Examination of Mediator composition and p53 in distinct breast cancer lines: MCF7 and MDA-MB-231

Honors Thesis
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Abstract

Found in all eukaryotic organisms, the Mediator complex is an essential component of the transcription machinery and acts as a molecular bridge between activators and other general transcription factors. The transcription factor p53 is a potent tumor suppressor and acts as a master controller of the cell cycle and programmed cell death. Binding of p53 to Mediator induces conformational shifts that activate RNA polymerase II transcription. In the wild type form, p53 aides in inhibiting growth of cancerous cells, but this is not the effect seen in mutant p53. Two breast cancer cell lines, MCF7 and MDA-MB-231 are clinically distinct with varying molecular backgrounds as the first expresses wild type p53 and the latter expresses hyperactive, mutant p53. In order to determine which transcription regulatory factors are common and different between the two cell lines, purifications were carried out to isolate the Mediator complex from each of these two cell types. Further, to analyze the p53 network and p53-Mediator interactions, a different set of purifications were completed using nuclear extracts from both breast cancer cell types. Mass Spectrometry analysis of these samples identified proteins that are similar and different in the two distinct breast cancer cell lines. In future work, these differences will be explored to determine whether they might contribute to clinical differences, which include metastatic growth and drug resistance.

Introduction

Transcription propagates the first step of the central dogma of molecular biology: DNA (deoxyribonucleic acid) to RNA (ribonucleic acid) to protein. The transcriptional machinery includes RNA polymerase II (pol II) which transcribes an RNA transcript from the DNA template. This RNA transcript is then edited and used to guide the synthesis of specific proteins that are needed for various cell processes. In addition to pol II, many other general transcription factors are required in transcriptional events. Included in these factors is the Mediator complex, an essential component in the composition of the preinitiation complex (PIC) at the transcription start site. The pre-elongation complex (PEC) is a more broad term to include genes that are regulated post recruitment of pol II where minimal to no initiation may have occurred (Taatjes, 2010). PIC and PEC are equivalent terms and PEC will be used for the remainder of this thesis. The composition of the PEC is shown in Table 1.

TFIIA: 2 subunits*
TFIIB: 1 protein, 33 kDa
TFIID: 16 subunits (TBP + 15 TAFs)
TFIIE: 2 subunits
TFIIF: 2 subunits
TFIIH: 10 subunits
pol II: 12 subunits
Mediator: 26 subunits

Table 1. The transcription initiation machinery that composes the PEC (Taatjes, 2010).

Contained within a gene are promoter regions of DNA to which activators and repressors can bind to either up or down regulate transcription. Activators are DNA-binding transcription factors that regulate the interactions between Mediator and pol II. Specific activators are recruited to different coding regions, depending on the gene that is being transcribed. One such activator is p53, a tumor suppressor protein. Often referred to as the "master regulator," p53 oversees the cell cycle and plays an essential role in abating the growth of cancerous cells.

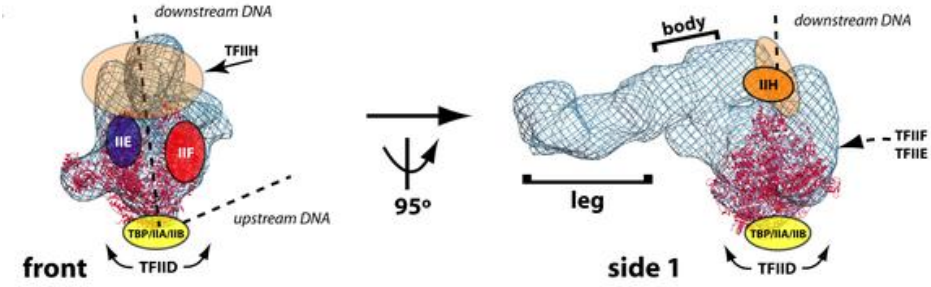

Figure 1. Front and side views of Mediator (blue mesh) interacting with the bound pol II (red ribbon structure) and the additional transcription initiation factors. (Bernecky, 2011)

I. The Mediator Complex

Found in both yeast and humans, Mediator is a large, 1.2MDa complex that consists of 26 subunits. Mediator functions at all pol II promoters in yeast and therefore, is thought to function at all human pol II promoters as well. It acts in part by binding to the C-terminal domain (CTD)

of pol II. Due to its necessity in transcription, Mediator can thus be considered a general transcription factor. As a target of a broad spectrum of activators, Mediator functions as an essential co-activator during transcriptional activities (Kornberg, 2005). This is greatly due to Mediator's size and shape, enabling a large surface area to be utilized for different, simultaneous protein-protein interactions. It is known that these interactions include the physical connection between mediator and many factors that comprise the PEC, including TFIIB, TFIID, TFIIE, TFIIH and most importantly pol II. While the specific structure of the entire PEC has yet to be elucidated, the cryo-EM structure of Mediator-pol II-TFIIF allowed a model of the human PEC to be proposed (Figure 1). This model suggests that Mediator acts as a fundamental scaffold to which activators and general transcription factors can bind, creating a molecular bridge between activators and pol II transcription machinery (Figure 2). The extensive composition of Mediator gives it the ability to integrate various signals received from other co-activators in addition to repressors (Malik, 2005).

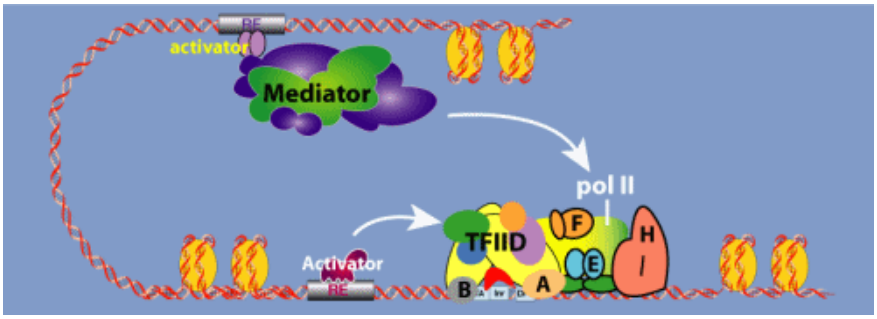

Figure 2. Mediator's associations with both activators and the pol II transcription machinery allow it to act as a bridge between the different factors (http://www.colorado.edu/chemistry/taatjeslab/background.htm).

The binding of activators to Mediator initiates a conformational change in Mediator, allowing for the activation of specific genes, depending on the activator that is present. The structure of Mediator is closely related to its functional capabilities (Figure 3), leading to the concept that activators themselves control the function of Mediator by changing its structural state, which can affect downstream interactions of Mediator with additional co-factors (Ebmeier and Taatjes, 2010). Interestingly, activator binding appears to be needed in order for Mediator-cofactor interactions to take place. An activator needs to be present in order to induce a structural shift in Mediator. This shift only takes place when

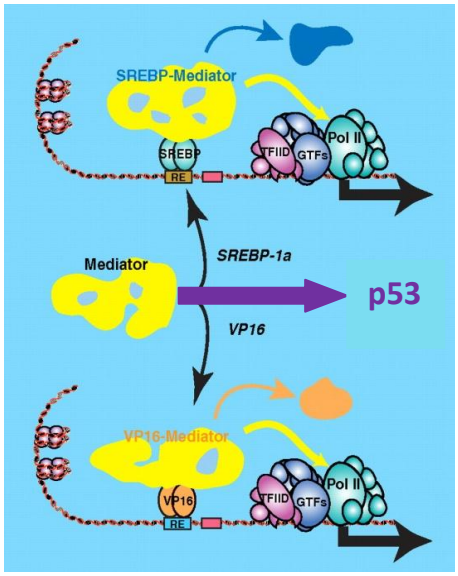

Figure 3. Different conformational states are induced in Mediator in the presence of different activators, leading to gene specific activation (Ebmeier and Taatjes, 2010).

Mediator is in close proximity with the promoter, because activators bind DNA at the promoter. Figure 3 represents this model of activator control with two different activators: SREBP-1a and VP16. Different conformations of Mediator have a higher affinity for different co-regulating factors, leading to direct communication with the transcription machinery and activation of specific genes. Similar events take place in the presence of p53. Mediator takes on different conformations depending on if it is in the active form or not and what activators are presently bound to the complex (Figure 4).

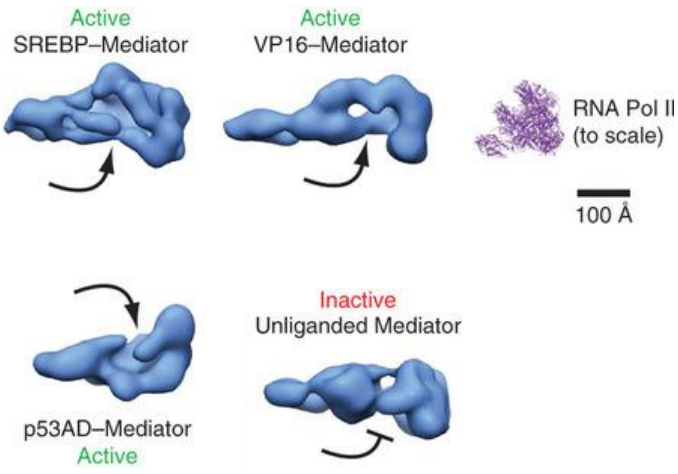

Figure 4. Viewed from the bottom, different Mediator structures bound to different activating. The arrows point towards the pol II binding pocket. The inactive conformation in which Mediator is not bound to an activator, lacks this pocket (Meyer, 2010).

All activated pol II transcription requires the presence of Mediator (Holstege, 1998). Gene specific activation requires the presence of distinct activators that interact with Mediator in such a way as to induce unique, conformational shifts. This suggests that by manipulating activator-Mediator interactions, gene expression can be managed. This implication reveals a store of possibilities relating to human health. Specifically, gaining control over the functions of diseased and cancerous cells in the body and determining their fate.

II. Tumor Suppressor p53

The protein p53 is a transcription factor that regulates the cell cycle at the G1 stage and helps control aging in mammals. In its full length form, p53 is 393 amino acids long and binds DNA as a tetramer (Figure 5). Two different subunits of the Mediator complex are bound by different regions of p53 (Meyer, 2010). The activation domain at the N-terminal of p53 (p53AD) operates with the Med17 subunit, while the C-terminal domain of p53 (p53CTD) interacts with

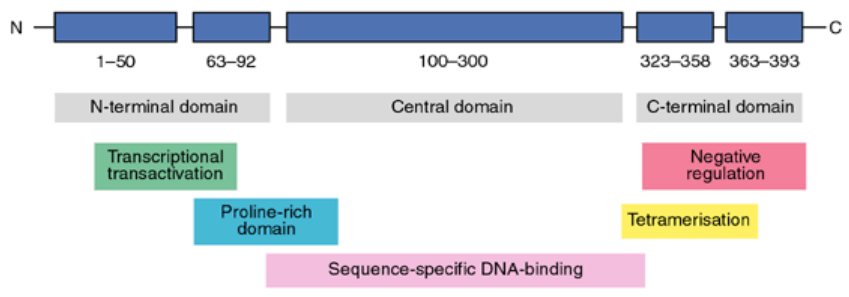

Figure 5. Protein map displaying the different domains of p53. The numbers represent amino acids (Anzola, 2003).

Med1. Upon binding of p53AD to Mediator, the complex undergoes structural shifts that apparently induce previously stalled pol II machinery to initiate elongation of a transcript (Figure 4). This is interesting to note, because certain genes targeted by p53 have promoters with pol II already preloaded but are inactive. Consequently, the conformational changes that Mediator undergoes after the binding of p53 are needed to stimulate the stalled pol II and to also oversee the post-recruitment events that take place in the activation of a gene. Furthermore, two p53 regulated genes, p21 and HDM2, do not require the presence of p53 to compile the transcription initiation machinery (PEC), and Mediator unbound by p53 is not sufficient to induce activation of the stalled pol II (Knuesel, 2011). Specifically, p53AD-Mediator interaction and its associated structural change is vital for activating stalled pol II at p21 and HDM2, whereas p53CTD-Mediator binding is unable to initiate transcription due to its inability to appropriately activate pol II (Figure 6). Genes responsive to p53 are highly dependent on the p53AD-Med17 interaction, thus alluding to numerous possibilities of manipulating p53 activity in cancer cells (Knuesel, 2011).

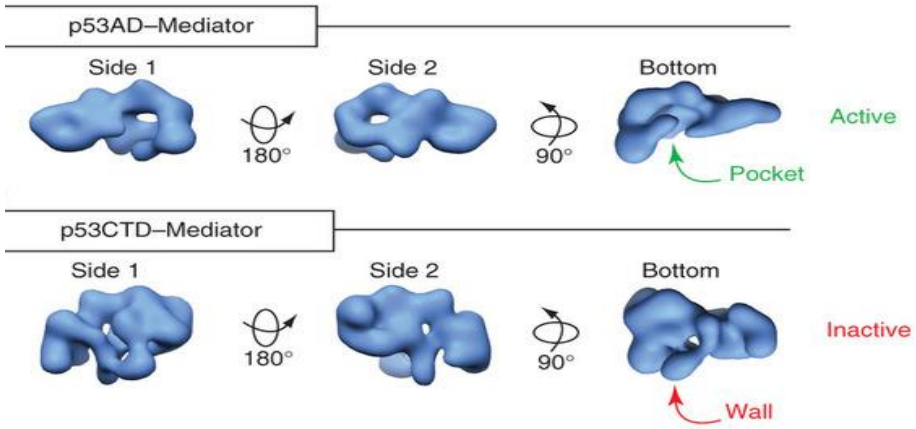

Figure 6. The active and inactive conformations of Mediator, depending on which region of transcription factor p53 the complex is bound to. The pocket indicates the pol II binding domain, and is not present in the p53CTD-Mediator structure (Meyer, 2010).

When regulating cancerous cells, p53 is considered one of the most important tumor suppressors and is known as a "master executioner," due to its ability to block angiogenesis, arrest the cell cycle and/or trigger programmed cell death in response to DNA damage, hyperactivation of oncogenes and other cellular stresses (Figure 7). In cancer, p53 is the most commonly mutated tumor suppressor gene. The most prevalent

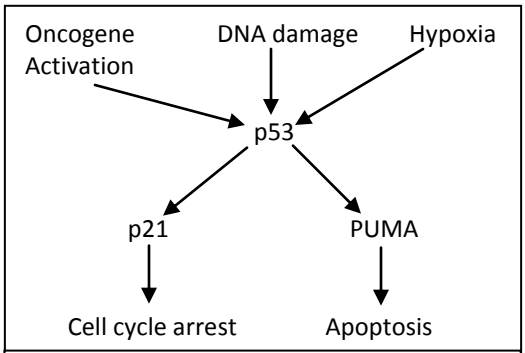

Figure 7. Summarized p53 pathway.

mutation is a missense mutation, which falls in the DNA-binding domain of the protein, suggesting that normal DNA binding activity is vital for maintaining tumor suppressor activity. Furthermore, in order for DNA binding to occur, all four p53 monomers are required. One mutant monomer can inactivate the entire tetramer complex. Mutant p53 in a non-cancerous situation can be equally as detrimental. The protein's function is essential in sustaining a healthy environment by promoting the proliferation of healthy cells and impeding the growth of detrimental ones.

III. Breast Cancer Lines: MCF7 and MDA-MB-231

Both of these cancers are adenocarcinomas, cancers of the breast epithelium tissue that originated in the mammary gland. The MCF7 breast cancer line was derived from an in situ carcinoma, meaning that the cancerous cells had not yet invaded surrounding tissues. It is hormone responsive in the sense that it expresses estrogen and progesterone receptors. Estrogen is a known tumor promoter. Mammary gland epithelium expresses estrogen receptor and the presence of estrogen stimulates the non-specific proliferation of cells. The advantage of a cancer being sensitive to hormones is that if the hormone receptor, in this case the estrogen receptor is blocked, then cell growth and cancer can be inhibited (Alkhalaf, 2003). On the other hand, the MDA-MB-231 breast cancer line was derived from a metastatic carcinoma and is not hormone sensitive; so, blocking the estrogen receptor in these cells will do nothing to inhibit the cancer. This line also expresses epidermal growth factor (EGF), an oncogene.

Clinically, the cancer of the MDA-MB-231 cell line is harder to treat (Sorlie, 2009). Relative to MCF7 cells, MDA-MB-231 cells grow faster and are more resistant to drug therapies. For instance, resveratrol is an antimicrobial substance that is naturally produced by plants when exposed to pathogens. It has gained attention due to its potential chemopreventative nature in fighting human cancers. While resveratrol hindered cell growth and activity in both MCF7 and MDA-MB-231 cells, only in the MCF7 cells was it able to induce apoptosis, the desired fate of cancerous cells (Pozo-Guisado, 2002). Furthermore, Akt is a downstream component of the PI3K intracellular signaling cascade, a key pathway in cellular survival. When Akt inhibitors are used in conjunction with the drug doxorubicin, the drug's anti-cancer effects are enhanced. MDA-MB-231 cells required Akt inhibitors in order for the growth inhibitory effects of doxorubicin to be maximized. On the other hand, the same level of growth inhibition is seen in MCF7 cells with use of solely Akt inhibitors, which did not augment the effects of doxorubicin (Wang, 2009). Noticeably, less aggressive methods are necessary when treating MCF7 cells than when treating MDA-MB-231 cells.

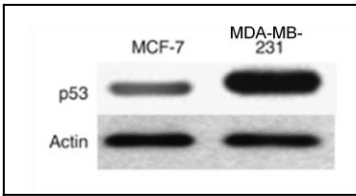

Figure 8. p53 protein expression levels in MCF7 and MDA-MB-231 cells. Same actin levels control for loading (Hui, 2006).

These MCF7 and MDA-MB-231 cell lines differ further in the p53 that they express. The MCF7 cells have wild-type p53, whereas the MDA-MB-231 cells have mutant p53 due to an arginine to lysine mutation at position 280 (Gurtner, 2010). In human cancers, when p53 is mutant, it is common for the mutant protein to be more stable than the wild-type one and therefore, is present at greater levels (Hupp, 2000). This appears to be the case when comparing p53 expression in MCF7 and MDA-MB-231 cells (Figure 8). Mutant p53 does not retain the tumor suppressing abilities of the wild-type. In fact, mutant p53 often exhibits oncogenic characteristics. For example, phospholipase D (PLD) aids in the survival of cancer cells. In MDA-MB-231 cells, PLD levels are increased in comparison to MCF7 cells, and their elevated activity is required to sustain the mutant p53 and their over expression (Hui, 2006). This is contrary to the MCF7 cells, where PLD activity actually inhibits wild-type p53. The clinical and molecular differences characteristic to each breast cancer will allow for significant comparison of Mediator and p53 interactions between MCF7 and MDA-MB-231 cells.

IV. Hypothesis

Both MCF7 and MDA-MB-231 cell lines have transcriptionally active p53. However, MDA-MB-231 cells have a p53 gain-of-function, hyperactive mutation, whereas MCF7 expresses wild type p53. Due to the pivotal role both wild type and mutant p53 play in cancer, it is essential to identify the factors that control p53's activity. The clinical features distinct to each breast cancer cell line are evidence of their dramatically different gene expression profiles (Kao, 2009). The implication follows that the identities and levels of p53 co-regulatory factors will also be very different in each cell type. Past studies have argued that different cancers possess different p53 activities, leading to different downstream responses depending on the cell type (Yu, 1999).

For instance, CXCR4 is a chemokine receptor that is likely to be involved in the motility and metastasis of breast cancer cells. Wild type p53 expressed in MCF7 cells repress the activity of CXCR4, while the cancer specific p53 mutation of MDA-MB-231 cells fails to repress CXCR4 expression (Mehta, 2007). A similar hypothesis is that the subunit composition of Mediator may vary from one cell type to the other. Due to previous research done on the interactions between p53 and Mediator, it can be presumed that since MCF7 and MDA-MB-231 cells have different p53 networks, the p53-Mediator relationships between the two will also differ (Meyer, 2010). To determine these differences and to

identify differences expected in the p53 network in each cell type, a comprehensive and unbiased approach will be taken to identify Mediator, p53 network and p53-Mediator complexes in both breast cancers, using Mass Spectrometry. By elucidating differences between the two cell types, distinct aspects of either the p53 pathway or Mediator can be targeted to change the clinical features of MCF7 and MDA-MB-231 and make treatment of these cancers more manageable.

Results

I. Project Overview

In order to analyze Mediator, nuclear extract needs to be generated. This was done by culturing the breast cancer cells on tissue culture plates and then isolating the nuclei from these cells. Purification steps carried out on the nuclei resulted in the final form of the nuclear extract (see experimental procedures). These extract samples were used in the prospective experiments.

To isolate Mediator in MCF7 or MDA-MB-231 cells, immunoprecipitations (IP) were carried out on the nuclear extracts using an antibody that binds the MED1 subunit of Mediator. Mass spectrometry (MS) analysis of these MED1 IP samples will define the exact Mediator subunit composition in each cell type. In addition, MS analysis might identify cofactors that interact with Mediator. Next, to identify the cofactors that specifically interact with Mediator when bound to p53, p53-Mediator samples were isolated using a GST-p53 affinity column. Comparative MS analysis of p53-Mediator and Mediator IP samples allows for identification of cofactors that selectively bind to the p53-Mediator complex.

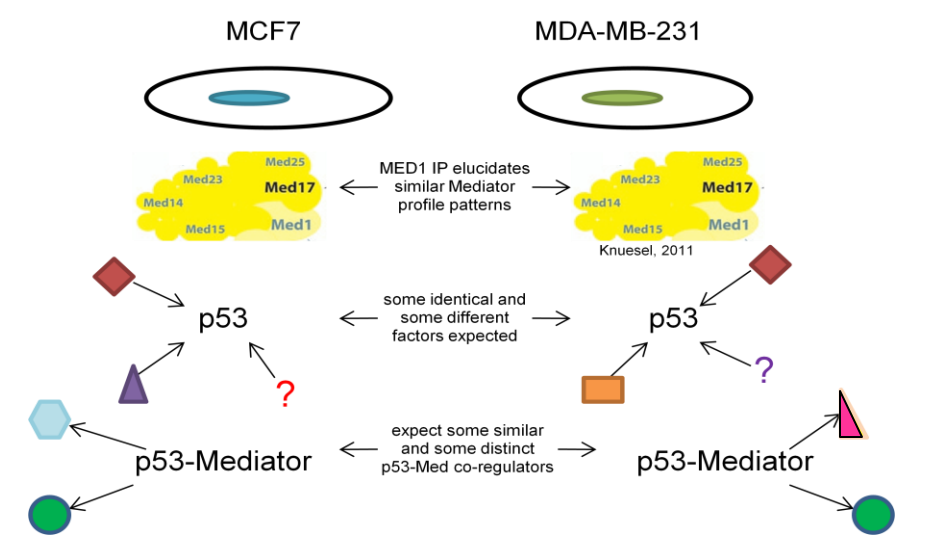

Figure 9. Visual representation of potential factors specific to either p53 or p53-Mediator and factors that may differ between each breast cancer cell line.

Additionally, other co-regulators may be present in the p53 network that do not interact with Mediator, but rather directly with p53 (Figure 9). Therefore, p53-Mediator complexes need to be separated from factors bound to p53 directly. Since Mediator is so large (1.2-1.8MDa), this separation is

possible by carrying out glycerol gradient sedimentations. An advantage of this protocol is that it allows for comprehensive analysis of potential changes in both the p53 network (i.e. factors bound directly to p53) and the p53-Mediator complex in each clinically distinct breast cancer cell line. Each cell type generated two sets of samples: the early fractions of the glycerol gradient that were comprised of p53 network factors, and the late fractions that included the larger p53-Mediator network.

II. Immunoprecipitation of the Mediator Complex

The technique of immunoprecipitation employs the use of an antibody to precipitate out a specific protein or protein complex in solution (see experimental procedures). TRAP220/MED1 antibody was used to isolate Mediator from the MCF7 and MDA-MB-231 nuclear extracts. Incubation of the antibody with the extract allows isolation of Mediator, because the antibody recognizes and binds specifically to the MED1 subunit of Mediator. Carrying out high salt washes on the immobilized antibody resin removes extraneous material and allows for isolation of relatively pure Mediator samples. The use of the detergent sarkosyl as the eluting agent removes Mediator from the antibody resin by denaturing all proteins bound to the resin (Figure 10A). As a control, samples of the A/G resin beads were loaded alongside a sample of the eluted material. Proteins bound and eluted from the resin were visualized with silver staining.

The use of the same antibody to precipitate out Mediator in each cell line allows for side by side analysis of the two cell samples. Both display the expected Mediator polypeptide bands, as shown in the silver-stained gels in Figures 10B and 10C. Twelve different samples of MCF7 and nine different samples of MDA-MB-231 extract were used to carry out twelve and nine separate IPs, respectively. This volume of material is need for reliable protein identification using MS.

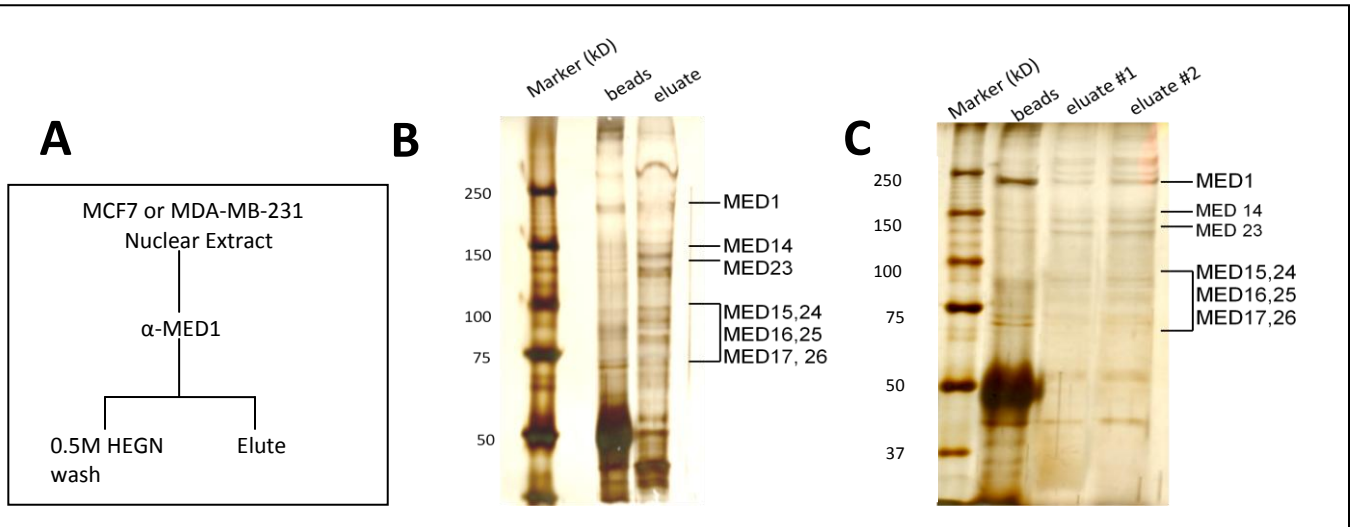

Figure 10. (A) Purification scheme showing how nuclear extract material was prepared for IP. SDS-PAGE and silver stain analysis of MED1 IP with MCF7 **(B)** and MDA-MB-231 **(C)** cells. Both cell types exhibit the distinct MED1 subunit band at 220kDa.

III. Isolation of p53-Mediator and other p53-associated factors

To observe p53-Mediator interactions in each cell line (MCF7 or MDA-MB-231), GST-p53 was first recombinantly expressed from E. coli. The activation domain of p53 (residues 1-70) was used instead of the full-length p53 protein (393 residues). This minimizes isolation of contaminants and

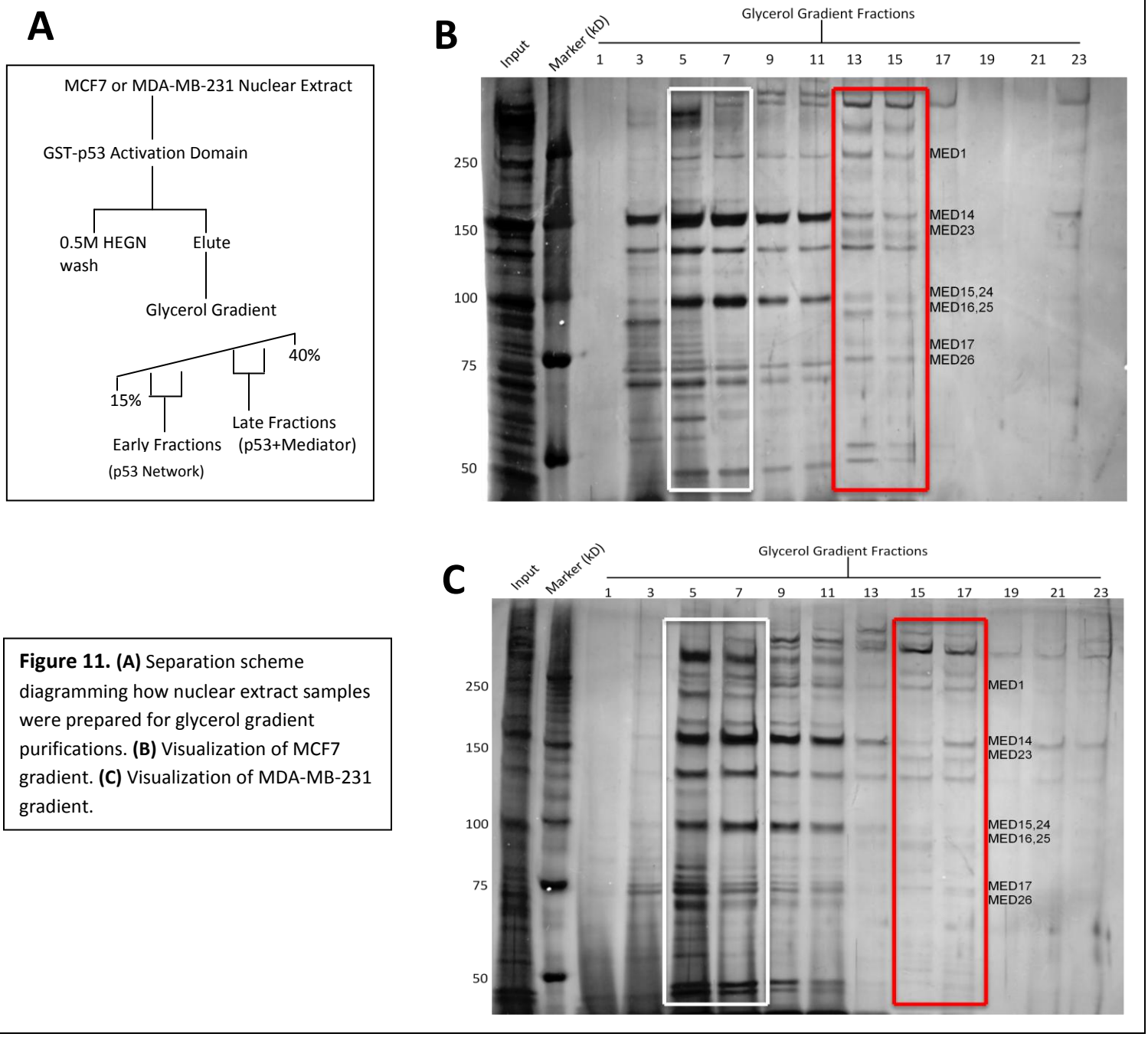

Figure 11. **(A)** Separation scheme diagramming how nuclear extract samples were prepared for glycerol gradient purifications. **(B)** Visualization of MCF7 gradient. **(C)** Visualization of MDA-MB-231 gradient.

ensures that coregulatory factors important for transcription, including Mediator, are isolated. The GST-p53 activation domain was purified and immobilized onto GSH-sepharose beads, which were then used to carry out pull downs from nuclear extracts derived from each cell type. The eluted p53-bound material was then loaded onto a 15-40% glycerol gradient (Figure 11A). Glycerol gradient centrifugation is another means of protein purification. In centrifuge tubes, a linear gradient is created with the highest concentration of glycerol (40%) at the bottom of the tube and lowest concentration (15%) at the top. When spun at high speeds (50,000RPM), the larger protein complexes (e.g. Mediator) migrate towards the bottom of the tube faster than the smaller protein complexes or individual polypeptides. By taking 100µL fractions, different p53 and p53-Mediator complexes are sequestered in each fraction. SDS-PAGE and silver stain analysis of these fractions allows for visualization of the distinct protein subunits present in the different fractions. Twelve different MCF7 samples and ten different MDA-MB-231 samples were used to yield twelve and ten different gradients. This volume of material is necessary for comprehensive MS analysis and protein identification.

The early fractions of the glycerol gradients, highlighted by the white boxed lanes in Figures 11B and 11C, are representative of the p53 network in each individual breast cancer. These samples represent individual proteins or smaller complexes bound to p53. This typically includes fractions 5-8 of each gradient. Since MCF7 and MDA-MB-231 cells express different sets of regulatory cofactors that affect p53 activity, the lanes representing these early fractions may have different band profiles between the two cell lines. Planned MS analysis of these fractions will identify these cofactors. The late fractions of interest, emphasized by the red boxed regions in Figures 11B and 11C, illustrate p53-Mediator complexes. Most commonly, Mediator appears in fractions 13-17, depending on analysis of each individual sample. These fractions exhibit the typical Mediator protein profile, as evidenced by the individual bands representing the different subunits that comprise the complex (Figures 11B and 11C). The differences in p53-Mediator interactions between MCF7 and MDA-MB-231 cells can begin to be assessed by examining the bands that differ between the two in the late fraction regions. However, to most reliably identify similarities and differences within p53-Mediator or the p53 network in each cell type, mass spectrometry will be used.

IV. Mass Spectrometry

Use of MS allows for comprehensive characterization and analysis of the protein components that compose the different samples: MED1 IP, early fractions (p53 network) and late fractions (p53-Mediator) of the glycerol gradients. Furthermore, comparison of the MS data of the breast cancer cell lines will elucidate factors that differ between the two. All samples were prepared equally with minimal variance in the conditions. They were first digested with trypsin to generate a peptide mixture that is able to be analyzed by liquid-chromatography-tandem mass spectrometry (LC-MS/MS). The trypsinized peptide mixture is first separated by the liquid chromatography system, allowing the mass spectrometer to measure the mass to charge ratio of each peptide and then determine the individual molecular masses of the peptides (Figure 12). Searching the results in a human protein database identifies the peptides present in the original sample. Mass spectrometry sample preparation and data generation was carried out by Chris Ebmeier of the Taatjes Lab.

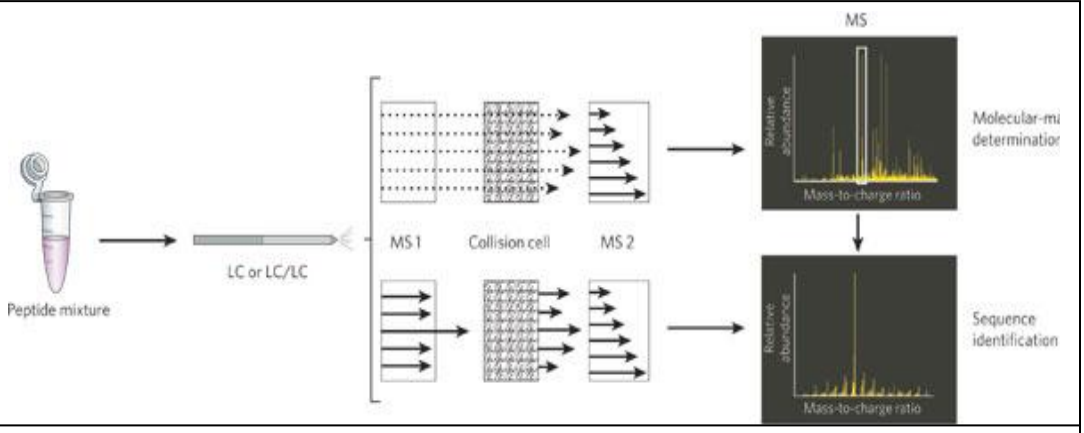

Figure 12. Sample preparation for mass spectrometry analysis (Cravatt, 2007).

The MED1 IP samples generated from MCF7 and MDA-MB-231 were subject to MS analysis. The results were searched against the human protein database ipi_Human_v3_75. Each peptide that is identified is given a spectral count of one. A 1% false discovery rate was used, meaning that each peptide has a 99% probability of being correctly identified (Old, 2005). The results obtained are qualitative and not normalized based on spectral counts. Between the two samples a total of 4424 unique peptides and 1431 unique proteins were found. Numbers of peptides and proteins specifically found in each breast cancer cell line

	MDA-MB-231	MCF7
unique peptides: 4424	unique proteins: 1431	
IP Samples	9	12
Peptides	12051	6721
Proteins	1181	712
Mediator Spectral Counts	460	429

Table 2. Number of peptides and proteins identified in each breast cancer cell line via MS analysis.

sample are listed in Table 2. More peptides and proteins are identified in the MDA-MB-231 cell sample than the MCF7 sample.

All of the Mediator subunits were identified (Table 3). The MDA-MB-231 sample produced 460 total Mediator spectral counts while the MCF7 samples produced 429. Data was then further separated by factors that were exclusively found in the MDA-MB-231 cell line and the MCF7 line (Appendices A and B). This allows for significant comparison and analysis of proteins and potential gene pathways that are similar and different between the two cell lines.

Discussion

Evidence compiled from the MED1 immunoprecipitation data suggests that both MCF7 and MDA-MB-231 cells express similar Mediator complexes. This was as expected, because all eukaryotes express Mediator as a part of the transcription machinery. As figures 10B and 10C show, both breast cancer cell lines possess similar peptide patterns resulting from MED1 IP purification. However, the silver stained gels do not quantify the amount of each Mediator subunit that is present. Previous data has shown that among different breast cancer lines, different Mediator subunits may be expressed at different levels (Kao, 2009). Consistency in Mediator subunits across the various cell types is not guaranteed.

MS data presented in Table 3 reveals that MCF7 cells and MDA-MB-231 cells share similar Mediator subunit profiles. Although analysis is qualitative and not quantitative, and spectral counts differ between the two cancer cell types, each line expresses nearly all of the Mediator subunits (Table 3). These results are consistent with expectations, because Mediator functions at all pol II promoters.

Gene	MDA-MB-231 Spectral Counts	MCF7 Spectral Counts	MW (Da)
MED1	**4**	**2**	168478
MED10	**6**	**6**	15688
MED11	**0**	**3**	13129
MED12	**60**	**71**	243081
MED12L	**0**	**2**	240032
MED13	**53**	**35**	239318
MED13L	**19**	**19**	242602
MED14	**24**	**28**	160607
MED15	**12**	**28**	86753
MED16	**24**	**8**	96793
MED17	**32**	**24**	72876
MED18	**3**	**0**	24453
MED19	**4**	**2**	26273
MED20	**13**	**10**	23222
MED21	**5**	**10**	15564
MED22	**1**	**6**	16480
MED23	**26**	**46**	156194
MED24	**59**	**45**	110305
MED25	**13**	**4**	84389
MED26	**10**	**2**	65446
MED27	**8**	**8**	35432
MED28	**0**	**3**	19520
MED29	**1**	**7**	23473
MED30	**7**	**5**	20277
MED31	**3**	**3**	15805
MED4	**28**	**14**	29745
MED6	**5**	**1**	29298
MED7	**11**	**10**	27245
MED8	**6**	**10**	32819
MED9	**7**	**2**	16403
CCNC	**4**	**4**	33243
CDC2L6	**12**	**11**	56802

Table 3. Mediator subunits identified by mass spectrometry in MED1 IP purified MCF7 and MDA-MB-231 samples.

Factors found exclusively in each cell line with a spectral count of 4 and above were separately searched against the HEFalMP database of genes that are relevant to pol II transcription in breast cancer. HEFalMP utilizes hundreds of publicly available genome databases to generate functional maps of the human genomes. When a list of genes, cellular processes and diseases are specified, the site is able to make educated predictions on possible gene pathways (Huttenhower, 2009). The relationship

between the MDA-MB-231 or MCF7 genes and the breast cancer gene set is given a p value, with a value of 0 indicating the most significant relationship. The most compelling gene relationships are presented in Table 4.

Gene	Name	MDA-MB-231 p-value	MCF7 p-value
PHB	prohibitin	0	-
TSG101	tumor susceptibility gene 101	0	0
PPM1D	protein phosphatase 1D magnesium-dependent	0	3.34E-06
PIK3CA	phosphoinositide 3-kinase, catalytic	0	2.00E-05
BRCA1	breast cancer 1, early onset	0	-
BARD1	BRCA1 associated RING domain1	3.34E-06	-
AKT1	v-akt murine thyoma viral oncogene	3.34E-06	-
RB1CC1	RB1-inducible coiled-coil 1	6.68E-06	-
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene	4.21E-03	0.0126

Table 4. Breast cancer genes that were found to have a significant relationship with the collection of factors that are exclusive to each breast cancer cell line.

One of the most noteworthy links presented in both breast cancer cell types is with the PPM1D gene (also known as Wip1), which encodes a protein that is a part of the PPM family of Ser/Thr phosphatases. This family is known to negatively regulate cell stress response pathways, including the p53 signaling pathway. The activation of p53 by DNA damage induces PPM1D. Selective inactivation of the p38 MAP kinase by PPM1D leads to suppression of p53 and negative regulation of subsequent tumor suppressor pathways. In many different cancers, including 11-16% of primary breast cancers, PPM1D is known to be amplified (Rayter, 2008). Other functions of PPM1D that may contribute to its oncogenic effects include regulation of the progesterone receptor, base excision pathway of DNA repair, and activation of ATM (ataxia-telangiectasia mutated). These factors in conjunction with the data gathered from the MDA-MB-231 and MCF7 samples suggest that PPM1D and/or associated factors could be a potential therapeutic target. The integral role that PPM1D plays specifically in the p53 pathway alludes to is likely importance in cancer growth and regulation in MDA-MB-231 and MCF7 cells.

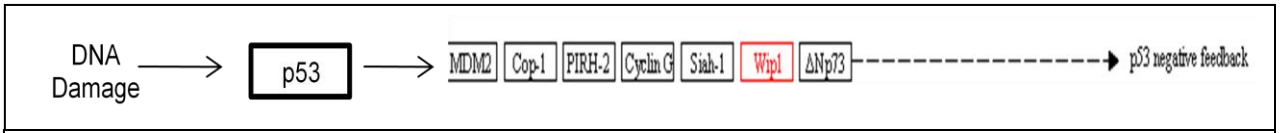

Figure 13. The role of PPM1D (highlighted in red as Wip1) in the p53 negative feedback pathway (http://www.genome.jp/kegg-bin/highlight_pathway).

Other important genes to note in Table 4 include BRCA1 (breast cancer 1, early onset), which is expressed in breast tissue cells, and helps repair damaged DNA or destroy cells if the DNA cannot be repaired. Abnormal BRCA1 greatly increases risk of breast cancer. Also, TSG101 may play a role in cell differentiation and acts as a negative growth regulator; it has been found to be frequently mutated in breast cancers. KRAS is a proto-oncogene that acts as a molecular switch in the Ras pathway. Although these genes are not directly integrated within the p53 pathway, they appear to have a significant impact on the molecular networks of the MDA-MB-231 and MCF7 cancer, and could be potential therapeutic targets, pending further research and analysis of their specific roles within each breast cancer cell line (Kao, 2009).

Comparison of MS data reveals that there are more factors exclusive to the MDA-MB-231 cell line versus the MCF7 cell line. This may be due to the fact that the first breast cancer cell type is overall more aggressive, and genes that are not usually expressed under normal conditions are now up regulated. Alternatively, more peptides were identified in the MDA-MB-231 MS analysis. In both MCF7 and MDA-MB-231 cells, the binding of p53-AD to Mediator induces similar, if not identical conformational changes in Mediator, thus revealing the same, previously unexposed protein domains. However, the differences between the two breast cancer lines lie in the fact that many more protein factors are present in the MDA-MB-231 cell. Different cancers have different gene expression patterns, as evidenced by the genes found solely in MCF7 cells versus MDA-MB-231 cells (Jones, 2008). The proteins that are exclusive to the MDA-MB-231 cells may be able to bind to the newly revealed domains of Mediator and alter global gene expression patterns. By contrast, such factors are absent from MCF7 cells. Thus, whereas the same Mediator domain becomes exposed upon p53AD binding, a subsequent interaction with the cofactor may not occur in MCF7 cells.

Future Directions

The conditions of glycerol gradient purifications allow larger protein complexes to be left whole and intact for analysis. For both MCF7 and MDA-MB-231 cells, the regions of the glycerol gradients highlighted as the early fractions show evidence of a diversity of factors within the p53 network. This is expected since both breast cancer cell lines express p53. MCF7 cells express wild type p53, while MDA-MB-231 cells express a hyperactive, mutant p53. In the later fractions of the glycerol gradients, it is apparent that some bands become fainter and even disappear as others appear and become darker. These changes represent the different protein complexes migrating throughout the gradient. The p53-Mediator interaction induces conformational shifts that may reveal previously inaccessible binding sites. This may stimulate the binding and activation of various co-activators that activate other genes.

To characterize the proteins that compose the "p53 network" and p53-Mediator fractions that were generated using glycerol gradient purifications, mass spectrometry will be carried out on these samples. Analysis of these samples will be similar to the analysis of the MED1 IP samples (Figure 12). This will identify additional protein factors that are similar and different between the two breast cancer cell lines. Further analysis of the proteins that are differentially represented between the two breast cancer cell lines will determine their role in specific gene pathways that may or may not be a part of the p53 network and/or Mediator interaction. These unique factors and potential transcriptional co-regulators may contribute to the clinical differences between the two cell lines (Sorlie, 2001). Such clinical differences include drug resistance, invasiveness and overall aggressiveness. The next step would be determining if manipulating the expression of the protein(s) in tissue culture will affect the clinical characteristics of the breast cancer, or if the protein is a potential therapeutic target.

Experimental Procedures

Protease Inhibitors : 1mM Benzamidine HCl, 1mM Sodium Metabisulfite, 1mM Dithiothreitol

Cell Cultures

MCF7:

Cells are cultured in Roswell Park Memorial Institute (RPMI) medium with 10% Fetal Bovine Serum (FBS) and 10% Penicilin-Streptomycin (Pen-Strep). Cell grow adhered to a surface and are incubated at 37°C in 5% carbon dioxide. When splitting cells to a higher passage number, wash cells twice with Dulbecco's Phosphate Buffered Saline (DPBS) and then use 0.05% trypsin to remove adhered cells from the surface of the culture plate.

MDA-MB-231:

Cells are cultured in Leibovit's L-15 medium with 10% FBS and 10% Pen-Strep. Cells grow adhered to a surface and are incubated at 37°C and 0% carbon dioxide. When splitting cells, wash cells twice with DPBS and then use 0.25% trypsin to release the adhered cells from the surface of the culture plate.

Isolating Cell Nuclei

For both MCF7 and MDA-MB-231:

Remove 90% of the media from the culture plate. Using a plastic cell scraper, scrape cells off of the plate and pipet into a 50mL conical tube. Keep cells on ice at all times. Centrifuge tubes at 4000RPM and 4°C for 20 minutes. Remove the supernatant and measure the packed cell volume (PCV). Resuspend the cell pellet in twice the PCV PBSM (1X phosphate-buffered saline, 0.5% $MgCl_2$) plus protease inhibitors. Spin the cells at 4000RPM and 4°C for 10 minutes. Thoroughly resuspend the cell pellet in five times the PCV Buffer A (10X Buffer A: 100 mM HEPES, 15 mM $MgCl_2$, 100 mM KCl, pH=7.9) plus protease inhibitors. Spin the cells at 4000RPM and 4°C for 10 minutes. Let the cells sit on ice for 20 minutes. Spin the cells at 4000RPM and 4°C for 10 minutes. Remove the supernatant. Resuspend the cell pellet in two times PCV Buffer A. Using a type A dounce homogenizer, dounce the cells five times. Spin cells at 2800RPM for 10 minutes. At this point, the upper and lower phases should be apparent. Remove the cytoplasm upper phase with a pipet. Save the cell nuclei lower phase. Flash freeze the lower phase in liquid nitrogen and then store the cell nuclei at -80°C.

Nuclear Extract Preparation

For both MCF7 and MDA-MB-231:

Thaw cell nuclei pellet and then keep on ice. Add 90% nuclei volume of Buffer C (20mM HEPES, 24% glycerol, 0.4M NaCl, 1.5 mM MgCl2, 0.5M EDTA, pH 7.9) plus protease inhibitors. Using a type B glass homogenizer, dounce the nuclear material twenty times. Rock at 4°C for 30 minutes. Spin at 15000RPM and 4°C for 30 minutes. Keep the supernatant. Place dialysis tubing in 1L of Buffer D (20 mM HEPES, 20% glycerol, 100 mM KCl, 2 mM MgCl2, 0.2 mM EDTA) plus protease inhibitors to equilibrate tubing. Then, place supernatant in dialysis tubing and dialyze to 0.1M salt (roughly two hours). Aliquot supernatant into 1.5mL tubes. Flash freeze in liquid nitrogen and then store nuclear extract at -80°C.

Immunoprecipitation

For both MCF7 and MDA-MB-231:

Combine 40µL of Protein A resin beads and 40µL of Protein G resin beads in a 1mL eppendorf tube. Wash beads twice with 0.15M HEGN (20mM HEPES, 5µM EDTA, 10% glycerol, 1mL 10% NP-40,

75mM KCl). Spin after each wash at 4000RPM and 4°C for one minute. Remove supernatant. Add 80µL of 0.15M HEGN to resin beads. Add 80µL of MED1 antibody. Flick eppendorf to resuspend beads and then rock at 4°C for 90 minutes. While tube is rocking, thaw 1mL of nuclear extract and then spin at 14000RPM and 4°C for 20 minutes. Keep nuclear extract supernatant. When eppendorf tube with beads and antibody is finished rocking, spin at 4000RPM and 4°C for one minute. Wash beads three times with 1mL of 0.5M HEGN plus protease inhibitors. Then wash beads once with 0.15M HEGN plus protease inhibitors. Spin between each wash at 4000RPM and 4°C for one minute. After washes are complete, add the 1mL of nuclear to the A/G resin beads. Rock at 4°C for three hours. After rocking, spin at 4000RPM and 4°C for one minute. Wash beads five times with 1mL of 0.5M HEGN. Then wash beads once with 1mL 0.15M HEGN. Spin beads between each wash at 4000RPM and 4°C for one minute. Make elution buffer by combing 800µL 0.15M HEGN and 200µL of 10% sarkosyl. Remove supernatant from beads after last wash and then add 80µL of elution buffer to the beads. Rock at 4°C for 30 minutes. After rocking, spin at 4000RPM and 4°C for one minute. Pipet off 70µL of supernatant and place into a filter tube. Spin filter tube at 4000RPM and 4°C for one minute. Add another 80µL of elution buffer to the tube with the A/G resin beads. Rock for another 30 minutes at 4°C. Spin tube at 4000RPM and 4°C for one minute. Pipet off all of the eluted material and place into the original filter tube. Spin filter tube at 4000RPM and 4°C for two minutes. To prepare samples to be run on the gel, add 10µL of 2X loading buffer to 10µL of the eluted material. Load 12µL in the gel. To the beads, add 10µL of 2X loading buffer. Load 3µL into the gel.

Silver Stain

Shake gel in 50% methanol for 10 minutes, 5% methanol for 10 minutes, 3.5µM DTT for 5 minutes and silver nitrate solution (250mg silver nitrate, 25µL formaldehyde in 250mL of water) for 10 minutes. Wash the gel twice with cold water. Then wash the gel twice with developing solution (15g sodium carbonate, 250µL formaldehyde in 500mL of water). Shake gel in developing solution until the desired intensity is reached. Quench with citric acid monohydrate until neutral pH. Transfer gel to water.

Growth/Expression of GST-p53 protein

Day 1: Take 20µL of BL21 E. coli cells with the p53 codon and add to 1mL of luria broth (LB) in a 1mL eppendorf tube. Shake at 37°C for one hour. Prepare an ampicilin agar plate. Prepare LB by placing a LB dry pellet in a 2.8L flask with 1L of mili-Q water. Autoclave for 45 minutes. After cells are finished rocking, place 3µL onto the ampicilin placte. Keep plate upside down and incubate at 37°C overnight.

Day2: Pick a single colony (large and isolated from other colonies) with a 10µL filter tip and add it to 5mL of LB in a culture tube. Let the culture grow by rocking at 37°C overnight.

Day 3: Prepare ampicilin by adding 100mg ampicilin to 1mL of water. Add this to the 1L of autoclaved LB. Add 3mL of the overnight culture to the LB. Shake the 1L culture at 37°C. Let the culture grow until the OD_{600}=0.6-0.8 (about 3-4 hours). When the OD_{600} reads the optimal absorbance value, reduce the temperature to 30°C and induce expression with 0.4mM IPTG (Isopropyl-β-D-thiogalactoside). Shake at 30°C for another 4 hours. Spin the cells at 4000RPM and 4°C for 10 minutes. Pour off supernatant into a container with bleach. Keep the cells on ice. Resuspend the cell pellet in cold 1X PBS (phosphate-buffered saline) plus protease inhibitors (45mL 1XPBS/500mL of cells). Spin resuspended cells at 2750RPM and 4°C for 10 minutes. Remove supernatant. Flash freeze the pellet in liquid nitrogen and store at -80°C.

GST-p53 Purification/GSH-Sepharose Immobilization

Thaw the cell pellet and then keep on ice. Resuspend the thawed cell pellet in H/E buffer (50mM Tris, 0.5M NaCl, 5µM EDTA, 10% glycerol, 25mL 10% NP-40) plus protease inhibitors (5mL H/E /250mL cells). Lyse cells by sonicating three times for 30 seconds, with one minute breaks in between. Centrifuge lysate at 4000RPM and 4°C for 30 minutes. While lysate is spinning, prepare GSH-Sepharose beads. In a 15mL conical tube, add 150µL of glutathione beads (300µL of the bead slurry). Add 5mL of H/E to the beads and spin at 2000RPM and 4°C for 5 minutes. Remove buffer from the beads. When lysate is finished spinning, pour supernatant over the GSH-Sepharose beads. Rock the beads at 4°C for one hour. Then spin beads at 2500RPM and 4°C for 5 minutes. Remove supernatant. Wash beads five times with 5mL each time of High Salt buffer plus protease inhibitors (1M NaCl, 50mM Tris, 5µM EDTA, 10% glycerol by volume, 50mL 10% NP-40 NP-40, 8mM CHAPS). Then wash beads two times with 5mL each of H/E plus protease inhibitors. Finally, wash once with 5mL of 0.15M HEGN. Spin between each wash at 2000RPM and 4°C for one minute. After last wash, remove most of the buffer, leaving ~250µL covering the beads. Store beads at 4°C for a maximum of seven days.

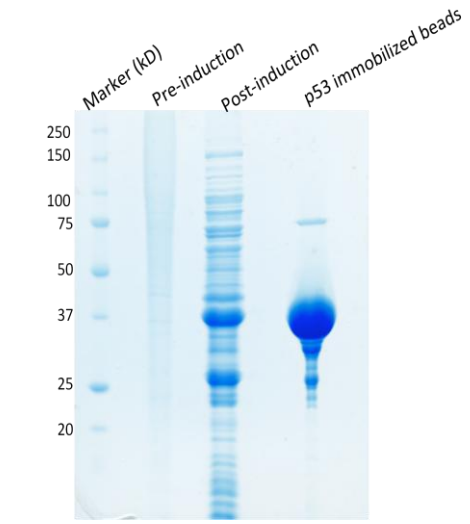

Figure 14. Work up of GST-p53AD.

Nuclear Extract Pull-down

For both MCF7 and MDA-MB-231:

Thaw 1mL of nuclear extract and then spin at 15000RPM and 4°C for 25 minutes. On ice, pipet 100µL of GST-p53 immobilized beads into a 1.5mL tube. Spin beads at 2000RPM and 4°C for five minutes. Remove the supernatant buffer on top of the beads. After nuclear extract is finished spinning, add the supernatant to the beads. Rock overnight at 4°C. Spin tube at 2000RPM and 4°C for two minutes. Was five times with 1mL each of 0.5M HEGN plus protease inhibitors. Then wash once with 1mL of 0.15M HEGN. Spin in between each wash at 2000RPM and 4°C for one minute. Remove supernatant from beads. Elute with bead volume of 30mM GSH in Tris Elution buffer (0.15M salt). Rock at 4°C for 30 minutes. Then spin at 2200RPM and 4°C for five minutes. Pipet off supernatant into a microfilter tube. Spin microfilter tube at 6000RPM and 4°C for three minutes. Add an additional bead volume of elution buffer to the beads. Rock again at 4°C for 30 minutes. Spin again at 2200RPM and 4°C for two minutes. Place supernatant into the original microfilter tube. Spin microfilter tube again at 6000RPM and 4°C for three minutes. To prepare a gel sample, add 5µL of eluted material to 5µL of 2X loading buffer. Load 9µL into the gel.

Glycerol Gradient

Pour a 15%-40% glycerol gradient into 2mL Beckman Centrifuge Tubes. Very carefully, transfer the eluted material from nuclear extract pull-down to the top of the gradient in the tube. Spin tubes at 55000RPM and 4°C for 6 hours. Pipet off 100µL fractions. To prepare gel samples, add 5µL 4X loading buffer to 10µL of the gradient fraction sample. Load 13µL.

Mass Spectrometry

All mass spectrometry work was done by Chris Ebmeier. The specific protocol can be found in the methods sections of "Molecular Architecture of the Human Mediator – RNA Polymerase II – TFIIF Assembly" by Carrie Bernecky et al. in *PLoS Biology* 9(3), 1-18.

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Appendix

Gene	MDA-MB-231 Spectral Counts	MCF7 Spectral Counts	MW (Da)	Gene	MDA-MB-2313 Spectral Counts	MCF7 Spectral Counts	MW (Da)
VIM	**156**	**0**	53652	HNRNPL	**14**	**0**	64133
FLNC	**98**	**0**	291022	PLIN3	**14**	**0**	45803
AKAP2	**35**	**0**	122071	SMN1	**14**	**0**	31849
AP2B1	**30**	**0**	104553	TRIP11	**14**	**0**	227639
TPM3	**28**	**0**	28922	EIF4G1	**13**	**0**	175619
DHX15	**27**	**0**	90933	MYO1B	**13**	**0**	
DST	**26**	**0**	633667	PPP1R12C	**13**	**0**	84881
HIP1R	**26**	**0**	119388	RBM39	**13**	**0**	59380
CKAP5	**25**	**0**	225495	RBM8A	**13**	**0**	19889
CALD1	**24**	**0**	93250	ATP2A2	**12**	**0**	109691
SNRPD2	**23**	**0**	13527	CDC42BPB	**12**	**0**	194315
RP11-631M21.2	**21**	**0**		CPSF6	**12**	**0**	59210
SFRS1	**20**	**0**	27745	RAD50	**12**	**0**	138432
SFRS2	**20**	**0**	25476	SNRPE	**12**	**0**	10804
TPM1	**19**	**0**		SQSTM1	**12**	**0**	47687
IMPDH2	**18**	**0**	55805	TLN1	**12**	**0**	269767
RPL18	**18**	**0**	21634	DDX23	**11**	**0**	95583
UHRF1	**18**	**0**	91100	LRPPRC	**11**	**0**	157905
EHD1	**17**	**0**	60627	LYAR	**11**	**0**	43615
LUZP1	**17**	**0**	120275	RAI14	**11**	**0**	110423
RPL4	**17**	**0**	47697	SERPINH1	**11**	**0**	46441
AP2A1	**16**	**0**	105370	STAMBPL1	**11**	**0**	52199
LEPRE1	**16**	**0**	90616	HSP90AB1	**10**	**0**	
MYLK	**16**	**0**	203128	NEXN	**10**	**0**	80658
THOC4	**16**	**0**	27558	RPL3	**10**	**0**	46109
AP2M1	**15**	**0**	49655	RPL32	**10**	**0**	15860
KRT7	**15**	**0**	51418	RPS2	**10**	**0**	31324
MYL9	**15**	**0**	13866	SKIV2L2	**10**	**0**	117805
RPL14	**15**	**0**	23787	SRRM2	**10**	**0**	299615
TOP2A	**15**	**0**	183124	U2AF1	**10**	**0**	27872
ANXA1	**14**	**0**	38714				

Appendix A. Factors receiving a spectral count of 10 and above found only in the MDA-MB-231 breast cancer cell line.

Gene	Protein description	MDA-MB-23l Spectral Counts	MCF7 Spectral Counts	MW (Da)
SPTBN2	SPECTRIN BETA CHAIN, BRAIN 2	**0**	**39**	271295
EPPK1	EPIPLAKIN 1	**0**	**22**	555621
SYTL2	SYNAPTOTAGMIN-LIKE PROTEIN 2	**0**	**16**	197919
MYO5B	MYOSIN-VB	**0**	**14**	213756
CGN	CINGULIN	**0**	**13**	137057
FAM83H	Family with sequence similarity 83, member H	**0**	**13**	127101
LAD1	LADININ-1	**0**	**11**	57131
MYO1D	MYOSIN-ID	**0**	**10**	116202
Septin8	PROTEIN SEPT8	**0**	**10**	50928

Appendix B. Factors receiving a spectral count of 10 and above found exclusively in MCF7 cells.

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