Genetic regulation within cellular stress response pathways

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Abstract:

Cellular stress is something that a cell deals with on a regular basis. Fortunately, there are several different mechanisms that are set in place in order to deal with them. Depending on the stress present in the cell there will be different stress response pathways that are activated in order to mitigate the stress or initiate cellular death mechanisms. Transcription factors (TFs) are key components within these pathways, and this will be the focus of this thesis. TFs within mammalian stress response pathways are tasked with the essential regulation of multiple genes within these pathways and are oftentimes linked with diseases when mutated. ATF4 and P53 are two central TFs that work within the Integrated Stress Response and DNA damage response pathways respectively. This thesis will highlight important aspects of gene regulation within cell stress pathways and highlight these two central TFs and their roles in stress response pathways.

Chapter 1: Cellular Stress

In order for humans to maintain healthy physiological states, it is necessary that our cells also do the same. This means that our cells are required to have systems in place in order to mitigate issues that may arise due to physiological or pathological conditions. These cellular stress response systems are diverse enough to be able to reestablish homeostasis under a variety of different stress conditions such as DNA damage or viral infection. Understanding how these stress conditions work is essential in understanding how our cells utilize information coded within DNA in order to promote the expression of genes that are essential in reestablishing cellular homeostasis.

DNA, or deoxyribonucleic acid is the hereditary material that organisms rely on in order to maintain proper function. Almost every cell in an organism's body has DNA and a majority of this DNA is found in the nucleus where it is protected from degradation (Fabrini et al., 2010). In order for an organism to properly develop, survive and reproduce, DNA needs to be converted into proteins. In order for this to occur, DNA needs to undergo transcription followed by translation. Transcription is the process by which DNA is duplicated into messenger RNA (mRNA) to allow for export out of the nucleus. This RNA strand differs from DNA because it is single stranded, and it replaces one of nucleic acid bases for another. Once the mRNA is exported from the nucleus, it can then be converted into amino acids via the ribosome during translation (*National Institute of General Medical Sciences*, n.d.). These amino acids are finally folded into proteins that aid in the structure, function and regulation of the body's tissues and organs.

In order for cells to form the proteins it needs in order to survive; it needs specific instructions from DNA in order to do so. Within the 3 billion base pairs found in DNA, are specific nucleotide sequences that code for genes. Genes are the specific instructions that are used to make proteins. Genes can vary in size from a few hundred base pairs, and all the way up to 2 million bases. Each organism will have a set of genes that are shared between species,

and these usually code for essential biological functions. It is within this set of genes that we find the genes that are responsible for mitigating different cellular stress conditions. These genes work in conjunction with one another to activate different mechanisms during cellular stress conditions. These different mechanisms are what allow cells to maintain homeostasis and have been useful when trying to find therapeutic approaches to deal with certain diseases.

The Heat Shock Response

One set of genes that are essential in reestablishment of cellular homeostasis are the heat shock proteins. The heat shock proteins are a set of proteins that aid in the refolding of misfolded peptides and restrain protein aggregation. The heat shock response (HSR) was initially described as a biochemical response to an increase in temperature around 3-5℃ above normal physiological growth conditions, but it has been recognized to be activated in response to a wide variety of different stimuli (Fulda et al., 2010). One of the main roles that the HSR plays within the cell is protein damage that leads to an accumulation of unfolded proteins within the cell. This is where these specific heat shock proteins take action to help alleviate protein aggregation and helps the cell build up thermotolerance where cells become more resistant to what would be lethal temperature elevations (Samali et al., 1999).

One of the most extensively studied and evolutionary conserved heat shock proteins is heat shock factor 1 (HSF1). HSF1 is a transcription factor expressed in most issues and cell types and is primarily regulated through posttranslational mechanisms (Hetz et al., 2018). Activation of HSF1 is primarily caused by an increase in temperature but it can also be activated in response to any cellular stress that can cause the accumulation of unfolded proteins such as oxidative stress and heavy metals (Fulda et al., 2010). Under normal cellular conditions HSF1 is shuttled between the nucleoplasm and the cytoplasm, but during heat shock this process is halted and the increased accumulation of HSF1 in the nucleus triggers the HSR. Activation of the HSR will pause general protein transcription and translation to alleviate the aggregation of

proteins and the cell will begin to upregulate the other HSF's (Hetz et al., 2018). These other HSFs are HSF1, which is essential in the HSR, but also in developmental processes, HSF2 & HSF4 which are important for differentiation in addition to development (Anckar et al., 2011). HSF1 has also been found to be essential in the HSR after mice who have had HSF1 genetically silenced suffer from sensitivity to stress and are unable to induce heat responsive genes upon heat shock.

The way in which the HSR is activated is still under investigation, even after more than 20 years of first being discovered. Current literature supports two models for HSF1 heat induced activation. The first model supports that HSF1 acts as a thermosensor which is kept monomeric by intramolecular leucine zipper formation and prevents trimerization with the other heat shock proteins (Rabindran et al., 1993). The second model proposes that chaperones prevent HSF1 activation and upon the sudden increase in temperature, the accumulation of unfolded proteins will draw the chaperone away from HSF1 which allows for induction (Morimoto, 1998). Three different chaperones that potentially inhibit HSF1 and allow activation of the HSR are Hsp70, Hsp90 or TRiC/CCT (Raychudhuri et al., 2014).

One of the most recent papers to be published proposes a mix of these two models where in response to stress monomeric HSF1 is released from an inhibitory complex and then is allowed to trimeritze. This allows HSF1 to undergo a series of post-translational modifications that facilitate its movement into the nucleus and conversion to an active DNA binding component. These post translational modifications include phosphorylation, sumoylation, acetylation, and deacetylation, all of which are important to the activation of the HSR (Dai, 2018). The DNA binding motif that HSF1 binds to is a conserved sequence known as the heat shock element (HSE) and is found at the promoter of target genes. A portion of these target genes are other heat shock proteins which are responsible for a number of different functions including aiding the refolding of unfolded proteins and inhibiting apoptosis (Fulda et al., 2010). Altogether the HSR is one pathway in which the cell takes in order to mitigate cellular stress.

The Unfolded Protein Response

The unfolded protein response (UPR) is another stress dependent pathway in which the cell utilizes to alleviate the aggregation of unfolded proteins in the cell. The UPR differs from the HSR in signaling pathways and the genes it activates in response to unfolded protein accumulation. The UPR relies on a complex network of interconnected signaling pathways initiated by three signal transducers located in the ER known as IRE1, activating transcription factor 6 (ATF6) and protein kinase RNA (PKR)-like ER kinase (PERK) (Wang et al., 2016). Once the UPR is activated, it works to reduce protein synthesis and increase the degradation of misfolded proteins. In addition to this the UPR will work to upregulate genes that are involved in global proteostasis control (Walter et al., 2011).

One of the signaling pathways within the UPR makes use of PERK, a transmembrane kinase that during endoplasmic reticulum (ER) stress it prevents general protein translation through the phosphorylation of eukaryotic translation initiation factor 2 (eIF2ɑ) at serine 51 (Walter et al., 2011). The prevention of general protein synthesis helps to reduce the number of proteins entering the ER and the phosphorylation of eIF2ɑ allows for the preferential translation of activating transcription factor 4 (ATF4) (Harding et al., 2000). This leads to an increase in the efficacy of ER protein folding, reinforcement of an antioxidant response and upregulation of macroautophagy (Harding et al., 2003). ATF4 is essential in inducing the expression of essential genes such as ATF3 and CHOP.

The IRE1 signaling pathway arm of the UPR is one of the most conserved and is widely expressed in mammalian cells. One isoform of IRE1, IRE1ɑ undergoes a conformational change upon ER stress which allows for the expression of a transcription factor called XBP1(Imagawa et al., 2008). XBP1 is unique because it is able to interact with a number of different genes depending on the tissue context due to its ability to form heterodimers with other transcription factors (Hetz, 2012). The spliced version of XBP1 is involved in mechanisms that allow for increased protein folding and chaperoning within the ER, in addition to an increased

flux of protein degradation via the proteasome and autophagy (Frakes, 2017). IRE1α is also involved in a process known as regulated IRE1-dependent decay (RIDD) which is able to degrade mRNAs that are localized to the ER (Hollien et al., 2009).

The last signaling pathway within the UPR is the ATF6 signaling pathway. ATF6 is a type II transmembrane protein that contains a bZIP transcription factor on the cytosolic domain. Upon the presence of ER stress, ATF6 translocates from the ER to the nucleus via the Golgi apparatus (Haze et al., 1999). Similar to RIDD, the exact mechanism by which ATF6 is able to migrate into the nucleus by way of the Golgi apparatus is still unknown. One theory is based on the way in which sterol regulatory element binding proteins (SREBPs), which are transcription factors important in the synthesis of cholesterol (Brown et al., 1997). These proteins rely on a SREBP cleavage activating protein (SCAP) to transport the SREBP to the Golgi complex where regulated intramembrane proteolysis (RIP) occurs to allow for migration into the nucleus (Sakai et al., 1998) (Duncan et al., 1997). Currently there has been no evidence of a molecule that acts in a similar manner to SCAP for ATF6, so while the SREBP transport model is one of the models which fits ATF6 transport the closest, there are still questions that remain.

DNA Damage Response

Since DNA is vital for our cells to have the correct set of instructions to synthesize proteins and other macromolecules, being able to repair DNA in case of damage or stress is essential. The most important mechanism in which DNA damage is mitigated is the p53 response pathway. This pathway is responsible for responding to stresses that can disrupt DNA replication, chromosome segregation and cellular division (Vogelstein et al., 2000). Upon the introduction of stress to a cell, the p53 pathway is activated via post-translational modifications and this leads to cell cycle arrest. If the stress remains unresolved, the pathway can also induce cellular senescence or apoptosis in order to prevent further division of mutated DNA sequences (Jin & Levine, 2001). Another interesting aspect of the p53 pathway is that while it is able to

induce specific single cell changes, it can also interact with neighboring cells through the secretion of molecules that can direct these neighboring cells. For example, p53 is able to signal immune cells to infiltrate tumors and aid in suppression by damaging and clearing tumor cells (Lujambio et al., 2013).

P53 is unique because while it is responsible for DNA damage, it is also activated in response to oncogene activation, hypoxia, cellular ribonucleotide depletion, mitotic spindle damage and nitric oxide production (Jin & Levine, 2001). Because there are many methods in which the p53 pathway can be activated, it is essential that it also has several methods available to regulate its action. One of the ways in which the p53 pathway is regulated by the Mdm2 E3 ubiquitin ligase. This ligase works by reducing the amount of p53 in the cell through degradation and ubiquitination, thus inhibiting p53's ability to halt the cell cycle and induce apoptosis (Kubbutat et al., 1997). In order for p53 function, the Mdm2-p53 interaction must be disrupted which can come from several different methods such as post-translational modifications, physical separation, and degradation (Brooks & Gu, 2006). These methods allow for proper function of the p53 response pathway and reestablishes cellular homeostasis.

As mentioned previously, the p53 pathway is able to induce cell cycle arrest or cellular apoptosis, but proper cellular function relies on p53 being able to choose which is best for the cell in specific circumstances. Because the p53 pathway is activated in response to many different types of cellular stress mechanisms, there are also many genes that are responsible for growth arrest and apoptosis. Pro-apoptotic genes such as Bax, PIG3, PUMA, and Pidd are useful because they will kill any cells harboring deleterious genetic abnormalities, but it is not the only line of defense a cell will have (Green & Kroemer, 2009).

Genes like GADD45, Reprimo, p21, and 14-3-3σ, are downstream gene targets responsible for inducing cell cycle arrest (El-Deiry, 1998). In addition to these genes p53 also has transcription-independent functions in mitochondria that can induce apoptosis (Green & Kroemer, 2009). Unlike the apoptotic route, cell cycle arrest allows the cell to assess DNA

damage that has occurred and avoid the accumulation of rogue genetic mutations. This time also allows the cell to repair damaged DNA, but if the damage is not properly resolved, then the abnormal cell can be allowed to replicate and further the development of tumorigenesis (Brooks & Gu, 2010). While the fine balance between both cell cycle arrest and apoptosis is key, the induction of cellular senescence is another option available via the p53 mechanism.

Genes that are involved in the cellular senescence pathway are p21 and PAI-1 which both have been shown to be physiological, in vivo response to DNA damage (Deng et al., 2008)(Kortlever et al., 2006). Cellular senescence is a multifaceted process that will prevent the proliferation of cells similar to apoptosis but while apoptosis is activated in response to overwhelming stress, senescence is usually a consequence of less severe damage (Vousden & Lane, 2007). The decision on which of these three pathways is still up to the cell to decide and ultimately may depend on both the length and severity of the damage itself. The cellular senescence arm of the p53 pathway also plays a role in organismal aging, but the exact mechanisms by which p53 functions to promote this arm remains poorly understood (Brooks & Gu, 2010).

Integrated Stress Response

The Integrated Stress Response (ISR) is an elaborate signaling pathway that is responsible for responding to various different cellular stressors such as amino acid deprivation, viral infection, heme deprivation, or ER stress. Because this pathway can be activated in response to a number of different pathological conditions, there are multiple different ways in which this pathway is activated. All of the different activators ultimately culminate on a central factor called eIF2. This in turn activates a number of different stress responsive genes, one of which is known as activating transcription factor 4 (ATF4) (Lu et al., 2004). Much like the p53 pathway if the cellular stress remains unresolved or is overwhelming, the ISR will initiate cell

death mechanisms. This allows the ISR to aid in prolonging cellular longevity and ensuring that defective cells are taken care of appropriately.

As mentioned previously the ISR is activated in response to a number of different cellular stressors. In order to accomplish this goal, the ISR relies on four different kinases to mitigate disturbances in cellular homeostasis. These kinases are PKR-like ER kinase (PERK), double-stranded RNA dependent protein kinase (PKR), heme regulated eIF2α kinase (HRI) and general control non-derepressible 2 (GCN2) (Ron, 2002). These kinases act as first responders to cellular stress and are important in initial activation of the ISR to allow for the preferential translation of specific cellular response genes. Additionally, these kinases will oftentimes work in conjunction with one another to fine tune stress responses, one example of this is GCN2 which is able to mediate ER stress in the absence of PERK by activation of the ISR (Hamanaka et al., 2005). While each of these kinases are activated by different kinds of cellular stress, they all trigger the activation of the ISR.

The central regulator of the ISR is eIF2 and under normal cellular conditions, eIF2 aids in initiation of translation and recognition of the AUG start codon (Jackson et al., 2010). Upon the presence of cellular stress, phosphorylation of the eIF2 alpha subunit triggers the preferential translation of downstream genes. The eIF2α subunit blocks the exchange of GDP to GTP which is essential to allow for the formation of the pre-initiation complex before transcription (Pain, 1996). The termination of the ISR is dependent on dephosphorylation of eIF2α and this allows the cell to reestablish protein synthesis and normal cell functioning (Novoa et al., 2001).

Conclusion

Because stress is a constant issue that cells experience on a regular basis, response pathways are essential in ensuring that homeostasis is maintained. The various types of stress that are present in an organism also requires that each of these cellular stress response

pathways are varied and work in conjunction with one another at times in order to deal with these conditions. As more research is done into different stress response pathways, there is a strong possibility that certain factors are shared between two or more different pathways. Understanding what factors are required in order for these pathways to be functional will allow medical professionals to target them to use for potential pharmaceutical treatments.

Chapter 2: Transcription Factors

Proper function within cells requires genes to be expressed in a timely manner, so the cell can respond to physiological, developmental, and environmental stimuli (Fulton et al., 2009). In order for these genes to be properly expressed, proteins known as transcription factors (TFs) work in conjunction with other factors to control the rate of transcription (Blair et al., 1994). By directly interacting with DNA and with other accessory proteins, these TFs are able to regulate the transcription of genes which are involved in the normal development of an organism, as well as for routine cellular function and response to disease (Voss & Hager, 2014). Because of this, many human diseases are correlated with mutations in TFs and TF binding sites. There are thousands of TFs within the human genome that regulate gene expression by controlling when, where, and how efficiently RNA polymerase functions. Ultimately, understanding how TFs are able to control transcription across different genes and cell types is essential in understanding how gene expression is regulated in multicellular pathways.

One interesting aspect of TFs is their versatility in terms of gene regulation. The same TF can regulate different genes in different cell types. An example of this is ESR1, a TF that regulates a different set of genes depending on whether it's present in breast cells or endometrial cell lines (Lung et al., 2020). This means that even a handful of TFs can control the transcription of hundreds of genes depending on the cell type or cell condition. TFs bind to specific DNA sequences known as “motifs” which are sets of short, related sequences that are preferred by a specific TF. These binding sites usually prove to be very beneficial in identifying other TF binding sites because TFs will work in conjunction with one another in order to manage transcription (Pettersson & Schaffner, 1990). This also means that TFs will sometimes have overlapping binding sites within the genome, which can lead to cooperation or competition.

We have been able to determine hundreds of TF binding motifs by utilizing in vitro assays such as systematic evolution of ligands by exponential enrichment (SELEX) and in vivo approaches such as chromatin immunoprecipitation followed by high throughput sequencing

(ChIP-seq) (Leporcq et al., 2020). The relative preference of the TF for each base in a binding site is shown in the form of position-weight matrices (PWMs). These PWM scores will give a score to each of the four bases for a particular position within a binding site, multiplying these scores for each base of a sequence generates a predicted affinity for a particular TF to bind to that site (D’haeseleer, 2006). These sites or motifs are then available in TF motif databases such as JASPAR where researchers can determine where a particular TF will bind. Ultimately even though these motifs have been experimentally determined, typically only a portion of the experimentally determined binding sites actually match these motifs (Inukai et al., 2017). Because of this, most TF binding sites are flexible which means that the typical human gene will contain multiple potential binding sites. These multiple potential binding sites are what allow one TF to bind to multiple different genes, while also allowing a multitude of other TFs to also bind to the same gene.

Identifying Transcription Factor Binding Sites

Identification of TF binding sites is crucial in understanding how gene regulation is managed. Thanks to projects like JASPAR, there are entire databases with TF binding site motifs available for public use. While these motifs are usually a good predictor to where TFs will bind, this does not definitively mark where TFs will actually bind. One reason this is the case is because there are often limitations to utilizing a singular assay to determine these sites. One example of an assay which has a number of different limitations is ChIP-seq assays. ChIP-seq or chromatin immunoprecipitation with sequencing, is an assay that analyzes protein interactions with DNA (Solomon et al., 1988). This method involves the crosslinking of DNA to proteins by treating cells with formaldehyde, followed by shearing of the chromatin into small fragments via sonication. These small fragments are then selected against an antibody specific to the protein of interest in order to select for the correct DNA-protein complexes. Finally, these complexes are unbound together, releasing the DNA and allowing for sequencing to be done in

order to determine sequences that are bound by the protein of interest (Park, 2009). ChIP-seq is a powerful tool for identifying TF binding sites but as mentioned previously, there are some limitations. For one, ChIP-seq can detect indirect binding, which can mislabel motifs for proteins other than the one ChIP-ed (Bailey & Machanick, 2012). Another issue is that because ChIP-seq requires the use of crosslinkers, the native state of DNA is disturbed and does not represent equilibrium binding (O'Neill & Turner, 2003). Lastly ChIP-seq data relies heavily on antibody quality, and certain antibodies cross-react so there are issues with definitely pinpointing a TF binding site (Park, 2009). Fortunately, as the field progresses, there are new approaches such as cleavage under target and release under nuclease, which have been created in order to address some of these issues and continue our understanding of TFs.

Another assay that is used to identify TF binding sites are EMSAs or electrophoretic mobility shift assays. These assays work by utilizing a DNA fragment labeled with phosphorus 32, a radioactive isotope. This fragment contains a specific DNA site which is incubated with a candidate DNA-binding protein. These DNA-protein complexes are then separated from unbound DNA by electrophoresis through a nondenaturing polyacrylamide gel. Because of the proteins size, it reduces the mobility of any DNA fragments its bound to, any DNA that is unbound will travel faster through the gel matrix. This allows researchers to reveal areas of unbound and bound radioactively labeled DNA and see where proteins bind to (Fried, 1989). Because this assay uses radioisotope-labeled nucleic acids, it is very sensitive, which allows it to work with small protein and nucleic acid concentrations and small sample volumes (Rye et al., 1993). Another advantage is that this assay can detect a wide variety of different nucleic acid sizes and complexes. This includes but is not limited to small circular DNAs, short oligonucleotides, and duplex structures (Fried & Daugherty, 1998). If EMSA's were perfect they would be the only assay we utilize to identify potential TF binding sites, but it's not the only assay. One of the most crucial setbacks when it comes to an EMSA is that the samples are not a chemical equilibrium during the electrophoresis step. This means that complexes that form

and dissociate quickly can result in an underrepresentation of binding density (Fried & Bromberg, 1997). Another limitation to this assay is the variety of different factors that affect electrophoretic mobility beyond just protein size. This means that a DNA-protein complex could potentially be limited in how far it travels through the matrix due to charge (Hellman & Fried, 2007). Third and probably one of the most important when it comes to current sequencing techniques, is that an EMSA is unable to provide little to no information as to what nucleic acid sequences are bound by protein (Brenowitz et al., 1986).

Another assay that has been proven useful when it comes to understanding TF binding in general is a yeast 1 hybrid (Y1H) assay. A Y1H assay relies on two individual plasmids referred to as “bait” plasmid and a “prey” plasmid (Li & Herskowitz, 1993). The bait sequence is composed of the DNA fragment of interest, which is cloned upstream of a reporter gene, while the prey sequence is composed of a TF of interest which is fused to the activation domain (AD) of the yeast TF Gal4. These two parts are introduced to a growing yeast strain and if the TF binds to the DNA sequence of interest, the AD will induce expression of the reporter gene. This allows the Y1H system to identify DNA-protein interactions with short cis-regulatory elements (Deplancke et al., 2006). When comparing a Y1H assay to other DNA-protein interaction assays, the Y1H assay has a couple of advantages over others. One advantage is that the Y1H system is able to identify multiple TFs binding to a specific DNA fragment of interest. This allows for the researchers to test out hundreds of TFs and see if they are able to regulate a certain gene of interest (Fuxman Bass et al., 2016). Another advantage of the Y1H system is that this assay can identify interactions with TF’s that may be in low abundances naturally within cells or tissues. This allows researchers to know definitively if the TF of interest binds to a specific DNA binding domain. While this works to bind TFs to specific regions, it could also introduce false positives. Because this assay is done in vitro, there is always the possibility that under vivo conditions, the TF of interest would not bind to the sequence. Another DNA-protein interaction that is unable to be fully captured by this assay are any interactions where the TF needs to

undergo post translational modifications that are not present in the yeast system (Fuxman Bass et al., 2016).

Transcriptional Activation

Transcriptional activation requires several different factors in order to ensure proper gene expression and cellular function. In order for TFs to bind to DNA regulatory regions, like promoters and enhancers, they need open regions of DNA not bound by nucleosomes (Spitz & Furlong, 2012). To accomplish this goal TFs often work with other co-factors in order to influence chromatin binding, remodel nucleosomes or modify histones and other proteins (Frietze & Farnham, 2011). These processes are essential in altering the shape of chromatin structure and allow TFs to bind to DNA regulatory elements. A special class of TFs, called pioneer TFs, are able to remodel chromatin to allow for other TFs to bind and begin the process of recruiting other transcriptional machinery to the site (Soufi et al., 2015). What makes these pioneers so special is their ability to bind to regions of DNA that are constrained by nucleosomes. Once pioneer TFs bind to DNA bound nucleosomes, they can disturb the structure by evicting histones within the nucleosome (Zaret, 2020). This allows for other TF target sites to become free and available for binding. The process by which TFs are able to bind to DNA and regulate gene expression is dependent on the structure of chromatin.

The most common way in which regions of open chromatin are created is via histone remodelers (Kolasinska-Zwierz et al., 2009). These enzymes are dubbed writers and they are responsible for modifications such as acetylation, methylation, phosphorylation, and ubiquitination. These four common histone modifications will then alter the state of chromatin to either create what is known as euchromatin, an open and accessible histone confirmation or heterochromatin, a closed histone conformation (Alaskhar Alhamwe et al., 2018). Depending on what the cell needs at that specific moment in time, histone modifying enzymes will work to facilitate transcription and allow for proper gene expression. Acetylation occurs when an acetyl

group is added to lysine residues on the N-terminal histone tail. Upon acetylation, the positive charge on the histone is neutralized and euchromatin is created, which allows for increased transcription (Gräff & Tsai, 2013). Methylation involves the addition of methyl groups to lysine or arginine residues on histones. This can either activate or repress genes depending on the target site and are important in development (Jambhekar et al., 2019). Phosphorylation is the addition of phosphoryl groups and ubiquitination is the addition of ubiquitin proteins (Watson & Higgins, 2016). Both modifications lead to increases in gene transcription and play a role in DNA repair. Ultimately there are many different enzymes that allow for the opening of chromatin for TF binding.

Under certain conditions, TFs perform their regulatory function as homo or heterodimers. Homodimerization occurs when dimers form between identical proteins and heterodimerization occurs when dimers form between different proteins (Funnell & Crossley, 2012). This dimerization can increase DNA binding affinity, it can create novel binding specificity, and can explain how TFs are able to bind to different sites depending on other TFs (Crothers, 2013). One of the earliest examples of TF dimerization came from the Fos and Jun protein families. These proteins regulate a variety of cellular processes such as cell proliferation, differentiation, apoptosis and oncogenesis (Abate et al., 1991). It was discovered that the Fos and Jun protein families bind to AP-1 regulatory regions found in a wide range of enhancers and promoters (Rauscher et al., 1988). According to the researchers, Jun proteins can form both homo and heterodimers and that the formation of these dimers leads to enhanced binding at AP-1 regulatory regions within DNA via alteration of the DNA helix.

The formation of homo and heterodimers is only one way in which TFs work together to initiate transcription. Multiple different TFs can cooperate with one another to allow for complex genetic processes to occur at individual regulatory elements. This cooperativity can happen via multiple different mechanisms. As spoken about before, TF proteins can bind to one another to enhance binding strength to DNA, but if the interaction between two TFs is weak, binding to

DNA can bring them close enough together to allow for interaction (Morgunova & Taipale, 2017). Alternatively, the binding of DNA can alter the conformation of one TF to increase the likelihood of binding with the other TF. DNA facilitated interactions are very common in the cell and there are many different ways in which proteins can interact with one another via DNA. Because two different TF proteins can be oriented towards each other in four different ways, and they can be located at different distances from each other, there can be a number of different interacting configurations possible (Morgunova & Taipale, 2017). DNA facilitated interactions also increase the strength of TF interactions between each other because only one or few hydrogen bonds are required.

Another way in which TFs are able to bind to DNA is through competition with nucleosomes. In order for TFs to bind to DNA bound by nucleosomes, they must compete with them or interact with nucleosomes or nucleosomal DNA to access their sites (Voss & Hager, 2014). This is usually accomplished by either initiating the displacement of nucleosomes or induce a conformational change. An example of this is the TF FoxA, which displaces the linker histone H1, which keeps enhancer nucleosomes accessible in chromatin and allows other liver specific TFs to bind (Lupien et al., 2008, p. 1). The most common mechanism by which these TFs are able to do this is by recruiting ATP-dependent chromatin remodelers and other TFs that help decrease nucleosomal occupation.

Once TFs are able to bind to DNA the next step involves the formation of the preinitiation complex. This process can involve different DNA binding proteins, DNA bound proteins and other general factors such as the TATA box binding protein (TBP). The TATA box is a DNA sequence that marks where a gene can be read decoded (Butler & Kadonaga, 2001). This marks where transcription begins, and it is named after its conserved DNA sequence which is most commonly TATAAA. The TATA box is usually located 25-35 base pairs before the transcriptional start site of a gene and is essential to indicate the direction of transcription. The main role of the TATA box is to serve as the site where the preinitiation complex will form

(Petrenko et al., 2019). The first step involves the binding of transcription factor II D (TFIID) to the TATA box on the basal promoter region. This binding is facilitated by TFIIA through the interaction with the central subunit of TFIID to form a complex. After this complex is formed, TFIIB is recruited and interacts with the TBP subunit of the TFIID and this addition is what allows for the recruitment for RNA polymerase II to the complex (Warfield et al., 2017). Ultimately once RNA polymerase II is attached to DNA, transcription and proper gene expression can occur.

TFs vary drastically in how they impact transcription upon DNA binding. As mentioned before, certain TFs work to directly recruit RNA polymerase, but other TFs will recruit other factors that promote specific phases of transcription. These cofactors are usually large multi-subunit protein complexes that regulate transcription using a variety of different mechanisms. One classic example of coactivator recruitment is the IFNβ enhanceosome. Activation of the INF-β gene requires the creation of a single composite element made up of the TFs ATF-2/c-Jun, IRF-3 and IRF-7, and NFkB (Panne et al., 2007). The INF-β gene has two nucleosomes that flank the enhancer region of the gene, one which masks the TATA box and thus the transcriptional start site. Once these TFs come together to form what is known as the enhanceosome, it allows for the recruitment of GCN5 and CBP/p300 which leads to nucleosome acetylation and chromatin remodeling by the SWI/SNF complex to reposition the nucleosome covering the TATA box (Agalioti et al., 2000). This allows access by the TBP and RNA polymerase II machinery to initiate transcription. The enhanceosome structure shows how different TFs are able to bind cooperatively because their binding sites overlap to create mutually compatible DNA interaction surfaces.

Conclusion

Studies involving TFs continue to advance as methods to investigate the function of TFs get better. These methods will help identify the various roles that TFs play in multicellular

eukaryotes and because TFs function as central controllers of many gene regulatory networks, this field will only continue to expand (de Mendoza et al., 2013). Understanding just how TFs affect these regulatory networks will be key in developing potential therapeutic treatments for diseases like type 2 diabetes and Parkinson's.

Chapter 3: DNA Regulatory Regions & Chromatin Structure

In order for our cells to fully utilize our genomic information, certain regions within our genome serve as markers to ensure proper physiological function. These markers, known as regulatory elements, are DNA sequences that are recognizable by transcription factors (TF) and indicate where these TFs should bind to DNA. In addition to dictating whether or not RNA polymerase can bind to DNA, TFs can even determine the rate at which genes are transcribed. Thus, regulatory regions determine the connectivity of molecular networks and aid in mediating a number of regulatory processes within the cell. Thanks to advancements made in genomic sequencing technologies, research into regulatory regions have become imperative as a number of human diseases can be caused by mutations found in these elements.

Promoters & Enhancers

One type of genomic regulatory region is called a promoter. Promoters are typically located at the beginning of genes and is where RNA polymerase binds to initiate transcription. These promoters will oftentimes work with other regulatory regions in order to ensure proper mRNA formation(Sharan et al., 2007). The site in which RNA polymerase binds within the promoter is known as the transcriptional start site (TSS). This site is selected by the presence and binding activities of other transcription factors, which work in conjunction with RNA polymerase in order to form the pre-initiation complex (Smale & Kadonaga, 2003). Within a 50-base pair (bp) region around the TSS, there are also promoter sequence elements. Two of the most well-known core promoter elements are the TATA box and the initiator (INR) element. These elements work within the promoter to ensure proper formation of the pre-initiation complex and proper transcriptional regulation. The TATA box is roughly 24-30bp upstream from the TSS and serves as a recognition sequence for the TATA binding protein (TBP), which is the central part of the pre-initiation complex (Breathnach & Chambon, 1981). Similar to the TATA box, the INR element also works to enhance binding affinity and strengthen the promoter,

ensuring that even promoters without TATA box elements can direct transcriptional initiation (Smale & Baltimore, 1989). These elements, in addition to the TSS, encompass what's known as the core promoter, but identifying which regions are essential within the promoter region has been the focus of several studies.

Another type of transcriptional regulator is known as an enhancer which acts to activate the transcription of a gene to higher levels than if the enhancer region were not present. These elements are unique because they are able to function up to 2-3 megabases from the TSS and require favorable folding of the genome (Lettice et al., 2003). This folding of the genome is what allows enhancers to interact with their gene target even at such long distances (Krivega & Dean, 2012). The first type of enhancer was a 72bp sequence found within the SV40 virus genome, and this enhancer could increase transcription rates of a reporter gene in HeLa cells by several hundred fold (Banerji et al., 1981). Similar to promoters, enhancers contain specific sequences that are recognized by TFs. These TFs then recruit co-activators and co-repressors that determine the activity of the enhancer (Shlyueva et al., 2014). Enhancers are able to act independently of their target genes and they can do so regardless of orientation or distance. One of the most important things about enhancers is their ability to add to each other in order to modulate the expression of their target genes. This is something that has been seen in certain reporter assays where combining multiple sequences leads to a pattern of expression that reflects their combined activity (Arnone & Davidson, 1997).

Enhancer Activation Models

Because enhancers play such a large role in expression of their target genes, understanding how enhancers directly interact with genes is essential in the development of therapeutics for diseases. The most common mechanism in which the enhancer functions is the transactivation model. In this model, TFs bound to the enhancer will interact with RNA polymerase II (RNAPII) on the promoter in order to initiate expression of the target gene.

Expression of the gene is facilitated through the formation of the pre-initiation complex or increasing the rate of transcription (Hughes, 2011). The distance between the enhancer and promoter is also important when taking into consideration how strong the connection between these regulatory regions will be. The addition of less than 100 nucleotides into the genome can disrupt the connection between enhancers and promoters; for example, the addition of 54bps between the VP16 effector domain and its promoter has been shown to reduce transcriptional activation (Roberts et al., 1995).

Another model for enhancer activity is known as the hit and run model. In this model looping interactions between enhancers and promoters cause enzymes that are bound to the enhancer to deposit histone marks and/or remodel nucleosomes at the promoter. In this model, the interaction between the enhancer and promoter establishes open chromatin and ensures transcriptional activation (Beagrie & Pombo, 2016). The Tet family of proteins, which can hydroxylate methylated DNA, has been found on certain enhancers which supports the model that enhancers aid in histone marking (Pulakanti et al., 2013). Alternatively, as opposed to adding histone markers at a target promoter, the enhanced could be removing repressive marks. An example of this is the Polycomb eviction model which proposes that distal enhancer elements remove repressive Polycomb protein complexes from the promoters of developmental genes (Vernimmen et al., 2011). The mechanism behind how these Polycomb proteins are cleared via enhancers is still unclear but one theory is that a demethylase such as JMJD3, is recruited to promoters by looping interactions from distal binding positions (Kondo et al., 2016).

Enhancers also play a very important role in directly recruiting RNAPII and other components of the pre-initiation complex to the promoter, thus initiating transcription (Vieira et al., 2004). The way in which these components are brought to the promoter are still under investigation but one method involves RNAPII binding to the promoter and then traversing the DNA until it reaches the promoter (Vernimmen & Bickmore, 2015). One study has proposed that this mechanism of RNAPII traversal is the cause of activation of embryonic ε-globin gene at the

human β-globin locus (Zhu et al., 2007). Because of large genomic gaps between enhancers and their target promoters, this “tracking” model has been disfavored (Lettice et al., 2002). One thing that aids in this model is that looping interactions have also been seen alongside this model. A theory that utilizes both looping and tracking is the “facilitated tracking” model which suggests that activating proteins bind to an enhancer and are delivered to the target promoter via a loop that gradually expands from the enhancer to the promoter (Blackwood & Kadonaga, 1998). This region also has been useful in explaining how enhancer-bound TFs can interact with the promoter before activation (Hatzis & Talianidis, 2002).

Enhancer Prediction Methods

Because enhancers are typically found hundreds of base pairs away from their target promoters, finding new enhancers can be a challenging task. New enhancers are discovered based on known characteristics of enhancers and thanks in part to the development of new next generation sequencing technologies there has been a rise in genome-wide studies of enhancer activities. In addition to this, the ability to synthesize longer DNA fragments or manipulate genomes in vivo has allowed even further tests to be carried out.

As mentioned previously, TFs bind to enhancer regions and these TF binding motifs can be utilized to discover new enhancers. Current approaches utilize computational matching of these motifs across the genome, either through enrichment of the TF motifs themselves or by searching for individual matches that are conserved across species (Bene et al., 2007). There are certain methods that also use both enrichment and conservation and/or identify only regions in which motif matches occur in specific combinations or in a particular order or arrangement (Aerts, 2012). Although it is true that TFs will bind to enhancers, the relationship between TFs motifs, binding, and enhancer activity is not as simple. Because some TF binding motifs are relatively short, these short motifs tend to frequently bind to genomic or random DNA sequences and an even smaller fraction of motifs are actually bound by the TF in vivo (Yáñez-

Cuna et al., 2012). Utilizing computer learning approaches to identify characteristic DNA sequence features in addition with flanking gene expression data can be useful when trying to identify novel enhancers (Hardison & Taylor, 2012).

As opposed to using TF binding motifs, researchers have also relied on ChIP-seq assays to highlight places where TFs bind to uncover the presence of active enhancers. In addition to ChIP-seq, different variants of this method have been used as well such as ChIP-exo and DNA adenine methyltransferase identification (DamID) (van Steensel & Henikoff, 2000). As mentioned in previous chapters, TFs are identified through ChIP-seq by chemical crosslinking of DNA to TFs and then antibodies are used to precipitate out fragments where TFs bind. These fragments are what allow researchers to utilize this and assays like this to determine enhancer activity. Unfortunately, while TF binding sites can be verified after methods such as ChIP-seq, the same cannot be said about the presence of functional enhancers (X. Li et al., 2008). There is no one sole reason why this is the case, but one reason is that the binding of one enhancer could not be sufficient to initiate transcription. In addition to this, TFs have a general affinity to DNA, which means that they could potentially bind to other regions in DNA that are outside of their binding motifs (Hammar et al., 2012). Another reason why this method is unreliable when it comes to functional enhancer discovery is that TFs can oftentimes bind to enhancers indirectly through interactions with other transcription factors. This means that TF binding data does not represent sequence-specific transcription factor binding or tissue specific enhancer activity (Lickwar et al., 2012). This means that while identification of TF binding sites can help to identify target enhancers but fall short when it comes to the identification of active enhancers. In combinations with other enhancer identification methods, this method can prove to be useful regardless.

Chromatin Dynamics

Both active enhancers and promoters are found in regions of chromatin that are depleted or free of nucleosomes. Chromatin acts as a genetic “gatekeeper” because of its ability to allow and restrict TF binding onto regulatory regions. Chromatin dynamics change throughout an organism's development and allows for different cells to respond to a wide variety of changes (Shlyueva et al., 2014). These changes in chromatin accessibility are characterized by nucleosome positioning, which is defined by the localization of an individual histone octamer with respect to a specific DNA sequence (Bell et al., 2011). The positioning of these histone octamers is dependent on different factors including post-translational histone modifications and ATP-dependent remodeling. The ability for chromatin to dynamically change structure and control DNA accessibility is what makes it essential in transcriptional initiation and elongation (B. Li et al., 2007).

As mentioned in previous chapters, TFs aid in the reorganization of nucleosomes by binding to sequence specific regions within the genome. While there is still no comprehensive understanding on how TFs are able to rearrange chromatin, it is understood that direct interaction between TFs and histones can lead to displacement of these histone octamers in vitro (Workman & Kingston, 1992). However, most TFs rely on ATP-dependent nucleosome remodeling activity and are cell type specific in order to remodel nucleosomes (John et al., 2011). For certain repressed promoters, there has been known to be at least one accessible binding site which allows for “pioneer” TFs to bind and allow for greater binding availability via histone displacement (Drouin, 2014). The interplay between TFs and nucleosomes allows for changes in the rates of transcriptional initiation, whether it be through TFs that increase transcriptional rates or reduce transcriptional rates.

Another way in which nucleosomes are rearranged is by using ATP dependent remodel complexes. These complexes are made up of multiple proteins that utilize ATP hydrolysis to slide or completely disassemble histone octamers (Clapier & Cairns, 2009). The most well

studied example is the nucleosome remodeling complex or RSC, which is required for transcriptional activation of many yeast genes (Parnell et al., 2008). The RSC is recruited to the nucleosome, usually after initial TF binding which allows for the creation of nucleosome depleted regions. In studies where the RSC is inhibited or blocked, these nucleosome depleted regions shrink in size and the flanking nucleosomes are poorly positioned (Hartley & Madhani, 2009). Positioning of nucleosomes is directed by these complexes after being influenced by the activity of trans-acting proteins such as TFs.

Another aspect that can alter chromatin structure is the presence of reversible post-translational modifications made onto histones. These modifications can function as docking sites for TFs or influence chromatin structure and thus DNA accessibility (B. Li et al., 2007). One of the most common modifications made is H3K4 trimethylation (H3K4me3) which is a typical mark for active and open chromatin (Rando, 2007). Some studies have suggested that histone modifications can facilitate nucleosome eviction by changing the net charge between neighboring nucleosomes (Shogren-Knaak et al., 2006, p. 16). A good example of this is H4K16ac which interferes with the electrostatic interactions between the amino terminal tail of histone 4 and an acidic patch on histone 2, thus preventing the formation of heterochromatin (Dorigo et al., 2003). The prevention of the formation of heterochromatin has been shown in *Drosophila melanogaster* to be critical for transcriptional upregulation as part of dosage compensation (Akhtar & Becker, 2000).

Conclusion

Genetic regulatory regions and chromatin dynamics are absolutely essential in understanding the genetic landscape on a more detailed level. Ultimately there are only two main things that I would like to care about and

Chapter 4: ATF4 & P53's Role in Stress Response Pathways

The cell maintains homeostasis and regular cellular functions by utilizing different stress dependent pathways in order to mitigate various types of cellular stress on a regular basis. These different pathways incorporate many factors that interact with one another to create interconnections between each pathway (Van Drogen et al., 2020). Two of the most important factors that are able to regulate a large number of downstream stress responsive genes are ATF4 and P53. While these two transcription factors (TFs) function in different manners in order to relieve cellular stress, there are certain similar characteristics between the two.

ATF4 and Its Role in The Integrated Stress Response

ATF4 is the central factor within the Integrated Stress Response (ISR). The ISR is activated in response to different stressors such as nutrient deprivation, viral infection, or redox imbalances and has been suggested to be the central regulator of protein homeostasis at both the cellular and organismal level (Costa-Mattioli & Walter, 2020). Activation of the ISR allows for scanning ribosomes to initiate translation at the canonical start site of the ATF4 open reading frame (ORF) (Harding et al., 2000). Once ATF4 is translated it reprograms the cell's transcriptional activities and regulate other genes essential in a wide number of processes including glucose homeostasis, energy expenditure, and neural plasticity (Pakos-Zebrucka et al., 2016).

Although phosphorylation of the eIF2 complex allows for preferential translational control of ATF4, there are selected stresses such as exposure to UV irradiation that do not increase ATF4 expression, even after eIF2 phosphorylation. When ATF4 mRNA levels were measured during ER and UV stress conditions, exposure to ER stress led to an increase in mRNA levels but under UV irradiation conditions, ATF4 mRNA was decreased (Dey et al., 2010). Researchers determined that in addition to translational control, ATF4 expression is subject to transcriptional regulation. This means that ultimately there are certain stress response

conditions that will lead to transcriptional and translational regulation of ATF4 expression. These differences in gene expression can be seen in interactions between ISR kinases and other stress response pathways. An example of this is GCN2, an upstream ISR kinase, is integrated with TORC1 during nutrient stress (Cherkasova & Hinnebusch, 2003).

mTORC1 has recently been shown to interact with ATF4 to promoter protein and gluthione synthesis downstream of growth signaling. mTORC1 is a core component that aids in the process of carefully controlling the growth of new cells by activating the chemical reactions cells need to grow (Torrence & Manning, 2018). Recently there has been evidence that ATF4 can be activated by pro-growth signals that stimulate mTORC1 signaling, in addition to cellular stress (Ben-Sahra et al., 2016, p. 1). It has been shown that genes involved in amino acid biosynthesis, transport and tRNA charging were induced by mTORC1-ATF4 signaling. The same study showed that ATF4 is a metabolic effector of mTORC1 involved in both promoting protein and gluthathione synthesis an cellular cystine uptake (Torrence et al., 2021, p. 1).

ATF4 has also been implicated in amino acid metabolism through the asparagine synthetase (ASNS) during the amino acid and unfolded protein response (UPR) signaling. This allows ATF4 to activate ASNS in response to amino acid deprivation or ER stress (Siu et al., 2002). Other stress responsive genes are activated via binding of ATF4 with C/EBP-ATF response element (CARE) sequences that can regulate transcriptional regulation in response to different conditions (Kilberg et al., 2012). This CARE element is unique because it can also function as amino acid response elements (AARE) during amino acid starvation, and similar to ASNS, ATF4 will bind to these sequences to reestablish homeostasis (Averous et al., 2004). This is only one example of how multiple pathways are activated in response to certain cellular stressors, although it is unclear if both pathways are activated at the same time or if one is preferentially activated over another.

The switch between adaptive and pro-apoptotic gene expression has been attributed to the formation of different ATF4 heterodimers that control specific targets and expression

patterns (Hetz, 2012). Another downstream target that ATF4 works with is another bZIP TF called CHOP. CHOP and ATF4 form a heterodimer together in order to induce autophagy in mammalian cells (Rouschop et al., 2010). The formation of this heterodimer also allows for greater binding affinity to AARE sites which has been reported as an early event in transcriptional induction of autophagy genes (B'chir et al., 2013). ATF4 is able to form heterodimers and/or homodimers with other proteins due to the presence of a leucine zipper domain (Podust et al., 2001). This allows ATF4 to influence the outcome of ISR signaling. While interactions with CHOP promote cellular autophagy, ATF4's interaction with ATF3 results in an enhancement in genes required to reestablish homeostasis (Wang et al., 2009). The ability for ATF4 to induce autophagy genes and genes important in mitigating cellular stress, showcases its importance in determining the fate of the cell under stress conditions.

In order for ATF4 to transcriptionally activate downstream targets it must first recruit the assistance of enzymes that modify chromatin structure in order to gain access gene regulatory regions. One study showcased that inhibition of deacetylase activity via HDAC inhibitors rescued transcription from certain genes in ATF4 knockout cells (Shan et al., 2012). This study showcases that histone acetylation is important for the transcriptional activation in certain genes but not others showcasing the flexibility in ATF4 induced transcription. ATF4 flexibility also extends to what type of cellular stressor caused initial activation. For example, ATF4 was only able to bind to the CARE sequence of the SNAT2 gene during amino acid deprivation where the amino acid response (AAR) was triggered. Under ER stress conditions, the unfolded protein response (UPR) was unable to induce histone acetylation and thus unable to create conditions for ATF4 to bind to the CARE region. When conducting the same experiment in a reporter plasmid, it was shown that the UPR was able to induce transcription at the SNAT2 promoter, which showcased how chromatin structure in vivo can contribute to ATF4 binding at promoters (Gjymishka et al., 2008). Based on these studies, it is essential for chromatin modifiers to allow access to regulatory regions for ATF4-DNA binding.

One aspect that is still not well understood is how ubiquitination plays a role in ATF4 expression. There are many types of post-translational ubiquitination that can occur on ATF4 that can alter its stability or transcriptional activity. One interaction of interest happens during cellular stress, where the bZIP domain of ATF4 and the deubiquitinating complex ABRO1-BRISC. This interaction allows the ABRO1-BRISC complex to be moved into the nucleus where it can de-ubiquitinate other factors (Ambivero et al., 2012). This is of interest because according to one study ABRO1 is able to stabilize another important stress dependent TF, p53 and thus regulate the DNA damage response (Zhang et al., 2014). This demonstrates how interconnected these TFs are in regulation of different stress dependent pathways.

P53 and the DNA Damage Response

Activated p53 is essential in transcriptionally regulating genes that are responsible for mitigating various cellular stressors such as DNA damage, hypoxia, oncogene activation and ribosomal stress. The genes that are activated in response to these cellular stressors help alleviate this and reestablish homeostasis by promoting cell cycle arrest, DNA damage repair, apoptosis and senescence (Hager & Gu, 2014). Because of its ability to activate these stress response mechanisms p53 has been dubbed as the 'guardian of the genome'. Ever since its initial discovery as a tumor suppressor in 1989, p53 has become one of the most studied human gene of all time (Baker et al., 1989, p. 17) (Dolgin, 2017).

As opposed to ATF4, p53 is activated in response to cellular stress through protein stabilization. In order to keep p53 levels low, the E3 ubiquitin ligase MDM2 ubiquitinates p53 which makes it a target for degradation (Honda et al., 1997). This means that MDM2 is not always present and that p53 is still produced, but under non stressed conditions it is quickly degraded. In the presence of different cellular stressors, phosphorylation of p53 weakens the interaction between MDM2 and p53, thus stabilizing p53 (Shieh et al., 1997). There are different cellular stressors that can initiate p53 activation including DNA damage, hypoxia, and nucleotide

deprivation. Interestingly enough, in addition to regulating p53 levels, MDM2 is also a downstream target gene which allows for autoregulation of p53 activity in cells (Barak et al., 1993). P53's ability to respond to a number of different cellular stress types makes it the most important tumor suppressor in cells and because of this p53 is frequently found to be mutated in cancer cells (Lawrence et al., 2014).

The list of genes that p53 is able to regulate is extensive and with many studies working with p53 in order to better understand its role in the DNA damage response, this list will only continue to grow. Some of the targets that p53 is able to transcriptionally regulate are DNA damage binding protein 2 (DDB2), GADD45A, PUMA, BAX, and TP53 induced glycolysis and apoptosis regulator (TIGAR) (Hafner, Bulyk, et al., 2019). Although these genes are all activated through p53 regulation, the type of damage and the cell type will dictate what specific sets of genes are activated in response to certain types of cellular stress (Madden et al., 1997). Additionally, it is not always clear what downstream gene targets are directly influenced by p53 as opposed to indirect regulation (Mirza et al., 2003). As the field of transcriptional regulation continues to expand, these questions will be investigated and will help researchers determine the interconnectivity between different stress dependent pathways.

The p53 gene has a number of functional domains including two tandem transcription activation domains (TADs) at the amino terminus (Candau et al., 1997). These were found to be required for proper p53 target gene induction in response to DNA damage in mice, but there were differences in how mutations in each TAD altered gene expression. In TAD1 mutants, there was a noticeable difference in gene expression when compared to the double TAD mutant and the TAD2 mutants indicating that these TADs could play a role in gene regulation (Brady et al., 2011). Further down from the TAD is the DNA binding core domain which is the site of most cancer associated mutations (Pavletich et al., 1993). P53 is able to bind cooperatively to its target site as a tetramer (Wang et al., 1995).

As one would expect, mutations in this binding core domain impaired p53 DNA binding and led to the loss of transcriptional activity (Davison et al., 1998). Since tetramerization is important for gene activation, researchers in another study looked at p53 mutants that generated monomeric, dimeric, or tetrameric species and noted that these mutants activated different gene sets which suggests that this tetramerization is important for regulating cell stress pathways (Fischer et al., 2016). These gene sets work to mitigate different types of stress and using a series of p53 mutants that differed in their cooperative binding to gene targets, induction of cell cycle arrest genes was found to be less dependent on cooperative p53 binding when compared to apoptosis genes (Schlereth et al., 2013).

Similar to ATF4, p53 is able to work with other cofactors in order to regulate the DNA damage response. One example of this is the acetyltransferase p300 was needed in the p53 dependent activation of another downstream gene CDKN1A in vitro and in cells (Barlev et al., 2001). P300 is required for inducing histone H4 acetylation which allows for p53 activation. These cofactors work in unison with p53 in order to rearrange chromatin so that it can activate. But unlike ATF4, P53 binding elements associated with DNA damage response genes were not associated with binding sites for other TFs (Akdemir et al., 2014). While there is little overlap between p53 binding sites and other TF motifs, there are several TFs that interact with the p53 DNA binding domain to modulate target-gene expression. One example is apoptosis stimulating of p53 protein 1 (ASPP1) and ASPP2, which are transcriptional targets of E2F and interact with the DNA binding domain of p53 (Patel et al., 2008). This highlights the importance that cofactors play in p53 regulation and activity, but with the understanding that p53 binding sites are often times found independently of binding sites for other TFs (Verfaillie et al., 2016).

While certain factors work in conjunction with p53 to induce activation, p53 has been known to bind to closed chromatin, classifying it as a pioneer TF (Laptenko et al., 2011). Because p53 binding sites typically do not cluster with the binding sites for other TFs, chromatin structure was suggested to be the main determinant of p53 binding patterns (Verfaillie et al.,

2016). This same study utilized different cell types and treatments to evaluate if chromatin structure was the main determinant for p53 binding sites and there was no change in p53 binding patterns. The chromatin remodeler RSF1 was found to be required for p53 binding (Min et al., 2018). The reduction of RSF1 led to a decrease in cell death following DNA damage which suggests that chromatin remodeling is important in the DNA damage response pathway. While p53 activation seems to be independent of the chromatin landscape, certain downstream genes still require remodeling in order to allow for proper p53 function (Hafner, Kublo, et al., 2019). There are still questions that remain on how chromatin structure and p53 interact with one another before and after DNA damage and solving these questions will be essential for developing cancer therapies.

Because of heterogeneity in p53 dynamics at the single-cell level, correlating p53 expression patterns with global gene expression dynamics is a challenge. Recent studies have sought to identify gene expression changes in response to p53 activation by utilizing ionizing radiation induction (Hafner et al., 2017). These studies highlighted the diversity of gene expression dynamics between p53 target genes, which had different timing, levels, and patterns of induction. According to one study in 2016, genes such as RPS27L were continually expressed, certain genes such as DDB2 reached a maximum expression level, and some genes such as CDKN1A only were expressed after p53 protein pulses (Porter et al., 2016). This makes it difficult to determine the connection between cell fates and gene expression because the length of expression could depend on the type of damage and the time point of measurement. Measuring gene expression too early could result in missing short lived mRNA fragments and too late could result in total loss of all gene targets.

Conclusion

Both ATF4 and p53 are essential TFs that work within their respective stress response pathways in order to mitigate stress by reestablishing homeostasis or initiating cellular death

mechanisms. As more research is conducted within stress response pathways, I believe there will be more connections between essential TFs like ATF4 and p53 will be uncovered. Having multiple ways to resolve issues within a cell, protects the cell even in the scenario where one pathway becomes defective.

Chapter 5: Continuing Cellular Stress Pathway Research

While there are numerous papers that outline different characteristics and features of cellular stress dependent networks, there are still questions that remain. Cellular stress is continually attributed as a factor that contributes to cancer and many other diseases like it (Hanahan & Weinberg, 2011). Investigating these cellular stress dependent pathways allows us to better understand the roles that transcription factors (TFs) play in the activation of downstream gene regulation.

The cross talk that happens between cellular stress dependent networks is essential in maintaining homeostasis in the cell and promoting longevity. TFs are unique because they can be activated across multiple different stress dependent pathways, but it is still uncertain just how many of these TFs exist or if they share activation methods. Determining if certain stress dependent networks regulate TFs differently depending on the stress that initially triggered it will be key. This will allow us to better understand how different stress pathways can trigger the same physiological response but activate different sets of genes. For example, ATF4 is a TF that can regulate the expression of many genes after being activated by the unfolded protein response, the amino acid response, or the ER response (Wortel et al., 2017). Genes that are activated downstream of ATF4 can be shared between these different stress response pathways but produce two different responses. Expression of the C/EBP Homology Protein (CHOP) is elevated in response to the accumulation of unfolded proteins in both the mitochondria and the ER. Even though CHOP is shared by the two responses, during the mitochondrial unfolded protein response, CHOP prevents the upregulation of chaperones from non-mitochondrial compartments (Kourtis & Tavernarakis, 2011).

Another interesting aspect to consider is just how much chromatin structure and gene regulatory elements play a role in determining what genes are selectively activated. We know that p53, a gene involved in regulation of homeostasis during the DNA damage response, can bind to closed regions of chromatin and make it accessible, a characteristic that can be seen in

pioneer TFs (Yu & Buck, 2019). This same pioneering ability is not shared across TFs and thus in order for genes to become transcribed they need to be in areas of open and accessible chromatin. An example of this is during nuclear and mitochondrial stress, the chromatin remodeling factor ISW-1 is needed to correctly activate stress response pathways (Matilainen et al., 2017).

One specific point that still needs to be investigated is the relationship between TF binding sites, motif composition, and chromatin structure. Previous genome wide studies have been able to identify regulatory regions where certain TFs bind, but these studies have always been in the context of genes that work downstream of central TFs. The direct investigation of how central TFs such as ATF4 and p53 are able to regulate genes in a stress dependent context remains largely unknown. It is generally understood that both chromatin structure and other TFs play a role in the activity of TFs, but most studies have not looked at these elements in detail. In addition to this, more research is required to understand how local and long-distance chromatin structure plays alter gene expression via TF activity. Similar to chromatin structure, the presence or absence of certain transcription factors can potentially alter TF binding that can determine the regulation of certain stress dependent genes. Insight into TF activation is impactful because of its ability to activate separate sets of genes in response to conditions such as DNA damage or amino acid deprivation (AAD), and thus knowing the role that it plays in mitigating stress will be imperative to constructing potential clinical therapeutics.

One TF that has been documented in regulating genes in response to ER stress and AAD is ATF4. Investigating what regulatory elements are bound by ATF4 and their connection to stress dependent genes is important to understand ATF4's role in regulating stress dependent genes. Currently one of the best sources of ATF4 binding data that we have comes from a study in 2015 that mapped ATF4 binding in HAP1 cells under AAD, using ChIP-seq (Gowen et al., 2015). This study focused on ULBP1 and its role in tumor suppression but understanding ATF4's role in regulating stress dependent genes within the ISR would expand

this and provide insight into how ATF4 is mitigating stress. By using an assay known as CUT&RUN (cleavage under target; cut under nuclease), one can identify ATF4 binding sites and produce a result that has increased resolution when compared to ChIP-seq. By utilizing an improved TF binding site assay, one can gain new insights what genes could be regulated by ATF4 in response to ER stress and/or AAD. In order to determine what genes are being expressed in ER stress and AAD conditions, RNA-seq can be used to measure gene expression across the genome. By investigating what genes are being activated in response to ATF4 binding one can begin to understand ATF4's role in mitigating cell stress.

As mentioned in previous chapters, for TFs to bind to DNA regulatory elements such as promoters or enhancers, these regions generally need to be free of nucleosomes. As a result, nucleosome structure reflects chromatin accessibility and it dictates TF binding sites through steric hindrance (Allis & Jenuwein, 2016). Because of this an overwhelming majority of TFs surveyed within the ENCODE project bind to open chromatin almost exclusively (Thurman et al., 2012). In addition to accessibility, chromatin flexibility plays a role in long distance gene regulation. Promoters and distal enhancers interact through a process called chromosomal looping (Kadauke & Blobel, 2009).

Although most TF binding sites tend to be open, few studies have investigated the relationship between chromatin remodelers and cellular stress response mechanisms. Investigating regions of chromatin that are remodeled in response to cell stress versus regions that are constitutively open will provide insights into how chromatin structure affects gene regulation via TF binding site accessibility. In addition to binding site availability, genomic regulatory elements need to interact with each other to manage transcription. Because of this, chromatin needs to be modified to allow for distal promoter-enhancer interaction. To determine what specific regulatory elements are interacting with one another, one could utilize circularized chromosome conformation capture. This assay is able to assess chromatin interactions within a specific genomic region of interest and allows researchers to investigate chromatin looping

events. By looking at regions like the ATF4 binding site within the ASNS promoter, one could learn about the different interactions between promoters and enhancers during viral infection or ER stress.

The interaction of these different regulatory elements is facilitated by multiple TFs that work together in order to regulate the transcription of genes. ATF4 and p53 are unique because they are able to regulate a large collection of stress dependent genes through their interaction with different TFs and regulatory elements. The main factor that dictates where a TF binds to is its sequence motif. Thanks in part to projects like JASPAR, the field has a collection of predicted binding motifs across the genome. But because these are just predicted binding site motifs, it is unclear whether these TFs bind to their respective motifs constitutively or only under certain stress conditions. The TFs that are predicted to bind to the same regulatory regions as ATF4 are important because they are more likely to contribute to ATF4's activation. ATF4's ability to regulate genes that respond to ER stress and AAD, makes it important to understand how the neighboring TF motifs affect ATF4 activity. By mutating TF binding sites that neighbor an ATF4 binding site you can test to see if there is any difference in downstream gene expression between that site being unmutated versus mutated.

Ultimately these are only a handful of the many questions that remain regarding stress dependent pathways. As researchers continue to develop this field, we will better understand the gaps in knowledge that we need to address. Incorporating the advancements made in cellular stress response pathways will lead to potentially discovering new medicinal drug targets and therapeutics.

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