# COFACTORS OF THE p65-MEDIATOR COMPLEX

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## Abstract

Regulation of gene expression is an essential process in all organisms. Research within the last decade has elucidated the central importance of the Mediator complex in the fundamentals of gene expression. Mediator has been shown to adopt various structural changes when bound to different transcriptional activators. As a part of the NF-κB family of proteins, p65 (RelA) is a diverse transcriptional activator highly involved in inflammation and immune response as well as other essential signaling pathways. Mediator and p65 have been shown to interact through the activation domain of p65. To further explore this interaction, the p65-Mediator complex was purified in vitro and analyzed by mass spectrometry to identify cofactors of the complex. Evidence is provided for the identification of both novel and previously described interactions.

# Introduction

Protein expression requires two distinct processes, transcription and translation. Transcription is the process of synthesizing RNA (ribonucleic acid) from a DNA (deoxyribonucleic acid) template, the genetic material of life. These RNA products are translated into a polypeptide chain.

There are three major types of RNA that are produced in cells: transfer RNA (tRNA), messenger RNA (mRNA), and ribosomal RNA (rRNA), which all work together to carry out the essential process of protein synthesis. My thesis work focused on the machinery involved in the production of mRNA, which is the form translated into polypeptides.

A sequence of mRNA encodes for a defined, unique sequence of amino acids called a polypeptide. Polypeptides are folded into distinct shapes and serve various physiological roles. The synthesis of the polypeptide (translation) occurs at the ribosome, which incorporates the individual amino acids into the polypeptide chain. But before any translation can occur, transcription of the target gene must first take place, resulting in the synthesis of the corresponding mRNA.

Specialization is extremely important for both differentiated and undifferentiated cells, and this specialization is accomplished by alterations in gene expression. For example, heart cells and brain cells differ in the proteins that they express, and this difference stems from a difference in the genes that are transcribed. For a certain mRNA to be produced, there are various steps that must occur. Within the nucleus, DNA is packaged by histones into a dynamic higher order structure called chromatin. Rather than simply being a long strand, chromatin is a tightly packed form of DNA. This structure inhibits the ability of the necessary components of transcription to access the gene of interest. This structure must therefore be modified in specific ways in order to make parts of the genome accessible (Li and Reinberg 2011; Voet and Voet, 2004).

Once the proper genetic sequence is made available by an activated DNA binding transcription factor (activator), the transcription machinery must assemble at the promoter (area just upstream of the

transcription start site) specific to the gene of interest. The transcriptional machinery is made up of RNA Polymerase II (RNAP II) and other proteins called transcription factors, of which there are three types. General transcription factors are necessary for all forms of transcription. They function by assembling at the gene promoter and serve to recruit RNAP II to the site of initiation, as well as a variety of other functions. Upstream transcription factors do not assemble at the core promoter, but instead bind to upstream DNA sequences (enhancers) and function to either activate or repress gene expression. Inducible transcription factors are analogous to upstream transcription factors except that they are under direct control of other systems (signaling pathways), either functioning to activate or repress transcriptional activity. (Voet and Voet, 2004)

The assembly of all of the necessary factors is termed the pre-initiation complex (PIC). At this point, initiation can take place and RNAP II will be released from the core promoter and travel along the DNA. As it travels, RNAP II incorporates the appropriate bases and a new mRNA strand is synthesized. It should be noted that further processing is necessary before this strand becomes mature mRNA which is capable of being translated by the ribosome.

## The Mediator Complex

Mediator is a human transcriptional coactivator that is essential for activated gene transcription. It is a 26-subunit complex that is 1.2 MDa in size. It has a very large surface area for protein-protein interactions yet has few predicted structural domains based on amino acid sequence. Mediator is thought to work by acting as an interface between DNA-bound transcription factors and the core promoter (Figure 1). Activator binding causes structural shifts within Mediator, thereby regulating its activity (Taatjes et al., 2002). This concept has been demonstrated with SREBP-1a and VP16-Mediator as shown in Figure 2. When activator-Mediator complexes were compared with the activator-free state, unique cofactors were identified that were specific to the activator-bound form of Mediator (Ebmeier and Taatjes, 2010).

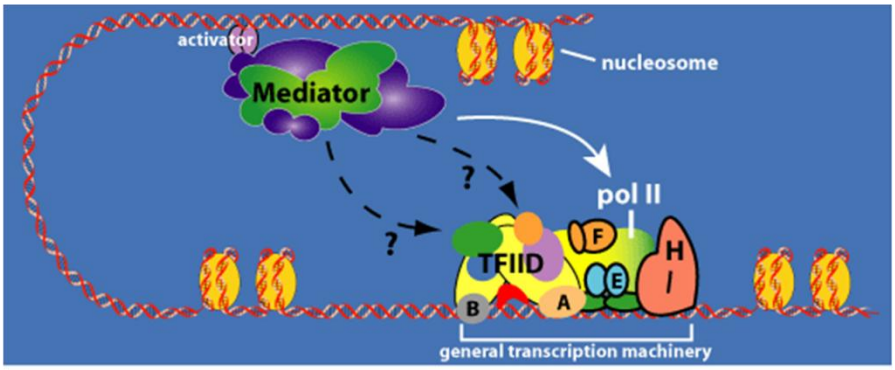


**Figure 1.** Mediator acts as an interface between activators and the general transcription machinery (adapted from http://www.colorado.edu/chemistry/taatjeslab/medpic.html).

In addition, Mediator has been shown to have activator-independent functions, as seen by basal levels of transcription (no activator present). It has been shown to be involved in RNAP II recruitment yet may also function in post-recruitment regulation of RNAP II (Knuesel and Taatjes, 2011). There is also sufficient evidence to suggest that Mediator should in fact be considered a general transcription factor due to its importance in transcription as a whole. Mediator is thought to function at all RNAP II promoters (Taatjes, 2010; Kornberg, 2005).

Understanding transcriptional regulation and gene expression in metazoans is very difficult in comparison to simple single-cell organisms (e.g. yeast) because of the greater complexity that each step incorporates. Despite these difficulties, recent advances in the bioscience fields have allowed further exploration. It is increasingly important to study various processes in the context of eukaryotes, especially because humans are eukaryotic organisms. Therefore research done in this context will be able to more directly relate to human health issues (e.g. cancer).

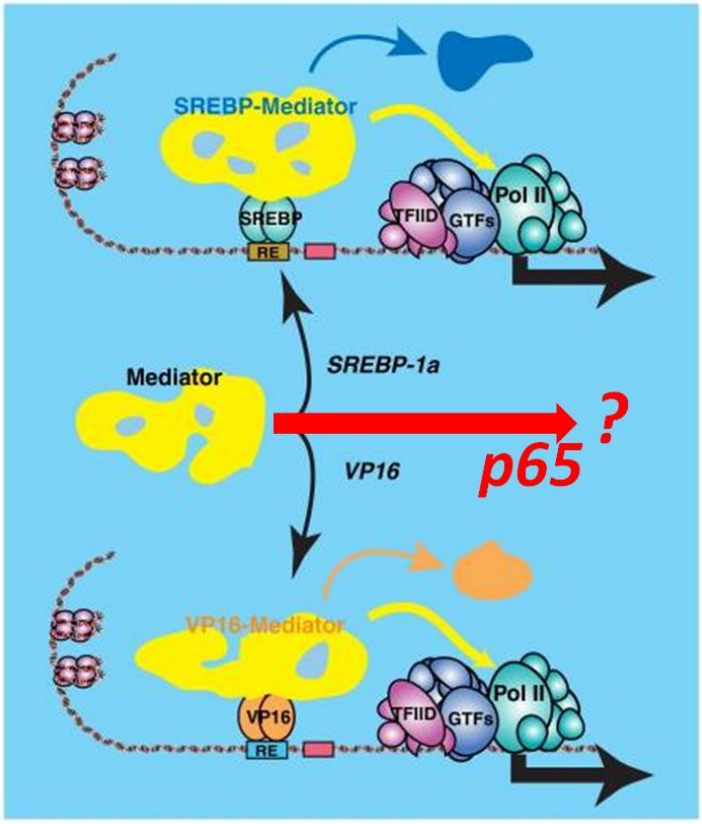


**Figure 2.** Different activators such as SREBP and VP16 cause different structural changes in the Mediator complex that act to regulate gene expression (Ebmeier and Taatjes, 2010).

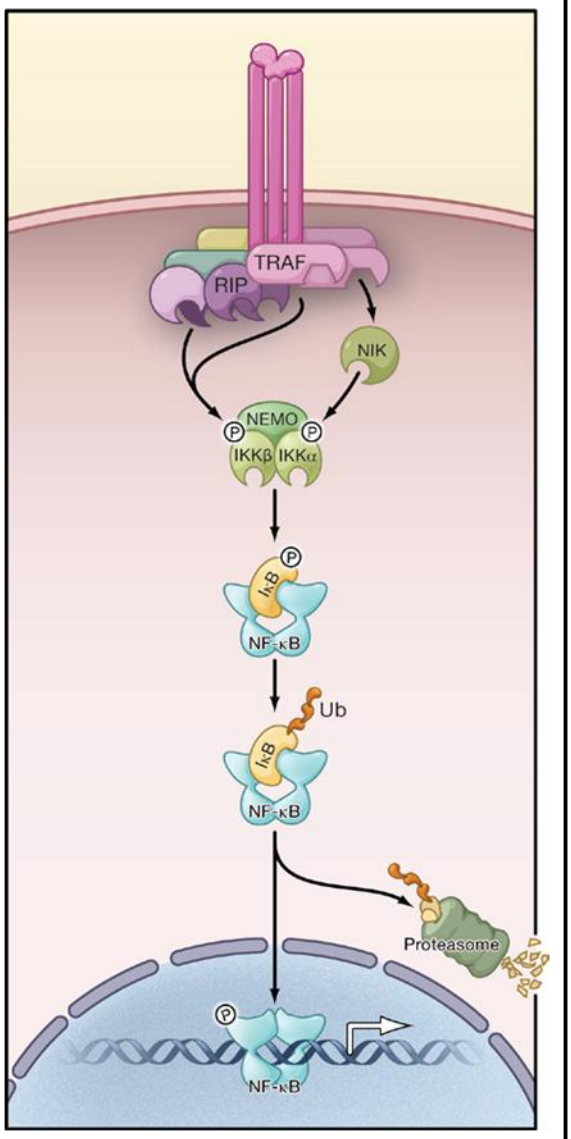


**Figure 3.** NF-kB signaling pathway (Hayden and Ghosh, 2008).

## The NF-κB Transcription Factor

The Nuclear Factor-kB (NF-κB) family of proteins are small signaling molecules that act as inducible transcription factors in mammalian cells. NF-κB family members are highly involved in the regulation of inflammation and immune response; however they are also important for regulation of genes that control cell growth and proliferation. Dysregulation of NF-κB has been shown to have major consequences in health issues such as chronic inflammation, immunodeficiency, and cancer (Hayden and Ghosh, 2008; Courtois and Gilmore, 2006).

One of the NF-κB proteins is p65. To form an active transcriptional complex, p65 forms a heterodimer with p50, another NF-κB family member. When inactive, the p65/p50 dimer is found in the cytoplasm of the cell bound to IκB. IκB inhibits the transport of the p65/p50 complex to the nucleus by binding in such a way that the nuclear localization signal (NLS) of p65 is no longer exposed. For activation to occur, IκB must be phosphorylated by IκB Kinase (IKK), which causes release of the p65/p50 complex and subsequent degradation of IκB through Ubiquitin-dependent proteolysis. The NLS is thereby exposed and the dimer translocates to the nucleus where it binds specific DNA

elements, recruits coactivators, and displaces repressors in order to activate transcription (Hayden and Ghosh, 2008). The NF-κB signaling pathway is shown in Figure 3.

With regard to the regulation of gene expression by the p65/p50 heterodimer, the transcription activation domain (TAD) of p65 is of the most direct importance, demonstrated by its ability to restore transcription activity of the full-length protein (O'Shea and Perkins, 2008). The Rel homology domain (RHD) is found in all NF-κB proteins and is responsible for DNA binding as well as dimerization (Hayden and Ghosh, 2008). As seen in Figure 4, the activation domain (TAD) is located between residues 428 and 551 (O'Shea and Perkins, 2008).


**Figure 4.** Schematic of p65 domains. The transcription activation domain (TAD) is made up of TA1 and TA2. The complete domain is found between residues 428 and 551 (O'Shea and Perkins, 2008).

## Hypothesis

Mediator is known to form a complex with p65 during transcriptional activation (Naar et al., 1999). Drawing from the data from other activator-Mediator complexes (as discussed above), we hypothesize that there are p65-specific cofactors that are bound to Mediator for regulation of activated transcription (Figure 2). The goal of this work is to isolate the p65-Mediator complex and identify associated cofactors. Comparison of p65-associated factors with other transcription factors should help identify key proteins that control expression of NF-κB target genes. These potential "p65-specific" cofactors must then be further validated so that conclusions and further hypotheses can be formed.

## Results

The hypothesis and direction for this project came out of two main ideas. First, p65 has been shown to interact with Mediator (Naar et al., 1999; O'Shea and Perkins, 2008). Secondly, previous data from our lab has shown that Mediator undergoes structural shifts in its activator bound state that are activator specific. Furthermore, these states have also been shown to recruit unique sets of cofactors (Ebmeier and Taatjes, 2010). To address this hypothesis the p65-Mediator complex would need to be purified with its associated cofactors.

Because the activation domain of p65 is responsible activating transcription of target genes, it is used here to evaluate the p65-Mediator complex. Its small size (~120 amino acids) allows for relatively straightforward recombinant expression in E. coli. It is clear from previous data (not shown here) that a

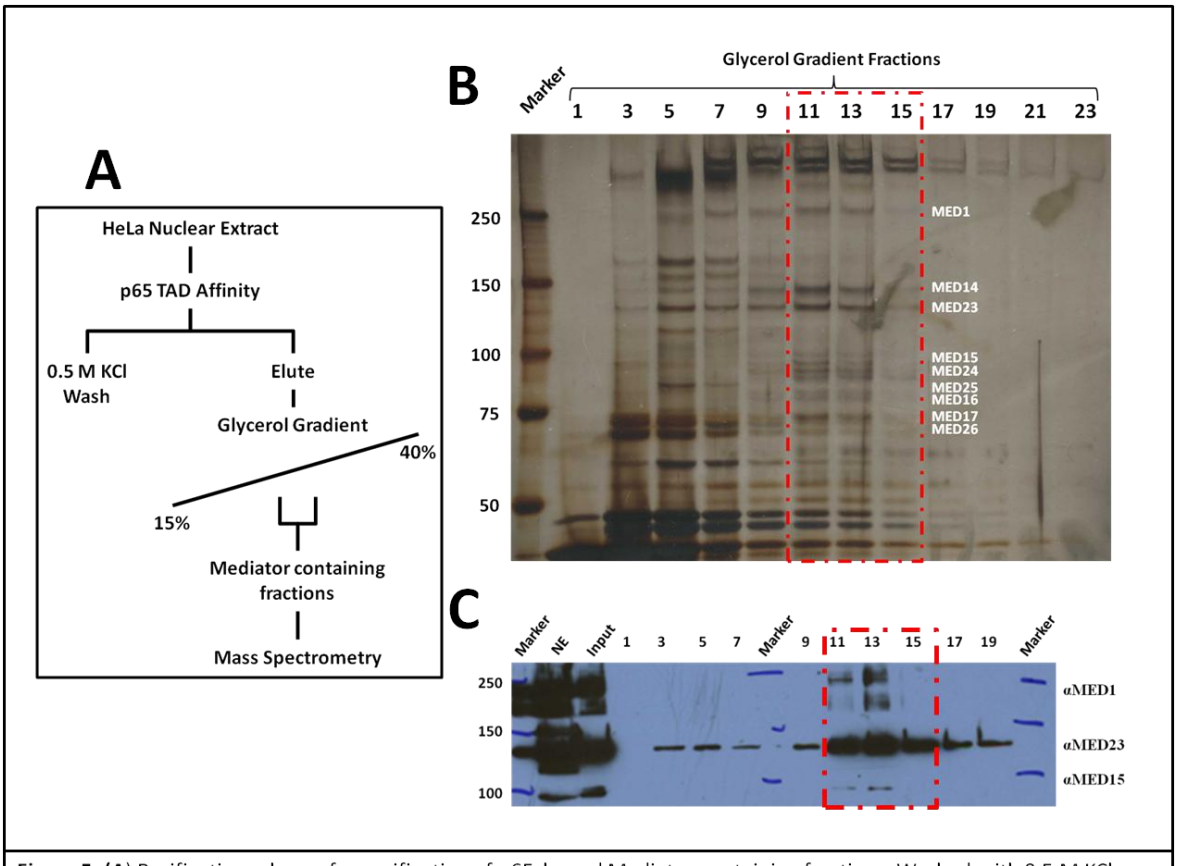


**Figure 5. (A)** Purification scheme for purification of p65-bound Mediator containing fractions. Washed with 0.5 M KCl after Nuclear Extract pull-down with immobilized GST-p65 residues 434-551. Eluate put over 15-40% glycerol gradient and Mediator containing fractions were identified by silver stain **(B)**. **(C)** Presence of Mediator was also evaluated by Western Blot, probing for MED1 (220 kDa), MED23 (150 kDa), and MED15 (105 kDa). The Mediator complex is seen most strongly in fractions 11 and 13. Fraction 11-15 were pooled for analysis by mass spectrometry.

high activator concentration is crucial during Mediator pull downs from Nuclear Extract. Starting with plasmid DNA coding for GST-p65 TAD (434-551), expression of the protein was optimized (see methods section). Recombinantly expressed GST-p65 AD was immobilized on beads and used to pull-down Mediator from HeLa nuclear extract. The elution of the GST-p65 pull-down was separated by a glycerol gradient (15-40%) to purify the intact complex in certain fractions (Figure 5A). The results of the gradient, as well as Mediator content, were analyzed by silver stain (SDS-PAGE) and western blot. Silver stain data revealed a clear Mediator profile (Figure 5B) in distinct fractions (most commonly fractions 11-15), providing validation of the strong interaction between the activation domain of p65 and the Mediator complex. Each gradient was analyzed individually in order to pool fractions that contained the highest amount of the Mediator complex. Western blot data further verifies above conclusions from silver stain analysis (Figure 5C) as fractions 11 and 13 indicate full assembly of the Mediator complex. Pooled fractions (5 from each gradient) were used for analysis by 2-dimensional liquid chromatography coupled to tandem

**Mediator Subunits Identified**

| Protein | Spectral Counts | MW |
|---|---|---|
| MED1 | 74 | 168478 |
| MED4 | 44 | 29745 |
| MED6 | 2 | 28425 |
| MED7 | 22 | 27245 |
| MED8 | 29 | 32819 |
| MED9 | 9 | 16403 |
| MED10 | 5 | 15688 |
| MED11 | 4 | 13129 |
| MED14 | 62 | 160607 |
| MED15 | 56 | 86753 |
| MED16 | 23 | 96793 |
| MED17 | 25 | 72876 |
| MED18 | 1 | 24453 |
| MED19 | 5 | 26273 |
| MED20 | 16 | 23222 |
| MED21 | 31 | 15564 |
| MED22 | 21 | 16480 |
| MED23 | 31 | 156194 |
| MED24 (MED5) | 95 | 110305 |
| MED25 | 14 | 84389 |
| MED26 | 16 | 65446 |
| MED27 (MED3) | 24 | 35432 |
| MED28 | 12 | 19520 |
| MED29 (MED2) | 27 | 23473 |
| MED30 | 11 | 20277 |
| MED31 | 13 | 15805 |
| **CDK8 Submodule** | | |
| MED12 | 89 | 243081 |
| MED13 | 72 | 239318 |
| MED13L | 28 | 242602 |
| Cyclin C | 31 | 33243 |
| CDK8/CDK19 | 22 | 53284/56802 |

**Table 1.** Mediator subunits identified by mass spectrometry in p65-Mediator purification. CDK19 is a paralog of CDK8 (Mukhopadhyay et al., 2010). MED13L is a paralog of MED13.

mass spectrometry (2D-LC/MS/MS). All mass spectrometry work was done by Chris Ebmeier of the Taatjes Lab.

Mass spectrometry was performed in duplicate and the results were searched against the human database ipi_v3_75 to provide a list of identified factors (using a 1% false discovery rate). Two runs combined gave 5759 individual peptides. Each peptide identified equals one spectral count. The data is presented as the number of spectral counts unique to each gene product. All Mediator subunits were identified. The spectral counts are displayed in Table 1. CDK19 is a paralog of CDK8 (Mukhopadhyay et al., 2010). MED13L is a paralog of MED13.

In order to address the question of whether there are cofactors that are unique to the p65-Mediator complex, the data set generated was compared to similar data sets generated in the lab. By comparing p65 data to other activators, it is possible to define p65-only cofactors. Based on this comparison, p65-only factors are defined as being only identified in the presence of p65 and not with p53. Using a cutoff of 5 spectral counts, this produced Table 2. Data from the three isoforms of SREBP

**p65-only factors**

| | | Spectral Counts | | | | | |
|---|---|---|---|---|---|---|---|
| **Gene** | **Protein** | **p65** | **p53** | **SREBP-1a** | **SREBP-1c** | **SREBP-2** | **MW** |
| GSTM3 | Glutathione S-Transferase | 62 | 0 | 1 | 1 | 13 | 26560 |
| CTSA | Cathepsin A | 22 | 0 | 0 | 0 | 0 | 54466 |
| GLB1 | Galactosidase | 14 | 0 | 0 | 0 | 0 | 76075 |
| JUP | Junction plakoglobin | 14 | 0 | 0 | 0 | 0 | 62616 |
| GRPEL1 | GrpE-like (mitochondrial) | 12 | 0 | 30 | 30 | 8 | 24279 |
| PSMA7 | Proteasome subunit | 10 | 0 | 6 | 4 | 6 | 27887 |
| SF3B4 | Splicing factor 3B | 10 | 0 | 0 | 15 | 0 | 44386 |
| NFS1 | Nitrogen fixation homolog | 9 | 0 | 0 | 0 | 0 | 50196 |
| KLHDC2 | Kelch domain containing | 8 | 0 | 0 | 0 | 0 | 46099 |
| SETD8 | Lysine methyltransferase | 8 | 0 | 0 | 0 | 0 | 42890 |
| TADA2A | Transcriptional adaptor 2A | 8 | 0 | 17 | 32 | 12 | 51496 |
| CDK9 | Cyclin-dependent kinase 9 | 6 | 0 | 1 | 7 | 4 | 42778 |
| HEXIM1 | Hexamethylene bis-acetamide inducible | 6 | 0 | 1 | 0 | 15 | 40623 |
| PSMD4 | Proteasome subunit | 6 | 0 | 0 | 0 | 9 | 40737 |
| SAPS1 | Protein phosphatase 6 | 6 | 0 | 3 | 4 | 6 | 103139 |
| KNTC1 | Kinetochore associated | 5 | 0 | 48 | 208 | 15 | 250749 |
| PLAUR | Plasminogen activator | 5 | 0 | 1 | 5 | 2 | 36978 |
| SP1 | Sp1 transcription factor | 5 | 0 | 19 | 5 | 3 | 80693 |
| | Total MED | 914 | 1145 | 7323 | 2674 | 1748 | |
| | Total Peptides | 5439 | 8556 | 26679 | 18965 | 26145 | |

**Table 2.** p65-only cofactors identified by mass spectrometry. These factors were determined to be p65-only when compared to similar data for p53. SREBP data is also shown. Five spectral counts was used as the cutoff. p53 and SREBP data courtesy of Chris Ebmeier and Jesse Goossens. Lysosome-related (Yellow). Proteasome (Green). Transcriptional regulators (Red).

(sterol regulatory element-binding protein), another activator, are shown for comparison purposes.

## Discussion

Evidence is compelling from the combination of silver stain, western blot, mass spectrometry data, as well as previously published data (Naar et al. 1999; O'Shea and Perkins, 2008) that Mediator is interacting with the activation domain of p65. Further purification (e.g. phosphocellulose column) of the nuclear extract, commonly used in laboratory protocols, was not used here to avoid eliminating some cofactors of the p65 complex from the purification. This method therefore allows for a broad screen of potential cofactors.

Under glycerol gradient conditions, complexes are left largely intact and migrate as one whole unit. This method thereby allows for the p65-Mediator complex to be isolated from other non-Mediator associated cofactors. Due to stringent wash conditions (0.5 M KCl), only associated cofactors with relatively strong affinity should remain bound to the complex. Mass spectrometry analysis identified 5759 peptides leading to a total of 435 different identified proteins, including every Mediator subunit. Based on this large data set, it can be said that the p65-Mediator complex has many further interactions with other factors. Despite even the strongest mass spectrometry data, cofactors must hold up to further biochemical validation before firm conclusions can be made about the interaction, and new hypotheses generated about its significance.

**Interaction with RNAP II**

| Gene | Spectral Counts | MW |
|---|---|---|
| POLR2A | 48 | 217206 |
| POLR2B | 15 | 133897 |
| POLR2C | 7 | 31441 |
| POLR2D | 29 | 16311 |
| POLR2E | 8 | 24551 |
| POLR2G | 2 | 19294 |
| POLR2H | 3 | 17143 |
| POLR2I | 1 | 14523 |

**Table 3.** Subunits of the RNA Polymerase II complex identified by mass spectrometry.

As displayed in Table 1, there are two major forms of the Mediator complex found in cells. Core Mediator is made up of all MED subunits except forCDK8, Cyclin C, MED12, and MED13. CDK8-Mediator has the addition of MED12, MED13, Cyclin C, and CDK8 and excludes MED26 (Taatjes, Marr, and Tijan, 2004). The CDK8-bound form of Mediator has been shown to inhibit interaction with RNAP II on certain promoters, which thereby inhibits transcription (Knuesel et al., 2009). CDK8-Mediator has also been

shown to have positive roles in transcription regulation (Galbraith, Donner, and Espinosa, 2010). Both forms of the Mediator complex are present in the mass spectrometry data; however it is difficult to assess which is more commonly associated with the p65 TAD based on this data alone. Additional experiments may help to elucidate the difference between factors associated with the p65-Core Mediator complex versus the p65-CDK8-Mediator complex. These differences may account for unique transcriptional events. Further evidence for the involvement of the p65-Mediator complex in the context of regulation of gene expression is provided by the interaction of the complex with RNAP II, displayed in Table 3.

## p65-only factors

From Table 2 come several interesting cofactors. Cathepsin A and galactosidase (a lysosomal-associated protective glycoprotein and lysosomal enzyme, respectively) function together to degrade cellular waste. In addition, cathepsin A has been shown to degrade p65/p50 (Masuhara et al. 2009). Various regulatory elements are also highlighted here, such as SETD8 (methyltransferase) (Shi et al. 2007) and TADA2A (transcriptional adapter, SAGA) (Gamper and Roeder, 2008). All of these proteins have not been shown previously to have p65-specific interactions, suggesting either novel or non-specific binding. Further biochemical data is needed for verification.

CDK9 is of particular interest. In complex with cyclin T1, CDK9 makes up P-TEFb, a positive transcription elongation factor. P-TEFb facilitates elongation by inactivating negative elongation factors and modifying the C-terminal domain of RNAP II to allow for more efficient elongation as well as cotranscriptional mRNA processing (Kurosu and Peterlin, 2004) Although CDK9 is seen in the SREBP data (Table 2), the fact that p53 shows zero spectral counts indicates that CDK9 may be an important factor specific to certain activators, such as p65. In the same vein comes HEXIM1 which is a known regulator of P-TEFb (Yik et al., 2003). Due to the positive effect P-TEFb has on elongation, it is reasonable to suggest that recruitment via an activator complex may facilitate transcription of target genes. Indeed, there has

been evidence to suggest that P-TEFb plays an integral role in transcriptional activation by the activator VP16 (Kurosu and Peterlin 2004). Furthermore, P-TEFb has been shown by multiple groups to interact with NF-κB proteins, acting to facilitate activation of target genes (Brasier, 2008). Inhibition of CDK9 was shown to inhibit expression of NF-κB target genes (Schmerwitz et al., 2010). CDK9 has been shown to interact with CDK8-Mediator (Donner et al., 2010), however whether or not this association is specific to (or enhanced by) p65-CDK8-Mediator or p65-Core Mediator is not known.

## p65-enriched factors

Another method of examining cofactors of the p65-Mediator complex is by fold enrichment. A comparison is found in Table 4. In this table the top spectral count hit for the p65 data set, PRKDC, appears. PRKDC is DNA-dependent protein kinase, which has been shown to play a crucial role in NF-κB target gene expression through phosphorylation of p50 (Ju et al., 2010). Its presence here may indicate p65-dependent recruitment of PRKDC which in turn helps to activate target gene expression. Filamin A is also of interest as depletion has been directly linked to a reduction in activation of NF-κB target genes (Hiyashi and Altman, 2006).

Furthermore, enrichment data emphasizes potentially novel p65-specific interactions. TIMM8A

**p65-enriched factors**

| | | | Spectral Counts | | | | | |
|---|---|---|---|---|---|---|---|---|
| Gene | Protein | Enrichment | p65 | p53 | SREBP-1a | SREBP-1c | SREBP-2 | MW |
| MED28 | Mediator subunit | 12 | 12 | 1 | 47 | 11 | 1 | 19520 |
| CSRP2BP | CRSP2 binding protein | 8 | 8 | 1 | 0 | 0 | 0 | 88802 |
| TIMM8A | Mitochondrial membranetranslocase | 8 | 8 | 1 | 0 | 0 | 0 | 10998 |
| PYCR1 | Pyrroline-5-carboxylate reductase | 8 | 15 | 2 | 21 | 7 | 112 | 35981 |
| TRIM24 | Tripartite motif family | 6 | 12 | 2 | 0 | 0 | 0 | |
| PSMC5 | Proteasome subunit | 5 | 5 | 1 | 1 | 1 | 12 | 45626 |
| TRAP1 | TNF receptor-associated protein | 5 | 5 | 1 | 11 | 23 | 10 | 80110 |
| PRKDC | DNA-dependent protein kinase | 5 | 492 | 102 | 92 | 221 | 265 | 469089 |
| DECR1 | 2,4-dienoyl CoA reductase | 5 | 200 | 43 | 109 | 75 | 96 | 36068 |
| FLNA | Filamin A | 4 | 13 | 3 | 6 | 6 | 14 | 280018 |
| BCL7C | B-cell lymphoma 7C | 4 | 4 | 1 | 0 | 0 | 0 | 23365 |
| GRINL1A | Glutamate receptor | 4 | 4 | 1 | 24 | 2 | 1 | 41740 |
| TIMM13 | Inner mitochondrial membrane translocase | 4 | 40 | 11 | 1 | 0 | 0 | 10500 |
| | Total MED | ~0.8 | 914 | 1145 | 7323 | 2674 | 1748 | |
| | Total Peptides | ~0.64 | 5439 | 8556 | 26679 | 18965 | 26145 | |

**Table 4.** p65-enriched cofactors identified by mass spectrometry. These factors were determined to be enriched in the context of p65 when compared to similar data for p53. Enrichment data is presented as fold increase over p53. SREBP data is also shown. The cutoff for this table was set at four times enriched over p53. Total MED (Mediator Subunits) and Peptide spectral counts shown for each activator.

and TIMM13 function together as translocases for the mitochondrial membrane (Beverly et al. 2008), yet data here indicates that they may also function as part of the transcriptional machinery responsible for transcribing NF-κB target genes. CSRP2 binding protein functions as an adapter as well as an acetyltransferase (Guelman et al., 2009). Acetyltransferases have a well known role in activation of transcription due to their ability to modify chromatin. TRIM24 is involved in transcriptional control of p53 target genes by acting to degrade p53 itself (ubiquitin ligase) (Jain and Barton, 2009). p65-specific recruitment of TRIM24 may have implications for regulation of p53.

## The Proteasome

The proteasome is responsible for degradation of proteins within the cell and functions as a crucial piece of the NF-κB signaling pathway by degrading IκB. The majority of proteasome subunits were identified in the mass spectrometry data set (Table 5). The alpha and beta type subunits, which comprise the core unit of the proteasome (20S), were all found to be p65-only factors when compared to p53. Figure 7 depicts the structure of the proteasome. The p65-specific recruitment of alpha and beta type subunits to the general transcription machinery may

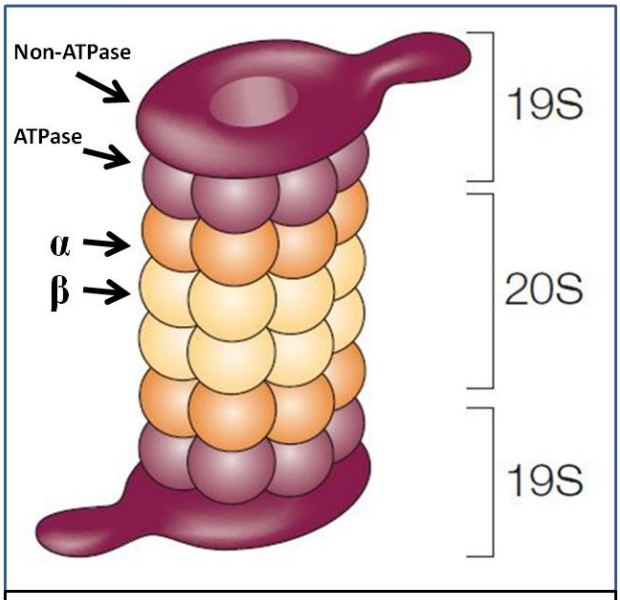


**Figure 6.** Proteasome structure. It comprised of one 20S subunit and two 19S subunits (Adams, 2004). The 20S is made up of alpha and beta subunits. The 19S (Regulatory) is made up of ATPase and Non-ATPase subunits.

**Interaction with the Proteasome**

| Gene | Subunit Type | Spectral Counts | | | | | MW |
|---|---|---|---|---|---|---|---|
| | | p65 | p53 | SREBP-1a | SREBP-1c | SREBP-2 | |
| PSMA1 | Alpha | 2 | 0 | 0 | 0 | 11 | 29556 |
| PSMA2 | Alpha | 2 | 0 | 0 | 0 | 13 | 25899 |
| PSMA3 | Alpha | 1 | 0 | 0 | 0 | 0 | 27647 |
| PSMA5 | Alpha | 3 | 0 | 0 | 3 | 15 | 26411 |
| PSMA6 | Alpha | 4 | 0 | 0 | 5 | 4 | 27399 |
| PSMA7 | Alpha | 10 | 0 | 6 | 4 | 6 | 27887 |
| PSMB1 | Beta | 4 | 0 | 0 | 0 | 4 | 26489 |
| PSMB2 | Beta | 2 | 0 | 0 | 0 | 7 | 22836 |
| PSMB5 | Beta | 2 | 0 | 0 | 0 | 13 | 28480 |
| PSMC2 | Regulatory | 10 | 15 | 0 | 5 | 6 | 48634 |
| PSMC3 | Regulatory | 4 | 3 | 2 | 3 | 15 | 49204 |
| PSMC4 | Regulatory | 3 | 0 | 1 | 3 | 10 | 47366 |
| PSMC5 | Regulatory | 5 | 1 | 1 | 1 | 12 | 45626 |
| PSMC6 | Regulatory | 1 | 10 | 1 | 2 | 4 | 44173 |
| PSMD1 | Non-ATPase Regulatory | 5 | 7 | 12 | 9 | 10 | 105836 |
| PSMD12 | Non-ATPase Regulatory | 2 | 8 | 0 | 1 | 0 | 52904 |
| PSMD2 | Non-ATPase Regulatory | 8 | 3 | 10 | 16 | 5 | 100200 |
| PSMD3 | Non-ATPase Regulatory | 4 | 4 | 3 | 0 | 25 | 60978 |
| PSMD4 | Non-ATPase Regulatory | 6 | 0 | 0 | 0 | 9 | 40737 |
| PSMD6 | Non-ATPase Regulatory | 8 | 17 | 13 | 11 | 14 | 45531 |

**Table 5.** Identified subunits of the proteasome. All alpha and beta types were only identified with p65 when compared to p53. SREBP data is also shown. Color coded subunits correspond to Figure 7 showing the structure of the proteasome.

have novel and far reaching implications. The proteasome has been shown to be involved in transcriptional activation, functioning to allow transcription factors to associate with the general transcription machinery, as well as to eliminate corepressors from the same complex (Walsh and Shupnik, 2009). The proteasome system is also important for regulating turnover and activity of transcription factors by use of ubiquitin, a small protein involved in the proteasome pathway (Muratani and Tansey, 2003).

## Future Research

Of particular interest for future research is the interaction between p65-Mediator, CDK9, and HEXIM1. To investigate this interaction further orthogonal purifications (utilizes multiple biochemically distinct methods in a single purification) will be performed. Purification of the p65-Mediator complex (with associated factors) will be followed by antibody purification of Mediator itself. This can be achieved by a nuclear extract pull down with GST-p65 TAD (as described previously) and subsequent immunoprecipitation (IP) using antibodies for MED1. The result will be a population of Core Mediator and CDK8-Mediator both in the p65-bound state. Figure 6 displays the purification scheme. This technique can be applied to any of the potential cofactors identified. The antibody used for the IP can also be varied. This data will give insight into activator-specific interactions that may be taking place.

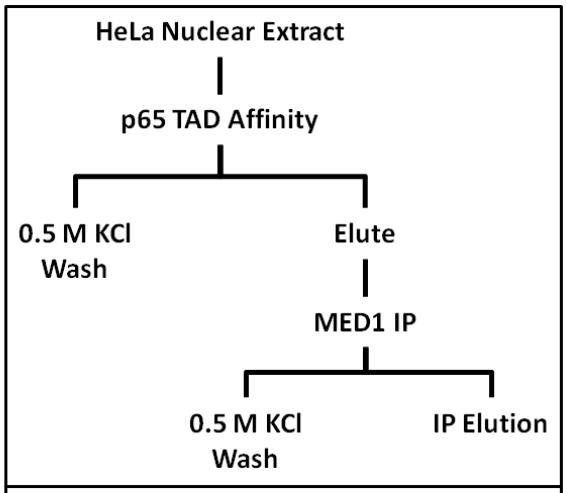


**Figure 7.** Orthogonal purification scheme. IP elution will be analyzed by Western blot.

In vitro transcription assays may be used to examine the relative transcriptional activity in the presence of different identified cofactors. NF-κB reporter assays may also be employed.

The two degradation pathways, lysosome and proteasome, that were identified will also be investigated further to determine their significance in regulation of gene expression. For example, similar experiments to those described above can be carried out with and without the presence of a proteasome inhibitor, helping to describe this potentially novel interaction.

# Experimental Procedures

*Protease Inhibitors (PIs): 1 mM Sodium Metabisulfite, 1 mM Dithiothreitol, 0.25 mM Phenylmethylsulfonyl Fluoride, 1 mM Benzamidine HCl, 1.7 mg Aprotinin/L

**Transformation:** Transformation by electroporation into XL1 (sequencing) and Codon Plus BL21 (protein expression) electro-competent E. coli cells. Chill electro-cuvette on ice, thaw bacteria on ice. Spot 1µL GST-p65 TAD plasmid DNA (from Naar Lab) into cuvette, add 50 µL bacteria, insert into BioRad Gene Pulser (settings: 0.2 cm electrode, gap=50, volts=2.5, capacitance=25, pulse controller=200 Ω).

**HeLa Cell Cultures:** HeLa cells cultured with JMEM (Joklik's Modification Minimum Eagle Medium). Cells harvested at 500,000 cells/mL or above. Wash once with PBSM (1X phosphate-buffered saline, 0.5% MgCl2). Swell with Buffer A (10X Buffer A: 100 mM HEPES, 15 mM MgCl2, 100 mM KCl, pH=7.9) 20 minutes. Dounce 7X with type A dounce homogenizer. Flash freeze pellet and store at -80°C.

**Nuclear Extract Preparation:** Thaw 100L nuclear pellet in water/ice bath. Add 0.9 times pellet volume of Buffer C (20mM HEPES, 24% glycerol, 0.4M NaCl, 1.5 mM MgCl2, 0.5M EDTA, pH 7.9) with protease inhibitors while stirring at 4°C. Dounce 20 times on ice with type B glass homogenizer. Stir at 4°C for 30 minutes. Spin at 4°C and 15K for 30 minutes. Keep supernatant. Dialyze in 4L Buffer D (20 mM HEPES, 20% glycerol, 100 mM KCl, 2 mM MgCl2, 0.2 mM EDTA) + PIs (no aprotinin) to 0.1 M salt. Spin at 4°C and 15K for 30 minutes. Flash freeze supernatant and store at -80°C.

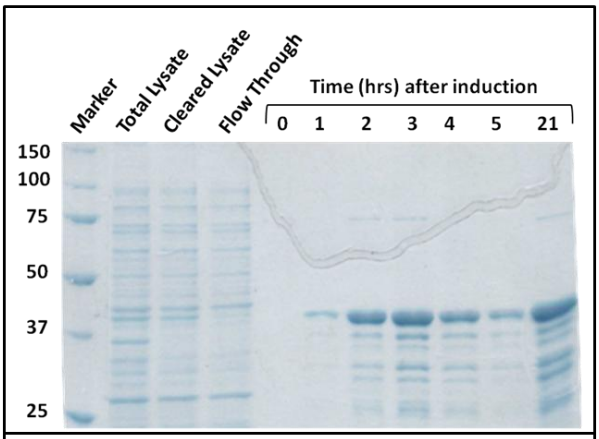


**Figure 8.** Time course for GST-p65 TAD expression at 16°C and 0.5 M IPTG. Lanes for "time after induction" were loaded with GSH-Sepharose immobilized beads. It is important to note the bands that change between the lysates and the flow through.

**Protein Expression:** *Starter culture.* Streak 2µL glycerol stock on LB/Ampicillin (Amp) agar plate. Overnight at 37°C. Prepare 5mL Luria Broth (LB) with 100µg Amp/mL and 50µg Chloramphenicol (CAM)/mL, inoculate with single colony from overnight plate. Grow at 37°C until saturated (approx. 7-8 hours). Prepare 100mL LB with 100ug Amp/mL and 50ug CAM/mL, inoculate with entire 5mL culture. Grow overnight at 37°C. *Expression Culture.* Prepare 1L LB with 100ug Amp/mL and 50ug CAM/mL, inoculate with 20mL starter culture. Grow at 37°C until $OD_{600}$=0.6-0.8, reduce temperature to 16°C, induce expression with 0.5mM IPTG overnight (approx. 21 hours). Harvest and wash with 1X PBS (phosphate-buffered saline) + PIs. Flash freeze and store at -80°C. Development work was carried out to optimize protein expression. Figure 8 shows an example of a time course at 16°C. Overnight expression was determined to be optimum. IPTG concentration was varied to increase protein solubility. Protein was soluble at 0.5 mM IPTG but insoluble at 1mM IPTG.

**GSH-Sepharose Immobilization:** Thaw 1L BL21 cell pellet on ice with 20mL H/E + PIs. Lyse cells via sonication for 30 seconds (1s ON, 1s OFF) on setting 3, repeat 3 times. Clarify lysate by centrifugation at 4°C, 40K rpm, 30 minutes. Equilibrate 250µL GSH-Sepharose Beads with 10mL H/E + PIs. Remove buffer from beads and transfer cleared lysate to beads. Rock/roll >3hr (<5hr). Wash beads 5X with High Salt (1M NaCl, 50mM Tris, 5µM EDTA, 10% glycerol by volume, 50mL 10% NP-40 NP-40, 8mM CHAPS) + PIs,

2X with H/E (50mM Tris, 0.5M NaCl, 5µM EDTA, 10% glycerol, 25mL 10% NP-40) + PIs, 1X with 0.15M HEGN (20mM HEPES, 5µM EDTA, 10% glycerol, 1mL 10% NP-40, 75mM KCl) + PIs. Remove buffer, leaving enough for 1:1 Buffer:Beads. Figure 9 shows analysis of immobilization effectiveness.

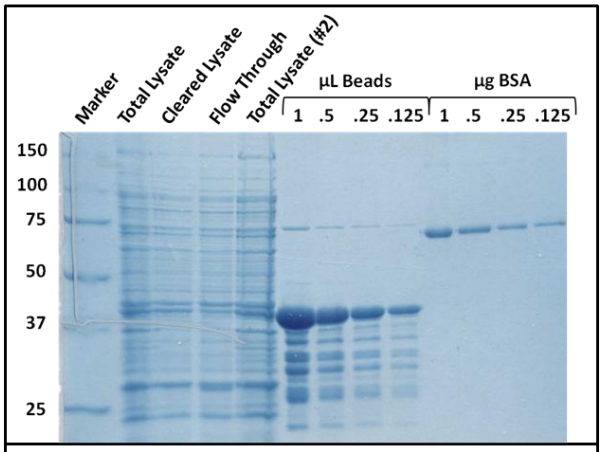


**Figure 9.** Full work-up for GST-p65 TAD expression (21 hrs). There are approximately 8 µg of protein bound per µL of beads.

**Nuclear Extract Pull-Down:** (Starting with 1L BL21 cell pellet) Thaw 4mL Nuclear Extract on ice. Centrifuge in 1.5mL tubes at 4°C, 14K rpm, 30 minutes. Transfer 1mL NE to ~50µL immobilized beads (see immobilization protocol), rock/roll overnight. Wash beads 5X with 0.5M HEGN + PIs and 1X with 0.15M HEGN + PIs. Elute with bead volume of 30mM GSH in Tris Elution buffer (0.15M salt) >30 minutes.

**Glycerol Gradient:** Pour 15-40% linear glycerol gradient in 2mL Beckman centrifuge tube. Transfer eluate (~200µL) to top of gradient. Spin at 4°C, 55K rpm, 6 hours. Pipet 100µL fractions.

**Silver Stain:** Shake gel in: 250mL 50% methanol 10 minutes, 250mL 5% methanol 10 minutes, 100mL 35uM DTT 5 minutes, 250mL with 250mg silver nitrate plus 25µL formaldehyde. Rinse 2X with water and 2X with developing solution (15g sodium carbonate, 250µL formaldehyde in 500mL cold water), develop to desired intensity and quench with citric acid monohydrate to neutral pH. Transfer to water.

**Western Blot:** Assemble blot with nitrocellulose and acrylamide gel in 1X transfer buffer (10% glycine/tris running buffer, 10% methanol). Transfer at 0.25 Amps for 45 minutes. Disassemble and block for 30 minutes with milk blocker (5% milk in PBST). Discard blocker and add appropriate primary antibody in fresh milk blocker. Rock overnight at 4°C. Wash 4X with PBST, 10 minutes each. Add appropriate secondary antibody in fresh milk blocker. Rock 45 minutes at 25°C. Wash 4X with PBST, 10 minutes each. Add developing solution and expose film at various time lengths.

**Mass Spectrometry:** See Figure 10 for flow chart. Mediator-containing glycerol gradient fractions were precipitated at 4°C using 20%(v/v) TCA, 0.067mg/mL insulin and 0.067%(w/v) deoxycholate. Precipitated protein pellets were washed twice with -20°C Acetone and air dried. Proteins were trypsin digested using a slightly modified Filter-Aided Sample Prep (FASP) protocol (Nat Methods. 2009 May;6(5):359-62). Briefly, protein pellets were suspended with 4%(v/v) SDS, 0.1 M Tris pH 8.5, 10 mM TCEP and incubated 30m ambient to reduce disulfides. Reduced proteins were diluted with 8M Urea, 0.1M Tris pH 8.5 and iodoacetamide was added to 10mM and incubated 30m in total darkness. Reduced and alkylated proteins were then transferred to a Microcon YM-30 spin concentrator and washed three times with 8M Urea, 0.1M Tris pH 8.5 to remove SDS. Three washes with 2M Urea, 0.1M Tris pH 8.5 were performed then trypsin and 2mM CaCl2 were added and incubated overnight in a 37°C water bath. Digested peptides were eluted and acidified with 5%(v/v) formic acid.

Peptides were desalted online and fractionated with a Phenomenex Jupiter C18 (5µm 300Å; 0.25 x 150mm) column using a two dimensional LC/MS/MS method (Agilent 1100). Seven steps of increasing acetonitrile (3, 6, 9, 12, 16, 20 and 100%B; A: 20mM ammonium formate pH 10, 4% acetonitrile and B: 10mM ammonium formate pH 10, 65% acetonitrile) at 5µL/minute eluted peptides for a second dimension analysis on a Dionex Acclaim PepMap C18 (3µm 100A; 0.075 x 150mm) running a gradient at 0.2µL/minute from 5 to 25% B in 100 minutes for steps one through six and 10 to 30% B in 100 minutes for step seven (A: 4% acetonitrile & B: 80% acetonitrile, both with 0.1% formic acid pH~2.5). PepMap eluted peptides were detected with an Agilent MSD Trap XCT (3D ion trap) mass spectrometer.

All spectra were searched with Mascot v2.2 (Matrix Sciences) against the International Protein Index (IPI) database version 3.75 with two missed cleavages and mass tolerances of m/z ±2.0 Da for parent masses and ±0.8 Da for MS/MS fragment masses. Peptides were accepted above a Mascot ion score corresponding to a 1% false discovery rate (1% FDR) determined by a separate search of a reversed IPI v3.75 database. Peptides were then filtered and protein identifications were assembled using in-house software (Chris Ebmeier).

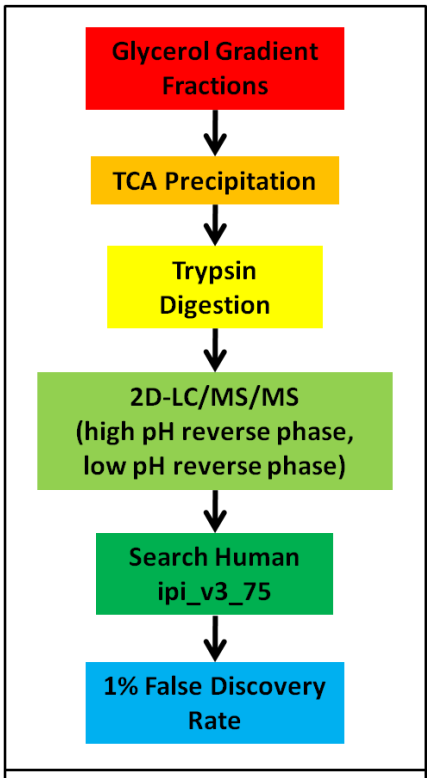


**Figure 10.** Flow chart of mass spectrometry analysis (C. Ebmeier).

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# Appendix

**Other Proteins of Interest**

| Gene | Protein | Spectral Counts | Function | MW |
|---|---|---|---|---|
| RUVBL1 | RUVB-like 1 | 61 | Interacts with RUVBL2 to regulate transcription | 50228 |
| RUVBL2 | RUVB-like 2 | 75 | Interacts with RUVBL1 to regulate transcription | 51157 |
| CBP | CREB-binding protein | 64 | Transcriptional coactivation, histone acetyltransferase | 265351 |
| EP300 | p300 | 37 | Similar to CBP | 264161 |
| IQGAP1 | Ras GTPase-activating like | 45 | Cell morphology and motility | 189252 |
| IQGAP3 | Ras GTPase-activating like | 52 | IQGAP family member | 184699 |
| PGAP1 | GPI inositol-deacylase | 47 | GPI biosynthesis | 105383 |
| SSBP1 | Single-stranded DNA binding protein | 27 | Genome stability, mitochondrial biosynthesis | 17260 |
| SUPT3H | Transcription initiation protein homolog | 20 | Transcription initiation | 35793 |
| TRRAP | Transformation/transcription domain-associated protein | 157 | Interacts with c-Myc, co-purifies with CDK8 | 437600 |
| ARHGEF2 | Rho guanine nucleotide exchange factor | 12 | GTPase activity | 111543 |
| EEF1D | Translation elongation factor 1D | 3 | Delivers aminoacyl tRNAs | 31122 |
| CCNT1 | Cyclin T1 | 3 | With CDK9 makes up P-TEFb | 80685 |
| CHUK | Inhibitor of IKKα | 3 | Inhibitor of NF-kB pathway | 84654 |
| IKBKB | Inhibitor of IKKβ | 1 | Inhibitor of NF-kB pathway | 86564 |

**Table 6.** Other p65-associated proteins identified by mass spectrometry that have functions that critical to cell maintenance and transcription.