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Probing a Role for TRAP150 in Gene Regulation

Divya Potabathula
Wright State University

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PROBING A ROLE FOR TRAP15O IN GENE REGULATION

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

DIVYA POTABATHULA
B.Tech., Jawaharlal Nehru Technological University, 2006

2009
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Divya Potabathula ENTITLED Probing a role for TRAP150 in gene regulation BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

Paula A. Bubulya, Ph. D.
Thesis Director

David L. Goldstein, Ph. D.
Department Chair

Committee on Final Examination

Paula A. Bubulya, Ph.D.

Barbara Hull, Ph. D.

Scott E. Baird, Ph.D.

Joseph F. Thomas, Jr., Ph.D.
Dean, School of Graduate Studies
ABSTRACT:
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Probing a Role for TRAP150 in Gene Regulation.

The mammalian cell nucleus is a highly organized cellular compartment. The nucleus is surrounded by the nuclear envelope and contains distinct domains such as nucleoli, nuclear speckles and Cajal bodies that may reflect nuclear functions such as transcription and RNA processing. Nuclear speckles are regions in the nucleus that are enriched in splicing factors and other RNA processing factors, as well as some transcription factors and the large subunit of RNA polymerase II. Mass spectrometry analysis of purified nuclear speckles revealed 146 nuclear speckle proteins of which 33 are novel nuclear speckle components. One such novel nuclear speckle protein is thyroid hormone receptor associated protein (TRAP150/THRAP3). Immunofluorescence of TRAP150 in Hela cells shows that it localizes predominantly in a diffuse distribution throughout the nucleoplasm that resembles the localization of transcription factors. Phylogenetics showed that TRAP150 and Btf (another novel speckle protein) are members of the same gene family. The amino acid sequence alignment of TRAP150 and Btf showed that they share 39.5% sequence identity and 62% similarity. High sequence similarity was observed at the C-termini, suggesting a second functional domain of unknown function.

TRAP150 and Btf associate with affinity-purified in vitro-spliced human mRNPs. Hence, I hypothesized that TRAP150 might have a role in transcription and/or pre-mRNA processing. Inhibition of RNA polymerase II transcription caused extensive
enrichment of TRAP150 in large, rounded nuclear speckles. I observed the association of TRAP150 and Btf with a reporter gene locus in vivo. Results showed that TRAP150 and Btf associate more frequently with the activated reporter locus than with the inactive locus. TRAP150 and Btf also localized in the vicinity of the reporter RNA. These results are all consistent with a role for TRAP150 and Btf in gene expression.
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LIST OF ABBREVIATIONS

Br-UTP- Bromo-Uridine triphosphate.
Btf- Bcl2 associated transcription factor.
CBC- Cap binding complex.
CXorf23- Chromosome X open reading frame 23.
DMEM- Dulbecco’s Modified Eagle Medium.
Dox- Doxycycline.
HDACs- Histone deacetylases.
HP1- Heterochromatin protein 1.
IGCs- Interchromatin granule clusters.
NLS- Nuclear localization signal.
RS domain- Arginine-Serine rich domain.
SF2/ASF- Splicing factor 2/Alternative splicing factor.
snRNP- small nuclear ribonucleoprotein.
TR- Thyroid hormone receptor.
TRAPs- Thyroid hormone receptor associated proteins.
TRAP150- Thyroid hormone receptor associated protein 150.
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I would like to thank Dr. Athanasios Bubulya for his assistance and insight.
I. INTRODUCTION

The mammalian cell nucleus is a highly organized cellular compartment. The nucleus is surrounded by the nuclear envelope and is arranged into distinct domains that may reflect functions such as transcription, pre-mRNA processing, and DNA replication (Spector, 1993). Some of these domains include the nucleolus, chromosome territories, nuclear pore complexes, Cajal bodies, Gems and nuclear speckles. (Spector, 1993; Lamond and Earnshaw, 1998; Misteli, 2001). These large regions in mammalian nuclei are functionally distinct from their surroundings and are unique because they are nonmembrane bound nuclear organelles. One idea is that these domains may serve as storage sites to supply functional complexes to regions of activity else where in the nucleus (Misteli, 2005).

Nuclear speckles are regions in the nucleus that are enriched in splicing factors and other RNA processing factors, as well as some transcription factors and the large subunit of RNA polymerase II (Misteli et al., 2005. Mintz et al., 1999; Saitoh, 2004). Nuclear speckles are not the sites of splicing and transcription, rather they are storage areas and/or assembly sites for these factors (Misteli and Spector, 1999; Caceres et al., 1997). Nuclear speckles are also called interchromatin granule clusters (IGCs) based upon their ultrastucture, with clusters of ribonucleoprotein particles seen interspersed between regions of chromatin. IGCs are one of the largest non-chromatin structures of the nucleoplasm. A proteomic analysis of IGCs purified from mouse liver showed that 81% of the proteins are involved in RNA metabolism among which 54% are involved in
pre-mRNA splicing (Mintz et al., 1999; Saitoh et al., 2004). Other components are thought to have a role in apoptosis, mRNA export, transcription and cleavage and polyadenylation (Saitoh et al., 2004). Mass spectrometry analysis of purified IGCs revealed 146 nuclear speckle proteins of which 33 are novel proteins that had not previously been identified as nuclear speckle proteins (Mintz et al., 1999; Saitoh et al., 2004). Assignment of these proteins to distinct functional groups was based upon motif analysis. For RNA processing factors, this included motifs such as RNA binding motifs, RNA helicase motifs, RNA recognition motifs, arginine serine (RS) rich domain motif and the D-E-A-D (Asp-Glu-Ala-Asp) box (DEAD box) helicase domain (Saitoh et al., 2004). These domains all serve specific functions in proteins that are involved in different aspects of RNA metabolism such as pre-mRNA processing.

Serine-arginine (SR)-rich splicing factors called SR proteins play a key role during constitutive and alternative splicing and are one of the major families of proteins found in nuclear speckles (Li and Bingham, 1991). During transcription, SR proteins and other RNA processing factors are recruited to transcription sites for co-transcriptional splicing (Misteli et al., 1997; Caceres et al., 1997). The arginine/serine rich (RS), domain found in pre-mRNA splicing factors is a speckle-targeting signal (Li and Bingham, 1991; Hedley et al., 1995; Caceres et al., 1997). Phosphorylation of serines in the RS domain regulates the release of SR protein complexes from the nuclear speckles and their subsequent assembly into spliceosomes at the transcription site (Hedley et al., 1995; Misteli et al., 1998). The dynamic movements of speckle components observed by expressing fluorescently tagged splicing factors in living cells may reflect the
recruitment of RNA processing machinery from nuclear speckles to transcription sites (Misteli et al., 1998; Caceres and Krainer, 1997).

SR splicing factors are divided into 3 groups according to the arrangement of the RS domain with respect to their other domains. Type I SR proteins have a RS domain and one to three RNA recognition motifs. Examples of type I SR proteins include “classical SR proteins” such as SF2/ASF, SC35 and 9G8. Type II SR proteins have a RS domain but do not have a RNA recognition motif. Examples of type II SR proteins include thyroid hormone receptor associated protein 150 (TRAP 150) and Bcl-2 associated transcription factor (Btf). Type III SR proteins are high molecular weight proteins that contain repetitive sequences in addition to the RS domain. Son and NP220 belong to this class of SR proteins (Saitoh et al., 2004).

**TRAP150**

TRAP150 was initially identified as a component of thyroid hormone receptor associated proteins. Thyroid hormone receptor (TR) is a nuclear receptor. In the absence of a thyroid hormone, thyroid hormone receptor binds to DNA and acts as a transcription repressor (Ito et al., 1999). In the presence of thyroid hormone, a conformational change occurs in the receptor, and it becomes a transcriptional activator (Ito et al., 1999). Thyroid hormone receptor associated proteins (TRAPs) co-activate TR-mediated transcription in a TATA-box-binding-independent manner. Some TRAPs have functions similar to the yeast mediator proteins that are responsible for transcription activation (Ito et al., 1999).
One among the potential nuclear speckle proteins is thyroid hormone receptor associated protein of 150 kDa (TRAP150). It was identified in proteomic analysis of purified nuclear speckles as a Type II RS domain containing protein (Mintz et al., 1999; Saitoh et al., 2004). Initially, TRAP150 was reported to have a role in transcription regulation acting as a co-transcriptional activator (Ito et al., 1999) but further work suggests that TRAP150 is not a bonafide component of the mediator complex (Dr. Robert Roeder, personal communication).

**Phylogenetics of TRAP150**

TRAP150 is a member of a small gene family that also includes Btf and Chromosome X open reading frame 23 (CXorf23) and their orthologs. A phylogeny of this gene family is shown in Figure 1. Orthologs of TRAP150 gene family are present in vertebrates including mammals, birds and fish (Figure 1). The lineage is not seen in invertebrates. One TRAP150-related protein Btf, is predicted to have roles in transcription regulation (Kasof et al., 1999). CXorf23 is a TRAP150 related protein that is uncharacterized. Within the gene family, TRAP150 is more closely related to Btf and its orthologs than to CXorf23.
Figure 1: Treefam phylogeny of TRAP150 gene. The three clusters of genes Btf, TRAP150 and CXorf23 hypothetical proteins are grouped together under one family of genes. Genes from the same species are denoted in the same color, the blue boxes denote nodes of the tree from which a specific gene is branched. The numbers at each node indicate the boot strap value of each clade.
Research from the Luhrman lab suggests that TRAP 150 and Btf might possibly be associated with affinity purified *in vitro*-spliced human mRNPs (Merz et al., 2007). For that study, mRNPs were synthesized *in vitro* under different conditions. For example, the mRNPs were synthesized either in the presence or absence of capping, splicing and polyadenylation and then purified for mass spectrometry analysis (Merz et al., 2007). The results show that TRAP150 and Btf loaded onto mRNPs during pre-mRNA processing. Btf and TRAP150 associate with mRNA in a way that is independent of pre-mRNA splicing events (Merz et al., 2007). While Btf remains as a stable component of affinity purified mRNPs, TRAP150 (and some RNA-binding proteins like YB-1 and NFAR1) dissociated from mRNPs under stringent conditions. One contrast between TRAP150 and Btf is that Btf was not found on purified mRNPs that lack a cap-binding complex (CBC), suggesting that the binding of Btf to mRNPs may be cap-dependent. It is unclear, whether Btf directly interacts with the CBC or the CBC indirectly influences Btf’s association with mRNPs (Merz et al., 2007).

**Transcription factories**

Nuclear compartments are large regions in the nucleus that are functionally distinct from their surroundings. The distribution and spatial organization of transcription complexes correspond to sub nuclear transcription centers or transcription factories (Cook, 1998, Chakalova et al., 2005). To visualize global transcription sites in cells, transcription sites can be labeled when nascent transcripts are extended in the presence of the nucleotide analog bromo-UTP, thus tagging the nascent RNA. Each focus of Br-UTP incorporation seen by microscopy would then represent a transcription
site. Many such nascent RNAs or transcription units are packed into a focus to form a transcription factory. The labeling is not from a single gene locus, but rather from a cluster of genes. In this study, we were interested to know if TRAP150 localization was consistent with localization of transcription sites. RNA polymerase II, transcription factors and splicing factors are also seen in a pattern of dispersed foci all over the nucleoplasm in an actively transcribing nucleus that is similar to the pattern seen by Br-UTP incorporation. However, when transcription is inhibited, the transcription factors and splicing factors accumulate in nuclear speckles. One idea here is that TRAP150 might be associated with transcription factories if its localization resembles that of transcription factors and RNA polymerase II. Determining the localization of TRAP150 with respect to RNA polymerase II or nascent mRNA would help determine the possibility of such a role.

**U2OS 2-6-3 locus:**

Labeling global transcription sites with BrUTP would be expected to show hundreds of sites throughout the nucleus. Co-labeling of TRAP150 would also show hundreds of foci throughout the nucleus. Spatial overlap between the two signals may not give a definitive conclusion whether TRAP150 is at every transcription site. The U2OS 2-6-3 locus is a special gene array that allows gene expression to be observed at three different levels by directly visualizing DNA, RNA and protein in the cell (Janicki et al., 2004). A diagram of the transgene array is shown in Figure 2.
Figure 2: Adapted from Janicki et al. (2004). Schematic representation of the U2OS 2-6-3 transgene array. The plasmid is composed of 256 copies of the lac operator, 96 tetracycline response elements, a CMV promoter, CFP fused to the peroxisomal targeting signal SKL, 24 MS2 translational operators (MS2 repeats), a rabbit β globin intron/exon module, and a cleavage/polyadenylation signal. Expression of CFP-lac repressor allows the DNA to be visualized and expression of pTet-On (rtTA) in the presence of doxycycline (dox) drives expression from the CMV minimal promoter. When MS2-YFP (YFP fused to the MS2 coat protein) dimerizes and interacts with the stem loop structure of the translational operator, it allows the transcribed RNA to be visualized. The labeling of peroxisomes with CFP in the cytoplasm confirm the translation and expression of the reporter protein. The U2OS 2-6-3 transgene array contains approximately 200 copies of this plasmid construct (Janicki et al., 2004).

The U2OS 2-6-3 cell line has ~200 copies of stably integrated transgene plasmid as estimated by Southern blotting (Janicki et al., 2004). Each copy contains a transcription unit that has 256 copies of lac operator, which serves a binding site for CFP lac repressor or YFP lac repressor. This region of the DNA is fluorescently labeled when plasmids encoding these fusion proteins are transfected into the cell line (Janicki et al., 2004). The locus also has 96 tetracycline regulatory elements that allow inducible regulation of transcription. To induce transcription of the array, a fusion of VP16 activation domain and tetracycline controlled transactivation domain (pTetOn) are transfected into the U2OS 2-6-3 cells. Doxycycline (Dox) addition to the culture medium induces binding of pTetON (transcription activator) binds to tetracycline response elements and activates transcription starting from the CMV minimal promoter (Janicki et
al., 2004). Transcription at the locus is switched ON and reporter gene expression takes place in the presence of doxycycline.

The locus can be visualized by fluorescence microscopy because of CFP-LacI that accumulates on lac operator repeats. In the OFF state, the chromatin at the locus appears as highly condensed as a single dot. The condensed locus is heterochromatic, as evident from its association with HP1 and methylated histone 3 at lysine 9 (H3MeK9) (Janicki et al., 2004). Upon induction of transcription, HP1 is removed and exchanged with histone H3.3 variant. The chromatin becomes highly decondensed and U2OS 2-6-3 locus becomes transcriptionally active.

The U2OS 2-6-3 reporter mRNA encodes a peroxisomal targeting signal-1 (Serine-Lysine-Leucine) fused to CFP (CFP-SKL). In the presence of dox, transcription of the reporter RNA begins. Accumulation of the nascent pre-mRNA containing MS2 stem loops can be visualized at the locus when YFP-tagged MS2 binding protein is expressed in the cells (Janicki et al., 2004). The appearance of CFP-SKL protein (that is targeted to peroxisomes) in the cytoplasm shows that all steps of reporter gene expression are complete. The localization of TRAP150 with respect to the U2OS 2-6-3 locus in its ON and OFF state was used to test for a possible role of TRAP150 in gene expression.
II. SPECIFIC AIMS:

The goal of this study is to determine the role of TRAP150 in gene regulation.

**Hypothesis:** TRAP150 is a transcription activator or a splicing factor. It is likely to be shuttling between its site of action (transcription sites) and storage areas (nuclear speckles).

**Specific Aim 1:** To identify potentially functional domains of TRAP150 and Btf. Amino acid sequence alignment of TRAP150 and Btf gives percentage similarity and also highlights conserved regions, which could lead to understanding the functionality of both the proteins.

**Specific Aim 2:** To determine if TRAP150 is associated with transcription.

a) To determine if the localization of TRAP150 is altered after inhibition of transcription. Immunofluorescence labeling of TRAP150 in Hela cells after inhibition of RNA polymerase II by α-amanitin treatment.

b) To determine if TRAP150 and Btf are recruited to transcription sites in vivo.

(i) Localization of TRAP150 and Btf at a specific gene locus

For this aim, I employed a specialized synthetic gene locus called the U2OS 2-6-3 locus. The condensed heterochromatic (or transcriptionally off) locus was compared to a completely decondensed and transcriptionally active gene locus to
determine if TRAP150 or Btf accumulated at the transcription site in either case.

(ii) Localization of TRAP150 and Btf with respect to nascent reporter RNA.

The U2OS 2-6-3 cells were transfected with a plasmid encoding YFP-MS2 to label nascent reporter RNA, and immunofluorescence was performed to label TRAP150 or Btf to determine if these proteins associate with the active gene locus.

**EXPECTED RESULTS:**

For **Specific Aim 1:** The percent identity/similarity is expected to be high at the amino termini because of the presence of the arginine-serine (RS) domain at the N-terminus. If stretches of identical amino acids are seen in both the proteins, perhaps conserved domains perform similar functions and homologs may be functional substitutes for each other. Conserved motifs could also serve as a binding site for some other protein involved in a process e.g. a phosphorylation site that binds to GSK3-β.

For **Specific Aim 2:** Upon transcription inhibition by α-amanitin, nuclear speckles would be enlarged and rounded. This is because all the speckle proteins will accumulate at nuclear speckles since the transcription sites will not actively produce pre-mRNA substrate that normally recruits splicing factors away from nuclear speckles. TRAP150 is expected to distinctly co-localize with the nuclear speckles since it is hypothesized to be a transcription and/or splicing factor. When looking for localization of TRAP150 at transcription sites, it is expected to co-localize with the decondensed U20S 2-6-3 gene locus just like any other transcription factor or pre-mRNA processing factor. It is also
predicted to co-localize with the reporter RNA. TRAP150 is not expected to associate with the transcriptionally inactive gene locus.
III. Methods:

cDNA constructs:

Dr. Robert Roeder, Rockefeller University, kindly provided cDNA of TRAP150. For sub-cloning, oligonucleotide primers were designed to amplify the open reading frame. The 5’ 203StartKpnI primer introduces a KpnI site immediately upstream of the start codon for translation. The 3’ 3070StopBamHI primer introduces a BamHI site immediately downstream of the stop codon. After PCR amplification, the product was sub-cloned into the pEYFP plasmid vector to create the YFP-TRAP150 fusion protein.

Cell Culture:

**Hela cells** were cultured in DMEM (Dulbecco’s Modified Eagle Medium) supplemented with 10% fetal bovine serum (Hyclone) and penicillin-streptomycin. The cultures were washed with 1X Phospahate buffered saline (PBS) trypsinized and seeded onto 100 mm plates with 10 ml of medium. Passage number was maintained.

**U2OS cells** were cultured in DMEM containing 10% Tet System Approved FBS (BD Biosciences Clontech, Palo Alto, CA) and penicillin-streptomycin. The cells were maintained in the same manner as Hela cells.

**Transient Transfections using the calcium phosphate method:**

1x10^5 Hela cells were plated on the previous day in a 35 mm dish in 2 ml of medium. After 24 hours, a mixture of 2M Calcium Chloride, dH2O and 3 µg of plasmid
DNA of interest plus 1.5 µg of carrier pBlueScript was added drop by drop to 1X Hepes buffer saline. The transfection mixture was then added to the cells in drop-wise fashion. 24 hrs post-transfection cells were washed with 1X PBS and fresh medium was added. Cells were fixed after 48 hrs to get maximum transfection efficiency.

**Transient transfection by Electroporation (for U2OS cells):**

Cells that reached ~70-80% confluency in 100 mm plates were trypsinized and centrifuged to pellet the cells. The pellet was resuspended in 600 µl of medium. 200 µl of cells were added to 4 mm gap cuvettes containing 40 µg of sheared salmon sperm DNA, 2 µg of pTetON, and 2 µg of either CFP-lacI or pSV2-YFP-lacI. In some experiments, such as for observing nascent reporter RNA, 0.5 µg of YFP-MS2 binding protein/YFP-TRAP150/YFP-Btf was also added. Electroporation was done using a Gene Pulser II (BioRad, Hercules, CA) (170 V, 950 µF). The electroporated cells were resuspended in 2 ml of medium, transferred to a 15 ml conical tube and centrifuged to pellet the cells. The cells were then resuspended in 5 ml of medium and plated 1 ml per well in a 6-well dish containing sterilized glass cover slips (rinsed with 70% EtOH and flamed). After 6 hrs of incubation at 37°C, doxycycline (dox) was added to the cells in half of the wells to activate transcription of the reporter locus. Immunofluorescence was performed 2h after dox addition.

**Immunofluorescence:**

Cells were rinsed briefly in PBS fixed for 15 min in freshly prepared 2% formaldehyde in PBS (pH 7.4) and washed three times for 5 minutes each in 1X PBS.
Cells were permeabilized in PBS +0.2% Triton X-100 and washed three times for 5 minutes each in 1X PBS+0.5% normal goat serum. Primary antibodies were added for 1 h at room temperature: anti-THRAP3 956A (anti-TRAP150 Bethyl Laboratories, Inc.), (1:2000), anti-SF2/ASF (1:2500). Cells were rinsed in PBS + 0.5% goat serum, then secondary anti-species-specific antibodies were added for 1 h at room temperature: donkey anti–mouse Texas red (1:500), donkey anti-rabbit FITC (1:500).

**Microscopy:**

U2OS cells were examined using a DeltaVision RT Microscope (Applied Precision) using Olympus IX70 inverted microscope, a 60X 1.4 n.a objective and a SONY ICX285 ER Progressive Scan CCD equipped with live cell imaging filter and CFP/YFP/mRFP and EGFP excitation and emission filters. SoftWoRx Explorer Image Viewer software was used to collect digital images. Images were acquired by collecting a stack of 0.28 µm increments in the Z-axis in all the required filters. Such a stack consisted of 4.2 µm-10.08 µm (15-36 sections) total depth and it contained 15-36 sections, depending upon the transcription status and size of the locus. Scrolling through the stack confirms that the co-localization even in the individual images within the stack. Images were deconvolved using 10 iterations of the conservative algorithm based on the point-spread function of the 60X lens. Stacked images were then compressed for display. The images were saved as TIFF images. Individual Z-images were also examined to confirm results obtained from projected images.

**Scoring the association of TRAP150 or Btf with the U2OS 2-6-3 locus:**

Starting from one corner of the cover slip, the cells were scored for having an open versus closed locus and also for the absence or presence of TRAP150 or Btf. The
morphology of locus was observed first under the microscope to determine the status of the locus (closed, partially open, open) by using the filter specific for the lac repressor. Then the localization of TRAP150/Btf was observed with respect to the locus by using the other filter. The cell was then scored accordingly. For example:

| # 45 | Open locus | TRAP150 absent |

**Sequence Alignments:**

Full-length amino acid alignment of TRAP150 and Btf was done using the SIM alignment tool at [www.expasy.org](http://www.expasy.org). The amino acid sequences of TRAP150 and Btf were used to perform the alignment. The resulting alignments can be viewed under LALNView that gives color-coded regions of similarity.
IV. RESULTS:

AIM 1: To identify the potential functional domains of TRAP150.

Proteins with similar amino acid sequences/conserved regions often share similar function. Hence, the higher the similarity in amino acid sequences between 2 proteins, the greater is the probability of their functional similarities. By comparing the protein sequence of newly identified speckle proteins, one can begin to understand the functions of the novel nuclear speckle proteins. In order to find potential functional domains of TRAP150, the amino acid sequence was compared with Btf (nuclear speckle component and a homolog of TRAP150) (Figure 3). Regions containing high sequence similarity suggest conserved domains, which in turn suggest regions of functionality of the protein.

The alignment in Figure 3 shows 39.5% identities, 62% similarities and 5% gaps. Several stretches of identical amino acids are seen at the N-terminus and the C-terminus. High sequence identity (≥50%) is seen at amino acids 1-150 (which is the RS domain for both of the proteins), 40% similarity is seen from amino acids 150-170 (Figure 4). Less similarity was seen in the middle region. 40% similarity was seen from amino acid 550-700, and from amino acid 820-920 there is a similarity range of 50%-80% (Figure 4). In this context, it is clear that TRAP150 and Btf share highest similarity at the N- and C-termini. Both TRAP150 and Btf have a RS domain at the N-terminus, which is a nuclear speckle-targeting signal. High similarity in the C-terminus suggests that this is a second functional domain that is not yet characterized. Motif analysis did not show up any results except for the RS domain and nuclear localization signal.
Figure 3: BCLF-1 is Bcl-2 associated transcription factor and TR150 is Thyroid hormone receptor associated transcription factor (TRAP150). The boxes in red and blue border a GSK3β site and a CHD1 interaction region respectively which are conserved in both the proteins. 39.5% identity in 945 residues overlap; Score: 1147.0; Gap frequency: 5.1%.
Fig 4 A graphical representation of local alignments between TRAP150 and Btf. The similarity scale at the top is the color code of the % similarity in the alignment. The alignment cartoon is obtained through LALN view software in BLAST search.
AIM 2: To determine if TRAP150 is associated with transcription.

Localization of TRAP150

TRAP150 was identified as a novel nuclear speckle protein (Mintz et al., 1999; Saitoh et al., 2004). In order to observe at the localization of TRAP150 with respect to nuclear speckles, dual-labeling immunofluorescence was performed using anti-rabbit anti-TRAP150 956A antibody from Bethyl Laboratories and anti-SF2/ASF (splicing factor2/alternative splicing factor is a bonafide nuclear speckle protein). The results showed that endogenous TRAP150 localizes all over the nucleoplasm in foci or punctate pattern that resembles the localization pattern of transcription sites (Figure 5). In some cases, enrichment of TRAP150 is also seen near the periphery of nuclear speckles. These are also sites that have been previously reported as regions of high transcription activity (Wei et al., 1999; Xing et al., 1993).
In order to further study TRAP150 localization, TRAP150 cDNA was PCR amplified sub-cloned into pEYFP-C1. The sub-cloned DNA was then expressed in HeLa cells and its localization was observed. TRAP150 localization was strictly nuclear. Some co-localization of TRAP150 with splicing factor SF2/ASF was observed in nuclear speckles (Figures 5 and 6). The localization of YFP-TRAP150 and endogenous TRAP150 was observed in order to validate the localization of YFP-TRAP150 with respect to nuclear speckles.

Figure 5. 1 X 10^5 Hela cells were plated in a 6-well dish and processed for IF after 24 hours using rabbit anti-TRAP150/THRAP3 956A polyclonal antibody (Bethyl labs) and mouse anti-SF2/ASF monoclonal antibody (AK103, A. Krainer). TRAP150 showed some overlap with SF2/ASF in nuclear speckles, and showed additional small foci throughout the nucleoplasm. DNA was stained with DAPI. Scale Bar= 5µm.
AIM 2A: To examine if the localization of TRAP150 is altered after inhibition of RNA polymerase II transcription.

Since I hypothesized that TRAP150 may have a role in pre-mRNA transcription, I examined the localization of TRAP150 upon transcription inhibition. Alpha-amanitin arrests transcription of pre-mRNA by inhibiting RNA polymerase II (Kedinger et al., 1970; Lindell et al., 1970). α-amanitin is a cyclic octapeptide that binds with a high affinity to the largest subunit of RNA pol II, RPBI. It blocks the binding among subunits, preventing them from forming the RNA polymerase II complex during the initiation of transcription (Cochet-Meilhac and Chambon, 1974; Lutter, 1982). After treating Hela cells with α-amanitin for 6h, immunofluorescence was performed to label U1-70K, a component of the U1 SnRNP that localizes to nuclear speckles (Figure 7).
Figure 7. 1X 10⁵ Hela cells were seeded onto cover slips for 24 hours, and 50µg/ml α-amanitin was added for 6h prior to processing for immunofluorescence localization of TRAP150 and U1-70K. After transcription inhibition, TRAP150 accumulates in enlarged nuclear speckles. DNA was stained with DAPI. Scale Bar= 5µm.

In amanitin-treated cells, nuclear speckles appear larger and rounder than untreated cells, as indicated by arrows in the insets in Figure 7 E and M. It appears that TRAP150 was targeted to nuclear speckles in the amanitin-treated cells contrary to its punctate or foci-like localization in the nucleoplasm in the untreated cells (shown by arrow-heads in the insets in Figure 7 G and O respectively). Hence, in a transcriptionally active nucleus, most of the components of nuclear speckles including TRAP150 are dynamic and localize throughout the nucleoplasm (Misteli et al., 1994). In transcription inhibited nuclei that are devoid of any splicing factor substrates, pre-mRNA processing factors are devoid of their sites of function, they tend to accumulate at their storage sites.
in the nuclear speckles. Here, TRAP150 exhibited similar enrichment in nuclear speckles as U-170K snRNP upon inhibition of RNA pol II.

**AIM 2B: To determine if TRAP150 and Btf are recruited to transcription sites in vivo.**

Since the previous results are consistent with a possible role for TRAP150 in gene regulation, we employed a more direct approach to study the localization of TRAP150 at a single gene locus. A synthetic gene locus called the 2-6-3 locus stably incorporated in human U2OS cells was used to determine if TRAP150 is present at the inactive and/or the active locus.

A range of morphologies is seen upon induction of transcription of the locus. These morphologies are illustrated in Figure 8. The U2OS 2-6-3 cells were transfected with YFP/CFP-tagged Lac repressor (YFP-LacI or CFP-LacI) and the tetracycline-inducible transcriptional activator pTetON. Doxycycline was added 6h post-transfection to induce transcription at the reporter locus. After treating the cells with doxycycline for 1.5-2h, the cells were fixed and processed for microscopy. The closed locus is highly condensed and heterochromatic (Janicki et al., 2004) and it appears like a single tightly condensed spot in the nucleus as seen in Figure 8A.
The transcriptionally active loci showed a range from partially decondensed (Figure 8B) to completely open and decondensed (Figure 8C). The locus in each individual nucleus is potentially induced or activated at different times following dox addition in the living cells. This is one explanation for why different morphologies of open loci were observed in fixed cells. The partially open morphology is likely a transitional state that might be either a transcriptionally active locus beginning to decondense or an open locus beginning to condense. Because it takes 1.5-2 hrs for this reporter locus to completely to decondense, the second possibility is unlikely only 2 hrs after inducing transcription (Janicki et al., 2004).
AIM 2B-i: TRAP150 and Btf at U2OS 2-6-3 gene locus:

Specific aim 2B of this project was to examine if TRAP150 and Btf localizes at the transcriptionally inactive or activated gene locus. U2OS 2-6-3 cells were transfected with YFP-LacI along with pTet-ON. Doxycycline was added to half of the coverslips 6h after transfection and cells were processed for microscopy 2h after dox addition. In the nuclei of cells treated with doxycycline, TRAP150 was enriched at the completely decondensed locus, where it distinctly localized at the vicinity of the locus as shown in Figure 9C.

Figure 9: U2OS 2-6-3 cells were transiently transfected with pSV2-YFP-lac repressor and pTet-ON (rtTA). Doxycycline was added 6h post transfection. Cells were fixed 2h after dox addition and immunolabeled for TRAP150. Panels A-D show dox-induced cells with an activated locus. Panels I-L show the inactive locus. In panel B, the locus is decondensed and open in comparison to panel J where the locus is closed (arrowheads indicate the position of the locus in B and J). Panels A and I show endogenous TRAP150. The overlay images in C and K show the localization of TRAP150 with respect to the lac repressor that labels the DNA at the locus. TRAP150 is enriched at the decondensed
active locus (panel C, arrow), but is not enriched at the condensed inactive locus (panel K, arrow). Panels (E-H) and (M-P) are enlarged insets to panels (A-D) and (I-L), respectively. DNA was stained with DAPI. Scale Bar = 5µm.

In contrast, at the heterochromatic locus (i.e., nuclei transfected with pTetON and YFP-lacI but without dox addition), no such enrichment of TRAP150 was seen (Figure 9K). Similar results were seen with TRAP150 homolog Btf. In the nuclei of cells treated with doxycycline, Btf was localized at the vicinity of the locus (Figure 10C), while Btf enrichment was not seen at the transcriptionally inactive locus (Figure 10K).

Figure 10: U2OS 2-6-3 cells were transiently transfected with pSV2-YFP-lacI, and pTet-ON (rtTA). Doxycycline was added 6h post transfection. Cells were fixed 2h after Dox addition and Btf was immuno-labeled with rabbit anti-Btf WU10 polyclonal antibody. Arrowheads indicate the position of the locus in panels B and J. Panels A-D show Btf enrichment at the activated locus, while panels I-L do not show enrichment of Btf at the inactive locus (arrow in I). E-H and M-P are insets of A-D and I-L respectively. DNA is stained with DAPI. Scale Bar = 5µm.
Mammalian gene regulation is controlled by regulatory mechanisms that modulate chromatin structure. The first level of chromatin condensation looks like beads on a string, which consists of DNA wound around histone octamers into the basic unit of chromatin called a nucleosome. There are eight histone proteins - two molecules each of H2A, H2B, H3 and H4 (Alberts et al., 2002). Two H2A-H2B dimers form a tetramer, which bind with a H3-H4 tetramer to form a core histone octamer (Alberts et al., 2002). The DNA wraps 1.65 times around the histone core to form nucleosomes that are separated by a short stretch of linker DNA. Formation of nucleosomes reduces the length of the DNA to 1/3 of its original length. Nucleosomes are further packed into a 30-nm fiber as histone NH$_2$-terminal tails interact with neighboring nucleosomes. Regulation of these interactions by chromatin remodeling enzyme complexes controls the accessibility of promoter and enhancer elements, and ultimately the activity of genes.

The U2OS 2-6-3 transgene array is stably integrated into a single region on chromosome 1. The large number of plasmid copies in the array results in makes it a huge gene locus. This transgene array is heterochromatic during its inactive state (OFF), when it exhibits higher order compaction and is inaccessible to the transcription machinery. The inactive locus is associated with histone methyl transferases and heterochromatin protein 1 (HP1) (Janicki et al., 2004). The methylation of lysine 9 on histone H3 creates a binding site for the chromodomain of HP1 hence silencing the gene (Jacobs and Khorasanizadeh, 2002). Observations of the inactive locus show that it appears as a single tight spot in the nucleus. When the 2-6-3 gene locus is turned ON upon transcription activation the methylated Histone 3 is exchanged with H3.3 variant. This exchange removes methylated histone H3, replacing it with H3.3 whose tail is not
methylated. This first step in locus activation decondenses the chromatin for it to be accessible for transcription machinery (Ahmad and Henikoff, 2002). The transcription activator (rtTA) binds to the tetracycline response elements (upon addition of doxycycline) and recruits gene expression machinery to the promoter region. When the locus is turned on completely, it has a large and irregular morphology with many lobes. Because not all copies of the transgene are simultaneously activated, there is usually a delay in the total activation of the locus. Some copies of the transgene persist in the inactive state hence, the intermediate morphologies of the locus that had only 2-3 lobes scored as partially open were observed. When transcription is activated and the locus is decondensed (either completely or partially), TRAP150 was frequently seen in the vicinity of the locus.

In order to confirm that TRAP150 is recruited to the transcriptionally active reporter gene locus, it was imperative to score a significant number of cells with enrichment of TRAP150 at the partially open or open locus. Also, to conclude that TRAP150 is recruited to the transcriptionally active reporter gene locus, it should be absent from the inactive locus. Cells were scored according to the status of the locus (active versus inactive) and the association with TRAP150 or Btf. Results are shown in Tables 1 and 2. For scoring, TRAP150 association with the U2OS 2-6-3 locus, +Dox/+pTet-ON (active) and -Dox/-pTet-ON (inactive) were scored for TRAP150 and Btf in separate experiments.

In a +Dox experiment, to examine TRAP150 association, a total of 87 cells were scored. Of these 15 cells had a completely open locus and 72 had a partially open locus (Table 1B). Out of the 15 nuclei with completely open/decondensed loci, 11 (73.3%) of
them had TRAP150 enrichment and 4 did not have TRAP150 enrichment. Out of 72 nuclei with partially open loci, 45 (62.5%) of these loci had TRAP150 enrichment while 27 nuclei (37.5%) of the loci had no TRAP150 association. In a –Dox experiment to examine TRAP150 association, 98 cells were scored. 30 of these loci were closed and 68 were partially open. Only 6.6% of these 30 closed loci had TRAP150 association, while 93.4% did not show TRAP150 association.

A

<table>
<thead>
<tr>
<th>Condition of the experiment</th>
<th>Status of the Locus</th>
<th>Number of cells</th>
<th>% of loci associated with TRAP150</th>
<th>% of loci with TRAP150 absent</th>
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<tr>
<td>+DOX</td>
<td>Open</td>
<td>87</td>
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<td>35.6%</td>
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<td>Closed</td>
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<td>6.6%</td>
<td>93.4%</td>
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B

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<tr>
<th>Condition of the experiment</th>
<th>Status of Locus</th>
<th>Number of cells/Number of cells with TRAP150 enrichment</th>
<th>% Recruitment</th>
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<td>15/11</td>
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<tr>
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<td>Partially Open</td>
<td>72/45</td>
<td>62.5%</td>
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<tr>
<td>-DOX</td>
<td>Close</td>
<td>30/2</td>
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<td></td>
<td>Partially Open</td>
<td>68/18</td>
<td>26.6%</td>
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</tbody>
</table>

Table 1: TRAP150 at the reporter locus. A. Comparison between +Dox and -DOX experiments regarding loci association with TRAP150. The number of cells scored with such association is shown for both cases. The percentages of the presence or absence of TRAP150 for each condition are also given. B. Further analysis of Table A. In a +Dox experiment, the numbers for an open locus containing cells is again divided into partially open and open loci. Also, for –Dox experiment, the types of loci observed were close and partially open. The number of cells scored and the percentages given in the Table 1.A are classified accordingly in Table 1.B.

TRAP150 was enriched on 26.6% of the 68 partially open loci. Comprehensively, 64.36% of nuclei with an open locus in a +Dox experiment have TRAP150 enrichment at
the locus, while 6.6% of nuclei in a –Dox experiment had TRAP150 association with a closed locus (Table 1A). While 35.6% of open loci containing nuclei in a +Dox experiment did not have TRAP150 at the vicinity of the locus, 93.4% of the nuclei with closed loci in a –Dox experiment were without TRAP150 enrichment. In an ideal experiment, when transcription is activated, the gene locus is decondensed and acquires an open conformation. If TRAP150 is predicted to be a transcription activator or a pre-mRNA processing factor, it would be expected to always co-localize at the gene locus. If TRAP150 is not involved in transcription/pre-mRNA processing, there would be no such enrichment. If TRAP150 is associated with transcription repression, it would be expected to co-localize with an OFF/condensed locus. Our experiment showed that there was a higher percentage of cells with TRAP150 enrichment to open loci than cells with closed loci. However, 35.6% of the cells with open loci were not seen with TRAP150 association. The reason could be that most of the cells counted had partially open conformation (Table 1B), suggesting that some of the (45 out of 72) partially open loci might not have been activated enough to associate with TRAP150 at the time of fixation. A higher percentage of nuclei with completely open loci associated with TRAP150 (11 out of 15) also reinforces the idea. If TRAP150 is predicted to be a transcription repressor, a high percentage of cells would have had TRAP150 enrichment at an OFF/inactive/closed locus. However, the finding that only 6.6% of closed loci had TRAP150 association would suggest that TRAP150 does not associate with inactive genes. While the locus should be completely turned off when its activity has not been intentionally induced by addition of Dox to the culture medium. The relatively small number of closed loci (6.6%) that showed TRAP150 association could be explained by
leaky transcription from the inducible promoter. I observed a significant difference in the percentage of partially open loci in a –DOX experiment versus a +Dox experiment with regard to TRAP150 association 62.5% of loci in a +DOX experiment had TRAP150 association with partially open loci, versus only 26.6% of nuclei in a –DOX experiment. Once again this suggests a difference in the extent of activation of the locus at the time of fixation, or could more importantly reflect that the intentionally activated loci were in some way more competent for recruiting gene expression machinery.

A

<table>
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<tr>
<th>Condition of the experiment</th>
<th>Status of the Locus</th>
<th>Number of cells</th>
<th>% of loci associated with Btf</th>
<th>% of loci with Btf absent</th>
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<td>-DOX</td>
<td>Close</td>
<td>72</td>
<td>7%</td>
<td>93%</td>
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</table>

B

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<tr>
<th>Condition of the experiment</th>
<th>Status of Locus</th>
<th>Number of cells/Number of cells with Btf enrichment</th>
<th>% Recruitment</th>
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<td>67%</td>
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<td>60%</td>
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<tr>
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<td>Close</td>
<td>72/5</td>
<td>7%</td>
</tr>
<tr>
<td></td>
<td>Partially Open</td>
<td>28/10</td>
<td>36%</td>
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</table>

Table 2: Btf at the reporter locus. A. Comparison of loci’s association with Btf between +Dox and –Dox experiments. The number of cells scored with such association is shown for both cases. The percentages of the presence or absence of Btf for each condition are also given. B. Further analysis of Table A. In a +Dox experiment, the numbers for an open locus containing cells is again divided into partially open and open loci. Likewise, for –Dox experiment, the types of loci observed were close and partially open. Accordingly, percentages of cells with Btf enrichment at each of the conditions of the loci in both +/-Dox experiments were noted.
Cell counts for the absence/presence of Btf were done in order to examine Btf association with the U2OS 2-6-3 locus. In a +Dox experiment, 67% of the nuclei scored showed Btf association with the open locus, while in a –Dox experiment, only 7% of the nuclei showed Btf recruitment at the closed locus. Also, in a –Dox experiment, Btf was absent from 93% of the closed loci while it was absent from 39% of the open/partially open loci. Ideally, if Btf functions as a transcription activator/pre-mRNA processing factor, we would expect it to always co-localize with the open/ON/decondensed locus. Our result showed 61.1% of open loci (scored in 90 nuclei) with Btf association. Of those 90 nuclei with open loci, most of them (72) were partially open. Considering the fact that partially open loci are not completely decondensed, the probability of transcription factors/pre-mRNA processing factors accessing the DNA is less. Hence, this may explain why fewer open loci than expected with Btf. 7% of nuclei in a –Dox experiment showed Btf association suggesting that there is possibly a leakiness in the system. Partially open loci are generally not expected in a –Dox experiment yet they were observed. The reason could be once again basal activity that could have decondensed the loci partially.

While 60% of nuclei in a +Dox experiment with partially open loci had Btf association, 36% of the nuclei in a –Dox experiment with partially open loci had Btf association. This suggests once again that the partially open loci that had intentionally been activated are somehow more competent for gene activation.
AIM 2B-ii: TRAP150 and Btf at RNA:

Next, the localization of TRAP150 and Btf with respect to U2OS 2-6-3 reporter RNA was examined. U2OS 2-6-3 cells were transfected with plasmids encoding YFP-MS2 and pTet-ON (rtTA). YFP-MS2 binding protein reporter labels the RNA upon induction of transcription by doxycycline. YFP-MS2 binding protein has a diffuse pattern all over the nucleoplasm in cells with inactive loci, but it accumulates at the activated RNA of the 2-6-3 reporter gene locus. No such accumulation of YFP-MS2 at the reporter locus was seen in a –Dox experiment (data not shown), and was not expected since transcription was not induced. The localization of TRAP150, Btf and SF2/ASF (a splicing factor) with respect to the reporter RNA was the objective of this sub-aim. SF2/ASF is an essential splicing factor that associates with nascent pre-mRNA and it was used as a positive control for these experiments. We would expect to see SF2/ASF exactly colocalized with nascent reporter RNA on the activated reporter locus.
Figure 11. U2OS cells were transiently transfected with pTetON (rtTa), YFP- or Cherry-MS2 binding protein. Doxycycline was added 6 hours post transfection and the cells were processed 2 hours later for immunofluorescence localization of A) Btf, B) TRAP150 and C) SF2/ASF. While all three proteins are recruited to the immediate vicinity of the RNA, there is only partial overlap between these proteins and the MS2-binding protein. DNA was stained with DAPI. Scale bar = 5µm.
Both Btf and TRAP150 accumulated at nascent reporter RNA as detected via binding by YFP-MS2 as shown in Figure 11 A and B. The arrows indicate Btf (part A) and TRAP150 (part B) that accumulate near nascent reporter RNA (arrowheads) labeled with YFP-MS2 binding protein. Co-localization between SF2/ASF and RNA was done by transfecting the U2OS 2-6-3 cells with YFP-SF2 and mCherryMS2 binding protein (Figure 11C). YFP-SF2 (Figure 11 J, arrowhead) co-localized with transcribing RNA (Figure 11 I, arrows). Interestingly, all of these proteins (SF2/ASF, Btf, TRAP150) were localized in the vicinity of the reporter RNA rather than precisely by which deviated from the expected result.

In summary, TRAP150 and Btf show a similar kind of co-localization with the reporter RNA as SF2/ASF. However, we cannot definitively say that there is a physical association between these proteins and the reporter RNA based on this result. The result can only suggest that there might be a physical interaction between the RNA and the proteins. Also, from the co-localization experiments it is difficult to track at which step of gene regulation these TRAP150 and Btf have functions. Transcription and pre-mRNA processing are parallel processes so it is difficult to identify and distinguish their factors in a co-localization assay without performing a time course experiment.
V. DISCUSSION

Thyroid hormone receptor associated proteins are generally recognized as co-activators of transcription (Ito et al., 1999). While TRAP150 was placed into this group, it has not been confirmed that TRAP150 participates in TR-mediated transcription co-activation. TRAP150 was also identified as a component of nuclear speckles, which are storage sites for pre-mRNA processing factors (Mintz et al., 1999; Saitoh et al., 2004). In addition TRAP150 was associated with in-vitro spliced affinity purified mRNPs (Merz et al., 2007). The above stated observations led to the hypothesis that TRAP150 might have a role in transcription and/or pre-mRNA processing.

Phylogenetics of TRAP150

A first step in understanding TRAP150 functions was to determine if TRAP150 has any homology with other known proteins. Phylogenetic study showed that TRAP150 has orthologs in vertebrates, but that TRAP150 is not found in invertebrates. Btf and CXorf23 are the only TRAP150-related proteins found in humans. There are no other proteins found similar to TRAP150 in humans, Btf and CXorf23 might have a crucial role in synergistic or antagonistic activities functions performed by TRAP150. While Btf is more similar to TRAP150 (it has 39.5% identity and 62% similarity with TRAP150), some similarity among TRAP150, Btf and CXorf23 (33% similarity at the C-terminal region of both the proteins) suggests possible overlapping functions. Interestingly, Btf and TRAP150 have a serine and arginine rich (RS) domain at the N-terminus that is absent in CXorf23. Because the RS domain is a speckle targeting sequence, CXorf23 may not be targeted to nuclear speckles, or it may have more important functions in lower
vertebrates that do not have distinctly organized nuclear speckles. Also, the C-terminal region of Btf is highly homologous to TRAP150, suggesting an important conserved function for the C-terminus of these proteins.

**Localization pattern for TRAP150 changes upon transcription inhibition:**

Endogenous TRAP150 localizes in a punctate pattern all over the nucleoplasm, including some localization around or near nuclear speckles, forming a punctate pattern that resembles transcription factors and RNA polymerase II. When transcription is inhibited, not only do the speckles look larger than in normal conditions, but TRAP150 also becomes enriched in nuclear speckles. According to Zeng et al. (1997), the transcription factors and all pre-mRNA processing factors reorganize within the nucleus when transcription is not taking place and accumulate at their storage sites in nuclear speckles. In this context, since TRAP150 also behaves in the same fashion upon transcription inhibition, this suggests that TRAP150 might have a role in transcription and/or pre-mRNA processing.

**TRAP150 and Btf at an actively transcribing gene locus:**

Initially Br-UTP incorporation experiments were attempted to examine co-localization of TRAP150 and global transcription sites. However, abundance of nascent RNA foci and limits of spatial resolution made analysis impractical. We therefore employed a transcriptionally-inducible gene locus for our studies. The U2OS 2-6-3 system allowed us to study the localization of TRAP150 and Btf at a single gene locus in both the inactive and active sites. Initial observations of the locus in fixed cells reflected
the dynamics that would be expected on the locus in living cells. There was a range of active states for the locus as reflected by changes in morphology of the chromatin as the locus switches from inactive to active status. We classified the locus morphology into 3 types: closed, partially open and open. The heterochromatic state of the locus (i.e. highly condensed/closed morphology) was observed as expected according to Janicki et al. (2004) when transcription was not induced. TRAP150 and Btf did not accumulate at the closed locus except in a few cases. TRAP150 and Btf were localized at the vicinity of the partially open locus as well as the open locus (i.e. completely decondensed loci), where they were seen in close proximity with the activated locus. In a previous study, the heterochromatic condensed U2OS 2-6-3 locus was measured at ~0.89±0.12µm in diameter while the transcriptionally activated decondensed locus was measured at ~2.0-4.5µm in diameter (Janicki et al., 2004). The average distance observed between the lac operator repeats of the locus and foci of accumulated TRAP150 in this study was 0.3 µm, while the average distance was 0.4 µm for Btf. The completely decondensed open locus did not exhibit 200 distinct foci, corresponding to the roughly 200 copies of reporter plasmid integrated in the array. This suggests that the individual transcription units within array do not decondense completely at one single time point, or that there is higher-order structure at the active locus that cannot be resolved by our microscopy methods. My experiment was conducted at a single time point, at which time a part of the locus might remain condensed. There remains a possibility that TRAP150 and Btf localize on only the part of the locus that has been activated. Most significantly, the data show that TRAP150 and Btf were associated with a higher percentage of transcriptionally active reporter gene loci than with inactive reporter loci. The data is consistent with the possibility that
TRAP150 and Btf are recruited to the transcriptionally active locus during the activation process. As such, TRAP150 and Btf could have either transcription activation or pre-mRNA processing functions as hypothesized. Alternatively, they could have a role in coupling transcription to downstream events such as mRNA export and mRNA stability (Bracken et al., 2008).

**TRAP150 and Btf colocalization with pre-mRNA:**

Pre-mRNA processing factors are recruited to nascent pre-mRNA during the activation process (Misteli et al., 2007). Precise overlap of TRAP150 and Btf with nascent pre-mRNA would support pre-mRNA processing functions for these proteins. YFP-MS2 binding protein was expressed in the U2OS 2-6-3 cells to label reporter RNA, then TRAP150 and Btf were observed for co-localization with the RNA. The localization of TRAP150 and Btf with respect to RNA was compared to co-localization of a known splicing factor SF2/ASF with RNA. All three proteins showed accumulation at reporter RNA. These studies showed extensive accumulation of these proteins at the vicinity of the reporter RNA. The localization of TRAP150 and the RNA, Btf and the RNA and SF2 and the RNA all showed the same patterns. This result is consistent with the findings of Merz et al., 2007 that both TRAP150 and Btf associate with mRNPs. It is also consistent with the possibility that TRAP150 and Btf have a role in pre-mRNA processing. However, further experiments need to be conducted to identify the exact step (e.g. capping, splicing, polyadenylation) where TRAP150 and Btf perform these functions.
Conclusions:

- TRAP150 localizes in speckles and also all over the nucleus in a punctate pattern resembling the localization of transcription sites.

- TRAP150 and Btf share 39% sequence identity and 66% sequence similarity, suggesting they may have related roles in transcription and/or splicing. This conclusion is supported by the similar localization patterns of TRAP150 and Btf in the nucleus and also by their similar recruitment to an activated reporter gene locus.

- TRAP150 and Btf are recruited to the vicinity of the active U2OS 2-6-3 reporter gene locus and the transcribed reporter RNA.

Future Directions:

- To see if TRAP150 and Btf show co-localization with transcriptional activators on the U2OS 2-6-3 reporter locus. This can be done by expressing a flagged pTetON transcriptional activator in the U2OS 2-6-3 cells and observing co-localization with TRAP150 and Btf.

- To study the kinetics of TRAP150 and Btf recruitment to the U2OS 2-6-3 reporter locus. So far, we were able to see the localization of TRAP150 and Btf at the gene locus and RNA. In order to confirm the presence of these proteins during transcription and pre-mRNA processing it is imperative to study the kinetics of TRAP150/Btf at the gene locus. Fluorescence Resonance Energy Transfer (FRET)
analysis of TRAP150/Btf and CFP-lacI of the gene locus would give a quantitative result of any interaction between the gene locus and TRAP150/Btf. Live cells microscopy would also be a useful tool to study the kinetics.

- To map the functional domains of TRAP150 and Btf for pre-mRNA processing and/or transcription. Deletion mutants of both the proteins would be helpful to see which regions are responsible for their functions.

- Btf was found to have an interaction with a chromatin remodeling protein CHD1 (Tai et al., 2003). CHD1 interacts with histone deacetylases (HDACs) and controls gene expression. It was also found out that CHD1 interacts with pre-mRNA processing proteins like SRrp20, SAF-B, p54 and modulates splicing. Tai et al., (2003) showed that CHD1 has an interaction with Btf. Since TRAP150 is homologous to Btf, the future direction would be to test for the interaction of TRAP150 with CHDI which suggests a role for pre-mRNA splicing.
VI. REFERENCES:


