Core Promoter Recognition Complex Switching in Liver Development and Regeneration

by

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Abstract

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The exquisite structural and functional complexity of the mammalian body requires the exactlying precise spatial and temporal regulation of gene expression. The most critically regulated step of which is the transcription of DNA to RNA by the elaborate interplay of sequence-specific activators and repressors, chromatin modifiers, coactivators, the basal machinery and RNA polymerase II. Until recently, developmental stage and tissue-specific gene expression patterns were believed to be regulated primarily by the combinatorial actions of DNA binding activators and repressors, while the core machinery was held to be invariant. New evidence suggests that significant differences in coactivator and core promoter recognition complex composition between cell types and developmental time points may facilitate the global changes in gene expression programs necessary to confer functional diversity. Because of its critical role in vertebrate development and homeostasis, the role of coactivator and core promoter recognition complex switching in the developing liver was examined.
This work shows that mouse liver progenitors or hepatoblasts contain significant levels of the canonical core promoter recognition complex TFIID, including the TATA binding protein and multiple associated factors or TAFs, and the canonical coactivator complex Mediator. These complexes and their constitutive proteins are significantly downregulated in adult hepatocytes. Furthermore, the promoters of several TFIID components become enriched for repressive chromatin marks in adult hepatocytes, and an in vitro model of liver development recapitulates the downregulation of these complexes observed in vivo. In contrast, expression of the TBP-related factor TRF3, the TAF7 paralogue TAF7l, and TAF13 is maintained or induced upon hepatic differentiation. Additionally, these proteins associate with high molecular weight complexes in vitro where they are enriched at hepatocyte-specific promoters, and their depletion attenuates hepatic gene induction. Together these observations support a model wherein liver development and the induction of hepatic gene expression requires the down regulation of TFIID and its replacement with one or more complexes likely containing TRF3, TAF7l and TAF13.
For my parents
Anne and Richard D’Alessio
who have supported all my adventures
and
with appreciation for Laurie McDonough
my best friend
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Chapter 1: Introduction

Summary

The precise spatial and temporal regulation of gene expression essential to metazoan development and cellular homeostasis requires a complex interplay between sequence-specific activators and repressors, coactivators, chromatin modifiers, general transcription factors (GTFs), and RNA polymerase II (PolII). The first and most regulated step in this process is the exacting recognition of unique gene-specific DNA regulatory sequences by diverse activator proteins. These activators then recruit the core promoter recognition complex TFIID consisting of the TATA box binding protein (TBP) and 12-15 associated factors (TAFs) to the site of transcription. Subsequently, coactivators including the Mediator complex (Med), chromatin modifiers, and the additional GTFs TFIIA-F are recruited to the core promoter to form the preinitiation complex (PIC). This allows for the recruitment of PolII and the start of productive RNA transcription.

Historically, developmental stage and tissue-specific patterns of gene expression were thought to be determined primarily by DNA regulatory sequences and their associated activators, while the general transcription machinery including TFIID was held to be invariant (reviewed by Thomas and Chiang 2006). However, recent studies have shown that during skeletal muscle myogenesis a novel mechanism of regulating global patterns of gene expression by switching of core promoter recognition complexes is employed (reviewed by Reina and Hernandez 2007). Whether this novel mechanism
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of transcriptional regulation exists in other tissues and developmental programs or is restricted to muscle is currently unknown.

Chapter two examines patterns of TBP and TAF expression in diverse adult mouse tissues and finds that they are significantly down regulated in the adult liver. Further by purifying fetal liver progenitors (hepatoblasts), partially differentiated neonatal hepatocytes, adult hepatocytes, and cycling regenerative hepatocytes, I demonstrate that TFIID is significantly upregulated in liver progenitors and entirely absent from the fully differentiated cell type, while at intermediate levels in slowly dividing or not fully differentiated populations. In striking contrast, the TBP-related factor TRF3 and TAF13 are expressed at the same levels throughout liver development and the unique TAF paralogoue TAF7l is highly induced upon differentiation. Importantly, these changes occur at both the transcriptional and protein levels and can be reconstituted in an *in vitro* cellular model of liver differentiation.

The third chapter of this thesis confirms the presence of TRF3, TAF7l, and TAF13 polypeptides in differentiated liver cells and provides evidence that they are components of one or more high molecular weight complexes *in vivo*. Furthermore, RNAi mediated depletion of each of these three proteins attenuates hepatic gene induction in an *in vitro* model of liver differentiation, and all three proteins show varying degrees of enrichment at the proximal promoters of hepatocyte-specific genes. Finally, an embryonic stem cell based hepatic differentiation protocol suggests that these changes in core promoter recognition complex architecture may extend beyond the murine model.
TFIID, TAFs, and the Basal Transcription Machinery

Proteins, the basic functional units of every living cell, are produced by the transcription of DNA to messenger RNA, the splicing and nuclear export of this mRNA, and the translation of mRNAs to polypeptides in a cell’s cytoplasm; a process known as the Central Dogma (Crick 1958). The most critical and regulated step in this process is the precise transcription of protein coding regions within a cell’s DNA by RNA polymerase II (PolII) to form mRNA. The recruitment of PolII and initiation of transcription at specific genes requires the exquisite coordination of hundreds of polypeptides including the core promoter recognition complex TFIID (Figure 1) (Thomas and Chiang 2006).

TFIID was initially identified as a biochemical activity required for in vitro transcription by PolII (Matsui et al. 1980). Upon further purification, it was identified as a 750kD complex containing the TATA box binding protein (TBP) and 12-15 associated factors (TAFs) that are required for activated transcription both in vitro and in vivo (Dynlacht et al. 1991; Pugh and Tjian 1991). It is now known that both TBP and most of the TAFs are highly conserved in yeast (S. cerevisiae), worms (C. elegans), flies (D. melanogaster) and humans (H. sapiens) and partially conserved in the metazoan predecessor M. brevicollis, suggesting an ancient evolutionary origin (Burley and Roeder 1996; Albright and Tjian 2000; King et al. 2008).

The process of regulated transcription takes place by the stepwise assembly of the preinitiation complex (PIC), followed by promoter escape, productive elongation, and termination. PIC assembly is initiated through the precise recognition and binding of
distinct DNA sequences or enhancers within a gene’s distal or proximal regulatory regions by a diverse set of tissue and developmental stage-specific transcriptional activators. These activators in turn position TFIID at the core promoter through binding of their activation domains either directly to TBP or a subset of specific TAFs (Stringer et al. 1990; Chen et al. 1994; Thut et al. 1995). TFIID’s affinity for the core promoters is further stabilized by its recognition of conserved motifs within the promoter itself through both TBP and a limited number of TAFs (Smale and Kadonaga 2003). The most widespread and important of these motifs include the TATA box (−30), the initiator (Inr, +1), and the downstream promoter element (DPE, +30), while unique core promoter elements continue to be discovered and their significance analyzed (Juven-Gershon et al. 2008). Currently, it is poorly understood whether distinct core promoter recognition complexes are selectively recruited to specific genes based on their preference for certain core promoter motifs (Muller et al. 2007).

TFIID’s association with promoter DNA is further stabilized through the sequential recruitment of heterodimeric TFIIA, which can bind directly to TBP, TAFs and activators, and the single subunit TFIIIB, which interacts with TFIID, PolII and promoter DNA itself (Maldonado et al. 1990). As part of this heterotrimeric complex, TFIIIB in turn recruits TFIIF, which maintains multiple contacts with, and thus brings to the promoter, the PolII holoenzyme (Ha et al. 1993). Subsequently, TFIIF recruits heterodimeric TFIIE, which stabilizes PolII binding and primarily functions to recruit the large multisubunit TFIIH, which posses multiple enzymatic activities required for the initiation of transcription (Maxon et al. 1994). Hence, TFIIH contains a helicase activity
necessary for the melting of promoter DNA, an ATPase activity necessary for formation of the first phosphodiester bond, and a kinase domain which phosphorylates the C-terminus of PolIII to signal the transition from initiation to productive elongation (Lu et al. 1992; Holstege et al. 1996). Thus, once recruited to the core promoter by sequence-specific activators, TFIID coordinates the stepwise assembly of all general transcription factors required for initiation of productive PolIII transcription.

Extensive structural and mapping studies have begun to provide a picture of how TBP and the TAFs are assembled and positioned within the larger TFIID holoenzyme (Cler et al. 2009). One of the first insights into TFIID structure came from the identification of histone fold domains within several of the TAFs, leading to speculation that TFIID may contain nucleosome like octomers. Biochemical studies have confirmed that TAFs with like histone fold domains do pair to form heterodimers that make up the basic structural subunits of the complex, including the pairs TAF4-TAF12, TAF6-TAF9, TAF8-TAF10, and TAF11-TAF13 (Selleck et al. 2001; Leurent et al. 2002). Though the exact stoichiometry of TAFs within the complex is unclear, biochemical studies suggest that certain TAFs, including TAF1, TAF2, and TAF7, are present as a single copy while the majority of TAFs are present as two or more subunits per TFIID molecule (Sanders et al. 2002). Though X-ray crystallographic structures of intact TFIID have not yet been solved, lower resolution electron microscopy and particle reconstruction has provided some insight to the three dimensional structure of the complex (Andel et al. 1999; Brand et al. 1999; Sanders et al. 2002). These structures reveal a tri-lobed horseshoe like structure in which TBP can be localized to the central cleft, suggesting that this is the
sight of DNA binding. Further, TAF12, TAF4, TAF6, and TAF9 appear to be present in each lobe, while TAF5 is present in the linker region joining all three lobes consistent with a scaffolding function (Leurent et al. 2004). RNAi and overexpression studies in insect cells suggest that TAF4, TAF5, TAF6, TAF9, and TAF12 are essential for the formation of a stable core complex in vivo, which is then decorated by additional TAFs to form holo-TFIID (Leurent et al. 2002; Wright et al. 2006).

Through a combination of in vitro and in vivo experiments, the specific functions of individual TAFs have been greatly illuminated (Albright and Tjian 2000). A subset of TAFs has been found to bind known sequence-specific activators and thus link these proteins’ activation domains to the rest of the basal machinery. Some of these observed interactions include binding of p53 to TAF6 and TAF9, binding of SP1 and Pygopus to TAF4, binding of VP16 to TAF9, and binding of multiple activators to TAF7 (Goodrich et al. 1993; Weinzierl et al. 1993a; Chiang and Roeder 1995; Thut et al. 1995; Wright and Tjian 2009). Yet other TAFs bind specific motifs within the core promoter to help stabilize TFIID position and PIC assembly. Hence, TAF1 and TAF2 can bind the Inr element while TAF6 and TAF9 contact the DPE (Verrijzer et al. 1995; Burke and Kadonaga 1997). More recently, some TAFs have been shown to interact directly with chromatin marks such as TAF1 with acetylated histone tails and TAF3 with trimethylated histone H3 lysine 4 residues (Jacobson et al. 2000; Vermeulen et al. 2007). Interestingly, TAF1 possesses kinase, ubiquitin ligase, and acetylase enzymatic activities which may act on histones or activators to further modulate transcription (Wassarman and Sauer...
2001). Thus, individual TAFs serve specific functions within the context of the larger complex, many of which are yet to be fully understood.

**Alternative TAF Complexes, TBP-Related Factors, and Tissue-Specific TAFs**

While TFIID is thought to be ubiquitously expressed and present at most genes, several complexes have been discovered which contain a subset of TAFs, are free of TBP, and likely act on a more limited number of promoters (Figure 2). The human TBP-free TAF-containing complex (TFTC) which contains many of the TAFs as well as additional proteins not found in TFIID, is able to bind multiple activators and facilitate *in vitro* transcription, though its role *in vivo* is not well understood (Wieczorek et al. 1998). The yeast Spt-Ada-GCN5 acetyltransferase complex (SAGA) contains both overlapping and unique subunits with TFTC and appears to be important in the activation and recruitment of free TBP to a subset of mostly stress responsive genes (Grant et al. 1998; Huisinga and Pugh 2004). Similarly, SAGA-like (SLIK) and the human Spt3-TAF9-GCN5L acetyltransferase complex (STAGA) have very similar composition to SAGA and likely many of the same functions but are less well studied (Martinez et al. 1998; Pray-Grant et al. 2002). The p300/CBP-associated factor (PCAF) complex has many similarities to yeast SAGA and is involved in both global histone modifications and locus-specific coactivation; additionally its disruption has been implicated in oncogenic transformation (Ogryzko et al. 1998; Nagy and Tora 2007). Hence, while the physiological roles of these alternative TAF containing complexes are not yet fully
understood, it is clear that TAFs have important functions outside the context of canonical TFIID.

To date three distinct TBP-related factors (TRFs) or TBP-like proteins (TBPL) have been discovered, from worms to humans, and their biological significance is beginning to be unraveled (Figure 2) (Aoyagi and Wassarman 2000; Hochheimer and Tjian 2003). TBP-related factor 1 (TRF1) was the first of these proteins to be identified and appears to be unique to Drosophila, where it is highly expressed in the nervous system and testes (Crowley et al. 1993). Similarly to TBP, TRF1 is found in a large complex, binds TFIIA, TFIIB, and TATA containing DNA, and can promote PolII transcription in vitro (Hansen et al. 1997). However, TRF1 appears to be distinct from TBP in that its expression is restricted to a limited number of tissues, it is present at relatively few promoters, and it also binds to a unique TC box core promoter element (Holmes and Tjian 2000). Initially identified based on homology, TBP-related factor 2 (TRF2) is found from worms to humans and is more closely related to TBP than TRF1 (Dantonel et al. 1999; Rabenstein et al. 1999). Though it does interact with TFIIA and TFIIB, TRF2 fails to bind a canonical TATA box and appears to bind novel promoters not occupied by TBP or TRF1 (Hansen et al. 1997). Hence, it has been suggested that TRF2 may also act as a repressor by disrupting PIC formation at TATA containing promoters (Moore et al. 1999). Further, TRF2 is part of a high molecular weight complex which lacks TAFs but contains known chromatin remodeling factors (Hochheimer et al. 2002). Knockdown studies in worms and frogs (X. laevis) showed that loss of TRF2 caused early embryonic arrest through the disruption of developmental
gene expression (Dantonel et al. 2000; Veenstra et al. 2000); however, TRF2 knockout mice are viable with the only apparent phenotype being disrupted spermiogenesis (Zhang et al. 2001).

The most recent and perhaps most interesting of these proteins to be identified is TBP-related factor 3 (TRF3/TBPL2). Initially isolated based on the near identity of its C-terminus to that of TBP including the DNA and GTF binding domains, TRF3 is conserved in vertebrates from fish (Fugu) to humans but is absent in lower metazoans (Persengiev et al. 2003). Initial reports showed that it was widely but variably expressed in adult mouse and human tissues, migrated as part of a larger 150-200 kD complex, and that its nuclear import was regulated during the cell cycle asynchronously from that of TBP. Subsequent studies showed TRF3 to be highly enriched in the ovary and to a lesser extent in the testes where it had a different pattern of nuclear import than TBP. However, these reports conflicted as to whether TRF3 is expressed in early embryogenesis following fertilization (Xiao et al. 2006; Yang et al. 2006; Gazdag et al. 2007; Jacobi et al. 2007). Additionally, one of these studies found that TRF3 and TBP occupied unique sets of promoters in mouse embryonic stem cells, while two others showed that TRF3 but not TBP was required for the transcription of a majority of embryonic genes. Knockdown studies in frogs and zebrafish (D. rerio) determined that TRF3 is required for embryogenesis and may be able to partially rescue loss of TBP in the early embryo (Bartfai et al. 2004; Jallow et al. 2004). Interestingly, regulation of the mespa gene by TRF3 is shown to be the earliest and an essential step in the commitment of mesoderm to hematopoietic precursors, suggesting that TRF3 plays a role in cell-type-specific
differentiation programs (Hart et al. 2007). Perhaps the best evidence for TRF3’s role in tissue-specific differentiation comes from studies of the transition from muscle precursors to mature muscle fibers. In this myogenic program it was found that canonical TFIID, including both TBP and most of the TAFs, is completely degraded, while a unique complex of TRF3 and TAF3 is retained in the adult cell type (Deato and Tjian 2007). Furthermore, knockdown of TRF3 blocks myogenic differentiation and TRF3 but not TBP is enriched at the promoters of key myogenic genes. Additional in vitro dissection of this mechanism found that the TRF3/TAF3 complex cooperates with the myogenic activator MyoD to initiate transcription of the myogenin promoter, and that this complex is necessary and sufficient for the process (Deato et al. 2008).

In addition to TBP-related factors, a series of novel tissue-specific TAF paralogs have been identified which further expand the diversity of core promoter recognition complexes (Figure 2). Apparently unique to flies are the testes-specific TAFs, no hitter (dTAF4b), cannonball (dTAF5b), ryan express (dTAF12b), and meiosis I arrest (dTAF6b), all of which are required for spermatogenesis and normal germ cell-specific gene expression (Hiller et al. 2001). Originally isolated from B-cells and highly expressed in the testes and ovary, human TAF4b is the best understood tissue-specific TAF. It associates with a slightly altered TFIID, which may or may not contain copies of TAF4 and is required for expression of a subset of ovary-specific genes (Freiman et al. 2001; Geles et al. 2006). Furthermore, TAF4b knockout mice display defects in folliculogenesis and spermatogenesis but no other apparent phenotypes, consistent with a germ cell-specific pattern of expression and function (Falender et al. 2005). Similarly,
abundant expression of TAF7l coincides with reduced TAF7 expression in mouse spermatids, and TAF7l associates with TBP and TAF1 presumably to form an altered TFIID like complex (Pointud et al. 2003). Additionally, TAF7l knockout mice produce abnormal sperm, have reduced fertility and overall weight, and show altered patterns of testes-specific gene expression (Cheng et al. 2007). However, to date the full range of phenotypes of these mice have not been characterized. The less well-studied TAF5l has been associated with an increased risk of type I diabetes, but its pattern of expression and association with TFIID remain to be determined (Cooper et al. 2007). Similarly, initial characterization of TAF9l shows that it is a component of both TFIID and TBP-free complexes, and that its loss affects the expression of a unique and overlapping set of genes from that of TAF9 (Frontini et al. 2005). Finally, cell-type-specific alternative splicing of TAF6 and possibly other TAFs may further expand the diversity of core promoter recognition complexes (Weinzierl et al. 1993b).

**TAFs in Metazoan Development and Differentiation**

The central role TAFs and canonical TFIID in organismal development is supported by the initial discovery of several TAFs in forward genetics screens and confirmed by the requirement for many others as shown in depletion and knockout experiments. Despite these findings, most TAFs, with the exception of tissue-specific paralogs, are assumed to be ubiquitously expressed and canonical TFIID present at a majority of promoters in nearly all tissue types. Hence, the first evidence of TFIID’s *in vivo* function came from mutations in TAF4 and TAF6, which were homozygous...
embryonic lethal and prevented proper complex formation in a sensitized *Drosophila* suppressor screen (Karim et al. 1996; Sauer et al. 1996). Further analysis of the heterozygous mutants showed a dosage dependent requirement for these TAFs as co-activators of a known transcriptional activator at two important embryonic promoters (Zhou et al. 1998; Pham et al. 1999). An independent mutation in TAF9 was found to have dosage dependent affects on the expression of multiple genes, and these different affects are potentially influenced by the genes differing core promoter architecture (Soldatov et al. 1999). Finally, loss of function mutations in TAF1 were found to affect the development of specific *Drosophila* tissues including the eye, ovaries, wing and bristle by altering cell cycle progression and tissue-specific differentiation (Wassarman et al. 2000).

Initial knockout studies in mice showed that loss of TAF8 or TAF10 destabilized TFIID resulting in apoptosis of the blastocyst's inner cell mass and embryonic lethality, though trophoblast viability appeared to be unaffected (Voss et al. 2000; Mohan et al. 2003). Conditional TAF knockout mice, which avoid the embryonic lethality phenotype, are providing greater insight to tissue-specific TFIID function. As expected, a conditional TAF4 allele failed to produce viable offspring when deleted in ES cells, but was successfully employed to generate proliferative embryonic fibroblasts devoid of TAF4 expression (Mengus et al. 2005). While these cell lines divided normally at routine serum concentrations they displayed altered morphology, accelerated serum independent growth, and disrupted expression of genes responsible for TGFβ signaling. Targeted deletion of TAF4 in basal keratinocytes demonstrated that it is absolutely required for
fetal epidermis differentiation but partially dispensable for adult skin maintenance (Fadloun et al. 2007). Furthermore, TAF4 inactivation increased the incidence of epidermal hyperplasia, and this phenotype was traced to upregulation of multiple EGF genes and downregulation of MAPK pathway components.

A similar approach to keratinocytes-specific deletion of TAF10 demonstrated slightly altered requirements for this subunit relative to that of TAF4 (Indra et al. 2005). Specifically, TAF10 is required for fetal epidermal differentiation, skin barrier formation, and normal patterns of fetal skin gene expression, but dispensable for adult keratinocyte homeostasis. Hence, adult epidermis lacking TAF10 showed normal gene expression patterns, responses to UV irradiation, and proper regeneration after wounding. Recently, the requirements of TAF10 in fetal liver development and adult homeostasis were analyzed by targeted deletion of the same allele (Tatarakis et al. 2008). These studies showed that TAF10 is required for the expression of most fetal hepatic genes and normal liver organogenesis, and that its deletion disrupts normal TFIID complex assembly along with general transcription factor promoter occupancy. Conversely, loss of TAF10 in adult hepatocytes has no detectable adverse phenotypes, causes downregulation of only a small subset of genes, and surprisingly derepresses genes that are normally silenced neonatally. These observations led the authors to propose a multiclass gene model in which some promoters require TFIID for their ongoing transcription, others are actively repressed by TFIID occupancy, and the majority employ TFIID for the first round of fetal transcription but continue to be activated in the adult despite its absence. New conditional knockout models and novel approaches to in vivo TAF ablation, including
virally mediated RNA, will continue to enhance our understanding of TFIID’s function in mammalian development and differentiation.

Embryonic and Neonatal Liver Development

The liver is unique to vertebrates and is responsible for many of the most critical metabolic and homeostatic functions of adult organisms in addition to being required for hematopoiesis during fetal and neonatal development. In the adult the liver is the primary site of carbohydrate, lipid, and protein metabolism and storage. Additionally, it is responsible for the synthesis and secretion of most serum proteins, including many growth and coagulation factors and several digestive enzymes. Finally, the liver is a critical site for the removal of endogenous waste products and exogenous toxins, including most pharmaceuticals. Thus because of its central and varied role in organismal function, there is great interest in understanding how the vertebrate liver develops and is maintained (Zaret 2002; Zaret and Grompe 2008).

While the molecular mechanisms of liver organogenesis are not yet fully understood, the morphogenetic steps of liver development have been well characterized. At mouse embryonic day 6.5-7.5 or 15-16 in humans, the primitive streak forms anterior to posterior along the ventral midline initiating gastrulation and leading to the creation of the three germ layers (Figure 3). Of these the definitive endoderm results from ingressation of epiblast cells through anterior portions of the streak and eventually gives rise to the organs of the embryonic gut including the liver, pancreas, lung, and thyroid; at the same time the extra-embryonic visceral endoderm is displaced laterally (Lewis and
Tam 2006). Cell lineage experiments show that the hepatic endoderm arises soon after gastrulation from lateral portions of the ventral foregut epithelium and a smaller group of cells along the ventral midline epithelium, which are joined upon tube closure (Lawson et al. 1991; Tremblay and Zaret 2005). As the hepatic endodermal cells rapidly proliferate, they migrate into the neighboring septum transversum mesenchyme to form the liver bud, which becomes increasingly vascularized and by embryonic day 10 develops the stromal spaces that will eventually form the sinusoids of the adult liver. By embryonic day 9 these cells already express important early hepatic markers, including albumin (ALB), α-fetoprotein (AFP), and transthyretin (TTR) and are now considered hepatoblasts, the bipotential progenitors that will differentiate into the major cell types of the adult liver (Nobuyoshi Shiojiri 1984; Germain et al. 1988; Gualdi et al. 1996). Hepatocytes differentiate from hepatoblasts between embryonic day 14 and birth, and the neonatal hepatocytes continue to proliferate and induce adult hepatic gene expression between birth and neonatal day 21.

Hepatoblasts are small stem-like cells that are defined by their expression of both hepatocyte and cholangiocyte markers and their ability to differentiate into both cell types. They expand as a homogenous progenitor population in the liver bud until embryonic day 14.5, when a subpopulation reduces its expression of hepatocyte genes and differentiates towards the cholangiocyte or bile duct epithelial cell lineage; the remaining hepatoblasts commit to the hepatocyte lineage between embryonic day 16 and birth. Primary hepatoblasts can be reproducibly purified from mouse embryonic day 13.5 livers by enrichment of hepatoblast-specific markers including E-cadherin (CDH1) and
delta-like 1 (DLK), or depletion of the hematopoietic markers TER119 and CD45 (Nitou et al. 2002; Tanimizu et al. 2003; Nava et al. 2005). Additionally human hepatoblasts have been successfully purified and characterized by a variety of techniques, and shown to expand and differentiate when transplanted to immunocompromised mice (Mahieu-Caputo et al. 2004; Wauthier et al. 2008). Several groups have reported the *ex vivo* culture of primary hepatoblasts and their faithful bipotential differentiation stimulated by extracellular factors; in particular the interleukin-6 family member oncostatin M (OSM) appears to be absolutely required for hepatocyte-specific differentiation (Kamiya et al. 1999; Kamiya et al. 2002). Further, immortalized hepatoblast cell lines that maintain progenitor gene expression patterns and the ability to differentiate into cholangiocytes and hepatocytes *in vitro* have been developed (Rogler 1997; Strick-Marchand and Weiss 2002; Tanimizu et al. 2004). Together these *in vitro* techniques have helped to elucidate the signaling and transcriptional mechanisms of *in vivo* liver development (Strick-Marchand et al. 2004; Tanimizu and Miyajima 2004; Ader et al. 2006). The extracellular signaling events that lead to hepatic endoderm specification and hepatoblast commitment have been extensively studied by tissue explant and gene knockout experiments. Hence, hepatic fate decisions are initiated by suppression of Wnt and fibroblast growth factor 4 (FGF4) signaling in the foregut mesoderm and suppressed by active Wnt signaling in the posterior gut mesoderm (Wells and Melton 2000). The hepatic endoderm’s anterior-posterior position is further refined by retinoic acid signaling from the paraxial mesoderm, and following tube closure, solidified by suppression of sonic hedgehog (SHH) signaling in the dorsal endoderm (Apelqvist et al. 1997; Kumar et al. 2003).
Foregut hepatic differentiation is further stimulated by multiple FGFs secreted by the cardiac mesoderm and bone morphogenetic proteins (BMPs) secreted by the septum transversum mesenchyme (Jung et al. 1999; Rossi et al. 2001). While it is known that specific extracellular signaling events alter intracellular mitogen-activated protein kinase activity (MAPK) and cause tractable changes in cellular transcription factor expression levels, the mechanism by which soluble factors transduce global transcription programs to specify hepatoblast cell fate are not well understood (Wandzioch and Zaret 2009).

The transcriptional control of hepatic differentiation is currently attributed to a large and dynamic network of liver-enriched classical sequence-specific activators (Cereghini 1996; Costa et al. 2003). These proteins are categorized based on the structural similarity of their DNA binding domains and include the onecut homeodomain family (HNF-1), leucine zipper family (C/EBP), winged helix family (HNF-3/FOXA), and nuclear hormone receptor family (HNF4, COUP-TFII, LRH-1, FXR, PXR). While these activators are widely detectable throughout multiple tissues and phases of liver development, their onset and degree of expression correlates well with their unique requirements in sequential phases of hepatic organogenesis (Kyrmizi et al. 2006). Hence, the initial specification of hepatoblasts within the definitive endoderm requires HNF-3α and HNF-3β, which are expressed as early as gastrulation (Duncan et al. 1998; Lee et al. 2005). HNF-4 and GATA-6 are expressed in the developing hepatic endoderm and are required for liver bud expansion and the hepatoblast to hepatocyte transition (Duncan et al. 2000; Li et al. 2000; Zhao et al. 2005). HNF-1b and HNF-6 are first expressed in the developing liver bud and are required for the lineage split of hepatoblasts to hepatocytes.
and cholangiocytes (Clotman et al. 2002; Coffinier et al. 2002). Further, C/EBPα, which is first expressed at birth, is necessary for the expression of metabolic genes which will function in the adult liver (Wang et al. 1995). The delicate spatial and temporal regulation of liver-specific gene expression in both the developing tissue and the adult organ is proposed to be achieved by the combinatorial activation and repression of each unique promoter by this diverse toolbox of proteins; regulatory diversity is further complicated by the ability of many of these factors to heterodimerize. Additionally, the liver-enriched transcription factors have been shown to autoregulate their own and each other’s expression throughout development and adult homeostasis (Odom et al. 2004; Odom et al. 2006). While the circuitry of liver-enriched sequence-specific activators has been extensively investigated, the role of core promoter recognition complexes, co-activators, and chromatin remodelers in liver specification is remains to be understood.

**Hepatic Regeneration**

The liver is distinct among metazoan organs for its intrinsic regenerative ability following acute physical, toxic, or viral injury (Fausto and Campbell 2003; Fausto et al. 2006). While much attention has been given to the potential role of resident liver stem cells or oval cells in maintaining the chronically injured liver, the primary source of liver regeneration in healthy animals is thought to be the limited proliferation of adult hepatocytes (Figure 3). This unique regenerative capacity has made the liver a target of human transplantation therapies as well as a model system for understanding adult stem cell differentiation and organ plasticity.
Within 36-42 hours of partial hepectomy (PH), the most accepted experimental model of hepatic injury, 95% of quiescent mouse hetapocytes synchronously transition from G0 to G1 and enter S-phase to begin DNA replication (Grisham 1962; Mitchell and Willenbring 2008). The hepatocytes divide once or twice and the full liver mass can be restored within one to two weeks in rodents or approximately one to two months in humans, although in most cases the original liver architecture is altered. Mouse models of chronic liver injury suggest that adult hepatocytes have an even greater replicative potential, which may allow for novel therapeutic applications. Hence, when wild type hepatocytes are transplanted into urokinase plasminogen activator (uPA) transgenic mice they undergo up to 15 cell divisions almost fully replacing the necrotic host liver (Rhim et al. 1994). Similarly, serial repopulation of the chronically injured fumarylacetoacetate hydrolase (FAH) knockout mouse liver with wild type hepatocytes suggests that at least 80 rounds of division are possible (Overturf et al. 1996; Overturf et al. 1999).

Much like developing heptaoblasts, hepatocyte regeneration depends on a complex network of extracellular signaling molecules and cellular transcription factors (Fausto et al. 1995). Hence, within hours of hepatic injury nonparenchymal cell derived tumor necrosis factor (TNF) and interleukin-6 (IL-6) stimulate the nuclear factor-kappa B (NFkB), signal transducer, and activator of transcription 3 (STAT3) pathways respectively, inducing immediate early gene expression, promoting the G0 to G1 transition, and priming the quiescent growth factor immune hepatocytes for activation by additional extracellular signals (Akerman et al. 1992; Webber et al. 1998; Iwai et al. 2001). Next mesenchyme derived hepatocyte growth factor (HGF) and hepatocyte
expressed transforming growth factor alpha (TGFα) activate the mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) cascades to promote progression past the G1-S restriction point and induce expression of additional transcription factors (Mead and Fausto 1989; Webber et al. 1993; Borowiak et al. 2004; Huh et al. 2004). The activation of NFκB and STAT3 is the result of post translational modifications and occurs independent of new protein synthesis, allowing these factors to induce immediate early gene expression within hours of hepectomy (Cressman et al. 1994; Cressman et al. 1995). The activated immediate early genes include transmembrane receptors required for continued mitogen responsiveness, cyclins and cyclin dependent kinases which mediate cell cycle progression, and additional transcription factors that drive the regenerative program (Mohn et al. 1991). Among these transcription factors are known mitogen dependent cell cycle regulators not normally expressed in the adult liver including c-Jun, c-Fos (AP-1), and c-Myc; the overexpression or deletion of these genes has been shown to promote or impair liver regeneration respectively (Thompson et al. 1986; Morello et al. 1990; Behrens et al. 2002). A second class of immediate early genes includes liver-enriched transcription factors such as C/EBPβ which are responsible for the reestablishment of normal hepatocytic function and coincident with these is an increase in the expression of liver proteins such as albumin (ALB), α1-antitrypsin (AAT) and Phosphoenolpyruvate carboxykinase (PEPCK) (Park et al. 1990; Diehl 1998). Intriguingly, gene expression profiles of regenerative and neonatal hepatocytes are similar suggesting, a common transcriptional program. More recently FoxM1B was shown to be an immediate late
gene in regenerative hepatocytes which is responsible for expression of cyclins regulating the G2-M transition and exit from the regenerative program; consistent with this overexpression, of FoxM1B can speed up liver regeneration or restore it in older hepatocytes that have lost their regenerative capacity (Ye et al. 1999; Wang et al. 2001). However, as with the embryo, the precise influence of ligands and signaling cascades on intercellular transcriptional events during hepatic regeneration is not well understood.

Chronic toxic injury, certain genetic abnormalities, and age related degeneration may limit or eliminate the hepatocytes proliferative capacity; under these conditions rare resident liver stem cells, or oval cells, can expand and differentiate, contributing to hepatic regeneration (Figure 3) (Fausto and Campbell 2003). While there has been considerable disagreement concerning the oval cell’s phenotype and capacity, a consensus exists that the portal region of the hepatic lobule contains a bipotential progenitor with large oval nuclei that expresses hepatic, biliary, and hematopoietic markers (Farber 1956; Dorrell et al. 2008). Though several groups have reported the purification of hepatic progenitors from healthy liver, most of the work on oval cells employs a model of chronic liver injury involving dietary deficiency, extended feeding of carcinogenic or hepatotoxic compounds, and partial hepectomy (Wilson and Leduc 1958; Dabeva et al. 1998; Preisegger et al. 1999; Wang et al. 2003a). Expanded oval cell populations from these injury models have been purified based on differential centrifugation or cell surface marker enrichment and used for in vivo bipotential differentiation studies as well as the derivation of immortalized cell lines (Wilson and Leduc 1958; Hayner et al. 1984; Yaswen et al. 1984; Germain et al. 1985; Dabeva et al.
1998; Preisegger et al. 1999; Petersen et al. 2003; Wang et al. 2003a). Furthermore, purified oval cells and immortalized cell lines have been successfully used to partially repopulate chronically injured liver models, providing the greatest evidence to date for the oval cells proliferative and regenerative capacity (Wang et al. 2003b; Song et al. 2004). While oval cell expansion is likely dependent on many of the same extracellular signaling pathways as hepatocyte proliferation, it also appears to require additional unique signaling events typically associated with hematopoiesis, such as the SCF/c-kit system (Fujio et al. 1994). Intriguingly, the oval cell’s temporal pattern of liver-enriched transcription factor expression has been shown to closely mirror that of the developing hepatoblast, suggesting that these two populations may differentiate through shared transcriptional mechanisms. However transcription in oval cells has not been investigated in any greater detail and warrants further study (Nagy et al. 1994).

Importantly, oval cell populations have been identified in the chronically injured human liver, and their bipotential and proliferative capacity are beginning to be understood (Haruna et al. 1996; Baumann et al. 1999; Roskams et al. 2004).

**Embryonic Stem Cells and Therapeutic Approaches**

The liver’s central place in many aspects of organismal function, combined with a restricted supply of human liver donors and the complications associated with whole organ transplantation, has generated considerable interest in cell based therapeutic approaches to liver repopulation (Grompe 2006; Nussler et al. 2006). Donor derived mature hepatocyte transplantation has been reported for the treatment of acute liver
failure, genetic metabolic disorders, and end stage cirrhosis with some success (Mito et al. 1992; Fox et al. 1998; Bilir et al. 2000). Hepatocyte transplantation, has considerable advantages over whole liver transplantation including the ability to treat multiple patients from a single donor, decreased immunogenicity, cryopreservation of donor cells, and reduced morbidity associated with the procedure itself. However, it also suffers from considerable drawbacks such as donor supply, low engraftment rates, limited proliferation of transplanted cells, and the inability to restore normal liver architecture (Fox and Chowdhury 2004). Thus, a principal aim of hepatology research is to create an indefinite source of autologous regenerative hepatocytes from patients’ own stem or progenitor cells. Understanding the transcriptional mechanisms of hepatic differentiation and regeneration is critical to this effort.

Embryonic stem cells (ES) hold great promise as a source of transplantable hepatocytes because of their indefinite proliferative capacity and ability to differentiate into every major cell type of the body (Keller 2005; Dalgetty et al. 2009). Mouse ES cells have been successfully differentiated into functional hepatocytes and used to partially restore liver function in a murine model of hepatic failure (Soto-Gutierrez et al. 2006). More critically, multiple groups have reported the directed differentiation of human ES cells into hepatocyte like cells which faithfully recapitulate the gene expression profiles and metabolic functions of primary hepatocytes (Duan et al. 2007; Hay et al. 2007; Hay et al. 2008b). In most cases these differentiation protocols are based on our current understanding of key signaling events required for hepatic development and employ the same timing and extracellular signals observed in vivo (Schuldiner et al.
2000). Furthermore, new discoveries concerning in vivo liver development have been successfully applied to improve the efficiency and accuracy of in vitro ES cell differentiation toward hepatocytes (Hay et al. 2008a). Recent reports demonstrate that these human ES cell derived hepatocytes can partially restore liver function when transplanted into an immunocompromised mouse model, further supporting their therapeutic potential (Basma et al. 2009). Similarly, multiple groups have transdifferentiated adult hematopoietic stem cells into functional hepatocytes by employing our understanding of key signaling events in liver developments thereby avoiding the ethical issues associated with ES cell based therapies (Alison et al. 2000; Lagasse et al. 2000). Thus, our expanding knowledge of the molecular mechanisms required for hepatic development is currently being employed to generate potentially therapeutic hepatocytes from non-hepatic stem cells.

The recent discovery that adult somatic cells can be reprogrammed into proliferative pluripotent stem cells termed induced pluripotent stem cells (iPS) could contribute greatly to our understanding of hepatogenesis, and hasten the advancement of therapeutic liver repopulation (Yamanaka 2007; Jaenisch and Young 2008). Intriguingly, iPS cells have been successfully generated from adult hepatocytes suggesting that the mature cell type possesses all but a few factors required for proliferation, and raising the possibility that damaged liver cells could be dedifferentiated, modified, and transplanted (Aoi et al. 2008). While iPS cells have not yet been differentiated into functioning hepatocytes, they have been specifically differentiated to cardiomyocytes, adipocytes, retinal cells, and multiple hematopoietic lineages (Schenke-Layland et al. 2008; Hirami et
al. 2009; Senju et al. 2009; Zhang et al. 2009). Furthermore, comparative studies suggest that iPS cells can be differentiated by the same protocols and with similar efficiency as ES cells (Taura et al. 2009). Importantly, directed in vivo differentiation of genetically modified autologous iPS cells followed by transplantation has been used to correct mouse models of human disease including sickle cell anemia, fanconi anaemia, and hearing loss (Nishimura et al. 2009; Raya et al. 2009). Such experiments raise the possibility that a patient’s own somatic cells could be used for therapeutic liver repopulation. Understanding the transcriptional mechanisms of liver development, regeneration, and dedifferentiation is essential to realizing this potential.
**Figure 1: Model of Preinitiation Complex Assembly**

Sequence-specific activators (green) bind proximal (PA) and distal (DA) enhancer elements within a gene's regulatory DNA and recruit TFIID (yellow) to the core promoter. These activators and/or TFIID in turn recruit chromatin remodeling complexes such as SWI/SNF (orange), coactivators including Mediator (MED, blue), and TFIIA and TFIIB (purple). The TFIID/TFIIA/TFIIB heterotrimer sequentially recruits TFIIE, TFIIF, PolII (red), and TFIIH (purple), allowing for promoter escape and productive transcriptional elongation.
Figure 2: Combinatorial Model of Core Promoter Recognition Complex Diversity

Canonical TFIID consisting of TBP (red) and 15 associated TAFs (yellow) is believed to be present at most promoters and in most cell types. Additional regulatory complexity comes from TBP-free TAF containing complexes such as TFTC which contain some TAFs plus additional unique proteins (burgundy), TBP-Related Factor complexes such as TAF3-TRF3 (green), and alternate forms of TFIID that use tissue-specific TAFs such as TAF5b and TAF7l (blue). Novel, as yet unidentified complexes may be present in specific tissues or developmental programs.
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TAF3-TRF3
Myogenesis

TAF4b-IID
Folliculogenesis/Spermatogenesis

TFIIA-TRF2
Spermatogenesis

Canonical TFII

TAF6-TRF3
Myogenesis
Figure 3: Hepatic Development and Regeneration

Hepatoblasts, the common bipotential liver progenitors, develop from the definitive endoderm between embryonic day 8.5 and 13.5. Starting at embryonic day 14.5 they differentiate to cholangiocytes or bile duct epithelial cells and hepatocytes. Hepatocytes continue to proliferate and mature from before birth until at least neonatal day 21. Following acute liver injury, mature hepatocytes reenter the cell cycle to regenerate liver mass and function. Rare resident liver stem cells, or oval cells, may also expand and differentiate contributing to liver regeneration.
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- Head
- Paraxial Mesoderm
- Somites
- Cardiac Mesoderm
- Septum Transversum
- Ventral Endoderm
- Dorsal Endoderm
- Septum Transversum
- Hepatic Endoderm
- Ventral Endoderm
- Hepatoblasts
- E8.5-E14.5
- Adult Cholangiocytes
- Neonatal Hepatocytes
- Adult Hepatocytes
- Oval Cells
- Cycling Hepatocytes
- Injury and Regeneration
- Fetal and Neonatal Development
Chapter 1: Introduction

References


Chapter 1: Introduction


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Chapter 1: Introduction


Chapter 2: TBP, TRFs, and TAFs in Liver Development and Regeneration

Summary

The precise spatial and temporal control of gene expression required for organismal development and cellular differentiation is primarily determined at the level of transcriptional regulation. Historically, diverse cellular programs of proliferation, terminal differentiation, and specialized function have been attributed to a repertoire of sequence-specific activators and repressors. General transcription factors including coactivators such as Mediator and core promoter recognition factors such as TFIID were assumed to be ubiquitous and invariant between tissues and developmental time points. Recent work from a variety of model organisms and mammalian differentiation programs supports an expanding role for TAF paralogs, TBP-related factors, and alternative core promoter recognition complexes in determining tissue and developmental stage-specific transcriptional programs. The vertebrate liver serves varied and critical functions in metabolism, growth control, and detoxification; however, the precise transcriptional mechanisms of hepatic development, differentiation, and regeneration remain elusive. Herein, I examine the expression of TBP, TRFs, TAFs and other coactivators in adult mouse tissues and purified liver cells. Furthermore, I demonstrate that during liver development and hepatocyte commitment canonical TFIID is in toto eliminated and its gene locus transcriptionally silenced coincident with retention of TRF3 expression. Importantly, an in vitro model of hepatic development recapitulates these observations.


**Introduction**

The highly specific and exquisitely complex patterns of gene expression required for organismal development and cellular differentiation have traditionally been attributed to sequence-specific DNA binding activators, while the general transcription factors including the core promoter recognition complex TFIID and the coactivator Mediator were assumed to be invariant (Thomas and Chiang 2006). In the mammalian liver developmental commitment and functional specialization are primarily attributed to a large group of heterogeneous nuclear factors (HNFs) and other liver-enriched activators, while changes in the basal machinery have remained largely uninvestigated (Schrem et al. 2002). Recent advances in genetic, biochemical, and cell culture techniques have allowed for a more thorough examination of core promoter recognition and coactivator proteins in diverse cell types and differentiation programs.

The first indication that the canonical core promoter recognition complex TFIID may not be essential at all developmental stages or for the transcription of all genes came from multiple gene disruption studies. Hence, while depletion of TBP in the mouse or zebrafish leads to eventual embryonic lethality it does not prevent the onset of zygotic transcription or reduce RNA PolII activity at the earliest embryonic stages, suggesting that an alternative mechanism of core promoter induction must take its place in this particular setting (Muller et al. 2001; Martianov et al. 2002). Similarly, disruption of the TBP associated factors TAF10 and TAF8 also led to eventual lethality but only after expansion of the inner cell mass, and staining experiments suggest differing requirements for these factors in the developing blastocyst (Voss et al. 2000; Mohan et al. 2003).
Conditional inactivation of the key TFIID subunit, TAF4 in embryonic fibroblasts showed it to be nonessential for cellular growth and differentiation and required for the transcription of many but not all fibroblastic genes, while depletion in developing keratinocytes suggested that it is essential to epidermal differentiation and maintenance, consistent with differential tissue-specific functions (Mengus et al. 2005). Intriguingly, TAF10 is also required for embryonic keratinocyte development but unlike TAF4 is dispensable for maintenance of the adult epidermis (Indra et al. 2005). Most recently, TAF10 was shown to be required for embryonic liver development but dispensable for the maintenance of adult hepatocytes and the ongoing expression of most liver-specific genes, suggesting that an alternative mechanism of core promoter activation may replace TFIID in the hepatogenic program (Tatarakis et al. 2008).

Additional evidence for tissue-specific differences in TFIID activity comes from several limited observations of differences in TBP and TAF expression throughout adult tissues. Hence, in an early study TBP and TAF12 expression were shown to vary widely between adult mouse tissue, being highly enriched in the testes and nearly undetectable in the heart and nervous system (Perletti et al. 1999). Furthermore, differences in TBP abundance were inversely correlated with TRF levels suggesting that TRFs may partially replace TBP in certain tissues. In another report, TAF4 was shown to be highly upregulated during neuronal differentiation and required for the induction of adult neuron-specific genes (Perletti et al. 1999). Furthermore, increased TAF8 (TBN) expression was shown to occur during adipogenesis, and functional TAF8 shown to be required for induction of the adipogenic gene expression program (Perletti et al. 1999).
Additionally, an investigation of the mouse TAF4 paralogue, TAF4b, demonstrated that TAF4 is significantly downregulated in the adult liver at least at the mRNA level (Freiman et al. 2001). While it is important to note that many of these studies are observational and may depend on the accuracy of specific reagents, collectively they provide a precedent for the idea that significant differences do exist in the expression of TFIID components between tissues and cell types.

The most recent indication of developmental stage-specific changes in the core promoter recognition machinery occurs in the myogenic program. Hence, in both primary cells and an in vitro model of myogenesis TBP and many TAFs are in toto degraded and their mRNA levels significantly decreased during the transition from myoblasts to myotubes (Deato and Tjian 2007). Importantly, levels of other general transcription factors including TFIIA-H and RNA PolII are unchanged as are levels of TAF3 and the TBP-related factor TRF3. Furthermore, TFIID was shown in vitro to be dispensable for the activated transcription of the myoube-specific myogenin promoter, suggesting that TBP and TAFs are at least in part functionally nonessential for the onset and/or maintenance of transcription in the adult cell type (Deato et al. 2008). Additionally, multiple components of the canonical coactivator complex mediator are also significantly downregulated during the myogenic transition, suggesting that like TFIID, Med activity may vary significantly between developmental stages and tissues. Importantly, downregulation of TFIID does not appear to be a hallmark of all developmental transitions, as TBP and TAF levels are shown to be unaltered at both the
protein and mRNA level during the differentiation of mouse ES cells to motor neurons (Francisco Herrera, personal communication).

Results

Varied Expression of TFIID Subunits in Diverse Mouse Tissues

The recent seminal finding that TFIID including TBP and many TAF subunits is wholly degraded and replaced by a novel core promoter recognition complex consisting of TRF3 and TAF3 during terminal differentiation has thus far been restricted to murine muscle development (Deato and Tjian 2007). To better understand if this intriguing transcriptional mechanism is unique to myogenesis or is a common feature of other distinct differentiation programs we investigated the disposition of TBP and multiple TAFs in diverse adult mouse tissues at both the mRNA and protein level.

To determine the relative levels of TBP and TAF protein in differentiated mouse tissues we subjected whole tissue lysates to Western blot analysis with specific antibodies against TBP, TRF3, TAF1, and TAF4 (Figure 1). Equal amounts of total protein were analyzed and GAPDH was used as a loading control. Importantly, TBP and multiple TAFs are enriched in proliferative C2C12 myoblasts and whole E13.5 embryos which contain multiple progenitor cell types including myoblasts and hepatoblasts, but at significantly reduced levels in differentiated C2C12 myotubes as previously reported. Additionally, TFIID components are highly expressed in the testes and spleen which poses significant stem and progenitor cell populations and were formerly shown to contain high levels of TBP (Persengiev et al. 1996; Perletti et al. 1999). Intriguingly,
TBP, TAF1, and TAF4 protein appear to be all but absent from many adult tissues and at exceedingly low levels in the whole liver; possibly at lower levels than in differentiated myotubes. Conversely, TRF3 protein is as abundant in many adult tissues as it is in C2C12 myoblasts or the whole embryo, and appears to be particularly abundant in the liver sample. Hence, core promoter recognition complex protein levels vary widely between tissues and TFIID protein levels appear to be particularly low in the differentiated mouse liver.

To further investigate the tissue-specific expression of TFIID we determined the relative levels of key subunit transcripts in the same tissues by reverse transcription and quantitative real-time PCR (qPCR) (Figure 2). Because no single mRNA is expressed at the same level in all tissues of the mammalian body, and the relative proportion of mRNA to total RNA varies considerably between tissues, accurate normalization of cross tissue qPCR is inherently problematic. To partially abrogate this problem, we used the same quantity of total RNA in reverse transcription reactions and normalized against both the 18s RNA (Figure 1A) and GAPDH transcript (Figure 2B), two of the most widely accepted total RNA and mRNA standards respectively. As with protein levels, significant differences are observed in TFIID mRNA levels between tissues, with adult liver showing some of the lowest levels of both TBP and multiple TAFs. Importantly, the whole embryo, testes and ovary, which contain more progenitors and proliferative cells than most adult tissues have the highest transcript levels of key TFIID components. While significant differences exist for TBP and TAF levels between the GAPDH and 18s normalizations, the general trend of reduced TFIID expression in the liver is the same.
As with the protein analysis, levels of TRF3 transcript do not correlate with TBP or TAFs and are at least as high in the liver and other adult tissues as the embryo or testes.

**Differential Expression of TBP, TRFs and TAFs in Highly Purified Liver Cells**

The embryonic and adult livers both contain multiple highly specialized cell types with unique functions and transcriptional programs (Zaret 2002; Zaret and Grompe 2008). While hepatocytes comprise sixty to eighty percent of the adult liver mass, significant populations of cholangiocytes and Kupffer cells, and smaller populations of several other specialized cell types are present and serve critical physiological functions. Similarly, the bipotential hepatoblasts that eventually give rise to hepatocytes and cholangiocytes are a minority population in the developing liver bud, where erythrocytes, leukocytes and other hematopoietic cells are highly abundant. Consistent with their specialized functions and gene expression patterns each of these cell types maintains a unique transcriptional program (Cereghini 1996; Costa et al. 2003). Hence, analysis of core promoter recognition complex subunits in whole liver tissue is likely complicated by differences in expression between the unique cell types that comprise the tissue mass, and may represent an average of different expression levels between the various cell types. More generally, most differentiated tissues are composed of multiple highly specialized cell types in addition to progenitors, such that any whole tissue expression analysis likely misrepresents the abundance of a given gene product in a specific functional cell.

To overcome this complication we chose to focus our analysis on highly purified embryonic and adult liver cells. Multiple protocols have been established for the
purification of hepatoblasts from the embryonic liver both by enrichment of hepatoblast-specific markers and depletion of hematopoietic cells (Weiss and Strick-Marchand 2003; Wauthier et al. 2008). We found that collagenase based dissociation of individually dissected E13.5 livers followed by magnetic bead based depletion of erythrocytes (TER119) and hematopoietic cells (CD45) consistently produced hepatoblast populations which were ninety five percent pure based on both negative and positive FACS analysis (Suzuki et al. 2000). Protocols for the purification of neonatal, partial hepatectomy, and adult hepatocytes by two step *in situ* perfusion with collagenase and differential centrifugation have been employed for more than 50 years (Wang et al. 2003a). The most excepted protocol for purification of regenerative cycling hepatocyte is 2/3 partial hepatectomy followed by *in situ* perfusion (Mitchell and Willenbring 2008).

To determine the expression of TBP, TRFs, and TAFs we performed quantitative RT-PCR using equal amounts of RNA from purified hepatoblasts and adult hepatocytes and normalized to the GAPDH transcript (Figure 3A). Intriguingly TBP, TRF2 and the majority of TAFs are significantly downregulated at the mRNA level in hepatocytes when compared to hepatoblasts. Importantly, this includes the downregulation of TAF3 which was previously shown to be unaltered in the myogenic differentiation program (Deato and Tjian 2007). Conversely, TRF3 and TAF13 expression appear to be relatively unchanged in the adult hepatocyte while the testes-specific TAF7 paralogue, TAF7l is significantly unregulated. Additionally, expression of a core component of a TBP-free TAF complex, PCAF is induced in hepatocytes.
The recent finding that components of the coactivator complex Mediator are downregulated during myogenesis led us to ask how mediator is affected in the hepatic program (Deato et al. 2008). Hence, we analyzed the expression of several key Mediator subunits including components of the head, middle, tail, and CDK8 domains in hepatoblast and hepatocytes by qPCR (Figure 3B). As with TFIID each mediator mRNA examined is significantly downregulated in hepatocytes relative to hepatoblasts. While approximately half of the known Mediator subunits were excluded in this analysis, none of these proteins has been yet been isolated in a complex other than canonical Mediator and those subunits which are shown to be downregulated are positioned throughout the known complex (Myers and Kornberg 2000; Taatjes et al. 2004).

These analyses demonstrate a clear difference in the expression of TFIID and Mediator subunits between progenitor hepatoblasts and adult hepatocytes. This led us to ask when in hepatic development TFIID is downregulated and how its expression might be altered following liver injury. While hepatocytes start to develop from hepatoblasts as early as embryonic day 14, they may not become fully differentiated until postnatal day 21. Hence, neonatal hepatocytes are smaller and less binucleate than adult hepatocytes, do not express all adult hepatic genes, and continue to proliferate. While not fully understood neonatal hepatocyte maturation likely involves transcriptional changes which are distinct from the embryonic or adult programs. Following toxic or mechanical injury adult hepatocytes reenter the cell cycle and proliferate to regenerate the liver mass. These regenerative hepatocytes undergo significant changes in gene expression without dedifferentiation and are thus a unique cell type from either quiescent hepatocytes or
embryonic and neonatal progenitors. While liver-enriched transcription factors and signaling molecules are known to be upregulated during liver injury, the transcriptional programs which govern hepatocyte proliferation are not well understood. Thus, we compared TBP, TRF, TAF, and Mediator mRNA levels from neonatal hepatocytes and 2/3 partial hepatectomy hepatocytes to hepatoblasts and adult hepatocytes by qPCR to further understand the role of these complexes in maturation and regeneration (Figure 4). Based on this analysis it appears the TFIID and Med expression in neonatal and regenerative hepatocytes mirrors that of adult hepatocytes as TBP and most TAFs are similarly downregulated relative to hepatocytes while TRF3, TAF13, and TAF7l are unchanged. Hence, canonical TFIID and Mediator are likely downregulated and TAF7l upregulated at the early stages of hepatocyte commitment prior to birth and their expression does not appear to be significantly reinitiated following liver injury and regeneration.

While spatial and temporal patterns of gene expression as determined by analysis of mRNA abundance often correlate with developmental and cell-type-specific function, differences in mRNA stability, translational efficiency, and protein turnover may cause significant fluctuations between mRNA levels and protein abundance. For this reason relative polypeptide levels are generally assumed to more accurately reflect the developmental stage and cell-type-specific requirements for a given protein product. Hence, we examined TBP, TRF and critical TAF subunit protein levels by western blot of whole fetal, adult liver, and purified liver cells (Figure 5). This analysis demonstrates that TBP and multiple TAFs are highly enriched in purified hepatoblasts and are possibly
expressed at higher levels than in C2C12 myoblasts or whole fetal liver. Conversely, TBP and every TAF examined is significantly downregulated in adult hepatocytes when compared to hepatoblasts including the critical structural subunits TAF1, TAF4 and TAF5 (Wright et al. 2006). Furthermore, neonatal and regenerative hepatocytes appear to have more TBP and TAF protein than do purified adult hepatocytes but clearly far less than hepatoblasts. Intriguingly, several TAFs such as TAF10 appear to be more highly expressed in the whole adult liver than they are in purified hepatocytes suggesting liver cells other than hepatocytes may possess higher TAF levels. Additionally, protein levels of TRF2 appear to be relatively unchanged between hepatoblasts and hepatocytes, suggesting that this protein may partially substitute for TBP in adult hepatocytes. In the case of TBP and several of the TAFs the fold reduction in protein levels may be greater than that for mRNA, but this comparison is limited by the qualitative nature of western blotting.

To further understand changes in Mediator expression during hepatic development we also conducted western blots of multiple Med subunits in whole liver and purified liver cells (Figure 6). As is the case with TFIID, every Mediator subunit examined is enriched in fetal hepatoblasts and significantly downregulated in adult hepatocytes while at reduced but intermediate levels in neonatal and regenerative hepatocytes. Importantly, this is true for components of the distinct head, middle, and tail domains, and the dissociable CDK8 module (Guglielmi et al. 2004). The apparent downregulation of Mediator protein during hepatic development closely mirrors what was reported for the myogenic program (Deato et al. 2008).
Transcriptional Silencing of TBP and TAF Promoters During Hepatogenesis

While qPCR and western blot analysis clearly show that TFIID levels are significantly reduced upon hepatic development, we wished to further confirm that TBP and TAF gene expression is specifically silenced at the promoter level. The enrichment or removal of highly defined histone marks is increasingly understood to correlate with the level or gene expression or silencing (Bernstein et al. 2007). Among the many recently identified histone modifications, promoter enrichment of histone H3 lysine 4 (H3K4Me3) trimethylation has been widely shown to correlate with gene activation, while enrichment of histone H3 lysine 9 trimethylation (H3K9Me3) is associated with transcriptional silencing (Li et al. 2007). Hence, we crosslinked E13.5 and adult livers and performed chromatin immunoprecipitation with specific antibodies against these two chromatin marks followed by qPCR of precipitated material with promoter-specific primers (Figure 7). This analysis shows that the proximal promoters of TBP, TAF3, and TAF5 are enriched for H3K4Me3 in the fetal liver sample and that levels of this mark are subsequently reduced in the adult liver. Similarly and more strikingly, H3K9Me3 enrichment at these promoters is low in the fetal liver and greatly increased in the adult. Importantly, the promoter of an adult hepatocyte-specific gene, CYP7A1, shows relative enrichment of H3K9Me3 in the fetal liver and of H3K4Me3 in the adult. Hence, TFIID promoters appear to be specifically upregulated in the fetal liver and transcriptionally silenced in adult cells through a mechanism which involves directed modification of histone tails in the proximal promoters of these genes.
Expression of TBP and TAFs in a Cell Culture Model of Hepatogenesis

Defined cell culture systems have contributed greatly to our understanding of multiple metazoan differentiation programs and provide the greatest opportunity for functional manipulation of cellular differentiation when a suitable whole animal model is absent. Several groups have reported systems for both the short term \textit{ex vivo} culture of primary hepatoblasts and the long term maintenance of immortalized hepatoblast derived cell lines (Strick-Marchand and Weiss 2003; Weiss and Strick-Marchand 2003). Hence, fetal mouse hepatoblasts purified by both negative and positive selection have been successfully cultured for multiple divisions on extracellular matrices in the presence of growth factors which mimic the hepatic endoderm niche (Nitou et al. 2002; Tanimizu et al. 2003). Furthermore, these cultures have been efficiently differentiated by defined factors into both hepatocytes and cholangiocytes that recapitulate the metabolic functions and gene expression patterns of \textit{in vivo} isolates (Kamiya et al. 1999; Kinoshita et al. 1999; Tanimizu et al. 2007). While these \textit{ex vivo} models have proven useful they require frequent isolation of fresh hepatoblasts and result in significant experimental variability. To date three groups have established defined immortalized hepatoblast cell lines which abrogate the complexities of hepatoblast culture while faithfully recapitulating embryonic liver development (Rogler 1997; Strick-Marchand and Weiss 2002; Tanimizu et al. 2004). Of these we chose to employ the Hepatic Progenitors Proliferating on Laminin (HPPL) cell line, which mimics proliferative hepatoblasts in the presence of epidermal growth factor (EGF) and hepatocyte growth factor (HGF) but is differentiated to functional hepatocytes following incubation with oncostatin M (OSM) and Matrigel.
We maintained and expanded the HPPL cell line for multiple generations on laminin-coated plates in the presence of EGF and HGF with no apparent change in morphology, proliferative capacity, or marker gene expression consistent with published reports. When confluent cultures were incubated for five days with 20ng/ml of recombinant oncostatin M followed by five days with growth factor reduced Matrigel cells took on an enlarged binucleate cuboidal morphology and acquired multiple intracellular vacuolar structures. Reverse transcription and qPCR revealed a significant decrease in the expression of bipotential and cholangiocytes markers by five days post induction and a greater increase by ten days (Figure 8A). Conversely, adult hepatic gene expression was significantly and rapidly upregulated. Similarly, western blot analysis showed a reduction of bipotential marker protein levels and an increase in proteins associated with terminal adult hepatocytes (Figure 8B). Thus the HPPL cell model faithfully mimics the hepatoblast to hepatocyte transition based on morphology and marker gene expression.

To assess changes in TBP and TAF expression during HPPL differentiation we performed reverse transcription and qPCR on RNA isolated from proliferating and differentiated HPPL cells (Figure 9A). These analyses clearly show that TBP, many TAFs, and at least one Mediator subunit are significantly downregulated by five days post induction with further downregulation by ten days. Conversely, TRF3, TAF13, and TAF7l transcript levels are maintained or increased coincidental with the downregulation of other subunits. Importantly the extent of transcript changes closely mirrors that seen between primary hepatoblasts and hepatocytes. Western blot analysis of TBP, TRF3, and
some TAFs displays a similar pattern with significant changes in TFIID but not TRF3 occurring by day five after induction. Hence, the HPPL culture system faithfully recapitulates the downregulation of TFIID observed the primary hepatogenic program.

**Discussion**

A growing body of functional data suggests that specific TAFs and potentially holo-TFIID may be dispensable for the activation of many promoters and the maintenance of at least some differentiated cell types. This, combined with two recent studies that demonstrate that TBP and multiple TAFs are specifically degraded during certain differentiation programs, cast doubt on the idea that TFIID is universally expressed and required for all cell-type-specific transcriptional programs (Brunkhorst et al. 2005; Deato and Tjian 2007). Because of its central role in mammalian biology, the transcriptional mechanisms that govern liver development and function are a subject of extensive study.

At least one previous study suggested that the adult mouse liver contains particularly low levels of the key TFIID subunit TAF4 (Freiman et al. 2001). Indeed our current data confirms that TAF4, along with TBP and TAF1, are significantly downregulated in the adult liver relative to the embryo and other adult tissues, suggesting that the hepatogenic program may also require the downregulation of canonical TFIID and potentially its replacement with an alternative core promoter recognition complex. While methods of normalization and differences in progenitor cell populations between tissues complicate cross tissue analysis, preliminary data suggest that the significant downregulation of TFIID at both the protein and RNA level that is observed in muscle
and liver may not be observed in all adult tissues and thus may be specific to a subset of differentiation programs. As new protocols for the isolation of tissue progenitors and fully committed cells become available, it will be interesting to explore how widely the phenomenon of TFIID downregulation and core promoter complex switching is employed or restricted throughout the mammalian body.

Because of the considerable cell type and functional complexity of most adult organs, changes in transcription factor expression and function are clearly best observed in highly defined pure cell populations. Hence, hepatocytes which represent sixty to seventy percent of the adult liver cell population may exhibit differences in transcription factor abundance from other liver cell types which would be masked in whole organ experiments (Daoust and Cantero 1959). Importantly, our results suggest that the hepatogenic downregulation of TFIID at both the protein and mRNA levels is most pronounced between hepatoblast progenitors and purified hepatocytes. Intriguingly, whole adult livers appear to retain at least some TAF subunits relative to hepatocytes, consistent with previous literature reports (Tatarakis et al. 2008). While the many minor cell types of the adult and developing liver are more difficult to purify and characterize, it will be interesting for future studies to determine if at least some of these populations retain and require canonical TFIID in their adult form, and if this accounts for at least part of the difference in TFIID abundance between whole liver and purified hepatocytes (Alpini et al. 1994). Specifically, it remains to be determined if bipotential hepatoblasts downregulate TFIID when committing to both mature hepatocytes and cholangiocytes or if selective downregulation is itself a determinant of this lineage split. While clear
differences in TFIID expression exist between hepatocytes and hepatoblasts, these two cell types lie at the ends of a differentiation process which requires at least two weeks and multiple intermediate stages in the mouse, making the exact timing of TFIID downregulation unclear (Zaret 2002). The finding that TAFs are already significantly degraded in neonatal hepatocytes suggests that this process primarily occurs early in differentiation and most likely prenatally, however the observation that some protein remains in late neonatal hepatocytes may imply that at least some TFIID is required for final maturation. Additionally, differences in the relative levels of TAFs in purified hepatocytes and the whole liver may indicate that some subunits are retained as part of characterized or novel TBP free TAF containing complexes including TFTC and SAGA; the role of these complexes in liver development and function remains to be determined. The significant decrease in TBP and TAF mRNA levels and the active silencing of these genes promoters supports the notion that long term reduction of TFIID in adult hepatocytes is primarily a transcriptional phenomenon. However, more extensive time course measurements of mRNA and protein levels during the differentiation process is needed to determine if an active mechanism of subunit proteolysis is also employed. One recent study suggests that in some differentiation programs, targeted ubiquitination and proteasome mediated degradation of TFIID subunits occurs independently of transcriptional changes (Perletti et al. 2001). Thus, it is possible that both transcriptional silencing and ubiquitin E3 ligase mediated degradation of specific TFIID polypeptides cooperate to establish immediate and prolonged reduction of TFIID levels. Most interestingly, it remains to be understood how known and as yet undiscovered
extracellular signaling events trigger the transcriptional silencing of TBP and TAF promoters or the targeted degradation of these subunits in precise spatial and temporal patterns. Recent advances in the ex vivo study of these critical signaling events may be fruitfully combined with existing transcriptional readouts to better understand these connections (Wandzioch and Zaret 2009).

Importantly, a cell culture model of hepatic differentiation recapitulates the downregulation of TFIID observed in purified primary cells. This model expands the range of functional and cell signaling experiments that can be performed to better understand the process of core promoter complex switching in hepatogenesis. Additionally, these observations also suggest that because core promoter changes that are observed in vivo are conserved in vitro, novel findings may be successfully employed to improve the efficiency and fidelity of potentially therapeutic differentiation and transplantation protocols.

Materials and Methods

Protein Extracts and RNA

Whole tissue lysates were generated by dounce homogenization in RIPA buffer of fresh CD-1 mouse tissues dissected in cold PBS, or purchased from Protientech Group. Tissue-specific total RNA was purchased from Ambion and used to generate cDNA by reverse transcription with the High Capacity cDNA Kit (ABI). Purified mouse liver cells were homogenized in RIPA buffer or used to generate cDNA by extraction with the RNeasy Plus Mini Kit (Qiagen) and reverse transcription with the High Capacity cDNA
Kit. Protein samples were clarified by centrifugation and total protein concentration normalized to BSA with the BCA Protein Assay reagents (Pierce).

**Quantitative PCR**

Relative qPCR of total cDNA was performed on an ABI 7300 with transcript specific FAM labeled Taqman probes and Universal Master Mix (ABI) or verified transcript specific primer sets and Power SYBR Green Master Mix (ABI). Input was normalized to 18s for whole tissue samples and GAPDH for all other experiments. Samples were quantified in triplicate and represent a minimum of two biological replicates.

**Western Blotting**

Equal amounts of total protein were separated by denaturing polyacrylamide gel electrophoresis and transferred to 0.22μm nitrocellulose, blocked with 5% milk or 5% BSA in Tris-buffered saline/0.1% Tween (TBST) and incubated overnight with appropriate antibodies. Membranes were washed extensively with TBST and probed with species-specific horseradish peroxidase conjugated secondary antibodies (Jackson). The following Antibodies were used: Rabbit anti-MED1 (Bethyl), Rabbit anti-MED6 (Bethyl), Rabbit anti-MED12 (Novus), Rabbit anti-MED18 (Novus), Rabbit anti-MED23 (Bethyl), Goat anti-CDK8 (Abcam), Mouse anti-TBP (Biodesign), Rabbit anti-TRF2 (Abcam), Rabbit anti-TRF3 (Deato and Tjian 2007), Goat anti-TAF1 (Santa Cruz), Rabbit anti-TAF3 (Deato and Tjian 2007), Mouse anti-TAF4 (BD Bioscience), Mouse anti-TAF5 (Eurogentec), Rabbit anti-TAF6 (Abcam), Rabbit anti-TAF7 (Abnova), Goat anti-TAF9 (Santa Cruz), Mouse anti-TAF10 (Chemicon), Rabbit anti-TAF12
(Proteintech Group), Rabbit anti-GAPDH (Cell Signaling), Goat anti-Albumin (Bethyl), Mouse anti-AFP (R&D Systems), Mouse anti-AQP1 (BD Bioscience), Rabbit anti-CYP7A1 (Santa Cruz).

**Histone ChIP**

Individual E13.5 livers were dissected from the embryos of timed pregnant CD-1 females and adult livers were dissected from 8-10 week old CD-1 females. Crosslinked chromatin was prepared essentially as described (Soutoglou and Talianidis 2002; Kyrmizi et al. 2006). Embryonic and adult liver tissue was minced in PBS and homogenized by 10-15 strokes of an A pestle in the presence of 1% formaldehyde. Crosslinking was continued for 10 minutes and quenched by addition of glycine to 125mM. Cells were collected at 1,000g for 10 minutes and washed twice for 20 minutes in hypotonic lysis buffer (25mM Hepes pH7.9, 10mM KCl, 0.250M Sucrose, 2mM EDTA, 1.5mM MgCl2, 0.1% NP-40, 1mM DTT, 0.5mM Spermidine, Protease Inhibitor Cocktail (Roche)). Nuclei were collected at 1,000g and resuspended in an equal volume of nuclear lysis buffer (50mM Hepes pH7.9, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, Protease Inhibitor Cocktail (Roche)). Chromatin was sheared to 300-600 bp fragments by sonication and cleared by centrifugation at 15,000g. Equal amounts of cleared chromatin were precipitated by overnight incubation with rabbit IgG, rabbit anti-H3K4Me3, or rabbit anti-H3K9Me3 (Active Motif). Immunocomplexes were incubated for two hours with pre-blocked protein A and protein G magnetic beads (Invitrogen). Samples were washed twice with nuclear lysis buffer, twice with nuclear lysis buffer plus 500mM NaCl, twice with Li was buffer (20mM Tris-HCl pH7.9, 1mM
EDTA, 250mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate), and once with TE. Chromatin was eluted by addition of 50mM Tris-HCl pH 7.9, 25mM EDTA, 1% SDS, 0.2mg/ml RNase A, 0.1mg/ml Proteinase K. After incubation at 37°C for 2 hours NaCl was added to 500mM and samples were incubated overnight at 65°C to reverse the crosslinking. DNA was purified using QIAquick spin columns (Qiagen) and used in quantitative PCR with primers surrounding the relevant proximal promoter.

Liver Cell Purification

Adult, day 15 neonatal, and partial hepatectomy hepatocytes were purified by two-step in situ perfusion with Liberase 3 (Roche) as described (Wang et al. 2003b). Partial hepatectomy of 8-12 week old male CD-1 mice was performed as described (Mitchell and Willenbring 2008). Indivitual E13.5 livers were dissected from 3-5 timed pregnant females in PBS and dissociated by incubation for 10-15 minutes at 37°C in the presence of 0.9mg/ml Liberase 3 (Roche) and 0.07% DNAse I (Sigma). Digestion was quenched by the addition of FBS to 3% and EDTA to 2mM. Cells were collected at 40g and resuspended in 1x Pharmalyse (BD Bioscience) for hypotonic lysis of erythrocytes. Cells were resuspended in PBS/10%FBS, passed through a 70μm filter and diluted to 1x10^8 cells/ml. Cells were incubated for 15 minutes at 4°C with CD45 microbeads (Miltenyi Biotec), and in the absence of hypotonic lysis TER119 microbeads (Miltenyi Biotec), after which labeled cells were removed by passage over an LS column attached to a MidiMacs (Miltenyi Biotec) separator according the manufacturer’s instructions.

HPPL Cell Culture and Differentiation

Hepatic Progenitors Proliferating on Laminin (HPPL) were cultured and
differentiated essentially as described (Tanimizu et al. 2004). Proliferating cultures were maintained in DMEM/F12 (Invitrogen), 10% heat inactivated FBS, 1x insulin/transferrin/selenium (ITS) (Invitrogen), 10mM nicotinamide (Sigma), 0.1μm dexamethasone (Novagen), 5mM L-glutamine (Invitrogen), 20ng/ml HGF (Peprotech), and 20ng/ml EGF (Peprotech) on laminin-coated plates (BD Bioscience). Confluent cultures were differentiated by incubation for five days in the same media minus HGF and EGF and containing 20ng/ml OSM (Peprotech). For complete differentiation OSM media was removed and cells were overlaid with fresh media containing 0.350 mg/ml growth factor reduced Matrigel (BD Bioscience) for an additional five days as reported (Kamiya et al. 2002).
Figure 1: TBP, TRF3, and TAF Expression in Mouse Tissues

C2C12 myoblasts and myotubes, CD-1 embryos, and whole mouse tissues were homogenized in RIPA buffer and equal amounts of total protein were analyzed by western blot with TBP, TRF3, TAF1, and TAF4 specific antibodies. GAPDH is used as a protein loading control.
Figure 2: TBP, TRF3, and TAF Expression in Mouse Tissues

Total RNA from diverse mouse tissues was reverse transcribed and used for relative qPCR with transcript-specific Taqman probes. CT values were normalized to 18s ribosomal RNA (A) or GAPDH mRNA (B). Values for each tissue are expressed as a percentage of the average value for all tissues combined.
Chapter 2: TBP, TRFs, and TAFs in Liver Development and Regeneration

Normalized to 18s

A

Normalized to GAPDH

B
Figure 3: TBP, TRF, TAF, and Med Expression in Purified Liver Cells

Total RNA was purified from mouse E13.5 hepatoblasts and adult hepatocytes, reverse transcribed, and used for qPCR. CT values were normalized to GAPDH and expressed as a percentage of hepatoblast values. (A) TBP, TRFs and TAFs. (B) Mediator subunits.
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A

B

Hepatoblast
Hepatocyte

Percent of Hepatoblast

10
20
30
40
50
60
70
80
90
100

Percent of Hepatoblast

0
10
20
30
40
50
60
70
80
90
100

Hepatoblast
Hepatocyte

MEDI1 MEDI4 MEDI6 MEDI7 MEDI12 MEDI13 MEDI16 MEDI17 MEDI18 MEDI21 MEDI23 MEDI26 CDK8
Figure 4: TBP, TRF, TAF, and Med Expression in Neonatal and Regenerative Hepatocytes.

Total RNA was purified from mouse E13.5 hepatoblasts, neonatal day 15 hepatocytes, regenerative hepatocytes following 2/3 partial hepatectomy, and adult hepatocytes; reverse transcribed; and used for qPCR. CT values were normalized to GAPDH and expressed as a percentage of hepatoblast values.
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% of Hepatoblast

Hepatoblast
Neonatal Hepatocyte
Adult Hepatocyte
Cycling Hepatocyte (2/3 partial Hep)

Percent of Hepatoblast
Figure 5: TBP, TRF and TAF Expression in Purified Liver Cells

Equal amounts of total protein from C2C12 cells, fetal and adult liver, and purified liver cells were analyzed by western blotting with TBP, TRF2, and TAF-specific antibodies.
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TBP, TRF2, TAF1, TAF3, TAF4, TAF5, TAF7, TAF9, TAF10, TAF12

- C2C12
- Fetal Liver (E13.5)
- Adult Liver
- Hepatoblast
- Neonate Hepatocyte
- Adult Hepatocyte
- Cycling Hepatocyte

Western Blot Analysis
Figure 6: Med Expression in Purified Liver Cells

Equal amounts of total protein from C2C12 cells, fetal and adult liver, and purified liver cells were analyzed by western blotting with antibodies against key Mediator subunits.
### Chapter 2: TBP, TRFs, and TAFs in Liver Development and Regeneration

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**Figure 7: Transcriptional Silencing of TBP and TAF Promoters During Hepatogenesis.**

Individually dissected fetal and adult liver cells were crosslinked and used for chromatin immunoprecipitation with histone mark-specific antibodies. Precipitated chromatin was used in qPCR with primers surrounding the relevant proximal promoters and values expressed as fold enrichment over an IgG control.
**Figure 8: Induction of Hepatic Gene Expression in a Cell Culture Model of Development.**

Hepatic Progenitors Proliferating on Laminin (HPPL) cells were differentiated by incubating for five days in media containing 20ng/ml Oncostatin M and followed for five days in media containing 0.350mg/ml Matrigel. Total RNA was reverse transcribed and used in qPCR to analyze bipotential and hepatocyte marker gene expression (A). Values are expressed as a percentage of undifferentiated control cells on a log scale. Whole cell lysates were used in western blots with antibodies specific for α-fetoprotein (AFP), albumin (ALB), aquaporin (AQP1), and CYP7A1 (B).
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### A

![Bar Chart](image)

- **X-axis**: Gene names (AFP, KRT19, AQP1, ALB, CYP7A1, TAT, TDO2)
- **Y-axis**: Percent Day 0 (Log Scale)
- **Legend**:
  - Day 0
  - Day 5
  - Day 10

### B

![Western Blot](image)

- **X-axis**: Day (0, 5, 10)
- **Y-axis**: Genes (AFP, ALB, AQP1, CYP7A1)
Figure 9: TBP, TRF, and TAF Expression in Differentiated HPPL Cells.

HPPLs were differentiated for five or ten days and total RNA was used for quantitative PCR of TBP, TRF3, and TAF expression levels (A). CT values were normalized to GAPDH and expressed as a percentage of an undifferentiated control. Whole cell lysates were made and equal amounts of total protein analyzed by western blot with antibodies specific to TBP, TRF3, TAF3, and TAF4 (A). GAPDH is used as a protein loading control.
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Figure A

Day             0             5            10

Day 0
Day 5
Day 10

Figure B

Day 0  5  10

TBP
TRF3
TAF3
TAF4
GAPDH
Chapter 2: TBP, TRFs, and TAFs in Liver Development and Regeneration

References


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Chapter 3: TRF3, TAF7l, and TAF13 in Hepatic Gene Regulation

Summary

The near infinite diversity of cell types and biological functions intrinsic to the mammalian body plan is primarily determined at the level of transcriptional regulation. In the mammalian liver, a diverse set of tissue-enriched and extensively studied sequence-specific activators are known to regulate embryonic and adult gene expression programs. While the spatial and temporal patterns of liver-enriched transcription factor expression have been determined, and their requirements in hepatic gene expression demonstrated, the exact mechanisms by which multiple factors collaborate to tune global patterns of expression are not well understood. In contrast, the molecular mechanisms of gene activation by canonical core promoter recognition and coactivator complexes such as TFIID and Mediator are well established in cell culture and in vitro systems, but have not yet extended to a tissue-specific context. Now that tissue-specific TAF paralogs, TBP-related factors, and alternative core promoter recognition complexes have been identified, it is critical to determine how these assemblies alter gene expression patterns to specify tissue and cell-type-specific functions in collaboration with known activators. To date, liver-specific core promoter recognition and coactivator complex subunits have not been specifically characterized. If canonical TFIID is indeed absent from the adult liver, than the isolation of such liver-enriched subunits and an understanding of their coordination with existing liver-enriched transcription factors would greatly elucidate our picture of liver development, disease, and regeneration.
Introduction

While TBP and the canonical TAFs which comprise TFIID were historically assumed to be invariant between tissues, cell types and developmental time points, tissue-specific TAF paralogs have since their discovery been known to exhibit highly restricted expression patterns and transcriptional functions. Though a variety of TAF paralogs were previously identified by bioinformatic means, the first concrete evidence of a tissue-specific TFIID subunit came with the discovery of a substoichiometric TAF4 paralog in cultured B cells (Dikstein et al. 1996). Subsequently, TAF4b was found to be highly enriched in mouse testes and ovary and to be an intrinsic component of an altered ovary-specific TFIID (Freiman et al. 2001). TAF4b knockout females, while viable, are infertile as a result of deficient folliculogenesis, and knockout males show age-dependent infertility resulting from a defect in spermatogenic maintenance (Falender et al. 2005). Consistently, gene expression analysis revealed a requirement for TAF4b in regulating a subset of ovarian granulosa cell-specific genes and genes required for spermatogonial stem cell maintenance (Geles et al. 2006). Furthermore, TAF4b containing TFIID does not necessarily require unique tissue-specific activators, but rather induces the expression of and cooperates with ubiquitous general activators such as c-Jun to regulate cell-type-specific gene expression (Geles et al. 2006). More detailed developmental analysis confirmed that TAF4b is the primary transcriptional integrator of extracellular signals that specify the developmental and proliferative program of granulosa cells (Voronina et al. 2007). While not explicitly required in B cells, TAF4b cooperates with known regulators of B cell-specific transcription such as OCA-B and NFκB, and may serve a
redundant function to TAF4 in this context (Yamit-Hezi and Dikstein 1998; Wolstein et al. 2000; Freiman et al. 2002). Promoter-specific differences in both basal and activated transcription by canonical and TAF4b containing TFIID were demonstrated in vitro, and these differences shown to correlate with TAF4b target genes identified in vivo (Liu et al. 2008). Intriguingly, a TAF4b homologue is enriched in Xenopus ovaries but absent from lower metazoans, suggesting that unlike canonical TAFs, tissue-specific paralogs may have evolved to direct species-specific transcriptional programs (Xiao et al. 2006).

The importance of tissue-specific TAFs was further validated by the identification of five testis-restricted (tTAF) subunits in Drosophila, no hitter (paralog of TAF4), cannonball (paralog of TAF5), meiosis I arrest (paralog of TAF6), spermatocyte arrest (paralog of TAF8), and ryan express (parologue of TAF12) (Lin et al. 1996). Disrupting each of these proteins alters expression of testis-specific genes, and together they form a stable complex, which is required for meiotic cell cycle progression and normal spermatid differentiation (Hiller et al. 2001; Hiller et al. 2004). Intriguingly, the tTAF complex mediates spermatid gene activation at least in part by displacing the repressive polycomb complex and promoting its sequestration to the nucleolus (Chen et al. 2005); hence, a derepressive tTAF strategy may represent a novel cell-type-specific mechanism of TAF dependent coactivation.

While tTAFs are restricted to Drosophila, a testis-linked parologue of TAF7 appears unique to vertebrates. High level expression of mouse TAF7l in late spermatocytes and haploid spermatids is coincident with a reduction in TAF7 expression, and as spermatogenesis proceeds TAF7l, is relocalized to the nucleus where it associates
with TBP and other TAFs to form an alternative TFIID-like complex (Pointud et al. 2003). Viable TAF7l knockout mice display altered patterns of spermatocyte gene expression and defects in spermiogenesis which lead to reduced fertility, consistent with a testes function (Cheng et al. 2007). Additional TAF paralogs, including TAF9b, while shown to associate with TFIID and promote alternative gene expression programs, are just beginning to be characterized, and the full repertoire of tissue-specific TAFs remains to be discovered (Frontini et al. 2005).

While noncanonical TAF paralogs were originally identified in a subset of tissues, TBP-related factors or TRFs were originally assumed to be widely expressed but are now known to exhibit tissue-specific expression patterns and regulatory functions (Hochheimer and Tjian 2003). Hence TRF1, the first identified TBP-related factor to be discovered, is restricted to Drosophila, where it is enriched in the nervous system and gonads and thus seems to have evolved to suite a specific purpose in these insect tissues (Hansen et al. 1997). Polytene chromosome staining and genome-wide promoter occupancy experiments suggest that TRF1 regulates a highly restricted set of genes relative to TBP, and that these loci are primarily associated with RNA PolIII transcription (Isogai et al. 2007b). However, TRF1 has been isolated in association with BRF and a larger complex containing novel proteins and been shown to direct both RNA PolIII and PolII transcription in vitro and in vivo, much like TBP (Holmes and Tjian 2000; Takada et al. 2000). Hence, TRF1 represents an evolutionarily restricted alternative core promoter recognition complex which directs promoter-selective transcription by multiple polymerases in a subset of tissues.
TBP-related factor 2 (TRF2, also called TLF, TLP, or TRP), though more widely distributed evolutionarily from *C. elegans* to humans, also exhibits tissue-specific expression patterns and possibly species-specific transcriptional specialization (Dantonel et al. 1999; Rabenstein et al. 1999). While TRF2 binds TFIIA and TFIIB and displays high conservation of the TBP DNA binding domain, it does not bind TATA box containing DNA and fails to complement TFIID in basal or activated *in vitro* transcription assays (Moore et al. 1999; Teichmann et al. 1999). Biochemically, TRF2 associates with the DRE binding factor (DREF) and components of the NURF chromatin remodeling complex, and has been shown to selectively regulate the transcription of DRE-containing promoters of cell cycle and proliferation genes in coordination with these proteins (Hochheimer et al. 2002). Intriguingly, several genes have been shown to contain tandem transcriptional start sites whose promoters are specifically and differentially regulated by TRF2 and TFIID. Disruption experiments in *C. elegans*, *Drosophila*, and *Xenopus* demonstrate a requirement for TRF2 in early embryonic development, but also confirm that TRF2 regulates a subset of developmental genes responsible for these phenotypes, while being dispensable for the expression of others (Dantonel et al. 2000; Kaltenbach et al. 2000; Veenstra et al. 2000; Kopytova et al. 2006). Conversely, mouse TRF2 is highly enriched in the testis, and while knockout animals display spermiogenesis defects and altered patterns of testes-specific gene expression, they show no discernable developmental defects, consistent with an evolutionarily divergent function in mammals (Martianov et al. 2001; Zhang et al. 2001). Recent genome-wide localization of *Drosophila* TRF2 showed that it occupies over a thousand
promoters, most of which are not bound by TBP and are enriched for DREs but lack a canonical TATA box (Isogai et al. 2007a). Intriguingly, the *Drosophila* histone H1 promoter is specifically regulated by TRF2 while the reminder of the histone gene cluster is TFIID dependent, suggesting that differences in core promoter recognition complex activity may alter histone ratios. Hence, TRF2 exhibits evolutionarily diverse roles, and clearly extends the diversity of core promoter recognition complexes in its regulation of both tissue-specific and promoter-selective gene expression patterns. Additionally, the existence of separate TRF2 and TFIID-dependent promoters within related gene clusters, or in some cases single genes, may allow a limited set of promoters and core promoter recognition complexes to produce dramatically varied expression patterns in response to signaling events or between tissues. This alternative regulation likely results from TRF2’s association with novel transcription factors through its divergent N and C-terminal protein interaction domains and its recruitment to novel core promoter elements not occupied by TBP.

The most recently identified and perhaps most intriguing member of the TBP-related factor family is the vertebrate-specific TRF3 (also called TBP2). Initially identified based on homology, mammalian TRF3 shows near identity to TBP in its C-terminus including, the TATA box, TFIIA, and TFIIIB binding domains, and considerable divergence from all known TBPs in its N-terminus (Persengiev et al. 2003). Preliminary characterization showed TRF3 to be widely expressed in adult mammalian tissues, though at varying levels relative to TBP, and to be primarily contained in a larger 150-200kD complex. Many studies of TRF3 function have focused on oogenesis and early
embryonic development. Hence, murine oocyte TRF3 expression has been shown to increase rapidly during oocyte growth from primordial follicle formation until preovulation at the same time that TBP expression is effectively eliminated (Yang et al. 2006; Gazdag et al. 2007). However, these observational reports reach conflicting conclusions on the role of TRF3 in murine embryogenesis, as one shows a steady increase in both TRF3 and TBP expression following fertilization, while the other concludes that TRF3 remains silent as TBP expression is induced post fertilization; this conflict in immunofluorescence data will likely be resolved by more extensive studies and additional approaches.

The role of TRF3 in early development has been most extensively investigated in Xenopus and zebrafish. Hence, Xenopus and zebrafish TRF3 bind TFIIA, TFIIB, and TATA containing DNA, and Xenopus TRF3 support TATA-dependent transcription in egg extracts (Bartfai et al. 2004; Jallow et al. 2004). While highly expressed in the Xenopus oocyte, TRF3 is expressed at lower levels in the developing embryo and is recruited to a subset of genes not occupied by TBP in a time-dependent manner. Importantly, TRF3 depletion causes defects in Xenopus gastrulation and blastopore closure and alters expression of many oocyte and embryo-specific genes, a subset of which also require TBP. Intriguingly, Xenopus TRF3 overexpression can partially rescue TBP knockdown phenotypes and gene expression patterns, consistent with a partial redundancy. Further genome wide analysis found that Xenopus TBP primarily regulates the synthesis of maternally deposited transcripts while a majority of embryonically transcribed genes require TRF2 or TRF3 (Jacobi et al. 2007). Additionally, TBP
dependent *Xenopus* genes are widely conserved from yeast to humans while TRF3-specific genes are generally restricted to vertebrates. *Xenopus* knockdown and ChIP experiments demonstrate that many promoters while exclusively dependent on either TRF3 or TFIID are occupied by both, suggesting that differences in complex-specific transcription may occur at the level of activation rather than recruitment. Expression of zebrafish TRF3 increases steadily from fertilization to gastrulation, after which it declines rapidly but remains detectable in all adult tissues and enriched in the ovary. As with *Xenopus*, knockdown inhibits normal gastrulation and results in mesodermal patterning defects. Intriguingly, both species show an enrichment of TRF3 in the ventral embryo consistent with a role in dorsoventral patterning. Additional knockdown studies in later stage zebrafish embryos demonstrate a specific requirement for TRF3 in the initiation of hematopoiesis, and partially define a lineage-specific transcriptional cascade in which TRF3 is the master regulator of this particular differentiating program (Verdorfer et al. 2007). Thus, multiple studies in lower vertebrates reveal a requirement for TRF3 in early development and elucidate both redundant and overlapping functions with TBP in embryonic transcription. However, it is intriguing to note that the striking embryonic phenotypes of TRF2 depletion in *Xenopus* and zebrafish are not conserved in the knockout mice and that the important embryonic functions of TRF3 in lower vertebrates likewise may not be preserved in mammals.

A study investigating the differentiation of mouse C2C12 cells found that while TBP, along with several TAFs, are degraded upon differentiation, at least some TRF3 is retained in myotubes, some of which is found in a complex with TAF3 (Deato and Tjian
Depletion of TRF3 and TAF3 by RNAi suggested that this putative core promoter recognition complex may be required for myogenesis. Consistent with a requisite role in differentiation, ChIP analysis found TRF3/TAF3 occupying a key muscle-specific gene promoter. Further biochemical analysis of the complex suggested that TRF3/TAF3 is sufficient for modest activation of the myogenin promoter \textit{in vitro} by the myogenic transcription factors MyoD and E47 (Deato and Tjian 2008). Protein-protein interaction studies, along with additional \textit{in vitro} transcription assays, revealed that MyoD directly targets TAF3, and that this interaction is necessary for \textit{in vitro} activity. It will be interesting to determine how widely the TRF3/TAF3 complex may be used in other tissues.

The Mediator coactivator complex was also recently found to display considerable subunit diversity, which ultimately may be shown to correlate with tissue and cell-type-specific transcriptional programs. Hence, biochemical fractionation of HeLa nuclear extracts yielded an altered CRSP/Med2 complex lacking two key canonical Mediator subunits previously shown to be critical for nuclear hormone receptor (NHR) dependent transcriptional activation (Taatjes and Tjian 2004). This complex, while unable to support NHR dependent transcription, supports transcription by activators which target other Mediator subunits and therefore may be responsible for limiting coactivation to a subset of extracellular signals or promoters. Similarly, the ARC-L/Med complex which contains most canonical Mediator subunits in addition to a dissociable CDK8/cyclin C/Med230/Med240 submodule may fine tune Mediator activity in response to environmental or developmental cues (Taatjes et al. 2002). Importantly, reversible
association of the ARC-L/Med submodule transitions C/EBPβ dependent Mediator activity from a stimulatory to a repressive state (Mo et al. 2004). While ARC-L/Med has generally been associated with transcriptional repression it appears to stimulate a limited subset of promoters, and thus the ratio of ARC-L/Med to Mediator may lead to the activation of some genes and subsequent repression of others (Akoulitchev et al. 2000; Zhou et al. 2002). Furthermore, the finding that the ARC-L/Med submodule enhances Mediator’s association with a ubiquitin ligase and may enhance its proteasome-dependent degradation suggests that submodule abundance may regulate coactivation globally by influencing Mediator turnover (Brower et al. 2002). Thus, combinatorial control of subunit composition could allow for cell-type and promoter-specific transcription dependent on distinct classes of activators. However, it remains to be seen whether these complexes are utilized in a cell-type-specific way.

Additional precedent for the idea of tissue and developmental stage-specific changes in transcription factor complexes that were previously held to be invariant comes from the recent observation that Swi/Snf chromatin remodeling complexes also vary greatly between tissues. Hence, the finding that specific subunits of the mammalian PBAF complex are enriched in cardiac progenitors and required for normal heart maturation provided the earliest support for the idea of cell-type-specific chromatin remodelers (Wang et al. 2004; Yan et al. 2005). Conversely, knockout studies of unique subunits from the related BAF-A and BAF-B complexes demonstrated overlapping deficiencies in ES cell pluripotency and lineage-specific differentiation, while disruption of PBAF showed no specific ES cell phenotype, consistent with distinct developmental
requirements for each complex (Gao et al. 2008; Huang et al. 2008; Yan et al. 2008). The first evidence of a requisite developmental stage-specific switch in subunit composition came with the discovery that homologous BAF45 and BAF53 subunits must be exchanged during the transition from neuronal progenitors to postmitotic neurons (Lessard et al. 2007; Wu et al. 2007). Moreover, this exchange is now known to depend on microRNA-mediated repression of the progenitor-specific subunits, adding an even greater level of regulatory complexity to the neuronal transition (Yoo et al. 2009). Most recently, a novel esBAF complex was isolated from embryonic stem cells which has a unique subunit composition, is required for pluripotency and self-renewal, and is downregulated upon differentiation (Ho et al. 2009b). Further genome-wide localization experiments demonstrate the existence of a cooperative transcriptional network between esBAF and known ES cell-specific transcription factors which defines self-renewal and pluripotency (Ho et al. 2009a). The extensive evidence that TAF1 can bind and modify histones, combined with the more recent finding that TAF3 shows a specific affinity for active chromatin marks, further suggests that diverse cell or developmental stage-specific chromatin remodelers and core promoter recognition complexes may cooperate with specific chromatin states to direct highly specialized transcriptional programs (Mizzen et al. 1996; Jacobson et al. 2000; Vermeulen et al. 2007).
Results

Hepatic Expression of TRF3, TAF7l, and TAF13

Because our previous qPCR analysis suggested that TRF3, TAF7l, and TAF13 expression may not be as dramatically downregulated as TBP and other TAFs in adult hepatocytes or may in fact be induced upon differentiation, we chose to further explore the expression and function of these specific subunits in hepatic transcription (Chapter 2, Figure 3). Hence, we performed western blots of purified hepatoblasts and hepatocytes with multiple antibodies specific for these three subunits (Figure 1). At least two separate anti-TRF3 antibodies recognize strong expression of TRF3 expression in both developing hepatoblasts and committed hepatocytes (Figure 1A). Within the quantitative limits of western blot analysis, TRF3 expression in liver cells does not appear to differ greatly from that in the whole ovary, a tissue in which TRF3 was previously shown to be highly enriched (Gazdag et al. 2009). While qPCR data suggest that TRF3 expression may be slightly greater in hepatocytes than hepatoblasts, quantitative western blotting or other techniques need to be employed to determine if this is true at the protein level.

Similarly, western blots with two different TAF7l antibodies suggest that this paralog is expressed at similar levels in hepatocytes and developing hepatoblasts and at comparable levels to the testes, the tissue from which TAF7l was first isolated and shown to have a specific function (Figure 1B). Again, more quantitative proteomic methods will need to be employed to determine if the significant upregulation of TAF7l transcript observed in adult hepatocytes leads to an increase in protein levels. Initial western blot comparison of TAF13 expression in developing hepatoblasts and mature hepatocytes also
suggests that this subunit may be retained or induced in the adult liver, in contrast to the majority of canonical TFIID components (Figure 1C). While further quantitation of TAF13 polypeptide is needed, initial data suggest that this subunit warrants further investigation.

**RNAi Mediated Depletion of TRF3, TAF7l, and TAF13 Attenuates Hepatic Gene Induction in HPPLs**

While spatial and temporal patterns of transcription factor expression often correlate with tissue-specific function, this is not always the case, and some factors may be inactive or dispensable even where they are highly abundant. To explore the possibility that TRF3, TAF7l, and TAF13 may play a role in adult hepatic gene transcription, we depleted these proteins by lentiviral RNAi in the HPPL cell line. pLKO.1 vectors which are part of the Broad Institute RNAi consortium and express short hairpin RNAs targeting these three transcripts under the U6 promoter, along with a puromycin resistance gene, were used to generate replication-incompetent lentiviral particles by transient transfection of HEK293T cells (Figure 2A). Proliferating HPPLs were infected for 24 hours with viral supernatants and then selected for four days in fresh growth medium containing puromycin to eliminate uninfected cells. Cells were then harvested to determine the extent of gene knockdown by qPCR or differentiated in the presence of Oncostatin M. By this approach we consistently achieved knockdown of TRF3, TAF7l, and TAF13 with sixty to eighty percent efficiency (Figure 2B). Importantly, infection with a control lentivirus targeting GFP had no discernible affect on
the levels of these three transcripts, and reduction of any one transcript did not influence
the expression of another, suggesting that off target effects and lentivirally induced
cytotoxicity are minimal in this system.

Because the most significant changes in both TFIID downregulation and hepatic
gene induction in the HPPL cell model were observed after five days of differentiation,
we reasoned that this represented the most critical period for transcription factor activity
in this particular system (Chapter 1, Figure 8 and 9). Following four days of selection,
confluent lentivirally transduced HPPLs were differentiated for five days in the presence
of Oncostatin M and RNA harvested for qPCR analysis. This data shows that even
moderate depletion of TRF3, TAF7l, and TAF13 has a significant impact on the
induction of multiple adult hepatic genes in the HPPL model, as judged by transcript
abundance (Figure 3). Qualitatively, differentiated TRF3, TAF7l, and TAF13 shRNA
expressing HPPLs contained fewer large cuboidal, binucleate, and vacuolated cells than
the GFP control, suggesting that these cultures failed to fully differentiate. Though it
cannot be concluded from this data that TRF3, TAF7l, or TAF13 specifically regulate the
hepatic genes in question, this approach suggests that these factors have some influence
on the HPPLs’ competence to differentiate towards a mature hepatic cell and that in their
absence, hepatic maturation is at least partially compromised.
TRF3, TAF7l, and TAF13 are Enriched at Hepatic Promoters in the Adult Liver and Differentiated HPPLs.

The physical recruitment of sequence-specific activators to their target enhancers and of the general machinery to a gene’s proximal promoters is a requisite step in transcriptional activation (Thomas and Chiang 2006). Hence, the ability to localize proteins and complexes at the regulatory elements of a given gene supports a role for such factors in that gene’s transcriptional regulation. Chromatin immunoprecipitation is a powerful technique for investigating the relative occupancy of defined DNA domains by specific proteins and has been extensively employed to elucidate the functions of single transcription factors and the complexities of large regulatory networks (Massie and Mills 2008; Park 2009). Specifically, this approach has been invaluable in unraveling the role of liver-enriched activators and general transcription factors in liver development and maintenance (Kyrmizi et al. 2006; Tatarakis et al. 2008).

To investigate the role of TRF3, TAF7l, and TAF13 in hepatic transcription we isolated formaldehyde crosslinked chromatin from individually dissected E13.5 and adult mouse livers, and subjected it to immunoprecipitation with antibodies against these three factors. Precipitated material was analyzed by qPCR with primers surrounding the promoters of adult hepatic genes (Figure 4). Intriguingly, TRF3, TAF7l, and TAF13 show overlapping and distinct patterns of promoter occupancy on multiple hepatocyte-specific genes. Most notably all three factors are significantly and specifically enriched on the promoters of α1-antitrypsin (AAT) and tryptophan-2,3-dioxygenase (TDO2), two genes which are completely silent in the embryo and are highly induced late postnatally.
Importantly, these promoters show no enrichment of the factors at day E13.5, a time when they are known to be transcriptionally silenced. Intriguingly, a second class of promoters, including apolipoprotein C3 (APOCIII) and possibly apolipoprotein B (APOB) and albumin (ALB), are occupied by TRF3, TAF7l, and possibly TAF13 in both the embryo and adult. In contrast to AAT and TDO2, these promoters are expressed in the embryo and continue to be expressed at high levels postnatally. Recruitment of TRF3, TAF7l, and TAF13 is less pronounced and varies between factors on the other promoters examined; however the extent of factor recruitment is significant and specific and its timing is consistent with the known developmental upregulation for these promoters.

The primary drawback of chromatin immunoprecipitation is its dependence on the specificity and efficiency of available antibodies for the transcription factor in question. Because of this, overexpression of exogenous epitope-tagged proteins followed by precipitation with well characterized antibodies against those tags has proven valuable to corroborate data obtained with antibodies against the endogenous factor. To this end, we generated recombinant TRF3, TAF7l, and TAF13 constructs with N-terminal FLAG tags in the doxycycline-inducible pTRIPZ vector; these vectors were used to produce lentiviral particles which were used to transduce proliferative HPPLs. Two days post infection confluent cells were differentiated for five days by the addition of oncostatin M, and pTRIPZ expression was induced by the addition of doxycycline to 2μg/ml on the last two days of differentiation. Cells were crosslinked and purified chromatin was immunoprecipitated with IgG or anti-FLAG polyclonal antibodies. Precipitated material
was analyzed by qPCR and results expressed as fold enrichment over the IgG control (Figure 5).

While the promoter enrichment of FLAG-tagged TRF3, TAF7l, and TAF13 in differentiated HPPLs does not exactly mirror that seen in adult liver, this data clearly demonstrates that all three factors are specifically recruited to adult hepatic genes (Figure 5). The efficiency of FLAG-tagged protein immunoprecipitation may be impacted by the presentation of the N-terminal FLAG epitope on these proteins in a native environment; likewise the extent of promoter occupancy may be impacted by the ability of these exogenous proteins to associate with other factors required for their recruitment or the presence of these factors in not fully differentiated HPPL. In particular, the reduced recruitment of TAF7l likely results from the relatively low expression and cytotoxicity that we observe with this construct and that may inhibit differentiation or adequate promoter binding. However, the data show that all three factors are enriched at multiple hepatic promoters by an approach that is independent of antibody specificity.

Furthermore, the relative fold enrichment is similar to that seen in whole liver and previously reported for TAFs, as well as general transcription factors on hepatic genes in whole liver and liver cell line samples (Tatarakis et al. 2008).

**TRF3 Associates with One or More High Molecular Weight Complexes in the Adult Liver.**

The finding that TRF3, TAF7l, and TAF13 are expressed in the adult liver, are enriched at overlapping hepatic promoters, and may be required for hepatic gene
expression raised the possibility that they interact to form a coordinated multisubunit regulatory complex. Indeed, one of the defining characteristics of known core promoter recognition and coactivator complexes including TFIID and Mediator is their existence as stable high molecular weight assemblies under native conditions (Albright and Tjian 2000; Myers and Kornberg 2000). Additionally, TRF3 has previously been shown to associate with a 150kD complex in HeLa cells and to form a 150kD complex with TAF3 in myotubes (Persengiev et al. 2003; Deato and Tjian 2007).

To explore this possibility, we prepared nuclear extracts from adult mouse livers and subjected them to size exclusion chromatography on a Superdex-200 column previously calibrated with known sizing standards. Western blot analysis of those fractions corresponding to molecular weights from 440 to 13.7kD reveal that TRF3 migrates at approximately 150kD as previously reported in the HeLa and myotube systems. However, TAF7l and TAF13 do not appear, under these conditions, to significantly associate with slower migrating TRF3, as they both migrate at or near their calculated monomeric molecular weights of 42 and 18kD respectively. Our finding that TAF3 is downregulated transcriptionally during hepatic development and seemingly absent from adult hepatocytes at the protein level led us to believe that the TRF3/TAF3 complex previously identified in myotubes would be absent in the hepatic system (Chapter 2, Figure 3 and 5). However, the observation that TRF3 remains associated with a 150kD complex in liver nuclear extracts suggests that either trace amounts of TAF3 remain, or that TRF3 is associated with other as yet to be identified subunits. While the slower migration of TRF3 clearly does not depend on its association with the
majority of TAF7l or TAF13 under these conditions, this does not rule out the possibility
that these three proteins interact more transiently in vivo or that minor amounts of TAF7l
and TAF13, which avoid detection in this setting, are associated with higher molecular
weight assemblies. It is important to note that neither TAF7l nor TAF13 is observed in
the void fraction that would contain canonical TFIID. Though many general
transcription factor assemblies survive biochemical fractionation under the conditions
employed, activator-coactivator interactions are typically weaker, more transient, and
refractory to efficient biochemical copurification. Clearly, further investigation is needed
to determine the full range of protein-protein interactions by TAF7l and TAF13.

Core Promoter Recognition Complex Changes in Human ES Cell Derived
Hepatocytes.

Canonical TFIID structure and function has been a subject of almost equally
intensive study in both the mouse and human systems, and its role in transcription has
been shown to be highly conserved between these and lower vertebrates. In contrast,
investigation of TBP-related factors and TAF paralogs, including TRF3 and TAF7l, has
been largely restricted to the murine, Xenopus, and zebrafish models. Furthermore,
significant differences in the mechanisms and processes of liver development and
regeneration have been identified between mice and humans (Wauthier et al. 2008).
Hence, novel mechanisms of hepatic regulation uncovered by murine experimentation
must be recapitulated and confirmed in the human system for their therapeutic potential
to be realized. The directed in vitro differentiation of human embryonic stem cells to
functioning hepatocytes with the potential for therapeutic transplantation has been reported by several groups and is a subject of intensive ongoing investigation (Duan et al. 2007; Hay et al. 2007; Hay et al. 2008a). Thus, we wished to determine if the changes we observe in core promoter recognition complex expression during murine liver development are conserved in an ES cell-based model of human hepatic commitment.

Human H9 ES cells were differentiated using a three step, sixteen day protocol which mimics *in vivo* signaling events and has a reported efficiency of 90% (Hay et al. 2008a). In the first stage, proliferating ESCs are committed to the definitive endoderm by incubation for five days with Activin A and Wnt3A, in the second stage they are differentiated to hepatoblast like cells in defined growth factor free media, and in the final stage matured to hepatocyte-like cells by the addition of hepatocyte growth factor and oncostatin M (Figure 7A). Analysis of known marker gene expression in these four cell populations by qPCR mirrors which is observed *in vivo* and is consistent with published reports of directed hepatocyte differentiation, suggesting that the procedure does faithfully model the hepatogenic process (Hay et al. 2008a; Hay et al. 2008b). Intriguingly, qPCR analysis of TBP, three critical TAF subunits, and MED17 in these four cell populations shows that TFIID and Mediator levels are held constant or induced during the first two stages and reduced dramatically during hepatogenic maturation (Figure 7B). Thus, as is observed in the purified mouse liver cell samples and HPPL protocol, this model suggests that human hepatogenesis requires significant changes in TFIID and Mediator expression or composition.
Discussion

Intensive and ongoing study of liver-enriched sequence-specific activators has contributed greatly to our understanding of the transcriptional changes that govern hepatic development and regeneration (Schrem et al. 2002; Costa et al. 2003). However, the molecular mechanisms by which unique combinations of activators alter global patterns of gene expression in response to developmental inputs or hepatic injury remains to be elucidated. Core promoter recognition and coactivator complexes are a critical link between activators, chromatin modifiers, core promoter DNA, and the general PolII machinery. Despite this, the composition and function of these complexes in liver development, regeneration, and homeostasis has not been thoroughly explored.

Our observation that canonical TFIID, including TBP and multiple TAFs, is significantly downregulated during hepatic development raised the possibility that alternative core promoter recognition proteins exist in the adult liver and at least partially replace the critical functions of TFIID (Chapter 2). This hypothesis was informed by several recent studies that demonstrate a role for TBP-related factors and TAF paralogs is defined differentiation programs or cell types. Specifically, muscle cell development and embryonic hematopoiesis were independently shown to require a novel TRF3/TAF3 core promoter recognition complex which at least partially replaces TFIID in the regulation of developmental stage-specific genes (Deato and Tjian 2007; Hart et al. 2009). Additionally, TRF3 was found to be a critical regulator of oocyte-specific genes during primary folliculogenesis, while TAF7l was shown to be essential for spermiogenesis, potentially as part of a novel TBP containing complex (Cheng et al. 2007; Gazdag et al.
Our qPCR analysis of known TRF and TAF transcripts in hepatoblasts and hepatocytes suggests that, unlike TBP and the majority of TAFs, TRF3, TAF7l, and TAF13, expression is maintained or induced in committed liver cells and that the role of these factors in hepatic transcription therefore warrants further investigation.

While more accurate quantitation of TRF3, TAF7l, and TAF13 polypeptide is required, initial data confirms expression of these three proteins in adult hepatocytes and suggests that they are therefore unique from the majority of TFIID subunits which are significantly downregulated. A more thorough understanding of liver organogenesis should include expression studies of TFIID and these three factors in the other major cell types, including Cholangiocytes and Kupffer cells, to determine if retention of these factors is specific to the hepatocyte lineage or a hallmark of multiple liver cells. Initial RNAi experiments demonstrate that depletion of TRF3, TAF7l, and TAF13 alters patterns of hepatic gene induction, suggesting that their presence is in fact functionally relevant. More detailed HPPL studies will be needed to determine if RNAi of these factors alters cell proliferation or competence prior to hepatocyte commitment, suggestive of a role in hepatoblast transcription, or if the observed phenotype reflects a specific requirement for these factors solely during the process of hepatic gene induction.

While gene expression is an important indirect readout of hepatic development, differentiated HPPLs have been shown to exhibit hepatocyte like metabolic functions such that it will be interesting to determine if RNAi impacts more functional readouts, including albumin secretion and cytochrome P450 metabolism (Kamiya et al. 2002). Additionally rescue experiments are needed to confirm the specificity of shRNA
targeting. Mouse knockout and knockdown experiments have helped to confirm the *in vivo* relevance of both liver-enriched transcription factors and TFIID components in other tissues (Lee et al. 2005; Mengus et al. 2005; Tatarakis et al. 2008) Whole animal knockdown studies are currently underway and should help to corroborate and expand the findings of TRF3, TAF7l, and TAF13 RNAi experiments conducted in the HPPL system.

Morphological and functional phenotypes resulting from gene ablation experiments often reflect secondary or tertiary effects and thus fail to prove that a given promoter is the direct target of the transcription factor in question. The chromatin immunoprecipitation data presented demonstrates that at least some adult hepatic genes are specifically occupied by TRF3, TAF7l, and TAF13 and suggests that the observed RNAi phenotype is not exclusively the product of secondary effects. In light of these results, ChIP and Re-ChIP analysis should be extended to determine more accurately the timing and potential co-occupancy of additional adult and embryonic promoters in multiple liver cell types. A major goal of hepatology research has been to uncover the overlapping and autoregulatory actions of all known liver-enriched transcription factors throughout development, and to use this data in defining an all inclusive regulatory network (Odom et al. 2004; Kyrmizi et al. 2006). If novel core promoter recognition complexes prove to be global regulators of hepatic transcription, then chromatin immunoprecipitation combined with recent deep sequencing techniques has the potential to identify all promoters regulated by these factors and refine or expand considerably our understanding of the hepatogenic transcription factor network (Park 2009). Reconstituted *in vitro* transcription using defined factors was recently employed to confirm the
regulation of a key myogenic promoter by the TRF3/TAF3 complex in coordination with a known myogenic activator, and was previously shown to be a valuable technique in the mechanistic study of a tissue-specific noncanonical TAF4 paralogs (Deato et al. 2008; Liu et al. 2008). Hence, if liver-specific core promoter recognition proteins and their target promoters can be sufficiently defined, then in vitro assays with known liver-enriched transactivators may prove valuable in further uncovering the mechanisms of hepatocyte-specific transcriptional activation.

While our data confirm the association of TRF3 with a high molecular weight complex in adult liver as previously reported for other cell types, initial experiments suggest that it does not physically associate with either TAF7l or TAF13 under the conditions employed (Hart et al. 2009). Clearly, further investigation is needed to determine if TAF7l and TAF13 possess unique or overlapping binding partners in committed hepatocytes and if such proteins are required for their promoter recruitment or coactivator function. One outstanding question is whether either of these factors associates with canonical TFIID in the fetal or adult liver and if this association is strengthened or eliminated during hepatogenic differentiation. While specific TAFs have been shown to bind known transcriptional activators and thus facilitate recruitment of holo-TFIID to the core promoter, no specific interaction has yet been reported between canonical TFIID subunits and liver-specific activators (Albright and Tjian 2000). It will be interesting for future studies to determine if known liver-specific activators exhibit preferential binding to TFIID or novel hepatocyte enriched core promoter recognition
proteins and if these interactions are consistent with previously identified target genes and patterns of expression for the activator in question.

Ongoing efforts to develop therapeutically relevant transplantable hepatocytes from human embryonic stem cells have benefitted greatly from basic advances in our understanding of liver development (Hay et al. 2007; Hay et al. 2008a). Our initial finding that hepatogenic in vitro differentiation of human ESC involves significant downregulation of TFIID as first observed in the mouse system, suggests that core promoter recognition complex changes are not unique to the murine system and may prove useful in the refinement of ESC differentiation protocols. In light of these observations and the recent generation of iPS cells from mature hepatocytes, it will be interesting to determine the function of TFIID and potentially novel core promoter recognition factors, not only in hepatocyte commitment but in the process of iPS induction, pluripotency, and self-renewal (Aoi et al. 2008).

It is now clear that TFIID, Mediator, and potentially other general transcription factor complexes, which were once thought to be a hallmark of all cell types and gene expression programs, are dispensable in at least some developmental strategies and tissue-specific cell types. The role of TBP-related factors, TAF paralogs, and noncanonical core promoter recognition or coactivator complexes have become increasingly important as new techniques have allowed us to study rarer and more defined populations of cells. The recent finding that myogenesis and early hematopoiesis employ a stripped down TRF3/TAF3 complex in place of canonical TFIID, and that germ cell development relies on multiple TFIID independent factors including TRF2, TRF3
and TAF7l raises the possibility that additional differentiation programs will require these and as yet unidentified proteins which supplant TFIID at critical developmental junctions. Our current data suggest that the commitment of hepatogenic precursors to mature hepatocytes likewise involves the downregulation of canonical TFIID subunits and their possible replacement with TRF3, TAF7l, or TAF13 and potentially other unknown core promoter recognition elements.

**Materials and Methods**

**Antibodies**

Fresh TRF3, TAF7l, and TAF13 antibodies were generated by multiple strategies. Rabbits, Guinea pigs, and chickens were injected with an N-terminal TRF3 peptide coupled to Keyhole Limpet Hemocyanin (Pierce) or a recombinant TRF3-Pseudomonas exotoxin fusion protein expressed from pVCH6 and purified under denaturing conditions. Rabbit and chicken anti-TAF7l antibodies were raised against a Pseudomonas exotoxin fusion-TAF7l fusion protein purified under denaturing conditions. Mouse anti-TAF13 antibodies were raised against full length recombinant *Drosophila* TAF13 purified under denaturing conditions. Rabbit and mouse polyclonal antibodies were purified on Protein A sepharose (Amersham) and chicken antibodies were purified using the IgY Purification Kit (Pierce). Mouse monoclonal anti-TBP/TRF3 antibody 58C9 was used for western blotting at a dilution of 1:1,000 (Sigma, (Hoey et al. 1993)).
Plasmids

shRNA constructs in the pLKO.1 plasmid are part of the Broad Institute RNAi library and were purchased from Open Biosystems. The lentiviral packaging plasmids pSPAX2 and pMD2.G were purchased from Addgene. The TRF3, TAF7l, and TAF13 open reading frames were PCR amplified to contain N-Terminal FLAG tags and C-Terminal HA, V5, and MYC tags respectively. PCR products were cloned into the AgeI and MluI sites of the Doxycycline inducible pTRIPZ vector (Open Biosystems). All constructs were sequenced and purified using Endotoxin Free Maxi or Giga kits (Qiagen).

Size-Exclusion Chromatography

Nuclear extracts were prepared from 8-10 week old CD-1 males as reported (Conaway et al. 1996) and fractionated on a 24ml Superdex-200 column (GE Healthcare) using an AKTAexplorer. Fractions were analyzed by western blot and calibrated using the Gel Filtration Calibration Kit according the manufacturer’s instructions (GE Healthcare).

Lentiviral RNAi

Recombinant lentivirus was generated by transient cotransfection of 293T cells with pLKO.1 or pTRIPZ (Openbiosystems) and pSPAX2/pMD2.G (Addgene) using Lipofectamine 2000 (Invitrogen). Viral supernatants were used to infect proliferating HPPL cells in the presence of 8ug/ml hexadimethrine bromide and 24 hours post infection cells were selected with 1.5ug/ml Puromycin (Sigma). RNA from
lentivirus infected cells was isolated using RNeasy kits (Qiagen) and used for RT-qPCR.

**Chromatin Immunoprecipitation**

Fetal and adult liver chromatin was prepared essentially as reported (Kyrmizi et al. 2006). Individually dissected adult and E13.5 livers were minced with razor blades in PBS and homogenized in 1% formaldehyde by 10-15 strokes of an A pestle. Crosslinking was continued for 10 minutes at room temperature and quenched by addition of glycine to 125mM. Cells were washed twice in PBS and twice for 20 minutes in cell lysis buffer (25mM Hepes pH7.9, 10mM KCl, 0.250M Sucrose, 2mM EDTA, 1.5mM MgCl2, 0.1% NP-40, 1mM DTT, 0.5mM Spermidine, Protease Inhibitor Cocktail (Roche) followed by passage through a 100μm filter. Nuclei were collected by centrifugation resuspended in two pellet volumes of nuclear lysis buffer (50mM Hepes pH7.9, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, Protease Inhibitor Cocktail (Roche)), and chromatin was sonicated for 10 minutes (30 second on cycles) on high with a Bioruptor (Diagenode). Chromatin was clearly by centrifugation at 12,000g for 10 minutes and equal A260 units were precipitated overnight with protein A purified rabbit anti-TRF3, TAF7l, and TAF13 antibodies or Rabbit IgG (Jackson Immunoresearch). Protein A and Protein G beads were blocked overnight with 5mg/ml BSA and 5mg/ml herring sperm DNA (Invitrogen), and added to the chromatin immunocomplexes for 1 hour. Beads were washed three times with IP buffer, once with IP buffer plus 500mM NaCl, twice with LiCl wash buffer (20mM Tris-HCl pH7.9, 1mM EDTA, 250mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate), and once with TE.
Complexes were eluted by incubation for 30 minutes at 37°C in elution buffer plus 10μg/ml RNase A (Qiagen) followed by two hours at 65°C in the presence of 10μg/ml Proteinase K (Invitrogen). Crosslinking was reversed overnight at 65 and DNA purified using Qiagen spin columns before qPCR with promoter-specific primers. Chromatin from pTRIPZ infected cell lines was prepared by addition of formaldehyde to the cell culture media to a final concentration of 1% for 10 minutes at room temperature followed by the addition of glycine to 125mM. Chromatin was prepared as above and precipitated with rabbit IgG or rabbit anti-FLAG polyclonal antibody (Sigma).

**H9 hESC Differentiation**

Human H9 hESC cells were maintained on irradiated mouse embryonic fibroblasts (MEFs) following WICell protocols and differentiated as reported (Hay et al. 2007; Hay et al. 2008a). Cells were passaged to Matrigel (BD Bioscience) coated plates in 10% MEF conditioned media and at 70% confluence induced to differentiate by the addition of priming media containing RPMI1640 (Invitrogen), 1xB27 (Invitrogen), 50ng/ml Wnt3a (R&D Systems), and 100ng/ml Activin A (Peprotech). After five days cells were split 1:2 onto fresh Matrigel and cultured in differentiation media containing knockout-DMEM (Invitrogen), 20% Serum Replacement (Invitrogen), 1mM glutamine, 1% nonessential amino acids (Invitrogen), 0.1mM β-mercaptoethanol, and 1% DMSO. Finally, cells were matured in L15 medium (Invitrogen) supplemented with 8.3% FBS, 8.3% tryptose phosphate broth (Sigma), 10μM hydrocortisone 21-hemisuccinate (Sigma), 1μM insulin, 2mM glutamine, 10ng/ml hepatocyte growth factor (Peprotech), and
20ng/ml Oncostatin M. After seven days RNA was harvested for reverse transcription and qPCR with validated gene-specific primers.
Figure 1: Expression of TRF3, TAF7l, and TAF13 in purified liver cells.

Recombinant TRF3, whole ovary lysate, adult liver lysate, hepatoblasts, and hepatocytes were subjected to western blotting with mouse monoclonal antibody 58C9 and rabbit anti-TRF3 antibodies (A). Recombinant TAF7l, testes lysate, fetal and adult liver lysate, hepatoblasts and hepatocytes were subjected to western blotting with two different polyclonal anti-TAF7l antibodies (B). Hepatoblast and hepatocyte lysates were subjected to western blotting with mouse anti-TAF13 antibody (C).
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A

Mouse Anti-TRF3(58C9)
Rabbit Anti-TRF3(22598)

B

Rabbit Anti-TAF7l(HYRB1)
Rabbit Anti-TAF7l(23266)

C

Mouse Anti-TAF13(31)
Figure 2: shRNA Mediated Depletion of TRF3, TAF7l, and TAF13 in HPPLs.

The pLKO.1 vector, which contains lentiviral 5’ and 3’ leader and other regulatory sequences, expresses short hairpin RNAs from the human U6 promoter and puromycin N-acetyl-transferase from the PKG promoter (A). Proliferating HPPLs were infected for 24 hours with pLKO.1 viral supernatants expressing the indicated shRNA, selected for four days with 1.5μg/ml Puromycin and knockdown assessed by qPCR (B).
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A

B

Percent of shGFP Control

TRF3  TAF7l  TAF13

shGFP  shTRF3  shTAF7l  shTAF13
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Figure 3: Expression of Adult Hepatic Genes in Differentiated and pLKO.1 Transduced HPPLs.

Following four days of selection, five days post infection, HPPLs were differentiated for five days in the presence of 20ng/ml Oncostatin M and the induction of hepatic genes was analyzed by qPCR.
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The bar chart shows the percent of shGFP control for various genes, including:
- Cyp2F2
- Cyp7B1
- Fasn
- IGF1
- Mup3
- Slco1a1
- FoxA2
- HNF1a
- HNF4a
- G6PC
- TAT
- TDO2

The chart indicates that shTRF3 and shTAF7l significantly reduce the expression of these genes compared to shGFP and shTAF13 controls.
Figure 4: Chromatin Immunoprecipitation in Fetal and Adult Liver

E13.5 and adult livers were individually dissected, crosslinked, and chromatin was immunoprecipitated with IgG or TRF3, TAF7l, and TAF13 antibodies. Crosslinking was reversed and precipitated DNA was analyzed by qPCR. Signals were normalized to input and expressed as fold enrichment over the IgG control.
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Figure 5: Chromatin Immunoprecipitation in Differentiated HPPLs.

Proliferating HPPLs were infected for 24 hours with pTRIPZ based lentivirus expressing FLAG tagged TRF3, TAF7l or TAF13 and differentiated for five days in the presence of 20 ng/ml Oncostatin M. Cells were crosslinked and chromatin was subjected to immunoprecipitation with IgG or anti-FLAG polyclonal antibody. Crosslinking was reversed and precipitated DNA analyzed by qPCR. Signals were normalized to input and expressed as fold enrichment of the IgG control.
Figure 6: Size Exclusion Chromatography of Adult Liver Nuclear Extract.

Adult mouse liver nuclear extract was fractionated on a Superdex-200 column and fractions analyzed by western blot with TRF3, TAF7l, and TAF13 antibodies. Relative molecular weights were calibrated with the standards in HMW and LMW Gel Filtration Calibration Kit.
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TRF3

TAF7l

TAF13
**Figure 7: Hepatic Differentiation of Human H9 ES Cells.**

Human H9 ES cells were differentiated to mature hepatocyte-like cells by a three-stage protocol that mimics *in vivo* signaling events (A). Analysis of known marker genes for ES cells, definitive endoderm, hepatoblasts, and mature hepatocytes by qPCR shows that this protocol faithfully mimics human hepatogenesis (B).
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**A**

**B**

![Graph showing gene expression levels across different stages and cell lines]

- Percent of hESC(H9) (Log Scale)
- hESC(H9) Stage 1
- hESC(H9) Stage 2
- hESC(H9) Stage 3
- Sox4
- Sox17
- CCR4
- Met
- Krt19
- Aqp1
- Prox1
- Afp
- Alb
- G6PC
- HNF3B
- AAT
- TDO2
- CPS1

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Figure 8: Expression of TBP, TRFs, and TAFs in H9 Differentiation

Expression of TBP, TAF1, TAF3, TAF5, and MED17 as judged by qPCR, remains stable during definitive endoderm and hepatoblast commitment, but decreases significantly during hepatocyte maturation in the H9 system.
References


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