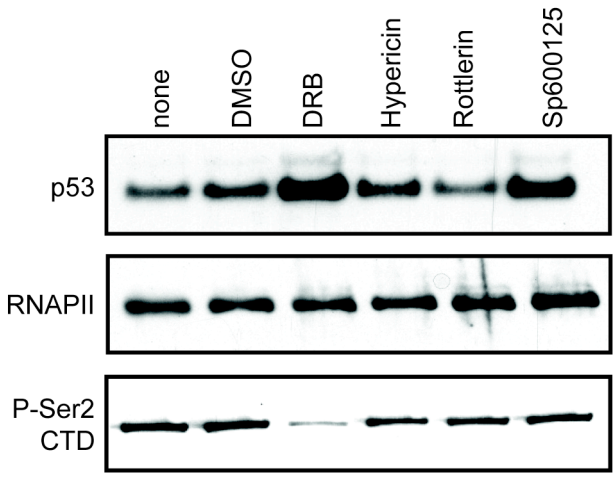

Figure 5.4 Western Blot analysis of drug treated HCT116 cells.

HCT116 cells were plated in 6-well dishes and treated with H_20 (none), DMSO (negative control), DRB (positive control), hypericin, rottlerin, or Sp600125 at 50μM final concentrations. Cells were collected 4 hours later and processed for SDS-PAGE. Immunoblots were probed using antibodies towards p53 (Calbiochem DO-1), RNAP II (H224 Santa Cruz), and P-Ser2 CTD (H5).

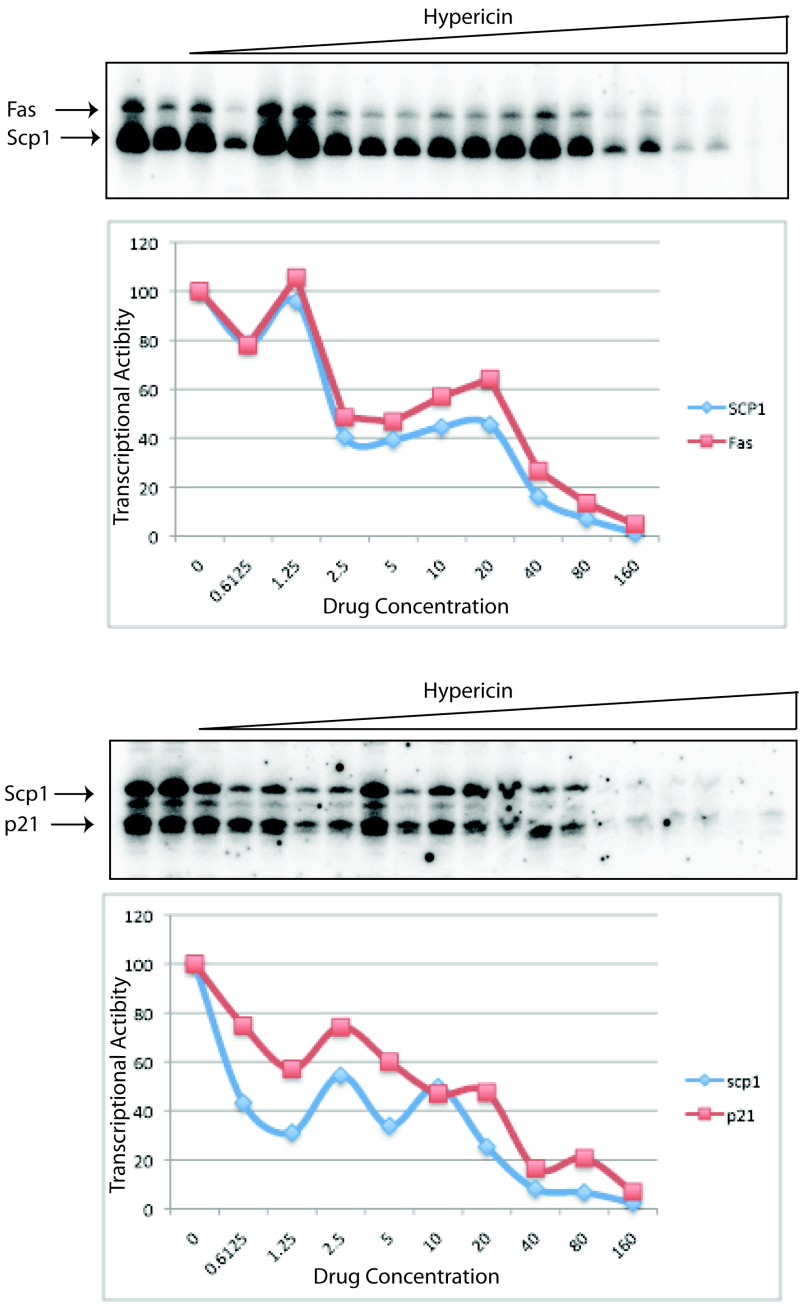

Figure 5.4 Analysis of the half maximal inhibitory concentration (IC50) for hypericin. *Increasing concentrations (0-160μM) of hypericin were added to in vitro transcription reactions using the indicated promoter templates. Reactions were analyzed as described and quantified using a phosphoimager. Hypericin has an IC50 of approximately 5-10μM concentrations.*

Table 1: List of compounds screened

KINASE INHIBITORS	INTENDED TARGET		KINASE INHIBITORS	INTENDED TARGET
PD-98059	MEK		KN-93	CaMK II
U-0126	MEK		ML-7	MLCK
SB-203580	p38 MAPK		ML-9	MLCK
H-7	PKA, PKG, MLCK, and PKC.		2-Aminopurine	p58 PITSLRE beta1
H-9	PKA, PKG, MLCK, and PKC.		N9-Isopropyl-olomoucine	CDK
Staurosporine	Pan-specific		Olomoucine	CDK
AG-494	EGFRK, PDGFRK		iso-Olomoucine	Negative control for olomoucine.
AG-825	HER1-2		Roscovitine	CDK
Lavendustin A	EGFRK		5-Iodotubercidin	ERK2, adenosine kinase, CK1, CK2,
RG-14620	EGFRK		LFM-A13	BTK
Tyrphostin 23	EGFRK		SB-202190	p38 MAPK
Tyrphostin 25	EGFRK		PP2	Src family
Tyrphostin 46	EGFRK, PDGFRK		ZM 336372	cRAF
Tyrphostin 47	EGFRK		SU 4312	Flk1
Tyrphostin 51	EGFRK		AG-1296	PDGFRK
Tyrphostin 1	Negative control for tyrosine kinase inhibitors.		GW 5074	cRAF
Tyrphostin AG 1288	Tyrosine kinases		Palmitoyl-DL-carnitine Cl	PKC
Tyrphostin AG 1478	EGFRK		Rottlerin	PKC delta
Tyrphostin AG 1295	Tyrosine kinases		Genistein	Tyrosine Kinases
Tyrphostin 9	PDGFRK		Daidzein	Negative control for Genistein.
HNMPA (Hydroxy-2-naphthalenylmethylphosphonic acid)	IRK		Erbstatin analog	EGFRK
PKC-412	PKC inhibitor		Quercetin dihydrate	PI 3-K
Piceatannol	Syk		SU1498	Flk1
PP1	Src family		ZM 449829	JAK-3
AG-490	JAK-2		BAY 11-7082	IKK pathway
AG-126	IRAK		DRB (5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole)	CK II
AG-370	PDGFRK		HBDDE (2,2',3,3',4,4'-Hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether)	PKC alpha, PKC gamma
AG-879	NGFRK		SP 600125	JNK
LY 294002	PI 3-K		Indirubin	GSK-3beta, CDK5
Wortmannin	PI 3-K		Indirubin-3'-monoxime	GSK-3beta
GF 109203X	PKC		Y-27632	ROCK
Hypericin	PKC		Kenpaullone	GSK-3beta
Ro 31-8220	PKC		Terreic acid	BTK
Sphingosine	PKC		Triciribine	Akt signaling pathway
H-89	PKA		BML-257	Akt
H-8	PKA, PKG		SC-514	IKK2
HA-1004	PKA, PKG		BML-259	Cdk5/p25
HA-1077	PKA, PKG		Apigenin	CK-II
HDBA (2-Hydroxy-5-(2,5-dihydroxybenzylamino)benzoic acid)	EGFRK, CaMK II		BML-265 (Erlotinib analog)	EGFRK
KN-62	CaMK II		Rapamycin	mTOR

REFERENCES:

1. Juven-Gershon, T., Cheng, S., and Kadonaga, J. T. (2006). Rational design of a super core promoter that enhances gene expression. Nature Methods 3, 917-922.

2. Ritz, R., et al., Photodynamic therapy of malignant glioma with hypericin: comprehensive *in vitro* study in human glioblastoma cell lines. Int. J. Oncol. 30, 659-667, (2007)

3. Miskovsky, P., Hypericin--a new antiviral and antitumor photosensitizer: mechanism of action and interaction with biological macromolecules. Curr. Drug Targets 3, 55-84, (2002)

4. Gschwendt M, Müller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G, Marks F. Rottlerin, a novel protein kinase inhibitor. Biochem Biophys Res Commun. 1994 Feb 28;199(1):93–98.

5. B.L. Bennett et al., SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase, Proc Natl Acad Sci, 98:13681-6, 2001.

ACKNOWLEDGEMENTS:

Collaborators subject to authorship deriving from this work include:

Beverly M. Emerson, Ph.D.

Ruo Huang, M.S.

Jose M. Morachis, Ruo Huang, Beverly M. Emerson. "Identification and characterization of three kinase inhibitors that block transcription initiation of *p21* and *Fas/APO1*." (In preparation)

Chapter 6

Conclusions

In unstressed cells, certain p53 target promoters, like p21, are “preloaded” with paused RNAP II, whereas pro-apoptotic promoters, among others, have negligible RNAP II association. Such striking variation in levels of promoter-bound RNAP II may have direct bearing on the differential activation kinetics observed after stress-induction of p53-responsive genes (1). The existence of such regulatory mechanisms acting before DNA damage to establish a default programmatic transcriptional response to stress is very intriguing. Our data suggest that the intrinsic properties of diverse p53 core promoters play a key role in regulating RNAP II affinity and dynamics to coordinate appropriate responses to different stress conditions. We find an unexpected level of transcriptional regulation governing RNAP II dynamics that is encoded within the DNA sequence of diverse core promoters that drives expression of p53-responsive genes (Fig. 6.1). The TATA box within the *p21* promoter has a critical role in recruiting the transcriptional machinery by promoting rapid formation of a functional PIC that is poised for initiation. However, the *p21* core promoter is intrinsically inefficient for re-initiation, which may be enhanced by signal-dependent components acting at other levels of regulation to facilitate PIC re-formation and prolonged RNA synthesis. In contrast to *p21*, the *Fas/APO1* promoter does not contain a TATA box or other well-characterized core motifs and the rate of PIC formation is very slow. A Fas downstream element that binds to NF-Y is essential for core promoter activity *in vitro* and may nucleate PIC assembly by direct interaction with the general transcription machinery. Surprisingly, once transcription is engaged, the *Fas/APO1* promoter is capable of efficient RNAP II re-initiation events. Published reports have demonstrated that initiation and re-initiation can be experimentally uncoupled and, in one example, re-initiation is faster than initiation (2), which we also observe with *Fas/APO1*.

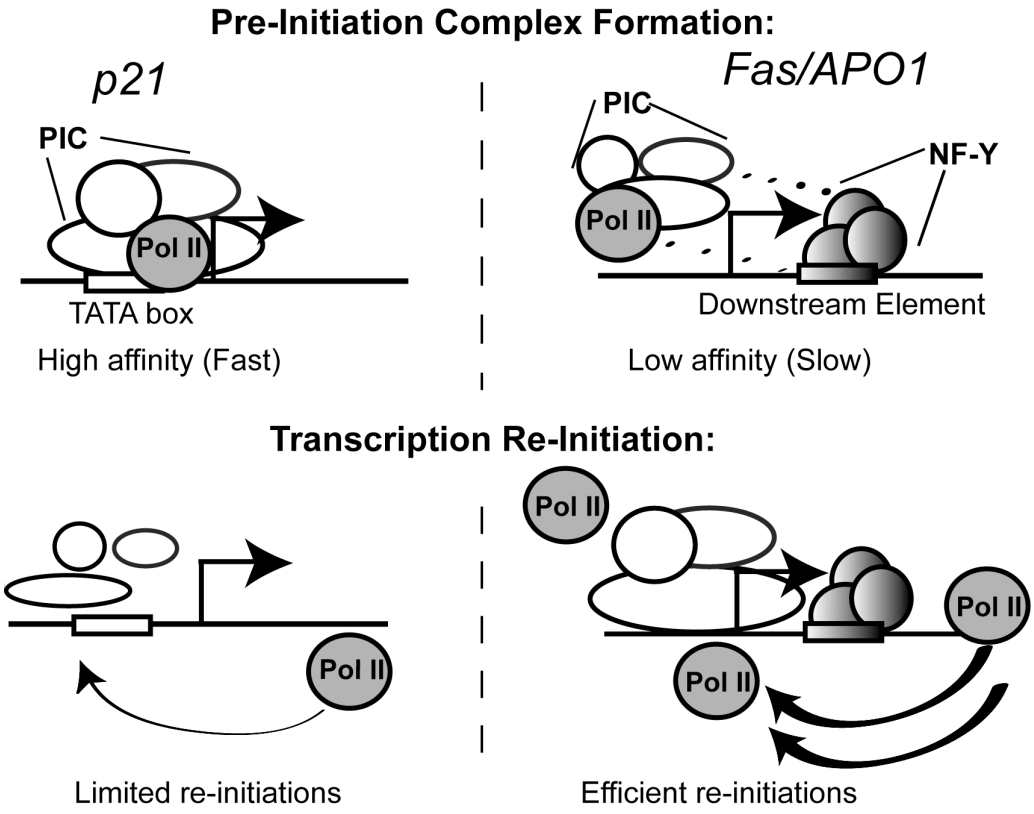

Figure 6.1. Schematic model of p21 and Fas/APO1 PIC formation and re-initiation kinetics. *The p21 core promoter supports efficient PIC assembly through the TATA box for rapid transcriptional activation, but only poor re-initiation capability. In contrast to p21, the Fas/APO1 core promoter has low affinity for PIC recruitment but supports multiple re-initiation events. The downstream NF-Y-binding element is required for core promoter activity and may facilitate nucleation of the PIC.*

Transcriptional activation of genes responsible for important cellular events such as cell cycle arrest and apoptosis must be highly regulated. For example, it is advantageous for the cell to have a poised RNAP II on the *p21* promoter to ensure its rapid activation upon stress signaling in order to quickly halt cell cycle progression. By contrast, it is equally advantageous to program pro-apoptotic genes, like *Fas/APO1*, for slow transcriptional initiation followed by rapid re-initiation as a safeguard against inappropriately timed cell death. Thus, a genetic system that is "hard-wired" within the DNA sequence of core promoters may be evolutionarily beneficial to protect cells against unwarranted cell proliferation after damage or apoptosis when damage cannot be repaired. For *Fas/APO1*, and other pro-apoptotic genes, having a fail-safe mechanism embedded in the promoter that prevents rapid transcription may be required to act as a buffer until critical signaling thresholds are surpassed. Once this point is reached, apoptotic genes can be actively transcribed and undergo multiple rounds of re-initiation to guarantee sufficient mRNA synthesis to drive cell suicide. Our data reveals that architecturally distinct core promoters, independent of chromatin or p53 binding, contain the information to impart these properties.

Role of the TATA box in *p21* promoter regulation

The TATA box was the first core promoter element to be identified and was originally believed to be required for all promoters. However, recent studies indicate that as few as 10% of all core promoters actually have a TATA sequence (3-4). For TATA-containing genes, this motif has been shown to be important in transcriptional activity and PIC formation, with TFIID recruited before other general PIC components (5-7). The core promoter of p21 contains a nearly perfect TATA sequence at about -30 bp, which is capable of interacting with either TBP or the TFIID holocomplex (Fig. 4.1). We show that

the TATA box is not only critical for p21 transcription but it enables the p21 promoter to efficiently assemble a poised PIC before transcriptional initiation. This intrinsic feature presumably facilitates the rapid stress-induced p21 expression kinetics observed *in vivo*. Interestingly, ChIP analyses of the p21 promoter in wildtype and p53-null cells show greatly reduced PIC formation in cells lacking p53 (1). In this case, p53 may function to relieve chromatin compaction through recruitment of co-factors, such as p300, to generate greater nucleosome accessibility for PIC formation before stress. In addition, specific post-translational modifications of p53 may also facilitate increased PIC interaction with the p21 promoter. In this regard, acetylation of lysines 373 and 382 on p53 has been shown to directly promote TFIID binding to the p21 core promoter (8).

Regulation of the *FasAPO1* core promoter downstream element by NF-Y

One explanation for the ability of the *Fas/APO1* promoter to undergo multiple rounds of transcription *in vitro* is through a particular multifunctional protein complex. Using immobilized DNA affinity assays, we captured and identified NF-Y as specifically binding to the Fas downstream promoter element. The physiological relevance was confirmed by demonstrating that NF-Y interacts with the endogenous *Fas/APO1* promoter and that overexpression of the NF-Y complex can stimulate basal *Fas/APO1* transcription *in vivo*. Interestingly NF-Y is known to activate or repress numerous promoters and associate with general transcription factors such as RNAP II, TFIID, TBP, and PC4 as well as cofactors, such as HATs and HDACs (9-13). Previous studies have shown that certain promoters have faster re-initiation rates by using a “scaffold”, consisting of TFIID, TFIIA, TFIIH, TFIIE and Mediator, which remains stably bound to the promoter after the first round of transcription (14, 15). This scaffold may facilitate RNAP II re-initiation by avoiding the requirement to re-assemble a full PIC after each round of

transcription. Our *in vitro* observations of slow initiation followed by efficient, multiple rounds of transcription from the *Fas/APO1*, but not *p21*, promoter are consistent with the existence of a scaffold. However, scaffold retention on *Fas/APO1* may be achieved by an unconventional mechanism since this promoter does not contain typical core elements but, instead, uses a near perfect NF-Y binding site (an inverted CCAAT box centered at +20). Because NF-Y can associate with TFIID, TBP, and RNAP II, it may facilitate nucleation of PIC components and/or help the core promoter to retain a partial-PIC "scaffold" to enhance re-initiation events. Another possibility is that NF-Y bends the DNA sequence in such a way as to increase transcriptional output from the core promoter. In this regard, the observation from chimeric promoters that the NF-Y binding motif (Fas downstream element) works positively with the TATA box in the *Fas/APO1* promoter but negatively when it is placed in the *p21* promoter suggests that NF-Y has a unique activity and must function in particular promoter contexts, underscoring its importance.

As a gatekeeper of cell growth and division, p53 must orchestrate the activities of numerous factors, such as NF-Y, that regulate transcription to ensure orderly cell cycle progression. Several studies have implicated a relationship between p53 and NF-Y in which p53 interacts directly with NF-Y to repress various cell cycle genes (16). Interestingly, NF-Y knockdown by siRNA causes apoptosis while activating many p53 target genes. We analyzed RNA levels in NF-Y knockdown cells and observed activation of *Fas/APO1, p21,* and *PUMA* genes, possibly by indirect induction. This is consistent with previous studies, although *Fas/APO1* expression was not measured (17). Activation of p53 target genes is not entirely surprising since Benatti et al. demonstrated that NF-Y depletion from HCT116 cells resulted in down-regulation of 478 genes and up-regulation

of 803 genes, indicating that NF-Y knockdown affects multiple genes in a global manner. We hypothesize that NF-Y is a critical, functionally diverse transcription factor and upon its cellular depletion a crisis ensues that results in apoptosis. The multifunctional nature of NF-Y on its distinct target genes may be conferred by phosphorylation of NF-YA by CDK2 (18). Interestingly, p53-dependent activation of *p21*, an inhibitor of CDK2, may create a regulatory loop that ultimately affects NF-Y phosphorylation and activity (19). With regard to *Fas/APO1*, we speculate that stress-dependent gene activation requires a positive interaction between promoter-bound p53 and NF-Y and the involvement of post-translational modifications as well as specific interactions with Mediator, TAFs, or chromatin modifying enzymes to generate a fully regulated transcriptional response (20-22).

Core promoter diversity

Our results provide insight into how TATA-less promoters are transcribed. Previous studies demonstrated that TATA-less promoters rely on other core elements that interact directly with various TFIID associated factors (TAFs) (23-25). Here we show that *Fas/APO1* can use an alternative promoter element that associates with NF-Y and potentially facilitates PIC assembly. In addition, our analysis of chimeric promoters demonstrates the importance of the relative context of core elements. An inserted TATA box within *Fas/APO1* increases transcription but cannot functionally replace the Fas downstream element. However, the Fas downstream element placed within the *p21* promoter negatively affects transcription and does not rescue a mutated TATA box. It is clear that the interplay between distinct core promoter elements confers another level of promoter regulation and can result in diverse transcriptional outputs. Recent studies have shed light on how these core regulatory circuits control RNAP II activity. One

seminal report demonstrated crosstalk between the TATA box and DPE in which factors bound specifically to one element had an inhibitory effect on transcription initiated at the other (26). Furthermore, TFIID has been shown to regulate promoters through two distinct motifs, the DPE and DCE, in a phosphorylation-dependent manner driven by CK2 (27). This is significant because it demonstrates that extracellular signals can impact gene activity by determining which core element a given transcription factor will function through.

The diversity of core promoter structures within p53-regulated genes implies that each gene may be uniquely regulated. However, the fact that most apoptotic target genes have much lower levels of poised RNAP II compared to cell cycle arrest genes suggests that there are some general similarities. A comparison of core promoters from several p53 target genes reveals that most cell cycle arrest genes have focused promoters (single start sites and typical core elements) whereas most apoptotic genes including *Fas/APO1, PUMA, and APAF-1* appear to resemble dispersed promoters (several start sites over 50-100 nucleotides and few typical core elements) (unpublished observations and (28)). We speculate that differences in core promoter structures exist to establish a default transcriptional state, with respect to PIC formation and dynamics, which insures an appropriate p53 programmatic response to diverse forms of stress.

Thus, two critical parameters of p53-dependent gene activation, the kinetics of induction and duration of expression through frequent re-initiation, are intrinsic, DNA-encoded features of diverse core promoters which may be fundamental to anticipatory programming of p53 response genes. The default mode, as seen at the *p21* promoter, is to rapidly form a PIC but undergo few rounds of re-initiation whereas that of *Fas/APO1* is

the opposite. Of course, sustained *p21* expression requiring multiple rounds of RNAP II re-initiation and reduced *Fas/APO1* expression by infrequent re-initiation can be achieved by overriding the default programming through sophisticated epigenetic processes that are tailored to specific stress environments. Considering the advantage of preserving flexibility to fine-tune cell fate decisions, having a default, genetic program embedded in core promoter DNA would safeguard against mis-regulation, particularly of apoptotic genes. This may reflect an evolutionary need to balance cell growth while limiting the ability to self-destruct. It is difficult to envision a cellular system that would evolve to activate cell cycle arrest and apoptotic genes identically. If this were true, apoptosis would likely override the cell cycle arrest program without allowing the cell to recover from stress or DNA damage. Further investigation into the default mechanisms utilized by structurally diverse p53 target genes may provide insight into how the programmatic response to stress is regulated and how it can be manipulated for targeted therapies.

Pharmacologic inhibition of transcription initiation

In chapter 5, I presented the initial findings from a small molecule screen to identify new factors that inhibit transcripton initiation. From a library of 80 commercially available kinases, I was able to identify three novel inhibitors of transcription at a concentration at or below 50μM. Our data suggests that these drugs are not inhibiting Ser2-CTD phosphorylation. Future experiments to test inhibition of Ser5-CTD phosphorylation are still required.

Additional studies will be required to fully understand the mechanism of action and the relevant kinases that they inhibit. The identification of these compounds adds to the limited toolbox available to scientists studying transcription regulation. Hopefully,

they will allow the dissecting of new mechanistic information beyond those drugs currently used to study RNAP II transcription. In addition, analogues of these compounds may provide new therapeutic candidates to treat some types of human diseases.

REFERENCES:

1. Espinosa, J. M., Verdun, R. E., and Emerson, B. M. (2003) p53 functions through stress- and promoter-specific recruitment of transcription initiation components before and after DNA damage, *Mol Cell 12*, 1015-1027.

2. Jiang, Y., and Gralla, J. D. (1993) Uncoupling of initiation and reinitiation rates during Hela RNA polymerase II transcription *in vitro*, *Mol Cell Biol 13*, 4572-4577.

3. Yang, C., Bolotin, E., Jiang, T., Sladek, F. M., and Martinez, E. (2007) Prevalence of the initiator over the TATA box in human and yeast genes and identification of DNA motifs enriched in human TATA-less core promoters, *Gene 389*, 52-65.

4. Suzuki, Y., Tsunoda, T., Sese, J., Taira, H., Mizushima-Sugano, J., Hata, H., Ota, T., Isogai, T., Tanaka, T., Nakamura, Y., Suyama, A., Sakaki, Y., Morishita, S., Okubo, K., and Sugano, S. (2001) Identification and characterization of the potential promoter regions of 1031 kinds of human genes, *Genome research 11*, 677-684.

5. Ranish, J. A., Yudkovsky, N., and Hahn, S. (1999) Intermediates in formation and activity of the RNA polymerase II preinitiation complex: holoenzyme recruitment and a postrecruitment role for the TATA box and TFIIB, *Genes Dev 13*, 49-63.

6. Mathis, D. J., and Chambon, P. (1981) The SV40 early region TATA box is required for accurate *in vitro* initiation of transcription, *Nature 290*, 310-315.

7. O'Shea-Greenfield, A., and Smale, S. T. (1992) Roles of TATA and initiator elements in determining the start site location and direction of RNA polymerase II transcription, *J Biol Chem 267*, 1391-1402.

8. Li, A. G., Piluso, L. G., Cai, X., Gadd, B. J., Ladurner, A. G., and Liu, X. (2007) An acetylation switch in p53 mediates holo-TFIID recruitment, *Mol Cell 28*, 408-421.

9. Peng, Y., and Jahroudi, N. (2003) The NFY transcription factor inhibits von Willebrand factor promoter activation in non-endothelial cells through recruitment of histone deacetylases, *J Biol Chem 278*, 8385-8394.

10. Caretti, G., Salsi, V., Vecchi, C., Imbriano, C., and Mantovani, R. (2003) Dynamic recruitment of NF-Y and histone acetyltransferases on cell-cycle promoters, *J Biol Chem 278*, 30435-30440.

11. Currie, R. A. (1998) Biochemical characterization of the NF-Y transcription factor complex during B lymphocyte development, *J Biol Chem 273*, 18220-18229.

12. Kabe, Y., Yamada, J., Uga, H., Yamaguchi, Y., Wada, T., and Handa, H. (2005) NF-Y is essential for the recruitment of RNA polymerase II and inducible transcription of several CCAAT box-containing genes, *Mol Cell Biol 25*, 512-522.

13. Bellorini, M., Lee, D. K., Dantonel, J. C., Zemzoumi, K., Roeder, R. G., Tora, L., and Mantovani, R. (1997) CCAAT binding NF-Y-TBP interactions: NF-YB and NF-YC require short domains adjacent to their histone fold motifs for association with TBP basic residues, *Nucleic Acids Res 25*, 2174-2181.

14. Yudkovsky, N., Ranish, J. A., and Hahn, S. (2000) A transcription reinitiation intermediate that is stabilized by activator, *Nature 408*, 225-229.

15. Zawel, L., Kumar, K. P., and Reinberg, D. (1995) Recycling of the general transcription factors during RNA polymerase II transcription, *Genes Dev 9*, 1479-1490.

16. Imbriano, C., Gurtner, A., Cocchiarella, F., Di Agostino, S., Basile, V., Gostissa, M., Dobbelstein, M., Del Sal, G., Piaggio, G., and Mantovani, R. (2005) Direct p53 transcriptional repression: *in vivo* analysis of CCAAT-containing G2/M promoters, *Mol Cell Biol 25*, 3737-3751.

17. Benatti, P., Basile, V., Merico, D., Fantoni, L. I., Tagliafico, E., and Imbriano, C. (2008) A balance between NF-Y and p53 governs the pro- and anti-apoptotic transcriptional response, *Nucleic Acids Res 36*, 1415-1428.

18. Chae, H. D., Yun, J., Bang, Y. J., and Shin, D. Y. (2004) Cdk2-dependent phosphorylation of the NF-Y transcription factor is essential for the expression of the cell cycle-regulatory genes and cell cycle G1/S and G2/M transitions, *Oncogene 23*, 4084-4088.

19. Yun, J., Chae, H. D., Choi, T. S., Kim, E. H., Bang, Y. J., Chung, J., Choi, K. S., Mantovani, R., and Shin, D. Y. (2003) Cdk2-dependent phosphorylation of the

NF-Y transcription factor and its involvement in the p53-p21 signaling pathway, *J Biol Chem 278*, 36966-36972.

20. Donner, A. J., Szostek, S., Hoover, J. M., and Espinosa, J. M. (2007) CDK8 is a stimulus-specific positive coregulator of p53 target genes, *Mol Cell 27*, 121-133.

21. Sullivan, A., and Lu, X. (2007) ASPP: a new family of oncogenes and tumour suppressor genes, *Br J Cancer 96*, 196-200.

22. An, W., Kim, J., and Roeder, R. G. (2004) Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53, *Cell 117*, 735-748.

23. Smale, S. T., and Kadonaga, J. T. (2003) The RNA polymerase II core promoter, *Annu Rev Biochem 72*, 449-479.

24. Burke, T. W., and Kadonaga, J. T. (1996) Drosophila TFIID binds to a conserved downstream basal promoter element that is present in many TATA-box-deficient promoters, *Genes Dev 10*, 711-724.

25. Martinez, E., Chiang, C. M., Ge, H., and Roeder, R. G. (1994) TATA-binding protein-associated factor(s) in TFIID function through the initiator to direct basal transcription from a TATA-less class II promoter, *EMBO J 13*, 3115-3126.

26. Hsu, J. Y., Juven-Gershon, T., Marr, M. T., 2nd, Wright, K. J., Tjian, R., and Kadonaga, J. T. (2008) TBP, Mot1, and NC2 establish a regulatory circuit that controls DPE-dependent versus TATA-dependent transcription, *Genes Dev 22*, 2353-2358.

27. Lewis, B. A., Sims, R. J., 3rd, Lane, W. S., and Reinberg, D. (2005) Functional characterization of core promoter elements: DPE-specific transcription requires the protein kinase CK2 and the PC4 coactivator, *Mol Cell 18*, 471-481.

28. Juven-Gershon, T., and Kadonaga, J. T. (2009) Regulation of Gene Expression via the Core Promoter and the Basal Transcriptional Machinery, *Dev Biol*.

ACKNOWLEDGEMENTS:

Collaborators subject to authorship deriving from this work include:

Beverly M. Emerson

Chris M. Murawsky

Ruo Huang, M.S.

Jose M. Morachis, Ruo Huang, Beverly M. Emerson. "Identification and characterization of three kinase inhibitors that block transcription initiation of *p21* and *Fas/APO1*." (In preparation).

Jose M. Morachis, Chris M. Murawsky, Beverly M. Emerson. "Regulation of the p53 transcriptional response by structurally diverse core promoters." (Genes & Development in press).

Appendix I

Materials and Methods

In vitro transcription assays

Nuclear protein extracts from Hela cells were prepared as described (Dignam *et al.* 1983)**.** Transcription reactions included 10µl (~5-6µg/µl) Hela Nuclear Extract (HNE), 15µl Hela Dialysis Buffer (HDB) (20mM Hepes-pH7.9, 50mM KCl, 1mM DTT, 0.2mM EDTA, 10% glycerol) and 25µl transcription mix (0.4mg/ml BSA**,** 20mM HEPES-pH 7.9, 70mM KCl, 3mM DTT, 1.2mM NTPs, 1-3mM $MgCl_2$, 0.5µl RNase inhibitor per reaction) and 500ng DNA templates. For the PIC kinetics analyses, NTPs were omitted from the transcription mix and the PIC was allowed to form for 0 min-2 hr at 25°C before adding 2µl of NTP mix to start the reaction (final volumes were adjusted with HDB). Sarkosyl (Sigma-Aldrich) was introduced in each reaction by adding 2µl of a 1% stock to 0.04% final concentration unless otherwise noted. Transcription reactions were incubated in a 30°C water bath or at room temperature. Reactions were stopped and processed using reagents from Zymo Research (RNA Clean-up Kit-5) by adding 200µl of RNA binding buffer, applying the mixture to columns, washing two times with wash buffer and then eluting with 8µl of RNAse-free water. Primer extension was performed by adding 3µl of primer annealing mix (10mM Tris, 1mM EDTA, 1.25M KCl) to each reaction and heating at 75°C for 2-3 minutes in heating blocks. Reactions were removed from the heat blocks and allowed to slowly cool to about 37°C. This was followed by addition of 23µl reverse transcription mix (20mM Tris-HCl pH8, 10mM $MgCl_2$, 0.1mg/ml Actinomycin D, 5mM DTT, 0.33mM dNTP) and 0.5µl M-MLV Reverse Transcriptase (Promega) per reaction and incubation at 37°C for 1 hour. Final reactions were precipitated and washed with ethanol and placed in a speedvac for 5 minutes. DNA pellets were each resuspended in 10µl formamide with EDTA (1mM)/NaOH(0.1mM) (2:1) and heated to 95°C for 2-3 minutes followed by snap cooling on ice. Samples were electrophoresed through 8%

polyacrylamide/TBE gels (SequaGel-8, National Diagnostics). For transcription reactions using immobilized DNA templates, plasmids containing the *p21* and *Fas* full promoters were first linearized by restriction enzyme cleavage with NotI, followed by cleavage with a second restriction enzyme, EcoRI, to generate sticky ends that were filled-in by Klenow DNAP with biotinylated dATP and dUTP. After removal of excess nucleotides, the biotinylated fragments were incubated with streptavidin-coated magnetic beads (Dynal, Invitrogen) and purified from un-biotinylated DNA using a magnet. The immobilized *p21* or *Fas/APO1* DNA templates (250 fmoles) were each incubated with Hela nuclear extract for *in vitro* transcription as described.

Plasmids and mutagenesis

The *p21* (-2.4 kb to +42 bp) and *Fas/APO1* (-1 kb to +700 bp) promoters were each cloned into pBSKII plasmids using EcoRI and XbaI and named p21DPE-A and FasMut2, respectively. For the "core" *p21* and *Fas* promoters, PCR was used to generate the sequence of -149 to 42 bp for *p21* and -50 to 78 bp for *Fas* and then subcloned back into pBSKII. All mutational analyses were performed by progressively generating 10 bp transversion mutations in the context of the full-length promoters using the GeneTailor™ site-directed mutagenesis system following the manufacturer's instructions (Invitrogen). Fas-TATA was created using *Fas/APO1* promoter sequence and inserting the *p21* ATATCAG sequence to replace -23 to -29 and create the Fas-TATA promoter. F-TATA was created using Fas-TATA and mutating the scan F region as in scan F of Fig. 3B. *p21* wildtype (TATA) or *p21* scan C (mutated TATA) promoters were mutated by inserting the F, G, or F+G elements of *Fas/APO1* at the same position with respect to the transcription start site (from +7 to +26 bp). All of the templates used

for *in vitro* transcription contained a 27 bp sequence from the luciferase gene (5'-gcgtcttccattttaccaacagtaccg-3') that was used for primer extension. For the luciferase reporter assays, the core promoters of *p21* (-134 to +42 bp) and Fas (-130 to +46 bp) were generated by PCR and each subcloned into pGL3 reporter plasmids.

EMSA

Protein-DNA binding reactions included: 2µg HNE, 250ng dI-dC, and 5µl pre-mix (1µl of 5X shift buffer (100mM HEPES, 350mM KCl, 25mM $MgCl_2$, and 15mM DTT), 0.5µl 4mg/ml BSA and 1.5µl 15% Ficoll adjusted to 10µl final volume with HDB. The pre-mix was incubated for 15 minutes at 4°C followed by the addition of 1µl (15fmol/µl) of ^{32}P-labeled oligonucleotide. Binding reactions were performed at room temperature for 30 minutes. For antibody "supershift" assays, 2µg of anti-NF-YA (C-18), anti-NF-YC (H-120), anti-YY1 (H-414), and anti-Bmi-1 (C-20) from SantaCruz were incubated with binding reactions for 15 minutes before gel loading. Samples were then electrophoresed through 4% PAGE (39:1 acrylamide/bis) in TBE and scanned using a Fuji FLA-5100 phosphoimager.

DNA-affinity protein chromatography

5'-biotin-labeled DNA primers (Supplemental table) were used in an efficient PCR method (Hemat and McEntee 1994) to generate ~500bp multimers of the wildtype and mutated DNA sequences used in the EMSA reactions. The stock bead reaction contained 5µg DNA, 2µg Streptavidin-coated magnetic beads (Dynal, Invitrogen) in 400µl final volume. For the recruitment assay, Hela nuclear extracts were first pre-

cleared by mixing 100µl (~600µg) HNE with 100µl “pre-mix” (similar to EMSA buffer) and passing the reaction through 30µl (~450ng) of immobilized mutated DNA three times. The pre-cleared extract was then separated from the beads, mixed with immobilized wildtype or mutated DNA, and allowed to bind at room temperature for 30 minutes. Reactions were washed three times with HDB containing 0.1mg/ml of single- and double-stranded E. coli DNA. Bound proteins were then eluted with 20µl of HDB containing 100mM, 250mM, 500mM, or 1M NaCl. These fractions were analyzed for protein composition by SDS-PAGE (Western blot, silver staining, and mass spec) and for specific DNA binding by EMSA.

Mass spectrometry

Silver-stained protein bands were excised and in-gel digested with trypsin (1) and analyzed by LC electrospray ionization MS as described (2,3). Briefly, samples were loaded onto a capillary column with integrated spray tip (75 um I.D., 10 um tip, New Objective, Woburn, MA), which was packed in-house with C18 reversed phase material (Zorbax SB-C18, 5 um particle size, Agilent, Santa Clara, CA) to a length of 10 cm. The reversed phase elution was achieved by a linear gradient of 0-60% acetonitrile in 0.1% formic acid within 60 minutes at a flow rate of 300 nl/min. The eluate was introduced into a Thermo LTQ-Orbitrap mass spectrometer (ThermoFisher, Waltham, MA) via a nano-spray source. Mass spectrometric analysis was conducted by recording precursor ion scans at a resolution of 60,000 in the Orbitrap Fourier-transform analyzer followed by MS/MS scans of the top 5 ions in the linear ion trap (cycle time approx. 1 s). An active exclusion window of 90 s was employed. Data were analyzed using the Mascot

algorithm (Matrix Science, London, UK) or on a Sorcerer Solo system running Sorcerer-Sequest.

References:

1. Shevchenko A, Wilm M, Vorm O, Mann M 1996 Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68:850-858

2. Schubert D, Herrera F, Cumming R, Read J, Low W, Maher P, Fischer WH 2009 Neural cells secrete a unique repertoire of proteins. J Neurochem 109:427-435

3. Herrera F, Chen Q, Fischer WH, Maher P, Schubert DR 2009 Synaptojanin-1 plays a key role in astrogliogenesis: possible relevance for Down's syndrome. Cell Death Differ 16:910-920

Transfection for dual luciferase assay and NF-Y over-expression

HCT116 cells were plated in a 12-well plate and grown at 70% confluency for transfection. For luciferase assays, 1.8µg pGL3, p21-pGL3 or Fas-pGL3 core promoter constructs and 200ng renilla (pRL-TK) were used/well for transfection with Fugene HD (Roche). After transfection, cells were maintained in 500µl DMEM growth media overnight. Cells were then lysed in 100µl 1X passive lysis buffer (PLB buffer, Promega) at room temperature for 15 minutes. For dual luciferase assays, 20µl lysate/well were aliquoted into a 96-well plate and analyzed using firefly luciferase (50µl LAR II) and

renilla (50µl stop- and glow-buffer). All analyses were performed in duplicate, with each experiment performed at least twice.

For overexpression of NF-Y, MCF7 cells were plated in 12-well plates and grown to 80% confluency for transfection. We used NF-YA(SC112917), NF-YB(SC116285), and NF-YC(SC112622) human cDNA clones in pCMV6-XL5 plasmids (Origen) and pCMV-GPF as a control. FuGENE HD (Roche) transfection reagent was used at an 8:2 DNA:reagent ratio, transfecting 20µl transfection mix and 800ng of total DNA. Cells were collected 24 hours after transfection and processed for mRNA purification (Qiagen) or lysed for Western blot analysis.

Real-time RT–PCR reactions

Cells transfected with NF-Y expression plasmids were collected 24 hours later and total RNA was prepared with the Qiagen RNAeasy Kit. RT reactions were performed with SuperScriptase III (Invitrogen) using the random hexamer protocol following the manufacturer's instructions. Primers for *Fas/APO-1, p21, and SDHA* (a control housekeeping gene) were used to analyze gene expression using SYBR green and values were normalized to *Beta Actin*.

ChIP assays

MCF7 cultures were grown to 50%–60% confluency and were treated with 50ng/ml of 5-FU (Sigma-Aldrich) for 14 hours. After washing with PBS, cells were cross-

linked with a 1% formaldehyde/PBS solution for 15 min at room temperature. Cross-linking was stopped by addition of glycine to 125mM final concentration. Cells were washed twice with cold PBS and harvested in RIPA buffer (150mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate, 0.1% SDS, 50mM Tris-HCl, pH 8, 5mM EDTA, 20mM NaF, 0.2mM sodium orthovanadate, 5μM trichostatin A, 5mM sodium butyrate, and protease inhibitors). Samples were sonicated to generate DNA fragments <1000 bp. For immunoprecipitation, 1mg protein extract was precleared for 1 hr with 40μl 50% A/G protein-Sepharose slurry before addition of indicated antibodies. The antibodies used were anti-NF-YA (H-209) or rabbit IgG (SantaCruz). Each antibody (2μg) was added to the samples and incubated overnight at 4°C in the presence of 40μl protein G-beads preblocked with 1mg/ml BSA and 0.3mg/ml salmon sperm DNA. Beads were washed once with RIPA buffer, three times with ChIP Wash Buffer (100mM Tris-HCl, pH 8.5, 500mM LiCL, 1% [v/v] NP-40, 1% [w/v] deoxycholic acid), once again with RIPA buffer, and twice with 1XTE. Immunocomplexes were eluted for 10 min at 65°C with 1% SDS, and cross-linking was reversed by adjusting to 200mM NaCl and incubating 5 hr at 65°C. DNA was purified, and a fraction was used as template in real-time PCR reactions.

Western blot analysis

Proteins were electrophoresed through 10% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences) before being probed with the following antibodies: anti-NF-YA (H-209), anti-NF-YB (FL-207), anti-NF-YC (H-120), and anti-RNAP II (H224) (Santa Cruz).

Table 1:

	In vitro transcription (primer extension)	
In vitro Txn	Luc2 primer	gcgtcttccattttaccaacagtaccg
	Gel Shifts	
Gel Shifts	Fas wildtype Fwd	atgcctcttctcccgcgggttggtggacccgctca
	Fas wildtype Rev	cctgagcgggtccaccaacccgcgggagaagaggc
	Fas mutated Fwd	atgcctcGGAGAAATATTTGGTTGTTCcccgctca
	Fas mutated Rev	cctgagcgggGAACAACCAAATATTTCTCCgaggc
	Immobilized multimers	
Immobilized multimers	Fas wildtype-biot-Fwd	ctgcctcttctcccgcgggttggtggacccgctcagcTgcctcttCTcccgcgggttggtggacccgctcag
	Fas wildtype-biot-Rev	ctgagcgggtccaccaacccgcgggagaagaggcagctgagcgggtccaccaacccgcgggAGaagaggcag
	Fas wildtype-biot-Fwd	ctgcctcGGAGAAATATTTGGTTGTTCcccgctcagctgcctcGGAGAAATATTTGGTTGTTCcccgctcag
	Fas wildtype-biot-Rev	ctgagcgggGAACAACCAAATATTTCTCCgaggcagctgagcgggGAACAACCAAATATTTCTCCgaggcag
	qPCR	
	Fas RT_qPCR_Fwd	GGGGTGGCTTTGTCTTCTTCTTTTG
	Fas RT_qPCR_Rev	ACCTTGGTTTTCCTTTCTGTGCTTTCT
	ccnb1_RT_Fwd	TCTGGATAATGGTGAATGGACA
	ccnb1_RT_Rev	CGATGTGGCATACTTGTTCTTG
	p21 RT_qPCR_Fwd	CTGGAGACTCTCAGGGTCGAAA
	p21 RT_qPCR_Rev	GATTAGGGCTTCCTCTTGGAGAA
	SDHA_RT_qPCR_Fwd	CCACCACTGCATCAAATTCATG
	SDHA_RT_qPCR_Rev	TGGGAACAAGAGGGCATCTG
	B_Actin_RT_qPCR Fwd	GAAACTACCTTCAACTCCATC
	B_Actin_RT_qPCR Rev	CTAGAAGCATTTGCGGTGGAC
	ChIP	
ChIP	*Q-ChIP_ccnb1F ((-57 to +30 bp)	GCCCTGGAAACGCATTCTC
	*Q-ChIP_ccnb1R	GCACTGCTCCCTCCTTATTGG
	FAS_INR-159F	CTTCCTTCCCATCCTCCTGAC
	FAS_INR-215R	GCGAGATCAGAGACGAGCTCA
	**p21TATA_F	gggcggttgtatatcagggc
	**p21TATA_R	cggctccaacaaggaactgactt
	CCNB1 1490 frw	CAGGGTCACACAGGTAAGTG
	CCNB1 1490 rev	GCAGAGATGGCTTGTAGTTG
	p21 distal F (p53 RE)	CGGCTGATTTTTGTATTTTTAATG
	p21 distal R (p53 RE)	TCACAGGGTCAGGAGTTTTGAGA
	Fas downstream 2-Fwd	ATCTCATAGTTTTTGGACAGCAGT
	Fas downstream 2-Rev	TGGTACAAAAGTTCAACAAGCATA

* = Hu, Q., Lu, J.F., Luo, R., Sen, S., and Maity, S.N. 2006. Inhibition of CBF/NF-Y mediated transcription activation arrests cells at G2/M phase and suppresses expression of genes activated at G2/M phase of the cell cycle. Nucleic Acids Res 34(21):6272-85, 2007.

** = Kaeser, M.D. and Iggo, R.D. 2004. Promoter-specific p53-dependent histone acetylation following DNA damage. *Oncogene* **23**(22): 4007-4013.