PARALLEL NETWORKS THAT GOVERN THE TRANSCRIPTIONAL RESPONSE TO STRESS

by

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ABSTRACT

The transcription factor, p53, plays a pivotal role in the oversight of many stimulus-dependent pathways. Its ability to respond to a wide variety of cellular stress stimuli by activating a broad range of target genes has led it to be characterized as a stress-dependent transcription factor. Our research focuses on deconvoluting the varied transcriptional response to distinct stress signals in an attempt to define the regulatory strategies leading to gene activation after cell stress. We have found that distinct stress response networks, some of which are p53-independent, are converging at activation of a common set of target genes. Our data suggest that Activating Transcription Factor 3 (ATF3), is a p53 target gene that is induced by multiple stress-dependent networks. We hypothesize that a group of direct and canonical p53 target genes are being activated in a p53-independent manner in response to other environmental conditions. We propose that the regulation of these p53 target genes is being achieved by the binding of stress-dependent transcription factors other than p53, at distinct regulatory regions such as enhancers and promoters. Researching the mechanisms of target gene activation induced by these parallel, stress-dependent pathways will provide insight into the regulatory paradigms employed by organisms to maintain and repair cellular homeostasis. Comparing and contrasting the transcriptional response to a variety of cellular stresses will allow for a better understanding of the general mechanisms used by the cell to respond to the vast array of environmental insults, which will provide insight into putative targets that may be attractive for future anticancer therapies.

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Chapter 1: The cellular response to stress

Introduction

Stress can be loosely defined as any disturbance to the natural elastic limit of cellular homeostasis. If homeostatic conditions in response to a particular stress stimulus do not exceed a certain threshold, the cell can mount an appropriate protective cellular response to survive this condition. Conversely, if the stimulus encountered is too severe, the damages cannot be repaired and homeostasis cannot be restored, the cell can initiate stress signaling cascades that eventually activate cell death pathways (**Fulda et al., 2010**). Several distinct stress responses can be distinguished, among them heat shock, unfolded protein, DNA damage, amino acid limitation, and oxidative stress responses (detailed hereafter) have been widely studied. Despite individual signaling components, these distinct stress stimuli can eventually incite general cell death effector mechanisms in the case that the cell is unable to successfully acclimate to the stress. Whether or not these forms of cellular stress trigger cell death or cell survival programs is determined by a set of different factors, including the severity and duration of the initial stress stimulus, the cell type encountering this perturbation, and various environmental factors preceding cellular contexts. Aberrant cellular stress responses are intricately linked to a plethora of human diseases, including diabetes, neurodegenerative disorders, cardiovascular disease, and many forms of cancer (**Costa-Mattioli & Water, 2020**). As such, investigations into the underlying molecular mechanisms involved in the response to various stress conditions are expected to provide insight into the development of targeted therapeutics and treatment strategies that will propel eukaryotic drug discovery.

Overview of Cellular Stress Responses

Heat Shock Response

Studies of a universal, prosurvival response, dubbed the Heat Shock Response, began in the early 1960s when researchers described a set of "puffs" on the salivary gland chromosomes of *drosophila busckii* that appeared to be induced by heat, dinitrophenol, or sodium salicylate (**Ritossa F., 1962**). As the name implies, the Heat Shock response was initially described as the biochemical response to cells undergoing mild heat stress, characterized by temperature elevations that exceeded 3-5C above normal conditions (**Craig EA., 1985 and Lindquist S., 1986**). It has since been recognized that many different stress stimuli, including oxidative stress and heavy metals, can induce the expression of heat shock proteins (Hsps), a set of highly conserved proteins that seem to have very general protective functions and have been implicated to play a role in normal growth and development. Hsps are grouped based upon similar molecular weights, approximately 110, 90, 70, 60, 40, and 15–30 kDa (**Samali A. & Orrenius S., 1998 and M. Jäättelä, 1999**). Some of these Hsps, for example, Hsp90, are constitutively expressed and act as molecular chaperones to reduce the premature folding of nascent polypeptides. Others are termed inducible Hsps, such as Hsp27 and Hsp70, which remain at relatively low basal levels until they are induced by environmental or physiological stressors (**Craig EA., 1985 and Lindquist S., 1986**). The induction of these Hsps inhibits apoptosis and promotes cell survival.

One of the main cellular consequences of these types of stress stimuli is the accumulation of unfolded proteins in the endoplasmic reticulum (ER). As a result, the heat shock response generally involves a global reduction in protein transcription and translation, along with the concomitant upregulation of chaperone proteins to alleviate the effects of misfolded protein accumulation. During this stress-induced rewiring of the transcriptome and

proteome networks, selective activation of a set of transcription factors, known as Heat Shock Factors (HSFs), occurs to enhance the expression of a subset of protective genes that will allow the cell to acclimate to these stress conditions, including the induction of Hsps (**Morimoto et al., 1996**). Vertebrates have three main HSFs: HSF1, shown to be essential for the Heat Shock Response and required for certain developmental processes, and HSF2 and HSF4, which are important for differentiation and development. HSF3 is only found in avian cells and is thought to be redundant in function to HSF1 (**Perkkala et al., 2001 and Shabtay & Arad, 2006**).

Inactive HSF1 is maintained in its monomeric form in the cytoplasm through interaction with Hsp90 and other chaperones. When the cell is exposed to heat or other stressful conditions, an accumulation of unfolded proteins occurs which outcompete HSF1 from binding to Hsp90. This release stimulates the oligomerization of HSF1 to its trimeric form where it relocates to the nucleus to interact with Heat Shock elements within the promoters of target genes, leading to induced expression of Hsps which promotes cell survival (**Voellmy R., 2004 and Shamovsky & Nudler, 2008**). The inhibition of stress-induced cell death by Hsps is largely achieved via direct modulations to the death receptor pathway and the intrinsic and extrinsic apoptosis pathways, including the interference of caspase activation and inhibition of pro-apoptotic factor release (**Samali et al., 2001 and Concannon et al., 2001**). Overall, Hsps can be activated by a range of stress stimuli, and act as pro-survival, anti-apoptotic molecules by influencing a variety of cellular processes which determine cell fate.

The Unfolded Protein Response (UPR)

Protein synthesis begins with the transcription and processing of pre-mRNA transcripts in the nucleus. The successful export of mature mRNA transcripts from the nucleus to the cytoplasm where translation occurs requires many regulatory checkpoints throughout this highly orchestrated process (**Kohler & Hurt, 2007**). Translational machinery synthesizes a polypeptide

chain which then undergoes further posttranslational processing in the endoplasmic reticulum (ER). Due to the critical cellular processes that occur in this organelle, including but not limited to, glycosylation, proper folding, oligomerization and disulfide bond formation, the ER environment must be tightly monitored to ensure the effective production and secretion of mature proteins. When cells are exposed to stressful conditions such as glucose starvation, hypoxia, and inhibition of protein glycosylation, an accumulation of unfolded proteins causes ER stress resulting in the activation of a set of pathways known as the Unfolded Protein Response (UPR). These signal transduction pathways are activated to either return the ER to its normal physiological state by increasing the folding capacity of the ER or to induce cell death via activation of the intrinsic and extrinsic apoptosis pathways.

Disturbances to the normal processes which ensure proteins are folded correctly, including those involving molecular chaperones, foldases, and lectins, lead to the initiation of ER-associated degradation (ERAD) pathways. If proper folding cannot be restored, and the accumulation of unfolded proteins continues, eukaryotic cells activate the UPR. In mammalian cells, the UPR is mediated by three ER-transmembrane receptors: Activating Transcription Factor 6 (ATF6), Inositol Requiring Kinase 1 (IRE1) and PKR-like ER kinase (PERK). This pathway has been characterized to have three main functions: adaptation, alarm, and apoptosis. During the initial phase of ER stress, or the adaptation phase, the goal of the cell is to restore folding homeostasis. This is done by inducing the expression of chaperones that assist in proper folding, as well as globally attenuating translation and degrading misfolded proteins to reduce the load on the ER. The PERK and ATF6 branches of the UPR are thought to largely promote this adaptation phase. If these measures fail to restore homeostasis, the cellular alarm program is initiated which reduces translational attenuation to promote an increase in the expression of pro-survival factors such as the B-cell lymphoma 2 (Bcl2) protein. After the alarm stage, the cell can undergo apoptosis or autophagy pathways. The IRE1 branch has been

shown to play a role in both pro-survival and pro-apoptotic signals, which is consistent with the idea that PERK and ATF6 branches are thought to be activated first, followed by IRE1 (**Szegezdi et al., 2006**).

In resting cells, all three ER stress receptors are maintained in an inactive state via their interaction with a molecular chaperone, GRP78/BiP. Among the UPR targets, glucose-regulated proteins (GRPs) are the best characterized and were initially identified as proteins induced by glucose starvation (**Shiu et al., 1977**). GRP78 is a family member of Hsp70, which as we described above, acts as a molecular chaperone during the Heat Shock Response (**Hightower & Hendershot, 1997**). Upon ER stress, GRP78 dissociates from each receptor leading to their sequential activation and subsequent triggering of the UPR. Upon activation of the first receptor, PERK, eukaryotic translation initiation factor 2 (eIF2a) is phosphorylated, blocking general protein synthesis as described above and preferentially enabling translation of certain ISR transcripts such as Activating Transcription Factor 4 (ATF4) (**Lu et al., 2004**). ATF4 then acts as a transcription factor to regulate expression of stress response genes required to restore ER homeostasis via interaction with cis-regulatory elements known as ER stress response elements (ERSE) (**Harding et al., 2000 and Yoshida et al., 1998**). Activation of PERK is rapidly followed by activation of ATF6 which is cleaved after its translocation from the ER to the golgi (**Chen et al., 2002**). Cleaved ATF6 then acts as a transcription factor, relocalizing to the nucleus to regulate the expression of ER chaperones, as well as X box-binding protein 1 (Xbp1), a transcription factor activated by the third branch of the UPR, where the Xbp1 protein is activated upon a splicing event carried out by IRE1 (**Yoshida et al., 2001a**). Activated Xbp1 then relocalizes to the nucleus to regulate expression of chaperones and genes involved in protein degradation (**Lee et al., 2003**). All three arms of the UPR induce the expression of basic leucine zipper (bZIP) transcription factors: ATF6, ATF4, XBP1, and growth arrest and DNA-damage inducible gene 153 (GADD153) also known as C/EBP Homologous Protein (CHOP).

(**Szegezdi et al., 2006**). CHOP acts as a transcription factor to regulate expression of pro-apoptotic genes such as BCL2 and GADD34.

The DNA Damage Response (DDR)

Upon cellular stress conditions that are caused by exposure to genotoxic agents, such as chemotherapeutic drugs, irradiation, or environmental stimuli such as ultraviolet (UV) light, a common initial result is damage to DNA (**Roos & Kaina, 2006**). The concerted action of sensors, transducers, and effectors to orchestrate an appropriate DNA repair and resolution of aberrant DNA structures has been deemed the DNA damage response (DDR). Until approximately 1996, the vast majority of what was known about the DDR came from research done in budding and fission yeast (**Elledge, 1996**). At that point, it was clear that the DDR was a signaling pathway activated by DNA damage and replication stress involving a phosphorylation cascade by regulatory kinases, however, it remained unclear how conserved these pathways were in mammals. Today, the past two decades of research in the DDR field have elucidated the highly conserved nature of this across mammals, defining homologs for each DDR pathway component identified in yeast. Additionally, it is now clear that the DDR is a multifaceted signaling pathway that regulates many physiological processes to ultimately repair any DNA damage and facilitate DNA replication.

To ensure proper protection of the genome, the cell must be able to detect a wide range of structural DNA alterations, also known as lesions, including nicks, gaps, single-stranded breaks (SSBs), double-stranded breaks (DSBs), and the myriad of alterations that block DNA replication. Distinct cellular repair mechanisms have evolved to specifically manage the type of DNA lesions encountered. For example, mispaired DNA bases or small chemical alterations to DNA bases can be repaired by mismatch repair (MMR) or base excision repair (BER) machinery, respectively, while more complex lesions such as pyrimidine dimers caused by UV

light must be repaired by nucleotide excision repair (NER) mechanisms (**Jiricny, 2006, Lindahl and Barnes, 2000, and Hoeijmakers, 2009**). SSBs are repaired via single-strand break repair (SSRB) while DSBs are processed by either non-homologous end joining (NHEJ) or homologous recombination (HR) (**Caldecott, 2008, and West, 2003**). The various DNA repair machinery is carried out by multiple enzymatic activities that chemically modify DNA to aid in these repair processes, including but not limited to: nucleases, helicases, topoisomerases, phosphatases, kinases, recombinases, and ligases (**Ciccia & Elledge, 2010**). This plethora of repair tools must be tightly and precisely regulated to orchestrate an appropriate DDR. As a result, eukaryotic cells have evolved strategies that manage the recruitment of specific DNA repair factors to sites of DNA damage, the activation of those factors, and the subsequent cellular decisions employed for efficient DNA damage repair.

DNA lesions and specific types of DNA damage are recognized by a set of at least five independent molecular complexes, of which the best characterized to date are proteins of the phosphatidylinositol 3-kinase-like protein kinases (PIKKs) family: ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR) and DNA-dependent protein kinase (DNA-PK) (**Valerie & Povirk, 2003, Jackson, 2002, and Meek et al., 2002**). ATM and DNA-PK are activated by DNA damaging agents that induce DSBs. ATM in its inactive form exists as a dimer that is recruited to broken DNA molecules by the Mre11-Rad50-Nbs1 (MRN) mediator complex which senses DSBs (**Lee & Paull, 2005**). Upon recruitment to DSBs, ATM dimers dissociate to a monomeric state and autophosphorylation events lead to the subsequent phosphorylation of one of its many substrates, such as checkpoint kinase 2 (CHK2). Unlike ATM which has hundreds of different substrates, DNA-PK regulates a smaller subset of proteins involved in DSB end joining. ATR is activated by DNA damaging agents that induce SSBs, achieving DDR via interaction with its regulatory subunit, ATR-interacting protein (ATRIP) (**Zou & Elledge, 2003**). ATR/ATRIP phosphorylates the cell cycle checkpoint protein, Rad17, which loads the

Rad9-Rad1-Hus1 (911) complex to the site of DNA damage. Stimulation of ATR activity by 911-associated protein, TOPBP1, activates the ATR signaling cascade leading to the subsequent phosphorylation of checkpoint kinase 1 (CHK1). In summary, upon recognition of DNA lesions, ATM and ATR activate mediator protein complexes which amplify the DDR by further recruiting ATM/ATR substrates. Effector proteins are then activated downstream of either ATM/ATR or CHK1/CHK2 kinases to activate the expression of DDR target genes that help the cell respond to this genomic instability.

Both ATM and ATR are required for the NHEJ, HR, and NER repair mechanisms which are mediated via both relatively fast posttranslational modifications or slower processes that involve transcriptional responses via effector proteins such as tumor suppressor protein, TP53 (p53). P53 is one of the best-characterized effectors of the DDR, and is activated by ATM/CHK2 in response to DSBs. In response to DNA damage, p53 induces important cellular programs such as cell cycle arrest, apoptosis and senescence via the transcriptional regulation of a broad range of target genes. In a prosurvival program, p53 can activate genes to induce cell cycle arrest, such as cyclin-dependent kinase inhibitor 1A (CDKN1A/p21), to allow time for these repairs to be made and ensure they are not further propagated. If in the case the damage is too severe or simply cannot be repaired, p53 can activate expression of proapoptotic proteins, Bcl-2 associated X protein (BAX), and p53 upregulated modulator of apoptosis (PUMA) (**Riley et al., 2008**). However, it is important to note that genetic studies in mouse models have demonstrated that p53-mediated acute DNA damage responses are indispensable for p53-dependent tumor suppression functions (**Li et al, 2012 and Valente, et al., 2013**). In summary, the response to DNA damage involves multiple repair pathways and surveillance mechanisms that allow the cell to activate cell cycle checkpoints as well as cell death programs.

The Amino Acid Response (AAR)

Amino acids (AA) are one of the main building blocks of life, and as such, mammalian cells and organism require efficient regulatory mechanisms to ensure a homeostatic balance of intra- and extracellular amino acid composition. A reduction in total dietary protein or a nutrient source with an imbalance in AA composition induces an amino acid deprivation response termed the Amino acid response (AAR) (**Kilberg et al., 2005**). To date, there are 22 genetically encoded AAs, twenty of which form the main building blocks for protein synthesis while the other two, selenocysteine and pyrrolysine, can be incorporated into nascent polypeptides via special translation mechanisms. Of these 20 proteinogenic AAs, 11 are classified as non-essential, as these can be synthesized by the majority of cells from metabolic intermediates, while the remaining nine "essential" AAs must be acquired from nutrients. Several factors regulate AA homeostasis including the entry and exit of amino acids in/out of the cell via transporters and, both AA and protein, biosynthesis and degradation (**Bröer & Bröer, 2017**). The human genome contains ~50 different amino acid transporters, many of which display cell-type specific expression and regulation (**Bröer & Palacin, 2011 and Perland & Fredriksson, 2016**). Consistent and highly expressed AA transporters include the Sodium-coupled neutral amino acid transporter (SNAT) proteins, SNAT-1, 2, 6 and -7, and alanine serine cysteine transporters (ASCT1 and ASCT2) belonging to the Solute carrier family 1 (SLC1) protein family (**Arriza et al., 1993, Utsunomiya-Tate et al., 1996, and Bröer et al., 2016**). Due to the constant turnover of proteins, cells recycle most AAs over time. The biosynthesis of non-essential AAs requires the cooperation of multiple signaling pathways, almost all of them being upregulated by a stress-dependent transcription factor, ATF4, which is induced upon amino acid deprivation (**Kilberg et al., 2005**).

Maintenance of AA homeostasis largely depends upon the ability of a cell to maintain a provision of the 20 proteinogenic amino acids within the cytosol, used to charge transfer RNA

(tRNA) molecules required to carry out protein synthesis. Intracellular concentrations of amino acids are dynamic and dependent upon the concerted actions of multiple tightly regulated signaling pathways, including the mechanistic target of rapamycin (mTOR) and general control nonderepressible 2 (GCN2) pathways (**Kilberg et al., 2005**). While the initial AA sensor of these AAR pathways has yet to be completely defined, it is generally accepted that the GCN2 kinase serves as a sensor of AA deficiency, as an increase in uncharged tRNA binds to the GCN2 kinase causing activation of the GCN2 pathway via phosphorylation of eIF2a (**Berlanga et al., 1999 and Sood et al., 2000**). As mentioned previously in our discussion of the UPR, eIF2a phosphorylation leads to a reduction in global protein synthesis with concomitant upregulation of specific transcripts, such as that encoding for ATF4. This was initially demonstrated for the yeast TF, GCN4, which was deemed a "master transcriptional regulator" of nutrient sensing in yeast, as it was observed to be upregulated in response to AA starvation and led to transcriptional changes in hundreds of genes' expression (**Natarajan et al., 2001**). This translation control in response to stress stimuli is modulated via short upstream open reading frames (ORFs) within these mRNA transcripts that permit translation of these TFs in the presence of translation inhibition by phosphorylated eIF2a (**Lu et al., 2004 and Vattem & Wek, 2004**). To date, studies in mammalian cells have implicated ATF4 as an important regulator of many cellular stress responses, including the ISR, which will be further detailed in Chapter 3: The Integrated Stress Response. (**Harding et al., 2003**). Along with the GCN2 pathway, the mTOR signaling pathway has been described to function as a checkpoint to confirm sufficient levels of AAs to support protein synthesis and cellular growth (**Fingar & Blenis, 2004**). Conversely to the GCN2 pathway which is activated in response to AA starvation, the mTOR pathway is activated in response to AA sufficiency leading to the subsequent phosphorylation of the ribosome-associated S6 kinase (S6K1) (**Kimball & Jefferson, 2004**). Activation of S6K1 allows for a high level of translation of mRNA transcripts encoding ribosomal proteins via the phosphorylation and inactivation of eukaryotic initiation factor 4E-binding protein (4E-BP1), an

inhibitor of mRNA translation (**Burnett et al., 1998**). The regulation of these two substrates, S6K1 and 4E-BP1, via mTOR signaling permits protein synthesis and cell growth rates to be maintained in a manner that is consistent with nutrient availability.

The Response to Oxidative Stress

Oxidative stress can be defined as an imbalance between oxidants and antioxidants, favoring the oxidants, which can lead to a lack of control in oxidation-reduction (redox) signaling and molecular damage to the cell (**Sies, 2015**). Years of research in the field of redox biology have uncovered the strategies employed by a cell to protect against the deleterious effects of reactive oxidants, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), generated via aerobic metabolism. ROS is a general term used to describe the various oxygen metabolites produced via redox processes including electron transfer and free radicals, including but not limited to: superoxide anion radicals, hydrogen peroxide, hydroxyl radicals, electronically excited states such as singlet molecular oxygen, as well as the nitric oxide radical and peroxynitrite (**Levonen et al., 2014**). These different types of ROS each have varying half-lives and affinities for different biomolecules, often binding with nucleic acids, enzymes, membrane lipids, proteins, and other small molecules (**Rajendran et al., 2014**). It is commonly accepted that the majority of ROS is produced via the mitochondrial respiratory chain, however, both exogenous (UV radiation) and endogenous (oxidases and oxygenases) sources of ROS can also contribute to the formation of oxidative stress (**Filomeni et al., 2015**). Low concentrations of ROS have an indispensable role in intracellular signaling pathways and the defense against pathogens, however, when levels of ROS reach a higher amount, these can contribute to many human diseases.

The cellular strategies employed to reduce damage caused by oxidative stress in response to ROS and RNS can be separated into three main processes: prevention, interception, and repair (**Sies, 1993**). The first line of defense against ROS is of course to

protect against their formation, a process carried out by many naturally occurring and diverse antioxidant compounds. The range of antioxidant compounds and enzymes that have evolved in biological systems can be grouped into non-enzymic (glutathione (GSH) and vitamins E and C), direct enzymic (superoxide dismutases (SODs), catalases, and GSH peroxidases), and enzymatic repair systems (Reduced nicotinamide adenine dinucleotide phosphate (NADPH) supply transport) (**Birben et al., 2012**). The interception process involves two main stages, one in which these ROS are sensed and upon formation, intercepted to prevent further damage from occurring via the process of deactivation. For radical compounds, this consists of the formation of a non-radical end product and typically occurs via a chain reaction wherein a compound carrying an unpaired electron will react with another compound to generate an unpaired electron in that compound, and so forth (**Sies et al., 2017**). The next stage of interception involves transferring the radical function away from further potential targets and towards a compartment of the cell where this oxidative species would be less damaging. As such, these antioxidant compounds and enzymes within the cell must be able to react with initial free radicals upon their formation, as well as interact with water-soluble compounds for their regeneration (**Sies, 1993, and Sies et al., 2017**). The fact that different subcellular sites and cell types have varying amounts of antioxidant compounds and enzymes reveals the requirement for a high level of regulation of these processes. Finally, the repair stage of the oxidative response is necessary, as these first two processes of prevention and interception are often not completely effective, and as such, damaging products may continue to form and accumulate. These damages can be in the form of DNA DSBs or SSBs, membrane damage via phospholipid oxidation, or other various macromolecular damages to proteins and compounds in the cell. Recent studies have linked the response to oxidative stress and redox-dependent signaling to numerous other stress-dependent networks and implicate its dysregulation in the causation of many human diseases (**Beckman & Ames, 1999, Wei et al., 2001, and Rahman et al., 2012**).

Evolution of the stress response: From single cells to multicellular organisms

Living organisms evolved in a hostile environment. Energy for organismal activities was derived from the oxygenated atmosphere via oxidative metabolism which in turn produced the many forms of potentially lethal ROS (**Trosco & Inoue, 1997**). As we've outlined in the previous section, fluctuations in environmental conditions such as temperature, ionizing and non-ionizing radiation, extrinsic environmental compounds, and intrinsic stresses shaped the cellular stress responses that have evolved to specifically manage these perturbations. During the transition from single-cell organisms, which adaptively survive by cell proliferation, to multicellular organisms, a unique gene expression program developed and the appearance of proteins that are required for intermembrane channels between contiguous cells, such as connexins, emerged (**Revel, 1988 and Kumar & Gilula, 1996**). The appearance of the connexin proteins correlates with the appearance of differentiated cells that allow the emergence of higher-order functions contributing to their adaptive responses and ultimately their ability to survive (**Loewenstein, 1979**). Multicellular organisms can therefore be thought of as, not just a collection of individual cells, but rather a well-coordinated collection of groups of different cells, each with unique functions, that must act together in concert with the fundamentals of all cells such as cell proliferation (**Trosco & Inoue, 1997**). As such, cellular stress responses have evolved in terms of the cellular goals and processes that result from these adaptations. For example, in unicellular organisms, quiescent states are essential for surviving adverse environmental conditions ranging from nutrient deprivation to antibiotic exposure (**Rittershaus et al., 2013**). In multicellular organisms, quiescence is essential for tissue homeostasis and the maintenance of adult stem cells (**Sun & Buttitta, 2017 and Li & Clevers, 2010**). While many of the stress-induced anti-proliferative activities of both single cells and multicellular organisms are shared, the specific pathways orchestrating these responses are very different.

Stress-induced anti-proliferative activities

Apoptosis

The term apoptosis was first used to describe the specific morphology of cell death common to the vast majority of dying cells. This morphology includes the shrinkage and blebbing of a cell, rounding and fragmentation of condensed nuclei, and in most cases margination of chromatin and phagocytosis of cell fragments without the accompaniment of an inflammatory response (**Kerr et al., 1972**). The morphology of cells undergoing apoptosis appeared distinct from that associated with other forms of cell death such as necrosis, therefore apoptosis quickly became one of the best-characterized forms of cell death and subsequently an attractive target for therapeutic intervention. Apoptosis became the focus of many research studies during the 1980s where researchers attempted to delineate the underlying biochemical and molecular pathways involved in this type of cell death. Today, apoptosis is generally accepted as caspase-dependent programmed cell death due to milestone discoveries in the field, such as the identification of pro-apoptotic proteins, death receptors, and caspases.

The morphological and biochemical changes associated with apoptosis can be largely explained by the activation of caspases, an evolutionarily conserved family of cysteine proteases that act as common death effectors during various forms of apoptosis. There are two main forms of caspases: initiator caspases (-2, -8, -9, -10, -12) containing a caspase activation and recruitment domain (CARD), and effector caspases (-3, -6, -7, -14) displaying death effector domains (DEDs) (**Ho & Hawkins, 2005**). As the name implies, initiator caspases function upstream within apoptotic signaling pathways and are capable of activating downstream effector caspases either directly, through proteolysis, or indirectly via a secondary messenger. Upon activation by an initiator caspase, effector caspases act as immediate executioners of the apoptotic program through cleavage of certain cellular components to cause demolition of the

cell. Together, these caspases are responsible for diverse cellular functions including apoptosis and inflammation (**Degterev et al., 2003**). This family of caspases can be further divided on a functional basis distinguishing between inflammatory and apoptotic caspases. For example, caspases -1, -4, -5, and -11 have been shown to play roles in cytokine maturation and inflammatory responses while the remaining family members are primarily involved in apoptotic signaling pathways (**Martinon et al., 2000**).

A hallmark of apoptosis is the proteolytic cleavage of a vast array of cellular proteins by caspase enzymes. Effector caspases cleave a variety of natural cellular substrates, including proteins that are responsible for the structural integrity of the cell, while initiator caspases cleave proteins that have more indirect roles in cellular morphology and metabolism. The cleavage of these cellular substrates by caspases can either functionally activate or inactivate their targets. For example, the proapoptotic Bcl-2 family member, Bid, becomes activated upon cleavage by caspase-8 initiating the formation of the apoptosome by the mitochondrial release of cytochrome C (**Gross et al., 1999**). Cleavage of another substrate, RIP1, by this same caspase leads to its inactivation and ultimately blocks survival signals mediated by NF-kb during FAS-induced apoptosis (**Martinon et al., 2000**). Due to the vital roles that these caspases play in maintaining the balance between apoptosis and survival, their activation and expression must be tightly regulated. Most caspases are constitutively expressed, however others require transcriptional regulation in certain contexts. For example, while transcription factor motifs present within the promoter region of caspase-8 have been shown to control basal expression levels, the presence of other *cis*-acting regulatory elements within these caspase transcripts can cause upregulation in response to certain conditions (**Liedtke et al, 2003 and Nishiyama et al., 2001**).

Necrosis

Apoptosis and necrosis are considered the two main forms of cell death and were initially considered mutually exclusive forms of programmed cell death. However, extensive studies into the cellular mechanisms regulating these forms of cell death have elucidated that there is a large amount of interplay between these two signaling pathways that must be balanced for successful cell death to occur (**Nikoletopoulou et al., 2013**). Morphologically, necrotic cells are characterized by the swelling of organelles, such as the ER and mitochondria, the rupturing of the plasma membrane and ultimately, lysis of the cell (**Schweichel & Merker, 1973**). Programmed cell necrosis, termed necroptosis, differs from apoptosis morphologically in that the nucleus typically becomes distended but remains largely intact. Other important differences between these two forms of cell death have been recognized. First, while apoptosis is largely regarded as an active and "programmed" process of cell death, necroptosis is defined as a passive, "accidental" cell death induced by deleterious cellular conditions (**Fink & Cookson, 2005**). Secondly, apoptosis generally avoids eliciting an inflammatory response, while necroptosis is characterized by the subsequent uncontrolled release of inflammatory compounds (**Los et al., 2002**). This inflammatory response of necrotic cells results in activation of the inflammasome and the subsequent release of pro-inflammatory cytokines, such as IL1β. Lastly, unlike apoptosis, necrosis is typically not associated with activation of caspases (**Kerr et al., 1972**).

Ferroptosis

Ferroptosis is an iron-dependent form of regulated cell death that is morphologically and mechanistically distinctive from other known forms of regulated cell death, such as apoptosis and necroptosis, discussed above (**Galluzzi et al., 2018**). Ferroptosis is triggered by the toxic buildup of lipid peroxides and other forms of ROS on cellular membranes (**Hadian & Stockwell,**

2020 and Jiang et al., 2021). Ferroptosis has been implicated as an important mediator of tumor-suppressive functions, as impaired ferroptosis has been shown to contribute to tumor development (**Jiang et al., 2015**). The role of p53 in mediating ferroptosis has been observed in response to high levels of ROS, although the mechanisms that underlie these responses remain poorly understood. Similar to the divergent effects of p53 activation during the DDR (promoting cell survival in response to acute DNA damage and signaling towards cell death upon severe DNA damage), it has been suggested that in response to low or basal ROS levels, p53 may prevent cells from accumulating lethal levels of ROS while also allowing survival and repair of moderate oxidative damage (**Jiang et al., 2015**). Conversely, in response to higher ROS levels, p53 may instead promote the removal of unsalvageable cells through activation of p53-mediated ferroptosis (**Zhang et al., 2018**). Unlike apoptotic cell death, activation of p53 alone is not sufficient to induce ferroptosis directly; rather, ferroptosis is modulated by p53 via expression of p53-activated metabolic target genes (Liu & Gu, 2022).

Cell cycle arrest

Cell cycle arrest is an active and generalized response to stresses that promotes cell survival under changing environmental conditions by inhibiting cell cycle progression. While many of the eukaryotic signaling pathways involved in this response have been elucidated, the putative core module responsible for orchestrating cell cycle arrest in response to various stress stimuli remains elusive (**Sun & Gresham, 2021**). Quiescence, a term used for cells in a nonproliferative state, can be regulated by various stress stimuli and distinct developmental signals (**Cheung and Rando, 2013**). In Eukaryotes, quiescence commonly occurs in a state where the activity of a proliferation-promoting kinase, CDK1, is low (low-CDK1 quiescence). In many organisms, however, cells can enter quiescent states even in the presence of high CDK1 activity (high-CDK1 quiescence), the mechanisms for which remain largely uncharacterized (**Sun and Gresham, 2021**). It has been suggested that Stress-activated pathways are good

candidates to promote quiescence regardless of Cdk1 activity due to their capacity to decrease global levels of translation and transcription and rewire cellular proteome networks (**Miles et al., 2013 and Marion et al., 2004**). As we will discuss in Chapter 3, the stress-dependent TF, p53, plays a crucial role in regulating cell cycle checkpoints via the upregulation of cell cycle inhibitors, such as CDKN1A/p21, as well as the downregulation of many cell cycle genes (**Engeland, 2018**). As mentioned above, unstressed, non-quiescent cells, either immediately increase CDK1 activity and enter the next cell cycle following mitosis, or exit the cell cycle entering a quiescent state, defined by low CDK activity and significantly higher levels of CDKN1A/p21 (**Pack et al., 2019**). Quiescence entry, therefore, depends on p21 expression, a canonical and direct p53 target gene. As such, cells devoid of CDKN1A/p21 rarely enter the low-CDK1 quiescence (**Overton et al., 2014**). Similarly, a component of the Integrated Stress Response (ISR), which will be discussed in detail in the following chapter, has also been implicated in regulating cell cycle arrest in response to diverse forms of cellular stress (**Miles et al., 2013 and Argüello-Miranda et al., 2021)**. Recent investigations into the initial stages of quiescence entry in response to AA starvation revealed that these signals are integrated by histone deacetylase regulator, X-box binding protein 1 (Xbp1) which was found to be essential for high-CDK1 quiescence in yeast (**Argüello-Miranda et al., 2021**).

Chapter 2. The Integrated Stress Response (ISR)

Introduction

The ISR is an elaborate signaling pathway present in eukaryotic cells that responds to both cell-extrinsic factors such as amino acid deprivation and viral infection, as well as cell-intrinsic factors such as endoplasmic reticulum (ER) stress. These cellular disturbances activate a set of regulatory kinases such as PKR-like ER kinase (PERK), double-stranded RNA-

dependent protein kinase (PKR), heme-regulated eIF2a kinase (HRI), and general control non-derepressible 2 (GCN2), that converge upon phosphorylation of eIF2a at the core of the ISR (**Donnelly et al., 2013**). Each of these eIF2a kinase family members shares homology in their catalytic regions, however, maintain distinct regulatory regions responsible for their unique functions in responding to specific stress stimuli (**Pakos-Zebrucka et al., 2016**). These stress signals detected by the regulatory domains of these distinct kinases trigger activation by dimerization and transautophosphorylation (**Lavoie et al., 2014**) to converge on activation of eIF2a via phosphorylation of Ser51 (**Wek, 2018**). Phosphorylation of eIF2a leads to global attenuation of cap-dependent translation in concomitance with preferential translation of ISR-specific transcripts, such as Activating Transcription Factor 4 (ATF4). ATF4 is a family member of ATF3, and is the best characterized transcriptional effector protein in the ISR pathway (**Pakos-Zebrucka, 2016**). The transcriptional, translational, and post-translational regulation of ATF4, as well as dimerization partners, can influence the cellular outcome of exposure to different stress stimuli, allowing the ISR to produce cellular outcomes tailored to the specific stress signal encountered (**Costa-Mattioli & Walter, 2020**).

Activation of the ISR

Four kinases converge on eIF2 to activate the ISR

PKR-like ER kinase (PERK)

PERK , is a transmembrane protein located in the endoplasmic reticulum (ER) membrane where its luminal domain is normally bound by 78kDa glucose-regulated protein (GRP78, also known as BiP/ HSP5a), a chaperone heat shock protein that serves as a master transcriptional regulator of the UPR pathway induced by ER stress. As described in Chapter 1, ER stress can arise from many cellular conditions including the accumulation of unfolded

proteins in the ER, perturbations in cellular energy metabolisms, calcium homeostasis, and/or imbalances in redox status. The response to these stress conditions is mediated via the activation of PERK which has been demonstrated to occur via two distinct mechanisms (**Korennykh & Walter, 2012 and Wang & Kauffman, 2016**). The classical model proposes that upon exposure to stressful conditions in the ER lumen, GRP78 dissociates from PERK, leading to its autophosphorylation and subsequent activation (Harding et al., 1999, Shi et al., 1998, and Bertolotti et al, 2000). In contrast to this classical model, more recent studies have suggested an alternative mechanism of PERK activation whereby PERK may be directly bound by unfolded or misfolded proteins at its luminal domain, causing subsequent activation (**Gardner & Walter, 2011 and Korennykh & Walter, 2012**). While further direct evidence is required for this updated model of PERK activation, it has been demonstrated that another ER sensor of the UPR, inositol-requiring enzyme 1 (IRE1), can be directly activated by unfolded or misfolded proteins in yeast, potentially bolstering this newly proposed model (**Carrara et al, 2015, Gardner & Walter, 2011, and Korennykh & Walter, 2012**). PERK activation has been demonstrated downstream of multiple other cellular stress pathways including the AAR pathway induced via glucose deprivation (**Moore et al., 2011**) and oncogene activation in cancer cells (**Hart et al., 2012**).

Double-stranded RNA-dependent protein kinase (PKR)

As the name suggests, mammalian PKR is a central component of the interferon antiviral defense pathway, activated mainly in response to double-stranded RNA (dsRNA) during viral infection (**Clemens & Elia, 1997**). Upon PKR activation in the presence of dsRNA, like all other ISR kinases, a dimerization and autophosphorylation step leads to the subsequent inhibition of viral and host protein synthesis through eIF2α phosphorylation (**Balachandran et al., 2000 and Dey et al., 2005**). Nevertheless, a plethora of other stress stimuli have also been shown to activate PKR in a dsRNA-independent manner, including but not limited to oxidative

and ER stress (**Shimazawa & Hara, 2006 and Lee et al., 2007**), growth factor deprivation (**Garcia et al., 2006**), and cytokine exposure in response to bacterial infection (**Williams, 1999**). Additionally, PKR can also be stimulated in response to caspase activity in the early stages of apoptosis, indicating a potential role for protein synthesis inhibition in the programmed cell death response (**Saelens et al., 2001**).

General control non-derepressible 2 (GCN2)

GCN2 is highly conserved from yeast to humans (**Castilho et al., 2014**) and contains a regulatory domain that shares homology with histidyl-transfer RNA synthetase (His-RS), an enzyme responsible for the synthesis of histidyl-transfer RNA (tRNA) which incorporates histidine into newly synthesized proteins (**Vasquez de Aldana et al., 1994**). Mechanistic insight into the activation of this kinase, largely derived from studies in yeast, elucidated that this kinase responds to amino acid depletion and nutrient deprivation by interacting with and binding to deacetylated tRNAs (**Harding et al., 2000 and Hinnebusch, 2005**). Recent work suggests that GCN2 may actively monitor mRNA translation and not just aminoacyl-tRNA availability, as it has been demonstrated to bind to a component of the P1/P2 stalk of the large ribosomal subunit, revealing another modality of GCN2 activation by not only tRNA but by the presence of stalled ribosomes (**Ishimura, et al., 2016 and Inglis et al., 2019**). It is now generally accepted that GCN2 can also be activated by other stresses, including ultraviolet light (UV), viral infection, serum starvation, and oxidative stress (**Costa-Mattioli & Walter, 2020 and Pakos-Zebruka et al., 2016**). However, the precise mechanisms underlying these vastly different activation modalities remain unknown.

Heme-regulated eIF2a kinase (HRI)

HRI, as the name suggests, is regulated via two heme-binding domains, one contained in the NTD and another in the kinase insertion domain (**Rafie-Kolpin et al., 2000**). The binding

of heme to these domains regulates HRI kinase activity, inhibiting the kinase by keeping it in an inactive dimer state. When cellular concentrations of heme are low, the absence of heme allows for non-covalent interactions between HRI molecules, resulting in an activated HRI dimer (**Hirai et al., 2007**). Given its regulation by heme and the fact that HRI is highly expressed in erythroid cells where it is involved in erythrocyte differentiation during erythropoiesis, HRI was long thought to have a specialized role in these cells dedicated to hemoglobin synthesis (**Han et al., 2001 and Chen, 2014**). It is now recognized that HRI is widely expressed in several cell types and organs (**Tabula Muris Consortium et al., 2018**) and responds to multiple other forms of cellular cell stress, such as oxidative and mitochondrial stress, heat shock, and cytosolic protein aggregation (**Guo et al., 2019 and Lu et al., 2001**). Interestingly, activation of HRI by these diverse stresses can occur independent of heme and is mediated by certain heat shock proteins, such as HSP90 and HSP70 (**Lu et al., 2001**); However, the exact mechanism of HRI activation in response to these various stimuli remains to be investigated.

Termination of the ISR

Dephosphorylation of eIF2a

Just as phosphorylation of eIF2α is central to the activation of the ISR signaling cascade, dephosphorylation of eIF2α is pivotal to ISR signal termination when restoration of protein synthesis and normal cell functioning is required after homeostatic conditions are stabilized in response to stress. Dephosphorylation of eIF2α is mediated by the protein phosphatase 1 (PP1) complex, recruiting a catalytic subunit (PP1c) and two regulatory subunits in mammals: PPP1R15A and PPP1R15B (**Novoa et al., 2001**). PPP1R15A, also known as growth arrest and DNA damage-inducible protein (GADD34), is induced by the ISR in response to stress, while the constitutively expressed paralogue, PPP1R15B, also known as constitutive repressor of

eIF2α phosphorylation (CReP), seems to be responsible for targeting the enzyme to eIF2α (**Jousse et al., 2003**). CReP generally operates in a complex with PP1c in unstressed cells to sustain translational homeostasis by maintaining low levels of eIF2α phosphorylation. In contrast, GADD34 expression is induced as a consequence of phosphorylated eIF2α, downstream of ATF4 during the later stages of ISR activation, subsequently leading to an increase in eIF2α dephosphorylation (**Kojima et al., 2003**). Thus, the GADD34–PP1 complex acts as an important negative feedback loop to restore protein synthesis once the particular stress has been resolved (**Ma & Hendershot, 2003**). In addition to promoting cell survival after stress, it may also facilitate the execution of cell death programs in the case that cellular homeostasis cannot be restored (**Liu et al., 2015**). The critical nature of successful eIF2α dephosphorylation is demonstrated by studies in knockout mouse models where PPP1R15A and PPP1R15B double-knockout mice exhibit early embryonic lethality, which can be rescued by mutations which prevent eIF2α phosphorylation (**Harding et al., 2009**). The mechanisms of ISR termination may not be limited to aiding in cellular recovery after protein synthesis cessation, but may also play important, independent roles in apoptosis induction (**Farook et al., 2013**).

The ISR pathway: The basics

eIF2a phosphorylation regulates the Ternary Complex (TC)

We have covered that the ISR's central regulatory switch centers around the modulation of phosphorylation states of eIF2a. This mechanism is tightly regulated via the cellular concentration of the eIF2 ternary complex (TC) composed of heterotrimeric eIF2 subunits (α, β, and γ), guanosine 5′-triphosphate (GTP), and charged methionyl-initiator tRNA (Met-tRNAi) (**Wortham et al., 2014**). While eIF2α, eIF2β, and eIF2γ together form the eIF2 TC, the eIF2α subunit is the main regulatory subunit of this complex since it contains both the phosphorylation

and RNA binding sites. Under normal conditions, eIF2 plays a key role in the initiation of mRNA translation and recognition of the AUG start codon (**Pain, 1996**). It forms the TC with GTP and Met-tRNAi binding the 40S ribosomal subunit, and together with two small initiation factors, eIF1 and eIF1A, subsequently forming the 43S pre-initiation complex (PIC) (**Aitken & Lorsch, 2012 and Lomakin & Steitz, 2013**). The exchange of GDP for GTP that is required for subsequent rounds of translation is catalyzed by the guanine nucleotide exchange activities of the eIF2β subunit. This exchange converts eIF2 back to its active form and dissociates the TC from the 40s ribosomal subunit (**Jackson et al., 2010**). In response to ISR activation, phosphorylated eIF2α blocks the eIF2β-mediated exchange of GDP for GTP, thereby preventing the formation of the 43S PIC, resulting in the global attenuation of 5′Cap-dependent protein synthesis and concomitant translation of selected ISR transcripts, such as those encoding *ATF4*, *CHOP*, and *GADD34* (**Harding et al., 2003, Lee et al., 2009 and Palam et al., 2007**). These preferentially translated mRNAs commonly contain a short upstream open reading frame (uORF) in their 5′ untranslated regions (5′UTR), and these select mRNAs do not require recognition of a 5'mehtylguanine cap, but rather, their translation relies upon a re-initiation mechanism or the direct recruitment of ribosomes to an internal ribosome entry site (IRES) (**Chan et al., 2013**).

ATF4 is the best-characterized effector of the ISR

A general consequence of eIF2a phosphorylation in response to stress signals is the global attenuation of cap-dependent protein synthesis, however, a known caveat to this response is the accompanying increase in translation of certain ISR transcripts.
During repression of global translational initiation, phosphorylated eIF2a selectively enhances the translation of ATF4 mRNA, encoding a basic leucine zipper (bZIP) TF that belongs to the activating transcription factor/cyclic AMP response element binding protein (ATF/CREB family) (**Karpinski et al., 1992 and Vallejo et al., 1993**). ATF4 is a key regulator of cellular fate decisions in response to ISR activation, and several dimerization partners may influence the

regulation of gene transcription that can ultimately influence cellular outcomes in response to stress (**Marciniak & Ron, 2003**). ATF4 is regulated at the transcriptional, translational, and post-translational level, and additionally, can be regulated via its ability to interact with other transcription factors to coordinate a gene expression program in response to stress (detailed in latter sections). A consequence of this intricate regulation is that despite the common mediator, the ISR produces distinct tailored responses to different cellular stresses, with the activation of different ATF4 target genes also being highly dependent on stress intensity and the cellular context (**Shroder & Kaufman, 2005, Ron & Water, 2007, and Pakos-Zebruka et al., 2016**).

ATF4 functions as a TF

ATF4 has been implicated as the main effector of the ISR, acting as a master transcriptional regulator during stressful conditions by facilitating the transcriptional upregulation of stress-responsive genes to ameliorate the deleterious effects of these conditions. Investigations into the TF activities of ATF4 have elucidated vital roles in many tissues, functioning in the regulation of obesity, glucose homeostasis, energy expenditure, and neural plasticity (**Costa-Mattioli et al, 2005, Rouschop et al., 2010, and Pasini et al., 2015**). In response to ISR activation, ATF4 acts as a downstream activator of stress-related genes involved in metabolism, the response to oxidative damage, and regulation of apoptosis (**Shroder & Kaufman, 2005 and B'Chir et al., 2013**). Elevated ATF4 levels can induce additional bZIP transcriptional regulators, such as CHOP/GADD153 and ATF3, which together direct a program of gene expression important for cellular remediation or apoptosis (**Wek, 2006 and Ye at el., 2010**). ATF4 regulates the transcription of its target genes through binding to C/EBP-ATF response element (CARE) sequences that can mediate the transcriptional activation in response to various stimuli (**Fawcett et al, 1999 and Kilberg et al., 2009**). ATF4 can form homodimers and heterodimers with several other bZIP transcription factors, including its downstream target CHOP (**Fawcett et al., 1999**). The contribution of other transcription

factors at cis-regulatory elements in regulating expression of ATF4 target genes in response to ISR activation remains largely unclear (discussed further in the next section).

Translational regulation of ATF4

The primary mechanism by which levels of ATF4 protein are modulated in response to different stresses, such as ER stress, hypoxia, or oxidative stress, is through translational regulation (**Blais et al, 2004 and Dey et al., 2010**). The structure of human ATF4 mRNA includes three short uORFs (uORF1, uORF2, uORF3) in the 5' UTR that precede the functional coding sequence (**Harding et al., 2000**). The organization of these uORFs, and the motifs modulating ATF4 protein stability, are essential for the appropriate response by ATF4 to stress conditions such as ER stress and hypoxia (**Ameri & Harris, 2008**). The preferential translation of ATF4 mRNA during ISR activation occurs via a mechanism involving two uORFs: uORF1 and uORF2. The 5' proximal uORF1 facilitates ribosome scanning and reinitiation at downstream coding regions in the ATF4 mRNA, while the uORF2 acts as an inhibitory element. During normal conditions when TC levels are abundant in unstressed cells, ribosomes initiate scanning at uORF1 and quickly reinitiate at the next coding region, uORF2, which overlaps the coding sequence of ATF4, in an out of frame manner, ultimately blocking ATF4 expression by preventing proper translation (**Vattem & Wek, 2004**). During cellular stress, limiting TC availability leads to longer ribosomal scanning along the ATF4 transcript allowing re-initiation of translation at the AUG start codon in the ATF4 coding DNA sequence (CDS).

Multiple TFs mediate signaling by the ISR

ATF4 can interact with other proteins forming homodimers and/or heterodimers due to the presence of a leucine zipper domain (**Hai et al, 1989**)). When ATF4 is not bound to its DNA target, it exists as a monomer (**Podust et al., 2001**). Transcriptional selectivity of ATF4 is modulated by the formation of heterodimers with other bZIP or activating protein family (AP-1)

members, influencing ATF4-mediated transcription (**Harding et al., 2000**). Thus, interactions of ATF4 with other transcription factors or binding partners can influence the outcome of ISR signaling. For example, interactions between ATF4 and Activating Transcription Factor 3 (ATF3), an ATF/CREB family member, have been shown to enhance cellular efforts in restabilization of homeostasis, while interactions with CHOP promote cell death upon ER stress (**Ohoka et al., 2005 and Wang et al., 2009**). Consequently, by association with other proteins and transcription factors, ATF4 can modulate the transcriptome of the cell in response to diverse stress stimuli. Global transcriptome analysis performed in mouse embryonic fibroblast (MEF) cells revealed that ATF4 is directly responsible for the upregulation of less than 50% of genes in MEF cells responding to ER stress (**Harding et al., 2003**). This result raises the question about the existence of other effectors that may be responsible for ISR target gene transcription. Here, we focus on the activity of TFs that are induced in response to ISR activation and have been implicated in contributing to the regulation of ATF4 target genes, such as Activating Transcription Factor 6 (ATF6), X-box binding protein 1 (XBP1) and growth arrest and DNA damage inducible protein 34 (GADD153) also known as C/EBP homologous protein (CHOP). The interactions between these transcription factors increase expression of distinct but overlapping sets of genes comprising both ER-specific and general cellular proteostasis pathways (**Yamamoto et al, 2004, and Adachi et al., 2008**).

C/EBP Homologous Protein (CHOP)

ATF4 can form heterodimers with several other bZIP transcription factors, including its downstream target CHOP (**Siu et al, 2002**). Recent studies have connected ATF4 and CHOP with autophagy induction in mammalian cells (**Rzymski et al., 2010 and Rouschop et al, 2010**). Similarly, studies done in mouse models confirm that upon ER stress and amino acid depletion in mouse cells, ATF4 alone, or together with CHOP, preferentially binds to proximal promoter regions of target genes. Although it is well established that ATF4 alone regulates the

expression of genes involved in amino acid transport and biosynthesis, in response to ER stress, ATF4 and CHOP interact to regulate common genes involved in cellular amino acid metabolic processes, mRNA translation, and the unfolded protein response (UPR) (**Shroder & Kaufman, 2005**). These gene targets often contain specific response elements within the gene promoter regions, such as CARE sequences (introduced in an earlier section) and amino acid response elements (AARE). The formation of an ATF4–CHOP heterodimer increases its binding affinity for AARE in the early stages of transcriptional induction of autophagy genes (**B'Chir et al., 2013**). The best-characterized mechanism of ISR-induced cell death is via ATF4-mediated activation of CHOP (detailed in the next section), found to be critical for stress-induced apoptosis. It is important to note that although there is a well-established role for CHOP in cell death signaling, CHOP expression alone is not sufficient to induce cell death, indicating the role of other factors in mediating the cell death response during stress (**Marciniak et al., 2004**).

Activating Transcription Factor 6 (ATF6)

Our discussion of the UPR in chapter 1 introduced an ER-localized transmembrane sensor of ER stress, Activating transcription factor 6 (ATF6). Similar to IRE1 and PERK, in the absence of stress BiP (GRP78) binds ATF6 to inhibit signaling (**Shen et al., 2005**). In response to ER stress, GRP78 is released, allows ATF6 to translocate to the Golgi where it is cleaved at the luminal and cytoplasmic sides via site-1 and site-2 proteases (S1P, S2P) in a process referred to as regulated intramembrane proteolysis (RIP) (**Ye et al., 2000**). These cleavage events release the N-terminal cytosolic fragment (ATF6-N), which then acts as a bZIP transcription factor, relocating to the nucleus in order to transactivate genes responsible for mitigating ER stress. ATF6-N homodimers bind to a conserved consensus motif called ER stress response elements (ERSE) found in UPR target genes that encode chaperones, ERAD proteins, and redox pathway components (**Kokame et al, 2001and Yoshida et al., 2000b**). In addition to ATF4-induced CHOP expression, ATF6 has also been shown to transactivate CHOP

in response to ER stress via an ERSE in the CHOP promoter (**Ma et al., 2002**). Similarly, while ATF4 has been reported to transactivate expression of GRP78 via interactions with other bZIP factors, ATF6 has also been shown to play a central role in GRP78 induction in response to ER stress (**Luo et al, 2003**). Critically, it has been demonstrated that ATF6 can transactivate, and form heterodimers with, X-box binding protein 1 (XBP1) to regulate expression of a distinct set of UPR genes (**Yamamoto et al, 2004 and Shoulders et al., 2013**).

X-box Binding Protein 1 (XBP1)

In Chapter 1, we discussed how the UPR is sensed via three ER-localized transmembrane receptors: ATF6, IRE1, and PERK. We have mentioned that activated N-ATF6 can act as an effector molecule in response to ISR activation, and we have discussed the kinase activities of PERK in regulating eIF2a phosphorylation at the core of the ISR. Here we reveal that IRE1, a highly evolutionarily conserved ER stress sensor, initiates unconventional splicing of XBP1 mRNA (XBP1s) in response to activation by ER stress (**Yoshida et al., 2001a**). As a result, XBP1s translated from the spliced XBP1 mRNA functions as a potent transcriptional activator of ISR target genes. Similarly to ATF6-N, XBP1s activates transcription of ER chaperone genes containing ERSE sites within promoter elements (**Lee et al., 2003**). Thus, the ATF6 pathway and IRE1-XBP1 pathway serve to activate the transcription of ER chaperone genes in response to ER stress in mammalian cells. These two TFs have been shown to occupy identical consensus sequences across the genome (**Yoshida et al., 2001a**), however, the biological dependencies on these transcription factors in organisms remain distinct. For example, XBP1s knockout mice are not viable, pointing to its critical nature for biological processes including development (**Reimold et al., 2000**). Alternatively, mice lacking ATF6α, the primary ATF6 homolog involved in the UPR pathway, develop normally, although deletion of both mammalian homologs, ATF6α and ATF6β, is embryonic lethal (**Adachi et al., 2008; Yamamoto et al., 2007**). The cooperation of ATF6 and XBP1, the putative redundancy

employed by these TFs, and the presence of TF-specific gene expression signatures in response to exposure to various stress stimuli is a fertile area for further investigation.

Cellular outcomes of ISR activation

Cellular recovery signaling by the ISR

Although multiple stresses converge on eIF2α phosphorylation to activate the ISR, the cellular outcomes of ISR activation vary depending upon a multitude of factors (**Pakos-Zebruka et al., 2016**). These factors include the nature of the stress, its duration and severity, but also, cellular outcomes are influenced by the extent of eIF2α phosphorylation and translational induction of ATF4 mRNA, as well as the activity of other bZIP transcription factors, discussed above (**Dey et al, 2010 and Guan et al, 2014**). It is generally accepted that an acute and rather short-lived ISR response is typically an adaptive, pro-survival one aiming at resolving the particular stress encountered and restoring homeostasis. Conversely, a chronic, or prolonged ISR response can signal towards the induction of cell death programs (**Rutkowski et al, 2006**). It remains largely unclear how exactly the ISR regulates this switch between pro-survival and pro-death signaling, however there is notable crosstalk between the ISR and other stress-dependent networks downstream of eIF2a phosphorylation, including: autophagy signaling, the UPR, the AAR, and the DDR (**Pakos-Zebruka et al., 2016**).

Through the activation of macroautophagy, hereafter referred to as autophagy, the ISR can regulate cell survival and cell death pathways. Autophagy is a self-degradative process important for balancing energy metabolism at critical times in development and in response to stress (**Glick et al, 2010**). Autophagy is generally thought of as a survival mechanism as it is responsible for the removal and degradation of misfolded or aggregated proteins, clearance of damaged organelles from the cell, such as mitochondria, ER and peroxisomes (**Deter & de Duve, 1967 and Nakatogawa et al., 2009**). Although the precise mechanisms by which

phosphorylated eIF2α leads to autophagy are still poorly understood, distinct stresses that lead to the phosphorylation of eIF2α have also been shown to induce autophagy via the activation of pathways that promote cell survival, such as the PI3K signaling pathway and its downstream target, mTOR complex 1 (mTORC1) (**Kazemi et al., 2007**). Cytoprotective functions of eIF2α phosphorylation in response to conditions that mimic viral infection or induce ER stress have been reported to lead to an increased expression of regulated in development and DNA damage response 1 (REDD1; also known as DDIT4), which can suppress mTORC1 activity leading to autophagy induction (**Dennis et al., 2013**). The global halt in translation downstream of eIF2α phosphorylation has also been implicated in the induction of the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) pathway in response to ER stress (**Deng et al., 2004**). ATF4, the main effector of the ISR, also activates the TF, nuclear protein 1 (NUPR), which regulates the expression of metabolic stress-responsive genes, particularly those involved in the DDR and cell cycle regulation, and as such, may be considered as a pro-survival factor activated in response to multiple stimuli that converge on eIF2a phosphorylation (**Jin et al., 2009 and Hamidi et al., 2012**).

Cellular death signaling by the ISR

The ISR is capable of activating pathways that can lead to the induction of cell death if the adaptive response is not successful in restoring homeostasis. These cell death pathways are mainly regulated through the transcriptional activity of ATF4, and some of its downstream targets, particularly, CHOP and ATF3. As discussed in a previous section, one of the best studied mechanisms of ISR-induced cell death is through ATF4-mediated activation of CHOP (**Pakos-Zebruka et al., 2016**). CHOP promotes cell death signaling through multiple mechanisms, most of which involve TF activities of CHOP which have been shown to induce cell death via the upregulation of various pro-apoptotic BCL-2 family members to promote ER stress-induced apoptosis (**Galehdar et al, 2010**). CHOP can also contribute to cell death by

enhancing the expression of one of the death receptors, DR5, that plays a role in the induction of apoptosis under ER stress (**Zou et al., 2008**). CHOP can further regulate gene expression by binding to other ATF/CREB family members, such as ATF4 or ATF3, thus altering their DNA binding specificity and subsequently, target gene activation. For example, CHOP–ATF4 heterodimers can upregulate ATF5 expression, amplifying cell death signaling by regulating the expression of several pro-apoptotic genes (**Teske et al., 2013**) while CHOP–ATF3 interactions can increase the expression of DR5 (**Liu et al., 2012**). It is important to note that although there is a well established role for CHOP in cell death signaling, CHOP expression alone is not sufficient to induce cell death, cells lacking CHOP are only partially resistant to ER stress-induced cell death, indicating the role of other factors in mediating the cell death (**Oyadomari et al., 2001 and Young et al., 2016**).

Ch. 3: The p53 Gene Regulatory Network (GRN)

Introduction

In 1979, it was discovered that the large T antigen, encoded by the simian virus 40 (SV40) virus, binds to a host protein with a molecular weight of 53-54kD in SV40-transformed cells (**Lane & Crawford, 1979 and Linzer & Levine, 1979**). This 53kD protein was aptly named p53, and was initially thought to be an oncogene until a decade later when it was found to be mutated in a number of diverse human tumors, hinting that it may have tumor suppressive functions (**Nigro et al., 1989 and Baker et al., 1989**). Concurrently, two labs reported that the wild-type (WT) p53 protein could act as a suppressor of transformation by mutant p53 and oncogene activation (**Eliyahu et al., 1989 and Finlay et al., 1989**). Shortly thereafter, Donehower and colleagues demonstrated that p53 -/- knockout (KO) mice models have a much higher propensity for developing spontaneous tumors than their WT counterparts, firmly

establishing that p53 does in fact act as a tumor suppressor (Donehower et al., 1992). The decades of research following this crucial discovery have led to the general acceptance of p53's ability to act as a tumor suppressor and master transcriptional regulator. We now know that the p53 gene is the most frequently mutated tumor suppressor gene in human cancers, approximately half of all human malignancies carry inactivating mutations in the p53 gene (**Soussi et al., 2000 and Hainaut & Hollstein, 2000**). The other half of malignancies that have retained the wild-type p53 gene frequently harbor defects either in the pathways regulating the stabilization of p53 in response to cellular stress or in the effectors required for the apoptotic activity of p53 in response to these stimuli (**Vogelstein et al, 2000**). Additionally, germline mutations in the p53 gene can result in Li–Fraumeni syndrome, hereditary cancer predisposing individuals to lymphomas, sarcomas, and breast, brain and various other tumors (**Malkin et al., 1990 and Srivistava et al., 1990**).

Beyond p53's role in cancer and tumor suppression, it has been named a master transcriptional regulator for its ability to respond to a wide variety of cellular stress signals by activating a broad range of target genes that allow the cell to orchestrate an appropriate cellular response to the particular stress encountered (**Kruiswijk et al., 2015**). For example, p53 is known to play a central role in the response to DNA damage (discussed in Chapter 1) by activating genes involved in cell-cycle arrest, DNA repair, and apoptosis **(Hager & Gu, 2014)**. As such, p53 is commonly referred to as the "guardian of the genome" and has become the most widely studied protein since its existence (**Dolgin et al., 2017**). Nevertheless, many open questions still remain regarding the regulation of p53 in response to cellular stresses (discussed further in Chapter 5). In summary, inactivation of the p53 pathway seems to be a general mechanism in tumor development, and possibly, a common feature of all human cancers, hence understanding the molecular mechanisms that regulate p53 function is critical for the study of cancer therapies.

The p53 family of proteins: p53, p63, and p73

Evolution and identification

The P53 family of proteins consists of three gene paralogs (p53, p63, and p73) all of which have been implicated in diverse human disorders. This gene family has conserved its structural and functional features for over one billion years of evolution. A common ancestor to these three p53 family members was first detected in the evolution of sea anemones, and most closely resembled a combined p63/p73-like gene. This ancestral gene was found to function in protecting the germ line from genomic instabilities in response to cellular stresses (**Belyi et al., 2010**). Two duplication events of this ancestral gene have been characterized, the first in early vertebrates (cartilaginous fish) which produced a gene most closely related to p53, and the second in bony fish, the first vertebrate lineage which contained all three members: p53, p63 and p73 (**Chang et al., 1997, and Lee & Kimelman, 2002, and Pan et al., 2003**). During the first duplication event, adult tissue regeneration via somatic stem cells was beginning to occur, therefore, while the ancestral p53 gene functioned solely to protect the germ-line, the vertebrate p53 gene acquired a tumor suppression function that protects somatic cells from mutations caused by DNA-damage (**Pankow & Bamberger, 2007 and Brodsky et al., 2007**). The second duplication event, occurring at the early stages of vertebrate evolution, allowed the structure and function of the p63 and p73 genes to diversify in higher vertebrates. Throughout evolution, the p63 and p73 genes have acquired more epithelial-specific and immune response functions, respectively (**Mills et al., 1999, Yang et al., 1999, and Nemajerova et al., 2018**); however, the ability of the p53 family to regulate the germ-line production of gametes in response to genomic instability was preserved nonetheless (**Flores et al., 2005**).

Domain structure of the P53 family proteins

The human p53 gene (*TP53*) is located at chromosome 17p13.1. It is composed of 19,198 nucleotides, spanning 11 total exons, the coding sequence of which begins in the second exon and ends in the last exon. The p63 gene (*TP63*) is located on chromosome 3q27-29. It is composed of 265,822 nucleotides divided up into 14 exons. The coding sequence can start in exon one and continue through exon 14, depending upon the isoform, which will be covered in the following section of this chapter. The p73 gene (*TP73*) is located on chromosome 1p36.3. It is composed of 80,728 nucleotides, and is divided up into 14 exons like its sibling, p63. The coding sequence can start in exon two and end in exon 14. None of these dimensions take into account the regulatory regions that control the synthesis of these gene products, a topic briefly described for p53 in the latter section of this chapter. The larger sizes of p63 and p73, in comparison to p53, are derived in large part due to longer introns with the addition of a few exons. As a result, a greater number of haplotypes exist for p63 and p73 than those known for p53, a fact largely attributed to a higher level of recombination events in these genes (**Belyi et al., 2010**).

TP53 encodes 393 AAs for the full-length isoform of the protein (FLp53), AAs one to 42, and 43-63, contain two transactivation domains (TADs) which presumably activate specific subsets of p53 target genes and harbor critical hydrophobic residues (**Lin et al., 1994 and Chang et al., 1995**). AAs 102-242 make up the DNA binding domain (DBD) which mediates the interaction between p53 and DNA sequence elements within p53-regulated genes (**El-Deiry et al., 1992**). The p53 protein functions and interacts with DNA as a dimer of a dimer, therefore, AAs 324-355 encompass the tetramerization domain or oligomerization domain (OD) (**Jeffrey et al., 1995 and Kitayner et al., 2006**). This is followed by AA residues 356-393 which span the carboxy-terminal (c-terminal) domain known for regulating the stability and DNA binding activities of the p53 protein (**Jayaraman & Prives, 1995**). Splicing at the amino- and/or carboxy-

terminal end of the p53, p63, and p73 genes can produce isoforms that have been shown to regulate the stability of these proteins, alter DNA binding activities, and transcriptionally repress certain target genes in the presence of altered amino-terminal TAD domains (**Yang et al., 1998 and Bourdon et al., 2005**). Thus, the p53, p63, and p73 genes each have many different isoforms with several diverse features, which will be covered in the following section. Organization of protein domain structures of p53, p63 and p73 are similar, and the p53 family shares a highly conserved DBD, through which these three proteins bind to very similar DNA motifs. Consequently, these family members bind both unique and shared sites across the genome (Lin et al., 2009). As a result, these proteins can regulate transcription of a common set of target genes as well as activate a subset of genes unique to each protein (**Ortt & Sinha, 2006, Meenk, et al., 2008, and Tozluoglu, et al., 2008**).

The p53 family isoforms

p53 isoforms

Until recently, only one promoter and three mRNA splice variants encoding FLp53, p53i9, and 40p53, respectively, were described (**Flaman et al., 1996, Chow et al, 1993, and Yin et al., 1992**). The identification that p53 transcription can occur via two distinct sites, one upstream of exon one and from an internal promoter located in intron four, elucidated that the p53 gene structure may not be that simple (**Bourdon et al., 2005**). This alternative promoter usage produces a truncated amino-terminal (n-terminal) isoform distinct from 40p53, and initiated from AA 133, therefore denoted 133p53 (**Bourdon et al., 2005**). Additionally, intron nine can be alternatively spliced to produce three p53 isoforms: α, β, γ - where the p53b (identical to p53i9) and p53y isoforms do not contain the OD (**Bourdon, 2007**). In summary, the human p53 gene can encode at least nine distinct protein isoforms due to alternative promoter usage and alternative splicing: FLp53α, FLp53β, FLp53γ, 133p53α, 133p53β, 133p53γ, 40p53α,

40p53 β, and 40p53γ. These different p53 isoforms can have unique biochemical activities, however, exploring the distinct functions of these isoforms has been challenging due to limitations in antibody specificity.

p63 isoforms

Not unlike p53, the human p63 gene expresses at least three alternatively spliced c-terminal isoforms: α, β, γ - which can be transcribed from an alternative promoter located in the intron three. The longer transactivating isoforms (TAp63) are transcribed from the promoter upstream of the exon one (P1), while the alternate intronic promoter (P2) produces the shorter n-terminal isoforms (Np63) with a distinct n-terminal domain (**Helton et al., 2006**). Consequently, the p63 gene encodes at least six different protein isoforms due to alternative promoter usage and alternative splicing: TAp63α, TAp63β, TAp63γ, Np63α, Np63β, and Np63γ. These unique p63 isoforms have been shown to play distinct roles in various tissues (**Woodstock et al., 2021**); For example, while the Np63 isoforms are highly expressed in epithelial tissues playing an essential role in epithelial cell development, morphology, and chromatin landscape (**Yang et al., 1999, Sethi et al., 2017, and Kouwenhoven et al, 2015**) the TAp63 isoforms are expressed in oocytes functioning to protect the female germ line (**Suh et al., 2006 and Livera et al., 2008**). Much less is known about the c-terminal protein isoforms and their specific functions, largely due to the same limitation met with studying p53 isoform function, antibody specificity, albeit to a much smaller degree.

p73 isoforms

Similar to p63, the human p73 gene can encode isoforms transcribed from an alternative promoter in the intron three. p73 expresses at least seven c-terminal isoforms: α, β, γ, δ, ε, ζ, η - and four n-terminal isoforms: TAp73, ex2p73, ex2/3p73, and Np73. Much like p63, the TAp73 isoforms produced from promoter P1 upstream of the first exon are longer while the Np73

isoforms produced from promoter P2 have truncated yet distinct n-terminal domains. The ex2p73 isoforms are due to alternative splicing of the exon two, while ex2/3p73 isoforms are due to alternative splicing of both exons two and three. Altogether, the p73 gene expresses at least 35 mRNA variants that can theoretically produce at least 29 different protein isoforms. The assignment of physiological functions for the p73 isoforms is much less clear; The TAp73 and Np73 isoforms are the best characterized of the four, having demonstrated functions in neuronal cell types (**Yang et al, 2000 and Tissir et al., 2009**).

The p53 pathway: The basics

Degradation and regulation of p53

Control of p53 activity via degradation of p53 by MDM2

The tightly regulated mechanisms that control p53 gene transcription remain largely unknown, however, regulation of p53 has been described at the level of transcription, translation, conformational change, and various covalent and noncovalent modifications (Ashcroft and Vousden, 1999). Many reports indicate that cellular levels of p53 are mainly regulated at the posttranscriptional level, and it is generally accepted that the principal mechanism through which p53 activity is governed is by controlling the stability of the p53 protein (**Kubbutat et al., 1997**). In normal cells, p53 is present at extremely low basal levels due to the rapid degradation of the protein following synthesis (**Kubbutat & Vousden, 1998**). One of the most important components of the p53 degradation pathway is known as mouse double minute 2 (MDM2), a gene that is a direct transcriptional target of p53, and as such is induced following activation of p53. However, unlike most p53 target genes, there is no evidence that MDM2 contributes to downstream p53 functions, such as cell-cycle arrest and apoptosis (**Reinke & Lozano, 1997**). The principal role of MDM2 in the p53 pathway is to interact with p53 proteins inhibiting their activity by binding to the N-terminus within the TAD. The binding of

MDM2 alone inhibits normal TF functions of p53, reducing its ability to activate gene transcription (**Momand et al., 1992 and Oliner et al., 1993**). However, this is not the sole mechanism used by MDM2 to control p53, it also achieves a more comprehensive control by directly contributing to the degradation of p53 protein levels in the cell, functioning as a E3 ubiquitin ligase to promote proteasome-mediated degradation of p53 (**Haupt et al., 1997 and Honda et al., 1997**). Besides direct ubiquitination of p53, MDM2 also plays a crucial role in the regulation of subcellular localization of p53 (**Boyd et al., 2000**). The efficient nuclear export of p53 requires a nuclear export sequence (NES) within the C-terminus of p53, possibly made more accessible upon interactions between MDM2 and p53 (**Stommel et al., 1999 and Geyer et al., 2000)**.

MDM2-independent mechanisms that negatively regulate p53 stability

While MDM2 has been well established as a key regulator of p53 abundance and location, additional MDM2-independent mechanisms have been described to regulate the stability of p53. In cervical cancer cells, ubiquitination and subsequent degradation of p53 are mediated via the human papillomavirus (HPV) E6 oncoprotein in complex with the E6AP protein (**Scheffner et al., 1993**) however, there is no evidence that E6AP participates in the degradation of p53 protein in normal cells or cells that do not express E6 (**Beer-Romano et al., 1997**). Other cellular proteins can play a role in regulating p53 stability in normal cells, such as c-Jun n-terminal kinase (JNK) which can interact with and regulate p53 ubiquitination and stability (**Fuchs et al., 1998a**). While the exact mechanisms of p53 degradation by JNK are unknown, JNK-directed degradation is independent of MDM2 and does not require the kinase activities of this protein and it has been proposed to be a regulator of basal levels of p53 in non-stressed cells (**Ip & Davis, 1998**). It is suggested that JNK can act as part of a ubiquitin ligase to regulate p53 stability (**Fuchs et al., 1998a**). In addition to degradation by the proteasome, other cellular proteases may play a role in regulating p53 stability. Calpain, a ubiquitously expressed

calcium-dependent protease, has been shown to cleave p53 in several systems and stabilize p53 protein levels upon inhibition, however the importance of this cleavage event in regulating p53 stability remains unclear (**Kubbutat & Vousden, 1997**) .

Activation and stabilization of p53

Phosphorylation of p53

Rapid degradation of p53 protein levels in the cell is essential to normal cellular functions. The activation of MDM2 by p53 is required to reverse the inhibitory effects of p53 on cell cycle progression, thus, the activation of MDM2 by p53 would automatically lead to the repression of p53 activity. This suggests that the p53-MDM2 pathway is an autoregulatory feedback-loop model, supported by investigations that demonstrated that MDM2 knockout mice are rescued from embryonic lethality by deletion of P53 (**Montes de Oca Luna et al, 1995**). Thus, p53 stabilization is likely to be mediated by cellular mechanisms that involve inhibition of MDM2. Activation and stabilization of p53 is a common response to a wide range of diverse stress stimuli (detailed in the following sections). The activation of p53 subsequently halts cellular growth via transcription of cell cycle arrest genes, which is thought to prevent the development and progression of malignancies by preventing the accumulation of genomic instabilities or other damages in aberrant or abnormally proliferating cells (**Levine, 1997**). Hence, the response to these various stress signals involves inhibition of MDM2-mediated degradation of p53, achieved via multiple independent pathways typically involving phosphorylation of p53 by several different kinases.

An abundance of kinases have been shown to phosphorylate residues in the N-terminus of p53 in-vitro, including the three main kinases activated in response to DNA damage: ATM, ATR, and DNA-PK, as well as JNK and CKI, a member of the casein kinase 1 family of kinases (**Jayaraman & Prives, 1999**). Endogenous p53 is phosphorylated at several sites following

DNA damage, including serine 15, 20, 33 and 37, and it has been demonstrated that ATM and ATR phosphorylate p53 at ser15 *in vivo* (**Siliciano et al., 1997, Shieh et al., 1997, Shieh et al., 1999, and Banin et al., 1998**). While it is clear that p53 is phosphorylated at multiple sites during the DDR, it remains largely unknown how each of these specific modifications contributes to p53 stabilization. It is suggested, however, that phosphorylation within the N-terminus of p53 can impede binding of p53 with its negative regulator, MDM2 (**Unger et al., 1999, and Fuchs et al., 1998b**). Phosphorylation at these residues certainly correlates with stabilization of p53 in response to some signals, although the patterns of phosphorylation that occur in response to distinct signals indicate that no individual site is responsible for stabilization in response to all signals. Investigations of p53 proteins in which all the known and putative N-terminal phosphorylation sites have been mutated indicate that phosphorylation is not essential for all forms of genotoxic stabilization of p53, and rather occurs in a stimulus-specific manner (**Ashcroft et al., 1999**).

Other methods of p53 stabilization that do not require phosphorylation…

Genotoxic stabilization of p53

As we discussed in Chapter 1, one of p53's best-characterized functions is to respond to different forms of DNA damage by promoting G1 cell cycle arrest, facilitating DNA repair, and when necessary activating programmed cell death and other apoptotic functions to eliminate damaged cells and prevent further genomic instability. Genotoxic, or DNA damage-inducing agents that activate p53 range from irradiation (UV, gamma rays, x-rays..etc.), carcinogens (mycotoxins, heavy metals..etc.), oxidative stress (hydrogen peroxide), cytotoxic compounds (5-FU), and topoisomerase inhibitors (etoposide) (**Pluquet & Hainaut, 2001**). Distinct differences in the extent and kinetics of p53 activation in response to these various agents suggest these different genotoxic stimuli may use distinct signaling pathways to activate p53 (**Ramet, et al., 1995**). It is generally accepted, however, that the induction of p53 in response to these agents is

mediated via concerted posttranslational modifications in the N- and C-terminus regions of p53 proteins (**Ljungman, 2000**). Modifications of p53 in the N-terminal domain (NTD) generally function to sense and transduce DNA damage signals via coordinated phosphorylation of critical residues in this region by DDR kinases ATM, ATR, DNA-PK, and CHK1/CHK2 (reviewed in Chapter 1). Changes in the phosphorylation state of several sites in the p53 NTD have been shown to stabilize p53 levels by destabilizing the interactions between p53 and its negative regulators, MDM2 and JNK, while increasing the affinity for this region to interact with essential transcriptional machinery and co-activators, such as members of the histone acetyltransferase (HAT) family, CBP/p300 and Tip60 (**Lill et al., 1997 and Tang et al., 2006**). Additionally, distinct modifications such as phosphorylation and acetylation of certain residues in the NTD have been shown to influence the specific set of target genes transactivated by p53, as well as cellular fate decisions between cell cycle arrest and apoptosis (**Oda et al., 2000 and Tang et al., 2006**). Modifications of p53 in the c-terminal domain (CTD) integrate multiple regulatory signals via phosphorylation of critical residues by cyclin-dependent kinases (CDKs) and protein kinase C (PKC), acetylation of lysine residues used as target sites for ubiquitins, and ubiquitination and sumoylation events that regulate p53 stability. While the exact contributions of each of these modifications in response to genotoxic agents remain largely unclear, these coordinated regulations are thought to be integral to p53 stability, DNA binding capacity, and crosstalk between the p53 N- and C-termini (**Pluquet & Hainaut, 2001**). Together, genotoxic stabilization of p53 involves the integration of tightly regulated and highly orchestrated posttranslational modifications that are likely essential for the p53-mediated DDR.

Non-genotoxic activation of p53

While it was initially thought that p53 was exclusively activated in response to DNA damage, studies performed in the early 1990's elucidated the activation of p53 in response to non-genotoxic stress. First, researchers demonstrated overexpression of the adenovirus 5

(E1A) protein induced the stabilization and accumulation of p53 leading to apoptosis (**Lowe & Ruley, 1993**). Next, others showed that p53 was activated in response to low-oxygen conditions (**Graeber et al., 1994**). Since, non-genotoxic activation of p53 has been described in response to various physiological processes, such as oncogene activation, cytokine expression, hypoxia, and senescence; As well as a variety of pharmacological compounds, including antimicrotubule agents (taxanes, nocodazole…etc.) and those that inhibit the interaction between p53 and its negative regulators (Nutlins, RITA…etc) (**Pluquet & Hainaut, 2001**). The observation that E1A activates p53 led to further research into the stabilization of p53 in response to oncogenic challenges, showing expression of p19 (ARF) downstream of oncogenic Ras, activated and stabilized levels of p53 via interactions with MDM2, leading to the suppression of epithelial cell transformation (**Zhang et al., 1998 and Lin & Lowe, 2001**). It is now emerging that p53 acts as a sensor of multiple forms of cellular stress, including but not limited to: senescence, microtubule and ribonucleotide depletion, and hypoxia. For example, perturbations to ribosome biogenesis can trigger a p53-dependent signaling pathway that acts independently of DNA damage and the tumor suppressor, ARF (**Bursac et al., 2014**). Evidence of p53-dependent cross-talk between ribosome biogenesis and cell cycle progression is accumulating, as it has been proposed that p53 senses nucleolar stress as a result of rRNA processing errors and is stabilized as a result to induce cell cycle arrest as a response to this stress (**Pestov et al., 2001**). Additionally, p53 is upregulated in many clinically relevant human ribosomopathies, a diverse group of pathological conditions in which genetic abnormalities cause defective ribosome biogenesis and/or mature ribosome function (**Narla & Ebert, 2010**). Recent studies of mouse models and clinical patient samples that harbor these ribosomopathies implicate aberrant p53 upregulation as a causative role in the pathogenesis of Treacher Collins syndrome (TCS) and Diamond–Blackfan anemia (DBA) (**Jones et al., 2008 and Watkins-Chow et al., 2013**). In conclusion, these reports support the generalization that while acute genotoxic stresses may preferentially induce apoptotic gene programs, non-genotoxic stressors may

selectively induce cell-cycle arrest, differentiation, and DNA repair target genes (**Giaccia & Kastan, 1998**). The involvement of p53 in the wide array of different types of non-genotoxic stresses substantiates its role as a master transcriptional regulator and adumbrates p53's critical role in the activation of cell cycle checkpoints, a function that is intimately and inextricably linked to various other biological functions such as cellular metabolism, nutrient use, the response to DNA damage, and many other stress-inducing conditions.

The p53-dependent transcriptional network

Transcription factor activity of p53

p53: a stress-inducible transcription factor

As discussed above, stabilization of p53 protein and nuclear localization are important mechanisms required for the activation of the p53 response. Activated p53 has been reported to have numerous biological functions, however, the best documented is its ability to bind to specific sequences of DNA and activate transcription of adjacent genes (**El-Deiry, 1998**). p53 is therefore a DNA-binding protein characterized as a stress-inducible transcription factor (TF) that responds to cellular conditions by activating transcription of hundreds of target genes. These genes can be grouped into four broad categories: cell cycle inhibition, apoptosis, genetic stability, and inhibition of blood vessel formation (**Vogelstein et al., 2000**). While p53 is frequently regarded as a transcriptional activator, its ability to transcriptionally repress certain target genes has been demonstrated (**Zhao et al., 2000**). While the molecular mechanisms involved in p53-mediated transcriptional repression remain largely unclear, it is generally accepted that p53 can indirectly downregulate many cell cycle genes via the p53–p21–DREAM–E2F/CHR pathway (p53–DREAM pathway) (**Engeland, 2018**).

DNA binding as a central requirement for p53 activity

Since its existence, the functional characterization of p53 has revealed a variety of important biochemical activities, however, its best-characterized function is its ability to act as a sequence-specific transcription factor that triggers activation of target genes that suppress cell proliferation. The importance of SSDB is highlighted by the fact that p53 mutations found in human cancers are most frequently located within the DNA binding domain (**Cho et al, 1994**) and often these mutations abrogate the SSDB of p53 (**Hussain & Harris, 1999**). Regulation of the sequence-specific DNA binding (SSDB) activity of p53 is complex and occurs at various levels (**Gohler et al., 2002**). The C-terminus has been shown to regulate binding of p53 to target DNA, and although the underlying mechanisms are not entirely understood, several models have been proposed to explain the influence of the C-terminal domain on SSDB by p53. The conformation model postulates that the p53 protein exists in two conformationally distinct forms: latent (for DNA-binding inactive p53) and activated (for DNA-binding active p53). According to this model, the C-terminal domain inhibits SSDB of latent p53 via a reversible allosteric inhibition that can convert p53 from the latent to an activated form (**Hupp et al., 1992**). However, structural studies do not support this model as they have demonstrated that the conformations of latent and active p53 forms are largely identical (**Ayed et al., 2001**). Another model has been proposed termed the competition model which explains the inhibitory effect of the C-terminal domain by its ability to bind DNA in a sequence-independent manner. According to this model, nonspecific DNA binding of the C-terminus interferes or competes with the sequence-specific interaction with DNA mediated by the core DBD (**Anderson et al., 1997**). Both of these proposed models suggest that SSDB is inhibited by the C terminus, either directly (conformation model) or indirectly (competition model). Additional mechanisms of p53 SSDB activity have implicated the role of structural organization of DNA within p53 target binding sites and the binding of other factors to the C-terminal region in this regulation (**Espinosa & Emerson, 2001**).

Transactivation: a key tenet of p53 function

Discussed in an earlier section of this chapter, we have briefly outlined the domain structure of the p53 protein, highlighting that p53 has two distinct transactivation domains (TADs): TAD1 and TAD2 (**Lin et al., 1994 and Chang et al., 1995**). The importance of p53 transcriptional activation in its ability to act as a tumor suppressor has been suggested by two major lines of evidence (**Jiang et al., 2011**). First, p53 mutations present in cancerous cells most commonly occur within the DNA binding domain, suggesting that inactivation of DNA binding is critical for tumor development (**Brady & Attardi, 2010**). These tumor-derived p53 mutations can be grouped into two types: contact mutants which alter residues that are essential for direct contact with p53 response elements, or structural mutants which impair proper folding of the p53 protein altering function. The six most common p53 amino acid residues altered in human cancers, known as 'hotspot mutations,' are: R175, G245, R248, R249, R273 and R282 (**Brosh and Rotter, 2009**). Second, genetic approaches and mouse models investigating the role of specific p53 transactivated target genes upon p53 functions conclude that many of these genes, such as *CDKN1A/p21*, *PUMA*, and *NOXA* are important for various p53-mediated functions, including cell cycle arrest, apoptosis, and senescence (**Riley et al, 2008 and Menendez et al., 2009**). However, it is important to note that while these specific target genes have been identified among the tumor suppressive components downstream of p53, they do not completely recapitulate p53 deficiency when deleted (**Valente et al., 2013**). The observation that tumor suppressor activity may be distributed across targets supports a model wherein p53-mediated tumor suppression relies upon the collective and cooperative activation of the p53 target gene network, and that it is the combined actions of these proteins encoded by the range of p53 target genes that mediate p53's tumor suppressive functions (**Andrysik et al., 2017**). In summary, transactivation potential is critical for many p53-mediated

biological responses and together, these studies investigating the role of p53 TADs provide great insight into how p53 serves as a tumor suppressor.

Cooperative DNA binding by p53

We have discussed that p53 can interact with DNA using two general mechanisms: in a sequence-specific manner via the p53 DBD, or in a sequence-independent manner via the C-terminal domain (CTD) (**Joerger & Fersht, 2008**). Like many other TFs, p53 requires an oligomerization step to become transcriptionally active, forming tetrameric complexes on DNA in the form of homotetramers (**Cho et al., 1994**). We have discussed in earlier sections of this chapter the domain structures of p53, and the region responsible for tetramerization located in the CTD of the p53 protein, known as the oligomerization domain (OD) (**Iwabuchi et al., 1993**). Structural studies have demonstrated that these tetrameric complexes, or "dimers of dimers," interact with two 10 bp half-site sequences, each binding to one p53 dimer and separated by a spacer of variable length, between 0 and 20 nucleotides (**Malecka et al., 2009 and Kitayner et al., 2006**). While it has been shown that, in contrast to other transcription factors which oligomerize upon interaction with DNA, p53 can exist as tetramers even in the absence of DNA (**Friedman et al., 1993**), the free energy and dissociation constant for tetramer formation have suggested that p53 exists predominantly as monomers in unstressed cells without DNA damage (**Sakaguchi et al., 1997**).

Many reports have shown that p53 mutants in which the OD has been deleted retain the ability to bind to DNA and stimulate transcription (**Bargonetti et al., 1993, McClure & Lee, 1998, and Nagaich et al., 1999**) however, their affinity for DNA is 10 to 100 times lower than that of the full length p53 protein (**Balagurumoorthy et al., 1995**). This observation can be explained by the fact that p53 monomers cooperatively bind to DNA, as one model suggests that one p53 dimer binds first to one half-site sequence, consisting of two separate monomers bound to adjacent quarter sites, which subsequently increases the probability for the second dimer to bind the

adjacent half-site (**McLure & Lee, 1998, 1999**). In conclusion, the OD is responsible for optimal transactivation potential and DNA binding activities of p53. Additionally, the OD has been implicated in regulating the sequence-independent DNA binding activities of the p53 CTD, and can influence both the strength of the interaction and the conformation of p53-DNA complexes (**Nagaich et al., 1999 and McLure & Lee, 1998**).

The p53 consensus motif

The DNA binding motif for p53 was first discovered in the early 1990's containing contains two decameric RRRCWWGYYY (R = A,G; W = A,T; Y = C,T) half-sites separated by spacers of 0–20 base pairs (bp) (**El-Deiry et al., 1992**). Within each 10 bp half site are two 5 bp quarter sites; these are palindromic sequences that can adopt a canonical head-to-head (HH) orientation, as well as head-to-tail (HT) or tail-to-tail (TT) arrangements. Researchers have shown that the tetrameric p53 protein can bind all three (HH, TT and HT) quarter-site orientations with equally high affinity, and in almost all naturally occurring p53-binding sites, the two half-sites share the same quarter-site orientations (**El-Deiry et al., 1992**). However, only a few of the experimentally validated p53bs identified in this initial analysis did not contain HH orientations. The subsequent characterization of genome-wide p53 binding sites (p53bs) in later years confirmed the binding preference and enrichment for this consensus site in-vivo using a variety of approaches (**Smeenk et al., 2008, Wei et al., 2006, Horvath et al., 2007**). These global analyses confirmed that p53bs containing HT and TT orientations were not enriched in the genome, suggesting that these non-canonical binding sites may be limited to specific genes (**Smeenk et al., 2008, Wei et al., 2006, and Verfaillie et al, 2016**).

The p53 cistrome

p53 occupancy at proximal gene promoter elements

Eukaryotic transcription initiation is directed by formation of the pre-initiation complex (PIC), composed of RNA Polymerase II (Pol II) and 6 general TFs: TFII-A, -B, -D, -E, -F, and -H, on DNA at a region surrounding the transcription start site (TSS) (**Roeder, 1996 and Levine et al., 2014**). To effectively stimulate transcription, p53 interacts with TFII members of the PIC and ultimately facilitates their assembly on the promoters of target genes (**Espinosa et al., 2003 and Coleman et al, 2017**). Studies examining the global binding of p53 to promoters by chromatin immunoprecipitation with sequencing (ChIP-Seq) have elucidated the vast differences in the kinetics and occupancy of p53 to target gene promoters *in vivo* (**Szak et al., 2001 and Kaeser & Iggo, 2002**). Differences in the affinity of p53 for its cognate binding sites or response elements (p53RE) have been attributed to cell fate decisions to undergo either growth arrest or apoptosis following p53 activation (**Vousden and Lu, 2002**). In addition, experiments have repeatedly shown that relative binding affinity is not the only relevant consideration when it comes to response elements (**Riley et al., 2008**). Additional important variables that may affect the functionality of a p53RE include adjacent cofactor binding sites (discussed in a later section), spacer length, distance from the TSS, and nucleosome positioning and chromatin structure (**Koutsodontis et al., 2005, Bourdon et al., 1997**). Enrichment of p53 at a single location is not the sole driver of stress-dependent gene expression, as it has been demonstrated that p53 binds to many more promoters than the total number of genes activated (**Andrysik et al., 2017**). Furthermore, the pausing of Pol II and other initiation factors at certain p53-dependent promoters prior to exposure to stress stimuli, suggests that additional regulatory steps may be required for stress-activated gene regulation. Open regulatory regions may therefore be poised for a potential stimulus-specific response prior to the stabilization of p53 protein levels (**Espinosa & Emerson, 2001**). As investigations into the molecular organization of eukaryotic genes progressed, it became increasingly clear that critical regulatory DNA sequences could be uncoupled from the confines of the promoter-proximal region, and the identification of distal sequence elements, termed enhancers, which could be located thousands

of base pairs from the TSS and the core promoter (**Banerji et al., 1981 and Moreau et al., 1981**).

p53 occupancy at distal gene enhancer elements

Although several genome-scale analyses of the p53 network have been performed, most of these studies have focused exclusively on interactions between p53 and gene promoters (**Nikulenkov et al., 2012, Menendez et al., 2013, and Andrysik et al, 2017**). While promoters direct gene transcription in a position- and orientation-dependent manner, enhancers characteristically function independently of their position and orientation with respect to their target gene or genes, as they can loop over long genomic ranges to engage distant promoters (**Kim et al., 2015**). Many models have been proposed to explain the ways that enhancers engage with their target gene promoters. One model proposes a tracking mechanism starting at the enhancer and moving towards the promoter, while another proposes that the intervening DNA sequence 'loops out' so that the enhancers and promoters are brought in close proximity. The mechanism of how distal enhancer elements interact with promoters over long distances remains largely unclear; However, research suggests that this process may involve both a looping and tracking mechanism, as looping has been demonstrated by chromatin conformation capture (3C) and related assays, tracking mechanisms from the enhancer to the promoter may also be used to set up the initial enhancer-promoter interaction (**Bulger & Groudine, 2011 and Kulaeva et al., 2012**). While it is now known that the vast majority of p53 binding sites occur outside of gene promoters, the functional impact of most of these distal sites on p53-mediated cellular responses remains unknown.

Recent studies investigating the function of p53 at distal gene regulatory elements have observed that p53 binds over 1000 responsive enhancer elements in the human genome to regulate transcription of key target genes (**Verfaillie et al., 2016**).

Recent investigations into the control of p53-dependent enhancer-driven transcription and cis-regulatory element activity have suggested that p53 binding to the genome is largely invariant (Verfaillie et al., 2016), proposing that p53 acts independently to drive gene expression of a core tumor suppressor network across all cell types (**Younger & Rinn, 2017 and Andrysik et al., 2017**) However, cell-type specific p53 binding and activity at cis-regulatory elements across the genome have also been observed (**Hafner et al., 2017 and Nguyen et al., 2018**). These conflicting observations may be explained by changes in the local chromatin environment and accessibility of these regulatory sites as binding of p53 appears to substantially increase the enrichment of these histone modifications at enhancers (**Sammons et al., 2015 and Karsli-Uzunbas et al., 2017**). The pioneering activity of p53 and its ability to bind structurally inaccessible regions of chromatin, along with the putative contributions of other TFs at regulatory regions, have been implicated in full transcriptional activation of p53-dependent targets. In summary, these observations support a model whereby p53 requires the combined activity of other transcription factors and chromatin modifying enzymes to discriminate its response elements under different chromatin contexts.

Dissecting the regulatory logic of a p53-dependent enhancer

Overview

The p53 transcription factor is a major effector of the DNA damage response (DDR) pathway. Upon recognition of aberrant DNA structures, p53 is activated by the ATM/ATR and CHK1/CHK2 kinases, and binds to genomic regulatory regions to activate transcription of key stress response genes (**Harper and Elledge, 2007**). One of these genes, Activating Transcription Factor 3 (*ATF3*), is an early immediate stress gene belonging to the Activating Transcription Factor (ATF) family of basic region-leucine zipper (bZIP) transcription factors (TFs) (**Hai et al., 1999**). This family of stress-dependent TFs are known to respond to a wide

variety of extracellular signals and has been implicated in playing a key role in adapting to and maintaining cellular homeostasis. Regulation of ATF3 is not limited to p53 and the DNA damage response (DDR); The ATF3 gene is induced by numerous physiological stresses across various tissues (**Chen et al., 1996**). Previous research has demonstrated that ATF3 and p53 work in a collaborative manner to regulate the expression of p53 target genes during the DDR by co-localizing at genomic DNAse hypersensitivity sites (DHS) including promoter regions and enhancer elements (**Li et al., 2016**). Promoters and enhancers, discussed briefly above, are similar in that they both contain multiple transcription factor binding sites, however promoters are required for transcription to occur and are located immediately upstream of the gene that they regulate. Promoters act as the start site of transcription, as RNA polymerase binds and the pre-initiation complex forms here, allowing transcription to begin (**Roeder, 1996)**. In contrast, enhancers are not required for basal levels of transcription and can be located either upstream or downstream from the gene or genes that they regulate; Often these distal regulatory elements are found up to one megabase away (**Banerji et al., 1981 and Moreau et al., 1981**). The transcription factors and other cofactors bound at enhancer regions interact with those bound at a gene's promoter to facilitate and enhance transcription. Furthermore, these enhancer sites are critical for the spatial and temporal control of gene expression (**Nakagawa et al., 2018**).

While much is known regarding the global occupancy of p53 at target gene promoters in response to DNA damage, the study of distal regulatory regions contributing to this response has been limited. To facilitate the study of p53's role in the DDR, the use of p53-activating drugs such as Nutlin-3A and Etoposide, allow for the simulation of natural cellular processes. Nutlin-3A is a non-genotoxic activator of p53 that inhibits the interaction between p53 and its inhibitor, MDM2 (**Vassilev et al., 2004**). In contrast, Etoposide is a chemotherapeutic agent that activates p53 in a genotoxic manner by inhibiting topoisomerase II (**van Maanen, et al., 1988**). DNA

topoisomerases are enzymes that are essential for the resolution of aberrant DNA structures and regulation of the proper topological state of genetic material. By inhibiting topoisomerase II, Etoposide effectively induces DNA double stranded breaks (DSBs) which are recognized by activated p53 via the DDR pathway (**Shieh et al., 1997**). By performing chromatin immunoprecipitation with sequencing (ChIP-Seq), we can obtain a global binding profile of p53 in response to these various forms of activation. Probing for characteristic histone modifications of known regulatory elements across the genome facilitates the identification of novel elements regulating expression of p53-dependent genes. Histone 3, lysine 27 (H3K27) acetylation (ac) is a commonly deposited mark of open and active enhancer elements. Histone 3, lysine 4 (H3K4) methylation (me) is a common feature of both promoters and enhancers, depending upon the level of methylation incurred (**Sammons et al., 2015**). While promoters typically harbor H3K27me2 and H3K27me3, enhancers commonly feature H3K4me2 and an absence of H3K4me3.

Results

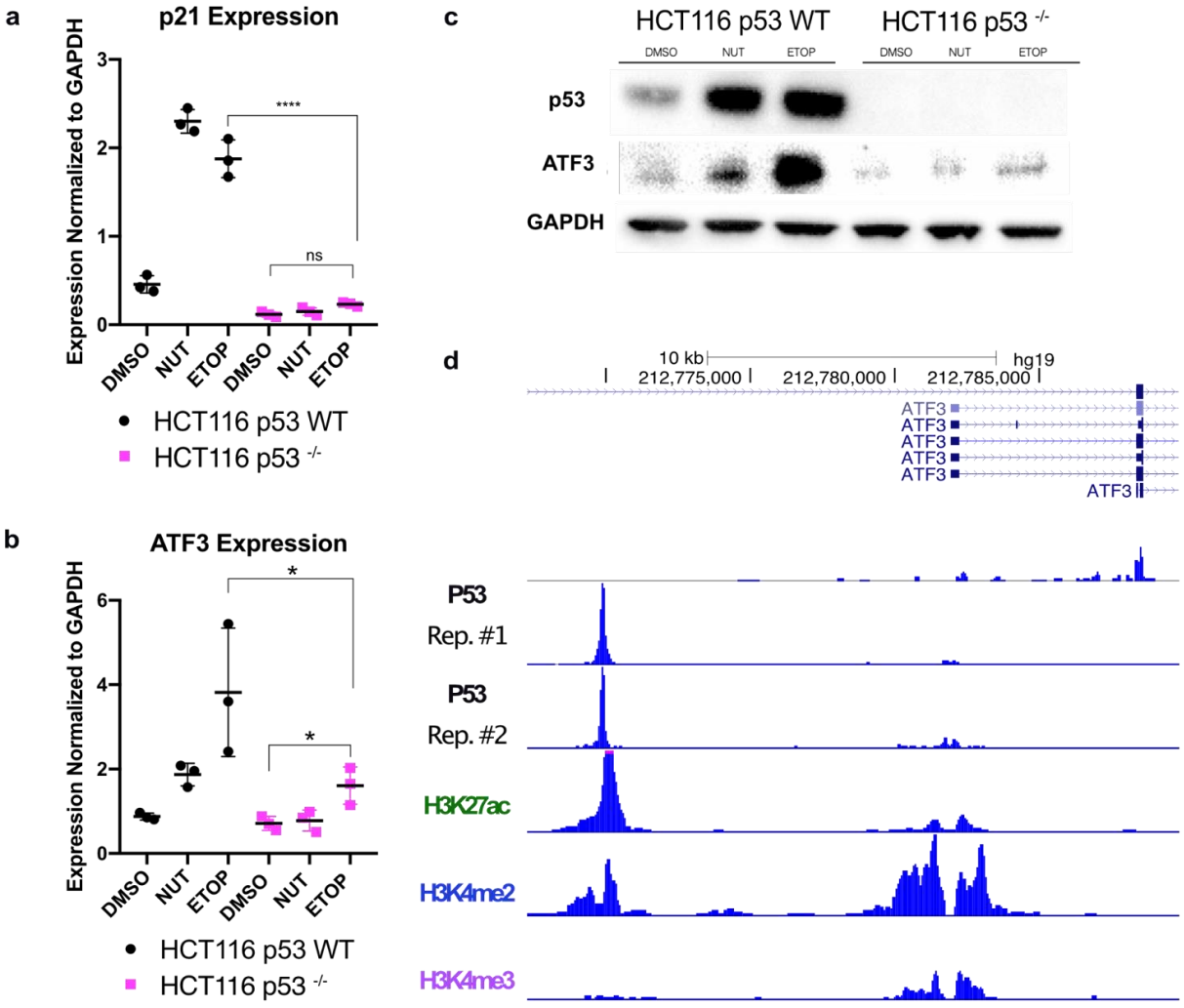


Figure 1. p53 occupies a DHS upstream of *ATF3*, a p53-dependent target gene during the DDR

RT-qPCR analysis of the A) *ATF3* gene and B) *p21* gene in HCT116 p53 WT (black) and p53 null cells (pink), following a 6 hr treatment with DMSO (vehicle control), 5uM Nutlin-3A (NUT), or 100uM etoposide (ETOP). C) Western blot analysis of p53 and ATF3 protein levels in HCT116 p53 WT and p53 null cells, following a 6 hr treatment with DMSO (vehicle control), 5uM Nutlin-3A (NUT), or 100uM etoposide (ETOP). GAPDH serves as a loading control. D) Genome browser view of the *ATF3* gene locus displaying ChIP-Seq data performed in HCT116 p53 WT cells, for p53 occupancy and enrichment of chromatin modifications, in response to 6 hr treatment with 100uM etoposide.

p53 occupies a DHS upstream of *ATF3*, a p53-dependent target gene during the DDR

ATF3 has been reported to be activated downstream of many stress-activated networks, including the DDR. We wanted to first confirm previous reports from the literature that the

expression of *ATF3* in response to genotoxic agents is dependent upon p53. To do this, we treated an isogenic human colorectal cancer cell line (HCT116), containing wild-type (WT) p53 and p53 (-/-) null backgrounds with nongenotoxic and genotoxic activators of p53: Nutlin-3A and etoposide. Using real-time quantitative reverse transcription PCR (RT-qPCR), we validated that both treatments were successful in stabilizing levels of p53 protein in HCT116 p53 WT, and confirmed that levels of p53 protein are not detected in our HCT116 p53 null cells (**Fig. 1c**). Next, we analyzed nascent mRNA expression of a canonical p53 target gene, *p21*, in response to these treatments (**Fig. 1a**). We observe an increase in *p21* expression in response to both p53-activating treatments in HCT116 p53 WT cells, and this induction is no longer observed in the absence of p53, suggesting that both treatments are working to stabilize cellular p53 levels and that p53 is functional and able to activate downstream targets in response to these stimuli. We next measured expression of *ATF3* mRNA in response to p53 stabilization (Nutlin-3A) or DNA DSBs (etoposide). Our results confirm that both mRNA and protein levels of ATF3 are induced in response to both non-genotoxic and genotoxic activation of p53 in HCT116 p53 WT cells (**Fig. 1b,c**). In the absence of p53, we no longer see induction of this gene in response to Nutlin-3A, indicating that p53 is required to induce expression of ATF3 in unstressed cells. Similar to p21, expression of ATF3 in response to DNA damage was significantly reduced in the absence of p53, indicating this gene has p53-dependent regulation in response to DNA DSBs. However, it is important to note that, unlike p21, we observe a slight, but statistically significant, increase in ATF3 expression in response to etoposide when compared to basal levels (DMSO) in HCT116 p53 null cells (**Fig. 1b,c**). This may suggest that while this gene is largely dependent upon p53 for transcriptional regulation in response to DNA damage, it may have some DNA damage-dependent, p53-independent activities during the response to DNA DSBs.

We have confirmed previous reports that ATF3 expression is induced in response to genotoxic agents in a p53-dependent manner, however the molecular mechanisms leading to this gene activation remain unknown. To determine if p53 is directly regulating expression of

ATF3 in response to DNA damage, we performed chromatin immunoprecipitation with sequencing (ChIP-Seq) to determine the occupancy of p53 across the genome in response to stabilization and DNA DSBs. To do this, we treated our HCT116 p53 WT cells with DMSO as a vehicle control, and 5uM Nutlin-3A and 100uM etoposide for 6 hrs, to stabilize p53 and induce DNA damage, respectively. In addition to probing for p53 occupancy, we analyzed the presence of certain chromatin modifications that are characteristic of active regulatory elements, such as H3K27ac, and H3K4 methylation which facilitates the identification of promoters and enhancers. Our results reveal that while p53 does not directly interact with the promoter region of the ATF3 gene, it is enriched at a DHS approximately 10kb upstream from the ATF3 TSS (**Fig. 1d**). This p53-bound DHS has chromatin modifications indicative that it may be a putative enhancer element, such as the presence of H3K27ac and H3Kme2, along with the absence of H3Kme3 (**Fig. 1d**). To determine if this p53-bound DHS was functional in facilitating gene transcription, we created a luciferase reporter for in-vitro studies on the transactivating potential of this genomic region.

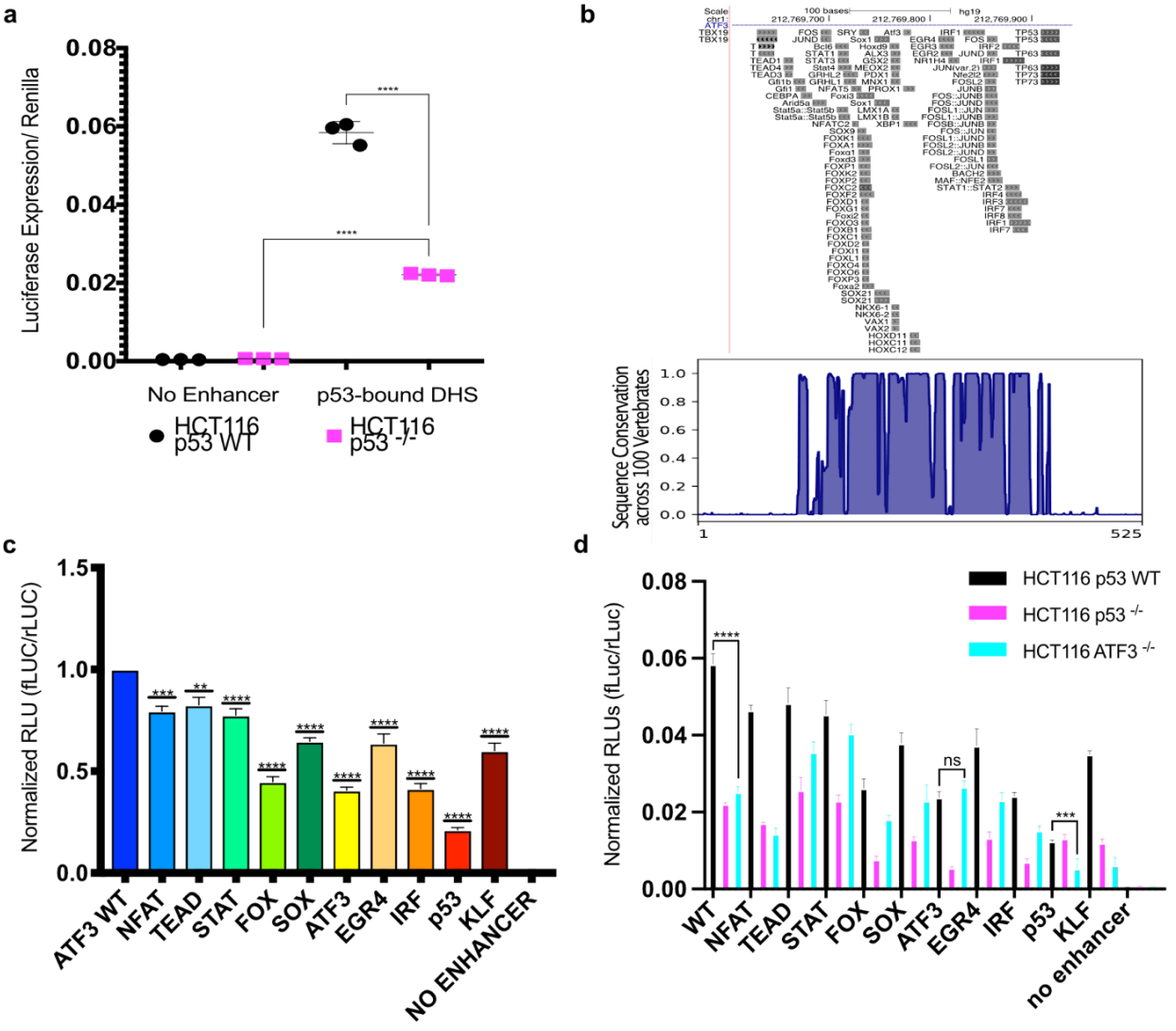


Figure 2. The p53-bound DHS acts as an enhancer element regulating expression of ATF3 in a p53-dependent manner

a) Relative luciferase units (RLUs) measuring activity of the p53-bound DHS upstream of ATF3 in HCT116 p53 WT and p53 -/- (null) cells relative to a negative control (No enhancer). **b)** Genome browser view of the p53-bound DHS locus upstream of ATF3 gene displaying a JASPAR track of putative transcription factor binding site (TFBS) motifs present in this sequence, along with a conservation map showing sequence conservation across 100 vertebrates. **c)** Normalized RLUs measuring the activity of mutated ATF3 enhancer sequences compared and normalized to the activity of the wild-type (WT) enhancer sequence in HCT116 p53 WT cells. **d)** Normalized RLUs measuring the activity of these mutated enhancers across multiple HCT116 cells, including: HCT116 p53 WT, p53 -/- (null), and ATF3 -/- (null), compared and normalized to the activity of the WT enhancer sequence.

The p53-bound DHS upstream of ATF3 is a direct p53-dependent enhancer element that also displays p53-independent activities

Using a plasmid-based reporter system known as a Luciferase assay, we cloned the entire p53-bound DHS into a firefly luciferase vector containing a minimal promoter to test the

ability of this construct to transactivate the reporter gene. To do this, we transfected HCT116 p53 WT and $p53^{-/-}$ (null) cells with our construct containing the WT p53-bound DHS and compared to expression resulting from the minimal promoter alone (No enhancer), as a negative control. Our results confirm that this p53-bound DHS significantly increased levels of luciferase expression in HCT116 p53 WT cells (black) when compared to the No enhancer control (**Fig. 2a**). This suggests that this p53-bound DHS is likely an enhancer element that regulates expression of *ATF3*. The expression of luciferase driven by this enhancer element is significantly reduced in HCT116 $p53^{-/-}$ cells (pink) when compared to activity in the presence of p53 (**Fig. 2a**). This suggests that this enhancer element is dependent upon p53 for full transactivation potential. Importantly, the absence of p53 did not completely ablate the transcriptional activity from this enhancer element, as levels of luciferase driven by this enhancer were significantly higher than those driven by the minimal promoter alone. This suggests that while this enhancer shows p53-dependent activity, it does not completely lose its ability to transactivate in the absence of p53, suggesting that TFs other than p53 that may interact with this enhancer element may be contributing to the expression of *ATF3* when p53 is not around. To investigate the activity of TFs other than p53 in regulating expression of *ATF3*, we used phyloP analysis (**Yang, 1995**) across 100 vertebrates and the JASPAR database (**Sandelin et al., 2004**) to determine a list of putative factors that may interact with this region (**Fig. 2b**). JASPAR provides genomic TFBS predictions for 8 organisms (A. thaliana, C. elegans, C.intestinalis, D. rerio, D. melanogaster, H. sapiens, M. musculus, and S. cerevisiae) with the JASPAR position frequency matrices (PFMs) associated with the same taxon. DNA sequences were scanned with JASPAR CORE TF-binding profiles for each taxa independently using PWMScan and selected TFBS predictions with a position weight matrix (PWM) relative score ≥ 0.8 and a p-value < 0.05. (**Castro-Mondragon et al., 2022**). PhyloP analysis supports several different methods for computing p-values of conservation or acceleration and is used to produce separate scores at each base considering all branches of the phylogeny rather than a

particular subtree or lineage. The scores were computed by performing a likelihood ratio test at each alignment column and scores for both conservation and acceleration were produced (**Siepel et al., 2005 and Pollard et al., 2010**).

JASPAR and phyloP analysis of the upstream ATF3 enhancer element revealed the presence of multiple putative TF binding site (TFBS) motifs within this region, all of which are highly evolutionarily conserved (**Fig. 2b**). To determine regions within this enhancer element that are contributing to its p53-independent activities, we have performed site-directed mutagenesis on our reporter construct (p53-bound DHS) to scramble putative TFBS motifs across this enhancer element. We scrambled ten putative motifs across this element, including the p53 binding site (p53bs) and the ATF3 binding site to confirm recent reports that i) ATF3 is an autoregulatory TF that can bind its promoter and/or enhancer elements (**Yin et al., 2020**), and ii) ATF3 can co-localize with p53 at genomic sites, regulating p53 target gene expression upon DNA damage (**Zhao et al., 2016**). Our results demonstrate that loss of any one of these putative sites (NFAT, TEAD, STAT, FOX, SOX, ATF3, EGR4, IRF, p53, or KLF) reduces overall ATF3 enhancer activity, albeit to varying levels (**Fig. 2c**). The p53 and ATF3 motifs were among the most important for transactivation, showing the lowest level of luciferase activity when these sites were mutated (**Fig. 2c**). These results suggest that p53 and ATF3 colocalize at this region, however, we wanted to confirm this occupancy by testing the activity of these mutated enhancers in cellular backgrounds where p53 or ATF3 have been deleted. To do this, we examined the effect of scrambling one of these putative motifs in our HCT116 $p53^{-/-}$ (null) and HCT116 $ATF3^{-/-}$ (null) cells. We report that loss of these proteins, in addition to loss of one of these motifs, resulted in a further decrease in overall enhancer activity (**Fig. 2d**). The validity of our experimental design is substantiated by the fact that we observe no significant difference in the activity of the ATF3 mutant in HCT116 p53 WT cells compared to that of this mutant in HCT116 ATF3 null cells, as the consequence of removing the ATF3 protein or simply mutating the binding site is equivalent, demonstrating a form of epistasis (**Fig. 2d**). Together, these

results indicate that other factors, such as members of the FOX, ATF, and IRF families, may be contributing to this p53-dependent enhancer-driven transcription.

Conclusions & Future Directions

Conclusions

We have confirmed that *ATF3* acts as a direct target, downstream of non-genotoxic p53 stabilization and DNA damage, via p53 occupancy at an upstream DHS site that we have determined functions as a distal enhancer element regulating *ATF3* expression in a p53-dependent manner (Figure 1). In-vitro analysis of this *ATF3* enhancer element suggests that it has both p53-dependent and p53-independent activities. This is mirrored in our qPCR analysis of the transcriptional induction of *ATF3* in response to p53-activation. We observe both p53-dependent and p53-independent activities of the *ATF3* gene in response to DNA damage. Further in-vitro analysis suggests that there are TFs other than p53 that may be contributing to overall enhancer activity, as measured via our plasmid-based reporter system. Our data support a model wherein the combinatorial activity of p53 and other TFs binding to this region facilitates the regulation of *ATF3* expression. We have performed mutagenesis of putative motifs across this region to identify candidate TFs involved in this coregulation of *ATF3* at this distal cis-regulatory element. Further research is required to determine what specific factors from these families are binding here and the mechanisms used by these factors to facilitate this enhancer-driven transcription.

Future Directions

The broad regulatory paradigms and logic used by parallel stress-dependent networks are largely understudied. Examining the cross-talk between cellular stress networks will provide insight into the mechanisms used by the cell to restore and maintain cellular homeostasis. Our

data suggest that there may be activation of parallel stress-dependent networks during the DDR that may contribute to the regulation of p53 target genes using p53-independent mechanisms. Transcriptome analysis of cell lines containing p53 WT and p53 null backgrounds in response to stress stimuli would greatly enhance the understanding of p53's role in stress-induced rewiring of the transcriptome. Many have noted the cooperation of distinct cell-signaling pathways to orchestrate a basic cellular response, however, the identification of the master transcriptional regulators required for a stimulus-specific response and the redundancy of these factors have not been fully characterized. Therefore, additional research is required to determine the functional regions of the genome, such as specific promoters and enhancers, which are responsible for p53-dependent target gene regulation during various forms of cellular stresses. Identification of the required trans-acting factors as well as the functional cis-regulatory regions involved in the regulation of target stress-response genes could offer answers to many remaining questions in this field of research. Furthermore, the identification of p53 target genes that are being regulated by different types of cellular stresses may lead to the discovery of a novel paradigm for the well-studied and already established p53 gene regulatory network. As such, investigations into the regulation of p53 target genes in response to ER stress, hypoxia, nutrient deprivation, and oxidative stress are an active area of future studies.

Future studies to investigate the regulatory regions employed by these effector proteins to activate expression of these shared target genes are prudent. In-vivo approaches such as CRISPR interference (CRISPRi), or traditional CRISPR/Cas9 gene editing may elucidate regions of functional importance providing a more native cell context. ChIP-Seq and other chromatin immunoprecipitation methods, such as Cleavage Under Targets & Release Under Nuclease (CUT&RUN) will allow us to characterize the genome-wide binding profile of TF effectors that we hypothesize may play a role in this gene regulation. Finally, identification of stress-stimuli that activate p53 target genes in a p53-independent manner may elucidate the putative stress-dependent networks also activated downstream of these stresses.

Chapter 4: Shared gene regulatory strategies for the p53 and ATF4-dependent transcriptional networks

Introduction

The ability to adapt to and thrive in changing environmental conditions has been a key factor driving the evolutionary success of organisms (**Wagner et al., 1996 and Babu et al., 2004**). Distinct molecular pathways have evolved to manage various perturbations to cellular homeostases, such as DNA damage, ER stress, and amino acid deprivation, amongst many others. Stress-dependent response networks share general themes, such as mechanisms that sense the perturbation, transduce the signal, and execute repair or programmed cell death responses (**Fulda et al., 2010**). This cellular stress response involves widespread changes to both anabolic and catabolic processes, including global changes in RNA and protein synthesis and turnover (**Vihervaara et al., 2016 and Advani et al., 2019**). This rapid and transient regulation of gene expression as a stimulus-specific response is largely mediated by the collective activity of transcription factors (TFs) at DNA-encoded regulatory elements across the genome, such as promoter and enhancer elements (**Shlyueva et al., 2014**). TF activity at cis-regulatory elements requires the concerted action of multiple binding events in a combinatorial manner, facilitated via direct and indirect cooperative mechanisms **(Spitz et al., 2012)**. The combinatorial nature of enhancer and promoter occupancy allows the coordination of gene expression programs through a series of complex and tightly regulated processes such as chromatin remodeling, mRNA biogenesis, and post-transcriptional regulation (**de Nadal et al., 2011**). Stress-dependent activation of TF networks leads to a global rewiring of the cellular transcriptome, presumably to combat the effects of the particular stress signal encountered. Many of the basic molecular mechanisms underlying these stress-dependent networks are

known (**Ciccacia & Elledge, 2010, Hetz, 2012, and Broer & Broer, 2017**), however, it remains unclear how the cell uses the plethora of TFs to orchestrate the expression of thousands of genes in response to the various stressors that a cell may encounter, often in parallel. It is therefore critical to consider not only the molecular basis of these distinct transcriptional responses but also, the potential crosstalk that may occur between the stress-dependent networks activated in response to these signals.

The DNA damage-inducible transcription factor, p53, is a well characterized effector of the DNA damage response (DDR) that activates transcription of a broad range of target genes involved in DNA repair, cell cycle arrest, and apoptosis in order to mitigate the potential cellular and organismal consequences of damaged or mutated DNA. p53 activity and engagement with the genome is highly cell-type intrinsic, wherein a conserved transcriptional response can be observed in response to both genotoxic and non-genotoxic p53 stabilization methods in primary human fibroblasts (**Catizone et al., 2019**). Comparative analysis of the transcriptional differences resulting from these contrasting methods of p53 stabilization suggested putative activation of a parallel DNA damage-dependent transcriptional response, likely involving the activation of DNA damage-inducible TFs other than p53 (**Catizone et al., 2019**). Furthermore, recent evidence supports the ability of p53 to cooperate with other stress-dependent TFs in regulating cis-regulatory element activity and downstream gene activation in response to stress stimuli. Using a massively parallel reporter assay (MPRA), we previously found that sequences flanking p53 response elements, and TFs other than p53, are required for p53-dependent transcriptional activation of certain gene targets (**Catizone et al., 2020**). Together, these data suggest that p53 signaling can be differentially regulated, depending on both cellular context and active crosstalk from parallel signaling pathways, to orchestrate an appropriate response to a specific stress stimulus.

In order to determine the potential role of parallel stress-dependent networks in modulating the expression of p53 target genes, we searched for p53 targets that respond to a wide variety of other stress agents. One of these p53 target genes, Activating Transcription Factor 3 (*ATF3*), has recently gained attention as a growing body of evidence suggests that it works as an immediate-early response gene acting as a hub of the cellular adaptive-response network (**Hai et al., 1999, Hai & Hartman, 2001, Jiang et al., 2004, Hai T., 2006**). *ATF3* expression is induced in response to various physiological conditions, most of which have been signals that presumably induce tissue injury (**Chen et al., 1996 and Yin et al., 1997**). *ATF3* can be induced in response to stress signals via both p53-dependent and p53-independent manners (**Amundson et al., 1999**). Together, these studies have confirmed that *ATF3* is a stress-inducible gene that plays an important role in the cellular stress response; however, the signaling pathways involved in the regulation of *ATF3* transcription in response to these extracellular signals remain unclear. In this paper we highlight the putative cross-talk between two distinct stress-dependent pathways, the P53 gene regulatory network (GRN) and the Integrated Stress Response (ISR) pathway. We address the regulatory paradigms employed by stress-dependent transcription factors, p53 and Activating Transcription Factor 4 (ATF4), to activate expression of key stress response genes common to each pathway. We discuss the molecular mechanisms employed across cell types leading to this gene activation in response to DNA damage and endoplasmic reticulum (ER) stress.

The present study aims to compare the varied transcriptional response produced by stress-dependent pathways to elucidate the molecular mechanisms and the potential cross-talk employed by these distinct GRNs to acclimate and survive stress conditions. Literature suggests that the activation of certain target genes, such as *ATF3* and Sestrin-2 (*SESN2*), are common to each of these parallel pathways (**Velasco-Miguel et al., 1999 and Garaeva et al., 2016**); However, the signaling cascades and factors required for this target gene activation, as

well as the regulatory regions used by these stress-dependent TFs, are thought to be unique depending upon the specific stress-stimulus encountered. We show that an upstream enhancer element regulating the expression of *ATF3* is required for induction in response to DNA damage and p53 activation, however is not directly required for transcriptional induction of *ATF3* in response to ISR activating stimuli. Collectively, these results identify p53 and ATF4 as effectors of distinct, stress-dependent networks and spotlight *ATF3* among the common downstream targets of these pathways. The current report characterizes the cis-regulatory elements required for the activation of common target genes activated by two parallel pathways, the p53 GRN and the ISR.

Materials & Methods

Cell Culture and Treatments

The human colorectal cancer cell lines, HCT116 p53 WT and HCT116 p53 null, were cultured in McCoy's 5A Media (Corning, #10-050-CV) supplemented with 10% Fetal Bovine Serum (FBS) (Corning, #35-016-CV) and 1% Penicillin-Streptomycin (PS) (Gibco, #15240-062). Human mammary epithelial cells, MCF10A p53 WT and MCF10A p53 null, were cultured in 1:1 Dulbecco's Modified Eagle Medium: Ham's F-12 (Gibco, #11330-032) supplemented with 5% horse serum (Gibco, #16050-122), 20 ng/ml epidermal growth factor (Peprotech, #AF-100-15), 0.5 ng/ml hydrocortisone (Sigma, #H-0888), 100 ng/ml cholera toxin (Sigma, #C-8052), 10 ug/ml insulin (Sigma, #I-1882), and 1% PS. The human near haploid cell line, HAP1 parental and HAP1 ATF4 null cells, were cultured in Iscove's Modified Dulbecco's Medium (Gibco, #12440-053) supplemented with 10% FBS and 1% PS. All cell lines were cultured at 37°C and 5% CO_2 in a water-jacketed incubator.

Cells were cultured with DMSO as a control, 5uM Nutlin-3A (Millipore Sigma, #45-SML0580) to stabilize p53 activation, 100uM Etoposide (Thermo Scientific, #J63651.MC) to induce DNA-

damage, 2uM Tunicamycin (Thermo Scientific, #J62217.MA) to induce endoplasmic reticulum stress and 2mM Histidinol (Acros Organics, #AC228831000) to induce amino acid deprivation. 50nM of ISR-IB (Millipore Sigma, #50-958-40001) was used to inhibit ISR signaling. All cells were treated with stress stimuli for 6h before processing cells for downstream experiments.

Lentivirus production, purification and transduction

Lentiviral shRNAs and dCas9-KRAB constructs were produced using HEK293T cells that were seeded in six-well plates. Mission shRNA oligos were purchased from Sigma Aldrich (ATF4: GCCTAGGTCTCTTAGATGATT).

1 µg of pLKO plasmid having either scramble shRNA or ATF4 shRNA was combined with 1 µg of mixture of packaging plasmids (pMD2 and psPAX2) and the mixture was diluted into jetPRIME buffer (Polyplus Transfection, catalog no. 89129-924) and reagents, following the manufacturer's protocol. Lentivirus-containing supernatants were collected at 24 and 48 h post-transfection and filtered through a 0.45-µm membrane and stored in aliquots at −80 °C. HCT116 or HAP1 cells were transfected with lentivirus supplemented with 8 µg/ml Polybrene. At 24 h post-infection with lentivirus, media were replaced with the 2 ug/mL puromycin selection.

Quantitative Real Time PCR (RT-qPCR)

Total RNA was isolated (Quick RNA miniprep, Zymo, #R1055) with an on-column treatment of 50U of DNase I for 30 minutes. Single-stranded cDNA was generated (High Capacity cDNA Reverse Transcription Kit, ABI#4368814) and qPCR was performed using the relative standard curve method and iTaq Universal SYBR Green Supermix reagents (BioRad). RT-qPCR primers are shown in Supplemental Figure S1. Table A.

ProteinSimple Wes System

Total protein was isolated using a RIPA buffer and protein expression was measured using the ProteinSimple® Wes platform. The 12–230 kDa Wes Separation Module containing 8 × 25 capillary cartridges, reagents, and consumables was used per manufacturer's instructions. Details on the antibodies used are in Supplementary figure S1. Table B.

Cut & Run

1.5x10^6 cells per Cut & Run reaction were prepared for batch processing using the Epichyper CUTANA ™ Cut & Run protocol v1.9 and reagents (Epicypher, #14-1048). Cells bound to activated Concanavalin A beads were incubated overnight with 0.5ug of primary antibody. DNA fragments were purified using phenol/chloroform extraction and 20ng of purified fragmented DNA was used to construct an Illumina-compatible sequencing library using the protocol developed by Nan Liu (**Liu, 2019**) which has been optimized for small fragments, and the NEBNext Ultra II DNA Library reagents (NEB#E7660).

Cut & Run and ChIP-seq Data Analysis

Raw paired-end sequencing reads for Cut &Run were aligned to the hg38 human genome reference using hisat2 (**Kim et al., 2019**). Single-end sequencing reads were aligned, BigWig files for visualization were produced via deepTools. ChIP-seq reads (**Andrysik et al., 2017**) for HCT116 input (GSM2296270), DMSO-treatment (GSM2296271), and Nutlin-3A-treatment (GSM2296272) were aligned downloaded from Gene Expression Omnibus and aligned to the hg38 human genome reference using hisat2.

RNA Sequencing

Cells were treated with various stress stimuli at 80% confluency in a six-well plate for 6 h and total RNA was isolated (Quick RNA miniprep, Zymo, #R1055). PolyA+ RNA was purified using Dynabeads Oligo $(dt)_{25}$ (Invitrogen, #61012) and fragmented at 94°C for 15 min. Fragmented RNA was used as the template for double-stranded cDNA production which was then used to

construct an Illumina-compatible sequencing library (NEBNext Ultra II Directional RNA Library Kit for Illumina, NEB#E7760). Libraries were quantified using qPCR (NEBNext Library Quantification Kit, NEB#E7630) and an Agilent Bioanalyzer and then pooled for sequencing on an Illumina NextSeq 2000 at the University at Albany Center for Functional Genomics or on an Illumina Hiseq 2000 at Azenta/GeneWiz. Transcript abundance from the ENSEMBL hg38 genome assembly (v.104) was quantified using kallisto in bootstrap mode (kallisto quant -b 100). Resulting transcript counts (TPM) were imported and processed via tximport (**Sonesen et al., 2015**) and differential expression was quantified using DESeq2 (32). Pathway enrichment analyses for differentially expressed genes were performed using enrichr (**Chen et al., 2013, Kuleshov et al., 2016, and Xie et al., 2021**).

Results

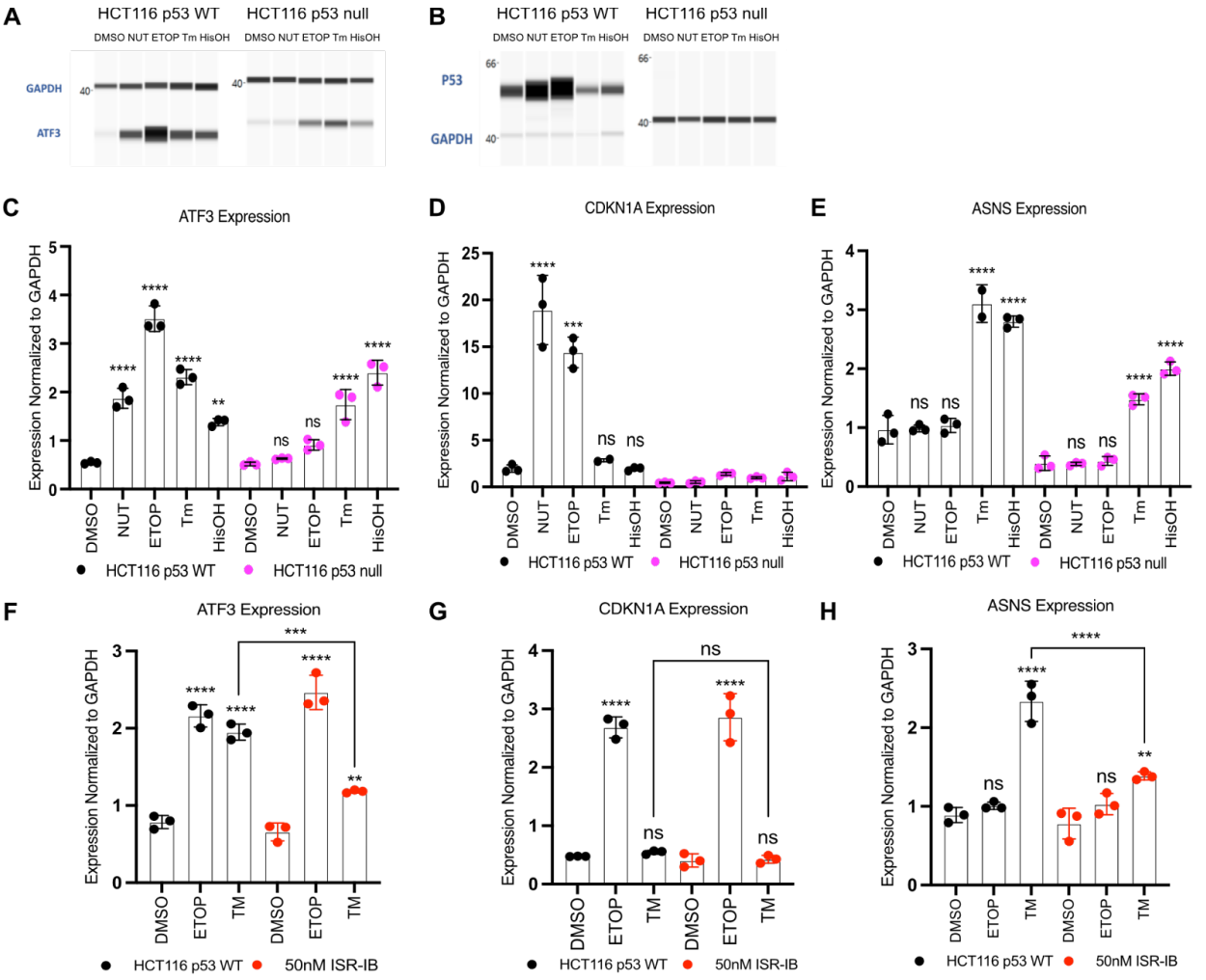


Figure 1. ATF3 is a p53 target gene that is activated via the Integrated Stress Response (ISR) in a p53-independent manner.

Western Blot analysis of **A)** ATF3 and **B)** p53 with GAPDH as a loading control in HCT116 p53 WT and p53 -/- (null) cells following a 6 h treatment with DMSO, 5uM Nutlin-3A (NUT), 100uM Etoposide (ETOP), 2uM Tunicamycin (TM) or 2mM Histidinol (HisOH). Gene expression analysis of the **C)** ATF3 gene **D)** CDKN1A gene and **E)** ASNS gene in HCT116 p53 WT (black) and HCT116 p53 null (pink) cells in response to 6 h treatment with DMSO, Nutlin-3A, Etoposide, Tunicamycin, or Histidinol. Gene expression analysis of **F)** ATF3 gene **G)** CDKN1A gene **H)** ASNS gene in HCT116 p53 WT cells in response to 6 h treatment with DMSO, Etoposide, Tunicamycin, with or without addition of concomitant 50nM ISR inhibitor (ISR-IB) treatment. All statistical comparisons were computed using a one-way ANOVA test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

ATF3, a p53 target gene, is induced via the Integrated Stress Response (ISR) in a p53-independent manner.

ATF3 mRNA expression is upregulated in response to several different stress conditions (**Hai et al., 1999, Hai et al., 2006, Lu et al., 2006, and Hashimoto et al., 2002**), but the specific transcription factors responsible are not fully characterized. We first confirmed previous reports

that ATF3 expression can be induced via activation of the tumor suppressor and transcription factor, TP53 (p53) (**Amundson et al., 1999**). We assayed ATF3 mRNA expression via quantitative reverse transcription polymerase chain reaction (RT-qPCR) after treatment of the isogenic human colorectal carcinoma cell lines, HCT116 *TP53*+/+ (p53 WT) or *TP53*-/- (p53 null), with vehicle control (DMSO), 100 µM etoposide, or 5 µM Nutlin-3A for 6 hrs. Etoposide activates several transcription factors, including p53, via induction of DNA double-strand breaks (DSBs) (**van Maanen, et al., 1988 and Shieh et al., 1997**). Nutlin-3A inhibits the negative p53 regulator, MDM2, leading to stabilization and activity of p53 in a non-genotoxic manner (**Vassilev et al., 2004**). We first confirmed that our treatments with Nutlin-3A and etoposide increased levels of p53 protein in HCT116 p53 WT cells (**Fig. 1B**). Similar to expression of the canonical p53 target gene *CDKN1A/p21* (**Fig. 1D**), *ATF3* mRNA expression increased in response to treatment with either Nutlin-3A or DSB-inducing etoposide in p53 WT, but not p53 null cells (**Fig. 1A, C**). These data suggest that *ATF3* mRNA expression in response to DNA DSBs is dependent upon the activity of p53 in HCT116 cells.

Our results using etoposide and Nutlin-3A confirm prior reports demonstrating that induction of *ATF3* in response to certain genotoxic agents such as ionizing radiation (IR) is p53-dependent, although the dependence on normal p53 function appears context-dependent, as *ATF3* mRNA can be induced in response to DNA damage caused by ultraviolet (UV) radiation in carcinoma cells containing p53-inactivating mutations (RKO/E6 cells) and TP53 -/- (knockout) mouse models (**Donehower et al., 1992**) (**Amundson et al., 1999 and Fan et al., 2002**). These conflicting results suggest that ATF3 can be regulated via p53-dependent and p53-independent means in a cell-context-dependent fashion. In addition, a growing body of evidence supports the involvement of parallel pathways, such as JNK/SAP and other members of the mitogen-activated protein kinase (MAPK) signaling pathway, in the induction of *ATF3* in response to stress signals (**Milne et al., 1995**, **Liang et al., 1996, Bulavin et al., 1999, and Sanchez-Prieto**

et al., 2000). Together, this research suggests that the induction of *ATF3* is regulated by complex mechanisms likely involving multiple signaling pathways. *ATF3* has been reported to be activated downstream of multiple stress-dependent networks, including the eukaryotic translation initiation factor 2 (eIF2) kinase stress response, more commonly referred to as the Integrated Stress Response (ISR) (**Jiang et al., 2004**). It is generally accepted that the primary function of the ISR is to promote cell survival in response to acute exposure to stress stimuli by activating a set of key stress response genes, however the cellular mechanisms underlying this observation have yet to be elucidated.

To determine if the ISR signaling pathway is involved in the regulation of *ATF3* in the cellular stress response, we treated our cells with additional stress stimuli known to activate the ISR. In vertebrates, the ISR is activated by stimuli that induce ER stress, nutrient and heme deprivation, and viral infection (**Taniuchi et al., 2016**). Therefore, we treated our HCT116 cell lines with 2 µM tunicamycin, an inhibitor of N-linked glycosylation which induces ER stress by causing an accumulation of unfolded proteins in the ER (**Wu et al., 2018**), or 2 mM histidinol, which initiates the amino acid response (AAR) via depletion of the essential amino acid, histidine (**Warrington, 1992 and Fu & Kilberg, 2013**). We first confirmed that expression of the canonical ISR target gene, asparagine synthetase (*ASNS*), increases in response to either tunicamycin (ER stress) or histidinol (AA starvation), validating that these treatments are effective in activating the ISR in both HCT116 p53 WT and p53 null cells (**Fig. 1E**). Importantly, neither Nutlin-3A nor etoposide treatments alter ASNS mRNA abundance in either genetic background, suggesting the ISR is not engaged after DNA DSB induction. Taken together, these two results suggest that tunicamycin and histidinol-mediated induction of the ISR in HCT116 cells does not require p53 activity. (**Fig. 1D,E)**. Similar to *ASNS*, *ATF3* mRNA expression is induced in response to either ISR-activating stimuli (tunicamycin or histidinol) in HCT116 p53 WT cells (**Fig. 1A,C**). Unlike that which we observed during the DDR, ISR-

activation leads to a significant induction of *ATF3* mRNA in the absence of p53 (**Fig. 1A,C**). Critically, neither tunicamycin nor histidinol stabilized p53 protein or induced expression of the canonical p53 target gene *CDKN1A/p21*, suggesting a completely parallel, p53-independent activation of *ATF3* via induction of the ISR pathway (**Fig. 1B,D**).

We next employed an inhibitor of the ISR pathway (ISR-IB) to confirm both the ISR-dependent activation and p53-independent activation, of ATF3 transcription in response to ER stress and AA starvation. ISR-IB suppresses the ISR by facilitating the assembly of active subunit eIF2b, rescuing translation in the presence of phosphorylated eIF2a (**Rabouw et al., 2019**). Inhibition of the ISR upon 6 hr treatment with 50 nM ISR-IB resulted in a significant reduction in the expression of both *ASNS* and *ATF3* in response to ER stress (**Fig. 1F,H**). Treatment with ISR-IB did not affect expression of *CDKN1A/p21*, further supporting the p53-independent mechanism of this gene regulation in response to ISR activation (**Fig. 1G**). Taken together, these data confirm that *ATF3* is a target gene for both the p53 GRN and the ISR pathway, and suggest that ER stress and essential amino acid starvation induce *ATF3* mRNA via p53-independent mechanisms.

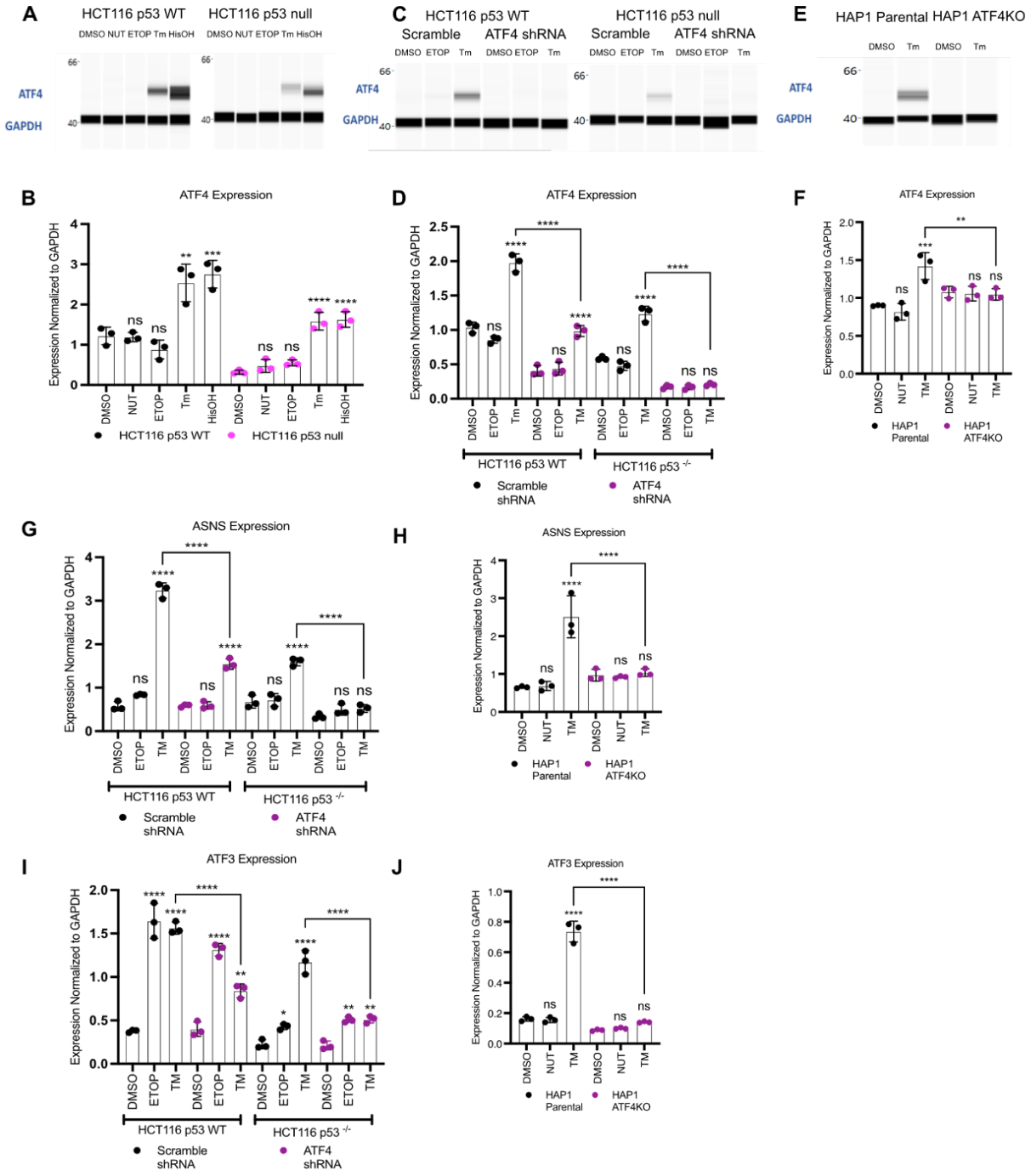


Figure 2. ATF4 and p53 independently regulate expression of ATF3.

Western Blot analysis of ATF4 with GAPDH as a loading control in **A)** HCT116 p53 WT and p53 -/- (null) cells and **B)** HAP1 parental and ATF4 null (ATF4KO) cells and **C)** HCT1116 p53 WT and p53 null cells containing shRNA constructs targeting ATF4 (ATF4 shRNA) or a non-targeting control (Scramble shRNA), following a 6 h treatment with DMSO, 10uM Nutlin-3A (NUT), 100uM Etoposide (ETOP), 2uM Tunicamycin (TM) or 2mM Histidinol (HisOH). Gene expression analysis of the *ATF4* gene in **D)** HCT116 cells **E)** HAP1 cells **F)** HCT116 ATF4 knockdown (ATF4KD) cells in response to 6 h treatment with various stress stimuli. Gene expression analysis of *ATF3* in **G)** HCT116 ATF4 knockdown cells and **H)** HAP1 ATF4KO cells following a 6 h treatment with various stress stimuli. Gene expression analysis of *ASNS* in **I)** HCT116 ATF4KD cells and **J)** HAP1 ATF4KO cells, in response to various stress stimuli. All statistical comparisons were computed using a one-way ANOVA test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

ATF4 and p53 independently regulate expression of *ATF3*

Our results and previous literature suggest parallel stress-dependent mechanisms regulate *ATF3* transcription in both p53-dependent and p53-independent manners. *ATF3* induction during the DDR is largely dependent upon the presence of p53 in HCT116 cells (**Fig. 1A,C**); however, the induction of *ATF3* mRNA in response to activation of the ISR occurs in the absence of p53 (**Figure 1**). ATF4, a member of the basic region-leucine zipper (bZIP) superfamily of stress-dependent TFs, is one of the main effectors of the ISR and a critical regulator of the transcription downstream of ISR activation (**Chen et al., 1996 and Han et al., 2013**). Prior work suggests that ATF4 regulates the expression of *ATF3* in other cellular contexts, therefore we tested whether ATF4 activity might underlie the p53-independent induction of *ATF3* mRNA expression in response to ER stress and AA starvation that we observe in HCT116 colon carcinoma cells (**Pan et al., 2007, Kilberg et al, 2009, and Fu & Kilberg, 2013**). We first characterized the activity of *ATF4* in response to ISR-activating stimuli in our HCT116 p53 WT and p53 null cells to confirm ISR-dependent ATF4 expression. As expected, *ATF4* mRNA and protein levels increase in response to both ER stress (via tunicamycin treatment) and AA starvation (via histidinol treatment), whereas, *ATF4* mRNA and protein expression were unaffected in response to p53 stabilization (via Nutlin-3A treatment) or DNA damage (via etoposide treatment) (**Fig. 2A,B**). The lack of inducible levels of ATF4 in response to p53-activating stimuli supports the possibility of these two factors in regulating expression of the same target gene, *ATF3*, using independent and parallel mechanisms.

To determine the putative role of ATF4 in regulating *ATF3* induction downstream of ISR activation, we created HCT116 p53 WT and p53 null cells expressing either a non-targeting control (scramble) or an ATF4-directed shRNA. *ATF4* mRNA and protein abundance are substantially reduced in ATF4 shRNA-expressing cell lines compared to those expressing the non-targeting shRNA control (Scramble) (**Fig. 2C,D**). mRNA expression of the canonical ISR target gene, *ASNS*, was significantly reduced after knockdown of ATF4, demonstrating the

effectiveness of these reagents in ablating both ATF4 expression and activity under basal and ISR conditions (**Fig. 2G**). In accordance with previous conclusions, the induction of *ATF3* in response to DNA damage was unaffected by the knockdown of ATF4, supporting the induction of this gene in response to DSBs as a highly p53-dependent response (**Fig. 2I**). Conversely, knockdown of ATF4 significantly reduced the amount of *ATF3* mRNA induction in response to ER stress, suggesting a direct role for ATF4 activity in mediating ISR-dependent *ATF3* expression (**Fig. 2I**). We extended our analysis to isogenic ATF4+ (HAP1 parental) and ATF4- (HAP1 ATF4KO) haploid leukemia cell lines. HAP1 ATF4KO cells lack detectable levels of *ATF4* mRNA and protein under basal conditions and in response to ER stress, confirming the full knockout of ATF4 (**Fig. 2E,F**). Deletion of ATF4 led to complete ablation of both *ASNS* and *ATF3* mRNA induction in response to ER stress, confirming that *ATF3* induction in response to ISR is ATF4-dependent (**Fig. 2H,J**). The present results demonstrate that ATF4 acts as the main effector of the ISR pathway by mediating the induction of key stress response genes, such as *ASNS* and *ATF3*, during exposure to specific stress stimuli, including ER stress; however, the molecular mechanisms underlying this gene regulation remain unclear. We have demonstrated that these two parallel stress-dependent networks converge at activation of a common target gene, *ATF3*, and we have confirmed the transcription factor effectors required for this gene activation in response to different stress stimuli such as DNA damage and ER stress. We next attempted to dissect the molecular mechanisms leading to this gene activation by defining the cis-regulatory regions used by stress-dependent TFs, p53 and ATF4, to orchestrate this stimulus-specific regulation of *ATF3*.

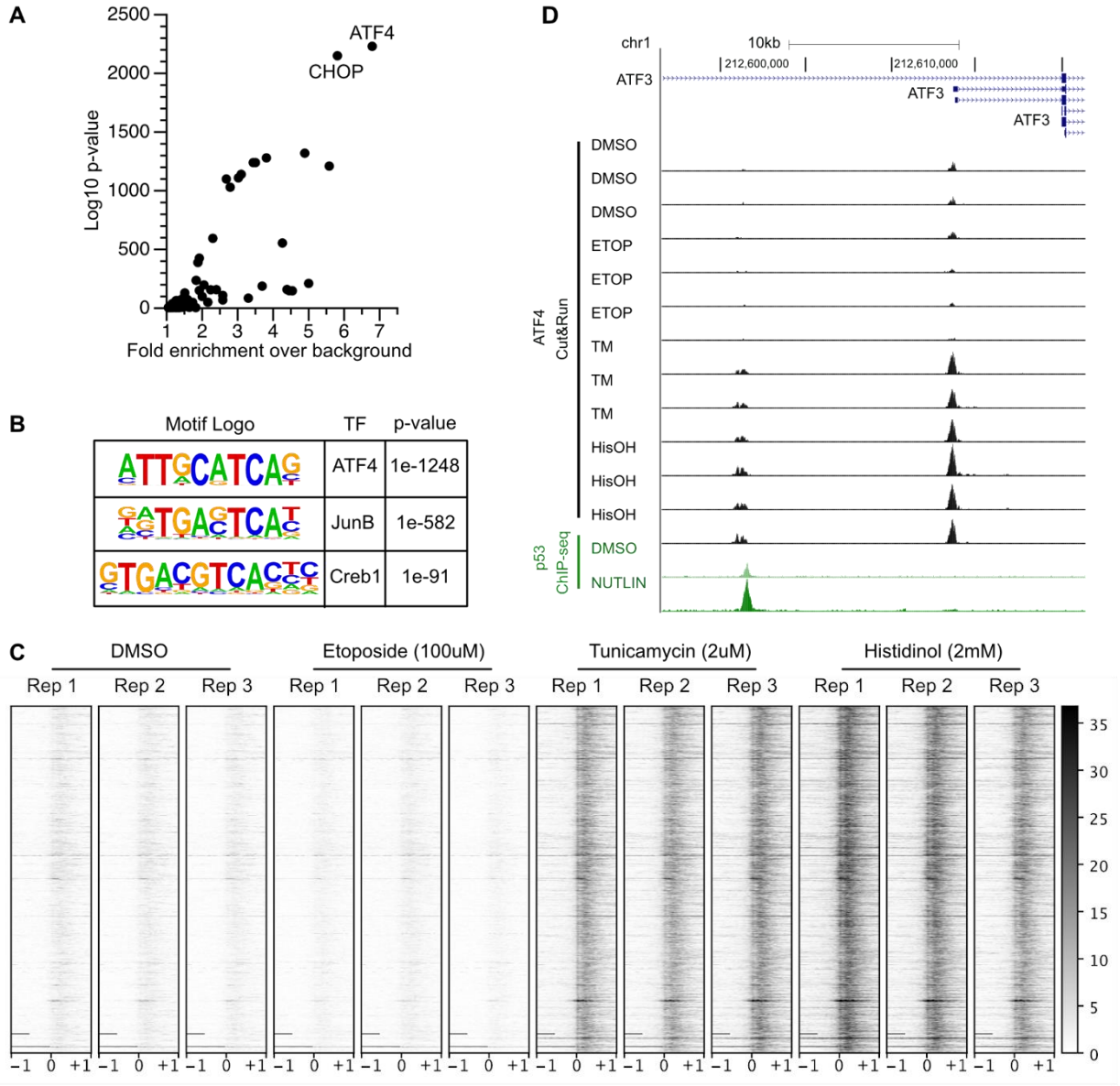


Figure 3. ATF4 and p53 occupy distinct regulatory regions in the *ATF3* gene locus

A) Known motif enrichment analysis of the high-confidence peak set reveals the predicted ATF4 motif as the most highly-enriched motif within this dataset. **B)** *de novo* motif analysis of high-confidence peak set shows enrichment of ATF4 motif. **C)** Enrichment of CUT&RUN sequencing tags following 6 hr treatment with DMSO (vehicle control), 100uM etoposide, 2uM tunicamycin, or 2mM histidinol. **D)** Genome browswer view of the ATF3 gene locus displaying ATF4 CUT&RUN data (black) and p53 ChIP-Seq data (green) following 6 hr treatment with various stress stimuli, including: DMSO (vehicle control), 5uM Nutlin-3A, 100uM etoposide, 2uM tunicamycin, or 2mM histidinol.

ATF4 and p53 occupy distinct regulatory regions in the *ATF3* gene locus

Our results demonstrate a genetic dependence for p53-mediated activation of *ATF3* transcription under DNA damage conditions, and a functionally distinct, ATF4-dependent

pathway that regulates *ATF3* transcription during the ISR. To understand whether this regulation occurs via direct or indirect binding to regulatory regions in the genome, we generated novel cleavage under targets & release under nuclease (CUT&RUN) (**Skene & Henikoff, 2017**) genomic binding data for ATF4. HCT116 cells (1.2x106 cells/IP) were treated with either DMSO (control), p53-activating (etoposide), or ISR-stimulating agents (tunicamycin or histidinol) for 6 hrs and then subjected to CUT&RUN analysis using either an ATF4-specific antibody or a non-specific IgG isotype control. Three biological replicates were generated for each condition. Regions of significant ATF4 enrichment (relative to IgG control signal) were identified using *macs2* (**Zhang et al., 2008**). We first created a set of high-confidence, ISR-activated ATF4 binding events by considering only peaks called from 5 out of 6 experiments from cells treated with either tunicamycin or histidinol (**Fig. S3A,B,C**). The rationale for filtering peaks in this manner is to allow examination and further analysis of putative ATF4 binding events that are universal to the ISR, while also accounting for potential variability within individual biological replicates or treatment conditions. In support of this approach, we observe 7,723 ATF4 binding events shared across ⅚ experimental conditions, with 5,093 (65%) existing across all observations. These 7,723 peaks were then examined for expected features of ATF4 binding, including specificity during ISR stimulation and enrichment of predicted ATF4 DNA binding motifs.

Known motif enrichment analysis revealed the predicted ATF4 motif as the most highly-enriched in the high-confidence peak set, followed by enrichment of motifs for the known heterodimer partner, C/EBP homologous protein (CHOP). (**Fig. 3A**). Similar enrichment of a motif most closely matching the known ATF4 motif was observed using *de novo* motif enrichment strategies on the high-confidence set (**Fig. 3B)**. Enrichment of CUT&RUN sequencing tags was highly specific for tunicamycin and histidinol-treated conditions compared to either vehicle (DMSO) control or under DNA damage (etoposide) conditions (**Fig. 3C**). We

first confirmed previous reports from literature which suggest the transcriptional activation of *ASNS* in response to ISR stimuli is mediated via binding of ATF4 to the promoter region of this gene in response to AA starvation (**Chen et al., 2004**). Our CUT&RUN analysis reveals a significant, ISR-specific enrichment of ATF4 at the *ASNS* promoter in response to both AA starvation and ER stress (**Fig. S3D**). These data suggest that our set of high-confidence, ISR-dependent ATF4 peaks are likely representative of true ATF4 genomic binding events. Therefore, we used this set of genomic locations engaged by ISR-activated ATF4, along with previously published p53 ChIP-seq data, to identify putative ATF4 and p53 binding events that might directly regulate *ATF3* transcription. We observe an ATF4 binding event within a DNAse-hypersensitive site (DHS) overlapping the first exon/transcriptional start site (TSS) of *ATF3* in response to both ER stress and amino acid deprivation, but not during the DDR or in vehicle-treated control conditions (**Fig. 3D**). This region corresponds with a previously reported promoter region regulating stimulus-dependent *ATF3* transcription (**Fu & Kilberg, 2013**). p53, on the other hand, binds to a DHS approximately 13kb upstream (p53-bound DHS) from the ATF4-bound *ATF3* promoter. ATF4 also occupies a spatially distinct DHS 15kb upstream (ATF4-bound DHS) from the *ATF3* TSS in an ISR-dependent fashion. In an attempt to probe these regions for regulatory function in response to stress-activating stimuli, we performed both *in vitro* and *in vivo* analyses of these regions to determine their contribution to the transcriptional regulation of *ATF3*.

Analysis of the enhancer: promoter pair regulating stress-dependent *ATF3* expression

Biochemical analyses and genetic loss-of-function experiments confirm that p53 and ATF4 likely regulate expression of *ATF3* independent of each other (**Fig. 2**). To determine if these distinct DHS bound by p53 or ATF4 were contributing to transcriptional activity of *ATF3* in response to stress, we first created a luciferase reporter gene assay where we could compare the transcriptional activity of the DHS bound by p53 to a construct where the p53 binding site

has been mutated (p53bs mutant). We also included the ATF4-bound DHS and a luciferase construct driven solely by a minimal promoter (miniP) as a negative control. We compared the levels of transcription induced by these constructs in HCT116 p53 WT and p53 null cells in response to our vehicle control (DMSO), p53 stabilization with Nutlin-3A, or ISR activation via tunicamycin-induced ER stress. Our results reveal that the ATF4-bound DHS does not act as an enhancer element, as there was no significant difference in the levels of transcription driven from this site when compared to those driven by the negative control (miniP), under any of the conditions and cellular contexts tested (**Fig. 4A**). Conversely, the p53-bound DHS induced significant levels of transcription in response to basal conditions and p53 stabilization, however no induction of transcription was observed in response to ISR activating stimuli (**Fig. 4B**). This putative enhancer element bound by p53 displays p53-dependent activity, as mutations present in the p53 binding site completely ablate the transcriptional activation from this enhancer in response to p53 stabilization with Nutlin-3A. Furthermore, analysis of this construct in the HCT116 p53 null cells resulted in a significant reduction in overall activity compared to cells containing p53, as well as an inability to induce transcription via Nutlin-3A treatment (**Fig. 4B**). These results suggest that this upstream DHS bound by p53 is a putative enhancer element that is dependent upon p53 for optimal transcriptional activation in response to p53 activating stimuli. Critically, our in-vitro reporter results very clearly demonstrate that ISR activation in response to tunicamycin-induced ER stress does not result in an increase of transcriptional activity from this enhancer regardless of p53 activity (**Fig. 4B**). Together, these results suggest that this upstream p53-bound enhancer element will likely not be contributing to the induction of *ATF3* in response to ISR activation, but may be involved in the regulation of this gene in response to p53 activation. This, along with the lack of transcriptional activity from the ATF4-bound DHS site, supports a hypothesis that ATF4 is likely activating transcription of *ATF3* in response to ISR activation via its occupancy at the *ATF3* gene promoter region.

Additionally, prior ChIP-seq and reporter gene assays suggest that ATF4 regulates *ATF3* via interaction with two canonical ATF/CREB family motifs within the *ATF3* promoter in hepatocarcinoma (HepG2) cells in response to ISR activation (**Kilberg et al., 2013**). In particular, these researchers highlight a CRE site (nt −93/−85, TGACGTCA) existing upstream of the CARE site (nt −23/−15, TGATGXAAX) within the *ATF3* gene promoter (−107/+35) (**Hai et al., 2010 and Weidenfeld-Baranboim et al., 2009**). To determine the role of these ATF4-responsive motifs in regulating the expression of *ATF3*, we created a luciferase reporter driven by the −107/+35 promoter fragment of the ATF3 gene - containing both the CARE and CRE sequences (WT ATF3 promoter), as well as constructs containing mutations in one (CARE and CRE) or both (CARE/CRE) of these sites. Previous observations by Kilberg et al. suggest that not only is the CARE site necessary for AAR activation, but the CRE site is also required to obtain the level of induction observed for the WT ATF3 promoter element (**Fu & Kilberg, 2013, and Pan et al., 2007**). We confirmed these reports in HAP1 parental cells, observing an increase in luciferase activity in response to both ISR-activating stimuli, ER stress and amino acid deprivation, driven by the WT ATF3 promoter (**Fig. 4C**). Mutations in the CRE site resulted in a significant reduction in overall transcriptional activity and a complete ablation in transcriptional induction in response to either ISR-activating stimuli. Conversely, mutations in the CARE motif resulted in ablation of solely the histidinol-induced AAR, but did little to affect the induction in response to ER stress. Promoter constructs harboring mutations to both the CARE and CRE site showed the lowest overall transcriptional activity, and did not result in induction via either treatment condition. (**Fig. 4C**).

In addition to confirming previous reports by colleagues, we provide a novel analysis of these promoter constructs in the absence of ATF4 (HAP1 ATF4KO). Overall WT promoter activity is significantly reduced in the absence of ATF4, and we observe no significant induction by the WT promoter upon treatment with either stress stimuli in HAP1 ATF4KO cells (**Fig. 4C**).

These results suggest that ATF4 plays a critical role at this promoter element and is required to induce transcription in response to ISR activation. Similar to that observed in HAP1 WT cells, constructs containing CRE and CARE/CRE mutations were unable to induce transcription in response to either stress condition in the absence of ATF4 (**Fig. 4C**). Interestingly, CARE mutants retained the ability to induce expression in response to ER stress in HAP1 ATF4KO cells, albeit, to reduced levels (**Fig. 4C**). Together, our in-vitro results suggest that ATF4-mediated regulation of ATF3 in response to ISR activating stimuli occurs through a combination of both CARE and CRE response elements within the ATF3 promoter. These data indicate that the CARE site is critical for transcriptional induction during the AAR, however is nonessential for transcriptional induction in response to ER stress. The ability of CARE mutants to induce transcription in the absence of ATF4 suggests the possibility that ISR factors other than ATF4 may be playing a role at specific response elements within his promoter region to regulate expression of ATF3 in a stimulus-specific manner.

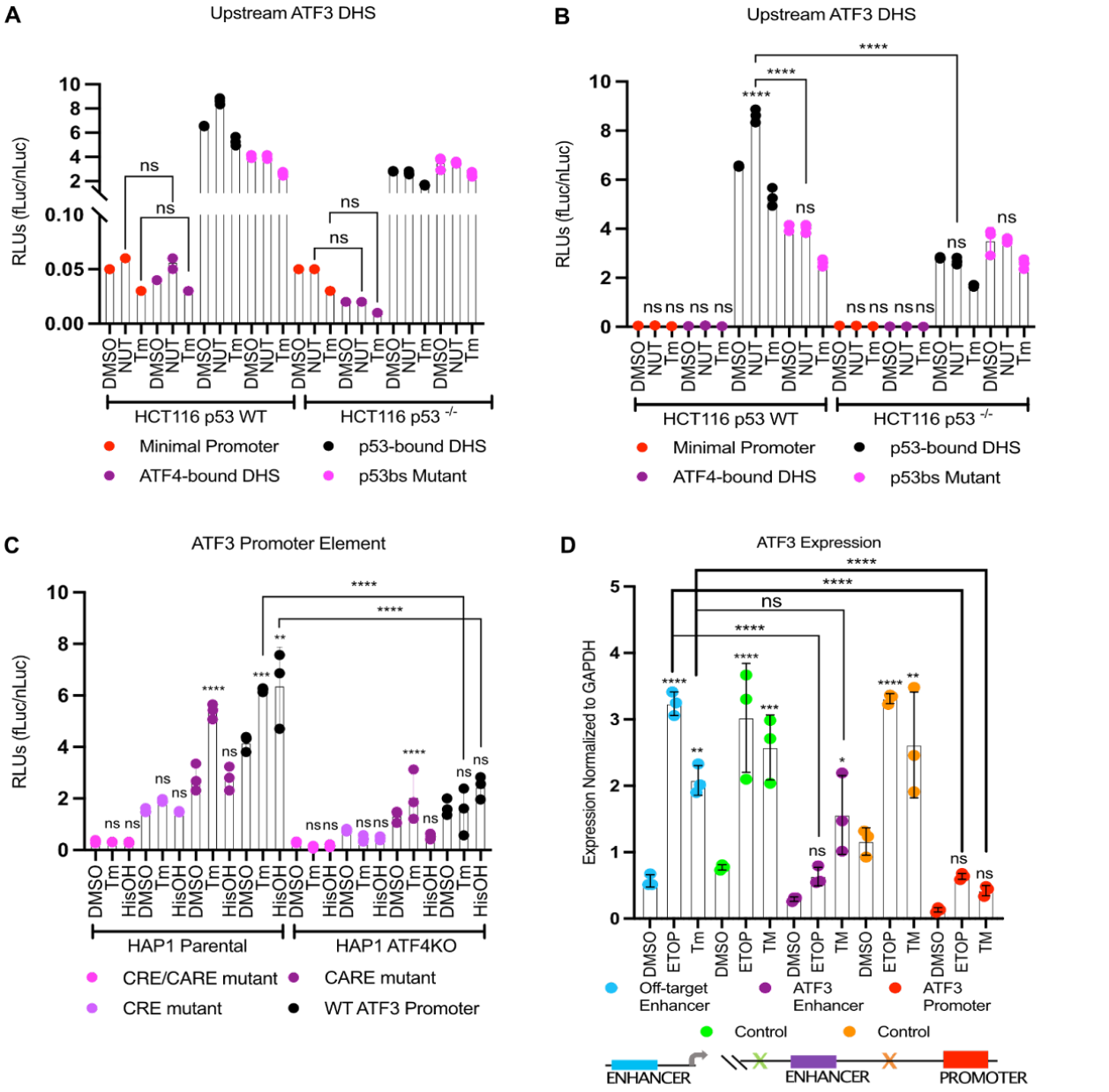


Figure 4. ATF3 induction by the ISR does not require the upstream enhancer element bound by p53.

A) Normalized luciferase values driven by the upstream *ATF3* DNA-Hypersensitivity sites (DHS): ATF4-bound DHS, p53-bound DHS, p53bs Mutant, and the minimal promoter (negative control), in response to 6 hr treatment with DMSO, 5uM Nutlin-3A (NUT) or 2uM tunicamycin (Tm) in HCT116 p53 WT and p53 null cells. The ATF4-bound DHS does not induce transriptional activity to levels that differ from the negative control. **B)** Normalized luciferase values driven by the upstream *ATF3* DNA-Hypersensitivity sites (DHS): ATF4-bound DHS, p53-bound DHS, p53bs Mutant, and the minimal promoter (negative control), in response to 6 hr treatment with DMSO, 5uM Nutlin-3A (NUT) or 2uM tunicamycin (Tm) in HCT116 p53 WT and p53 null cells. C) Normalized luciferase values driven by the (-107/+35) *ATF3* promoter sequence (WT ATF3 promoter) and constructs containing mutations in specific ATF4 response elements: CARE, CRE, CARE/CRE, in response to 6 h treatment with DMSO, 2uM tunicamycin (Tm), or 2mM histidinol (HisOH) in HAP1 parental and ATF4KO cells. **D**) RT-qPCR analysis of the *ATF3* gene in response to 6 h treatment with DMSO, 100uM Etoposide (ETOP) or 2uM Tunicamycin (TM) in HCT116 p53 WT cells where dCas9-KRAB is targeting regions across the *ATF3* gene locus for transcriptional repression. Statistical comparisons for nascent expression levels were computed using a one-way ANOVA test. Statistical comparisons for fold change induction levels were compute using an unpaired t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Stress-mediated induction of *ATF3* in response to ISR activation does not require the upstream enhancer bound by p53

Our data demonstrate that ATF4 and p53 independently regulate expression of *ATF3* (**Figure 2**) and occupy distinct putative regulatory regions in a stress-dependent manner **(Figure 3)**. Our *in-vitro* reporter assays provide direct evidence for the role of ATF4 and p53 at specific cis-regulatory elements to induce transcriptional activity of ATF3 in response to distinct stress stimuli (**Figure 4A,B,C**). In order to further characterize the mutual independence of p53 and ATF4 and to demonstrate whether these binding events control *ATF3* transcription *in-vivo*, we utilized a CRISPR interference (CRISPRi) system to block effector protein binding (**Gilbert et al., 2013, and Qi et al., 2013**). We chose the dCas9-KRAB CRISPRi system which fuses the catalytically inactive form of the *Streptococcus pyogenes* Cas9 protein with the KRAB transcriptional repressor domain **(Margolin et al., 2004)**. dCas9-KRAB targeting to cis-regulatory elements has proven an effective strategy for blocking effector protein binding and inhibiting regulatory elements and linked gene expression (**Thakore et al. 2015, Yeo et al., 2018, and Catizone et al, 2020**). We first targeted dCas9-KRAB to the *ATF3* promoter as proof of principle, since repression of a gene promoter should inhibit transcription initiated from that element. Targeting of dCas9-KRAB to the putative *ATF3* promoter, approximately 100bp upstream of the TSS (**Fig. S4A**) significantly reduced *ATF3* mRNA levels compared to all three separate off-target controls (**Fig. 4D**). This repression was observed under basal (DMSO) and both DNA damage and ER stress-inducing conditions, demonstrating the effectiveness of using the CRISPRi system to inhibit transcription of *ATF3* via specific targeting of regulatory elements.

Confirming our observations with non-genotoxic p53 stabilization *in-vitro*, targeting of dCas9-KRAB to the DHS bound by p53 upstream of *ATF3* significantly ablated *ATF3* mRNA levels in response to genotoxic p53 activation when compared to all non-targeting controls (**Fig.**

4D). Targeting of dCas9-KRAB to any of the three control locations (FGF2 enhancer, 5' control, and 3' control) did not significantly alter either basal or DNA damage-induced *ATF3* expression, demonstrating that targeting and activity of dCas9-KRAB is specific to this region alone. This opposes what we observe for induction of *ATF3* in response to ISR-activating stimuli; While we do observe an effect on basal levels of *ATF3* mRNA expression in the presence of dCas9-KRAB inhibition of the enhancer element, *ATF3* mRNA was still inducible in response to tunicamycin-induced ER stress (**Fig. 4D**). Additionally, expression of *ASNS* was still inducible in response to ISR-activating treatments in the presence of transcriptional repression of this enhancer element, suggesting the ISR is still functional when this site is inhibited (**Fig. S4C**). The expression of a canonical p53 target gene, *CDKN1A/p21*, can also be induced in response to P53-activating stimuli in the presence of dCas9-KRAB inhibition of this p53-bound enhancer element, suggesting the activity of this enhancer is specific to regulation of *ATF3* (**Fig. S4D**). These results indicate that while this p53-bound upstream enhancer region is required for both basal and p53-mediated transcriptional activation of *ATF3*, it is not directly required for the induction of *ATF3* in response to ER stress. These opposing observations suggest that the upstream enhancer element bound by p53 is required for optimal induction of *ATF3* in response to p53 stabilization and DNA damage, but not for ATF4-mediated induction of this gene in response to ISR activation.

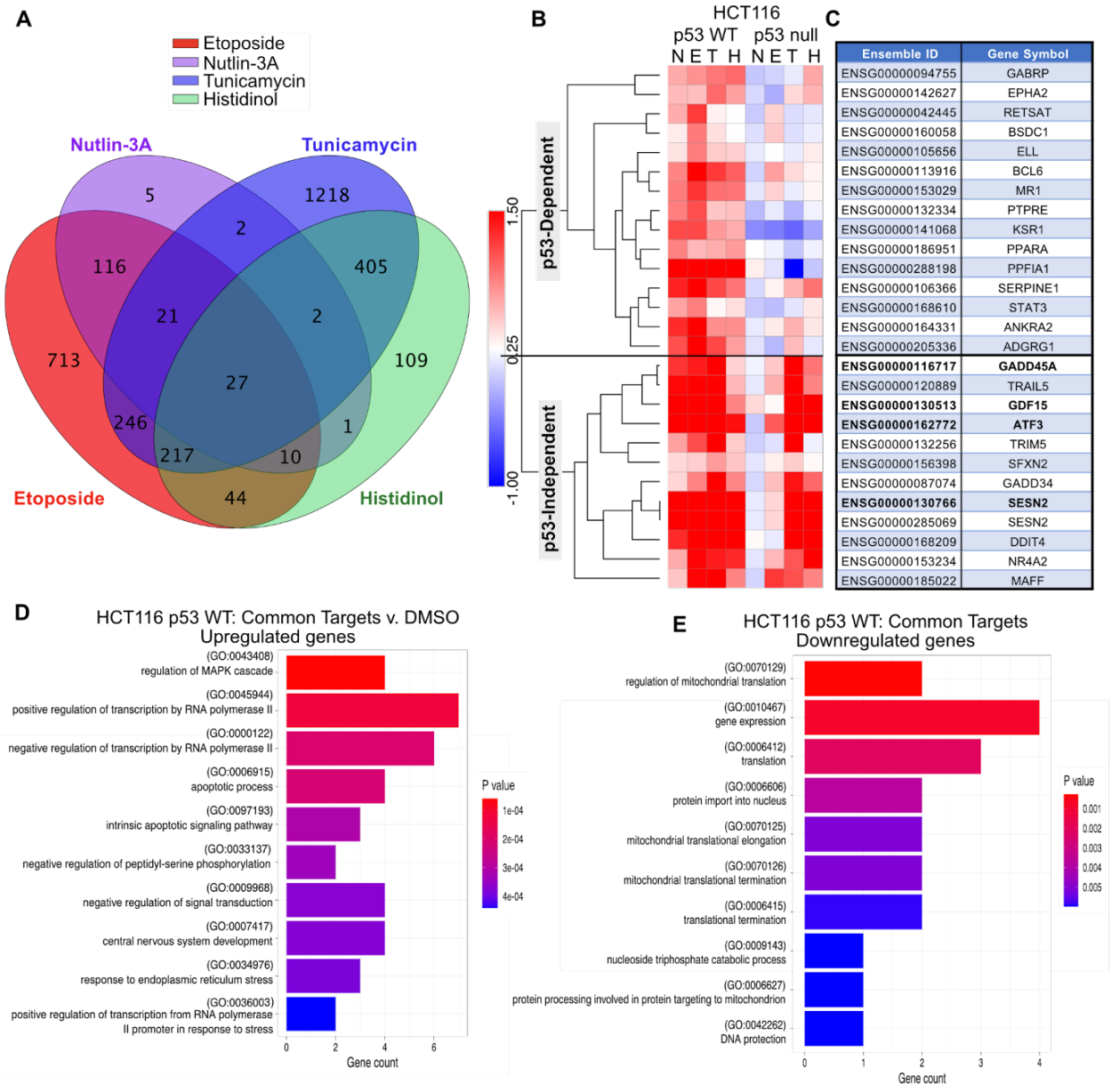


Figure 5. Global transcriptome analysis identifies common gene regulatory targets of the p53 GRN and the ISR

A) Intersection of genes upregulated in HCT116 p53 WT cells treated with 5uM Nutlin-3A, 100uM Etoposide, 2mMTunicamycin, and 2uM Histidinol, when compared to vehicle control (DMSO) for 6hrs. There are 27 common targets upregulated during activation of these various stress stimuli. **B)** Heat map displaying fold change values for the 27 common targets identified in panel A, in HCT116 p53 WT and p53 null cells treated with various stress stimuli for 6hrs. **C)** Table displaying the gene symbols for the 27 common target Ensembl gene IDs identified in panel A. Gene ontology analysis of the genes commonly **D)** upregulated and **E)** downregulated, in response to these various stress stimuli.

Global transcriptome analysis identifies common gene regulatory targets of the p53 and integrated stress responses

Our data demonstrate that the DNA damage response (via p53) and the Integrated Stress Response (via ATF4) both activate transcription of *ATF3,* although they do so independently of one another through two different gene regulatory elements. To determine if this parallel activation of *ATF3* by these two critical stress response pathways was more widespread, we performed polyA+ RNA-seq on HCT116 p53 WT and p53 null cells after 6 hours of treatment with p53 or ISR-activating stimuli: DMSO (vehicle), 5uM Nutlin-3A (p53 alone), 100uM Etoposide (p53 via DNA damage response), 2uM Tunicamycin (ATF4 via ER stress), and 2mM Histidinol (ATF4 via amino acid starvation). Three biological replicates were performed for each treatment condition and analyzed by performing transcript counting via pseudoalignment (*kallisto*, 100 bootstraps), followed by calling of differentially expressed genes using *deseq2.* We first confirmed that our treatments were effective in eliciting an expected stress response by performing gene ontology analysis of the genes upregulated (**Fig. S5A-D**) and downregulated (**Fig. S5E-H**) when compared to vehicle control (DMSO) using Enrichr (**Chen et al., 2013, Kim et al., 2019 and Xie et al., 2021**). Treatment with either Nutlin-3A or etoposide led to the significant upregulation of ontological gene categories consistent with a functional p53 response, including those related to known p53 signaling and the cellular response to DNA damage (**Fig. S5A,B).** Treatment with tunicamycin led to upregulation of genes consistent with ER stress and transcriptional regulation (**Fig. S5C**) and downregulation of genes involved in translation and ribosome biogenesis (**Fig. S5G**). Similar ontology groups were enriched in differentially regulated genes after treatment with histidinol (**Fig. S5D,H**), although we note expected treatment-specific enrichment of ER stress-associated genes after tunicamycin treatment and metabolic regulation after histidinol exposure. Taken together, these broad analyses of gene regulation via gene ontology groupings demonstrate that each of these treatments recapitulates expected cellular responses to each of these cell stress conditions.

After confirming the validity of our treatments in activating the specific stress responses, we sought to identify genes regulated like our observations for *ATF3*. This set of genes would be i) significantly upregulated in response to both p53- and ISR-activating stimuli in HCT116 p53 WT cells relative to DMSO, ii) p53-dependent in response to Nutlin-3A treatment, a stimuli that specifically activates and stabilizes p53 (**Fig. S5I**), and iii) significantly upregulated in the absence of p53 in response to ISR-activating treatments, Tunicamycin and Histidinol. Limiting our analysis to genes with these behaviors, we identified a set of 27 genes that are commonly upregulated in response to p53 activation, DNA DSB, ER stress, and AA starvation when compared to our vehicle control in HCT116 p53 WT cells (**Fig. 5A**). *ATF3* was among the genes upregulated in response to all four treatment conditions relative to DMSO, providing support for the methodology and the quality of the data set (**Fig. 5C**). Gene ontology analysis of the 27 commonly upregulated targets suggest that this set of commonly upregulated genes are involved in the regulation of transcription by RNA polymerase II, apoptotic signaling pathways and processes, as well as regulation of the mitogen-activated protein kinase (MAPK) signaling cascade, a pathway known to integrate and amplify signals from a diverse range of stimuli to produce an appropriate cellular response (**Zang & Dong, 2007 and Chavel et al., 2010**) (**Fig. 5D**). Ontology analysis of the genes downregulated in response to these 4 treatments suggest that activation of both the p53 and ISR pathways commonly downregulate protein synthesis-associated genes, likely reflecting a cellular switch from an anabolic to catabolic state and numerous prior reports of broad translational control by these pathways (**Fig. 5E**).

We next analyzed the behavior of these commonly upregulated genes in HCT116 p53 null cells to determine whether their induction in response to stress was dependent on p53 or, similar to our observations for *ATF3*, can still be upregulated in response to ISR-activating stimuli in the absence of p53. Interestingly, our RNA-seq analysis suggests that 11 of the 27 commonly regulated genes behave similarly to *ATF3* (**Fig. 5B**). These genes can be classified

as ISR target genes that are activated in response to both ER stress and AA starvation in a p53-independent manner (**Fig. 5B**). Somewhat expectedly, genes in this group displayed evidence of remaining somewhat dependent upon p53 for full induction in response to ISR-activating treatments (**Fig. 5B**). Importantly, p53 protein levels are not stabilized in response to ISR-activating stimuli (**Fig. 1B**) and we observe no evidence that canonical p53 target genes like *CDKN1A*/p21 respond to ISR-activating stimuli (**Fig. 1D**). Together, these data suggest that the p53-dependent nature of these common target genes do not reflect a direct dependence upon p53 itself, but rather, reflect p53-dependent, indirect mechanisms, such as regulation of target genes that feed forward onto ISR/ATF4 target genes.

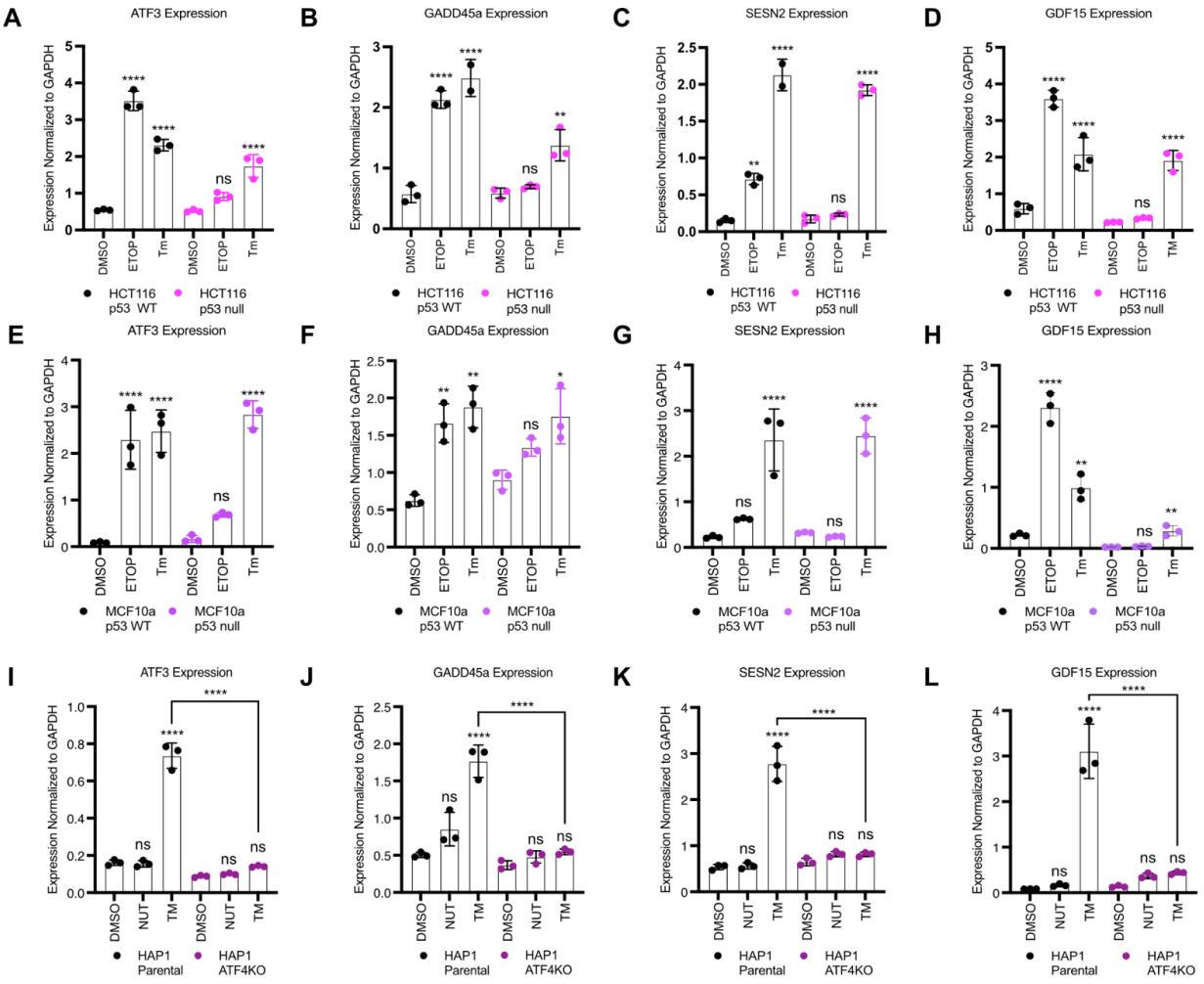


Figure 6. Parallel stress-dependent networks converge at activation of a common set of target genes.
RT-qPCR analysis of the *ATF3, GADD45a*, *SESN2,* and *GDF15* gene in **A-D**) HCT116 p53 WT and p53 null cells, **E-H**) MCF10A p53 WT and p53 null cells, and **I-L**) HAP1 parental and ATF4KO cells, following a 6 h treatment with DMSO, 100uM Etoposide (ETOP), or 2uM Tunicamycin (Tm). All statistical comparisons were computed using a one-way ANOVA test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Parallel stress-dependent networks converge at activation of a common set of ATF4-dependent target genes

To determine if this mode of regulation was unique to HCT116 cells, we performed validation of a few of the p53-independent target genes identified in our RNA-seq analysis in a non-malignant mammary epithelial (MCF10A) cell line. To do this, we treated our MCF10A p53

WT or p53 null background cells with DMSO, 100 micromolar Etoposide (ETOP) to induce DNA damage and activate p53, or 2 micromolar Tunicamycin (Tm) to activate the ISR and induce ER stress. Using RT-qPCR, we confirmed that these common target genes are transcriptionally induced by activation of these two parallel pathways in response to DNA damage and ER stress, and more importantly, the induction of these targets via ER stress-induced ISR activation is achieved in the absence of p53 in both HCT116 cells (**Fig. 6A-D**) and MCF10A cells (**Fig. 6E-H**). These results suggest that these distinct stress-dependent networks are displaying crosstalk at the level of target gene activation to upregulate a set of co-regulated target genes across multiple cell types. Together, this data confirms that the regulatory paradigm we have observed is not unique to *ATF3*, nor specific to HCT116 cells; rather, our results suggest that these two parallel pathways converge at activation of a common set of target genes in a broad and conserved manner.

Our transcriptome analysis has allowed us to identify a core set of p53 target genes that can also be activated by a parallel stress-dependent network, the ISR, in both p53-independent and p53-dependent manners (p53/ISR target genes) (**Figure 5**). We have demonstrated that, like ATF3, multiple other genes are upregulated in response to ER stress using p53-independent mechanisms, and this manner of regulation is observed across multiple cell types (**Figure 6**). The activation of one of these common target genes, *ATF3*, is dependent upon ATF4 for induction in response to ER stress (**Figure 2**). We have provided a molecular basis for the activation of this gene, *ATF3*, in response to p53 and ATF4 activating stimuli using ChIP-seq and Cut & Run to define the global binding profiles of these stress-dependent effectors (**Figure 3**). Using the ATF3 gene locus as a model, we have proposed a mechanism for the regulation of ATF3 in response to various stress stimuli using a CRISPRi approach to probe distal and proximal regulatory regions for function (**Figure 4**). To determine if the set of genes we have previously identified are activated via the ISR in a p53-independent manner, and are dependent

upon ATF4 for induction in response to ISR activation, we validated these targets in our HAP1 parental and ATF4KO cells using RT-qPCR. HAP1 parental or ATF4KO cells with vehicle control (DMSO), 5 micromolar Nutlin-3A to specifically stabilize and activate p53, or 2 micromolar tunicamycin to induce ISR activation via ER stress. Treatment with Nutlin-3A fails to activate ATF3 or three newly identified shared p53/ISR target genes: *GADD45A* (**Zhan et al., 1994 and Ebert et al., 2020**), *SESN2* (**Budanov & Karin, 2008 and Garaeva et al., 2016**), and *GDF15* **Osada et al., 2007 and Li et al., 2018**) (**Fig. 6I-L**) in either WT or ATF4KO HAP1 cells, as expected, given the single, mutant p53 allele present in the HAP1 parental cell line (Ser215Gly). Treatment of ATF4KO HAP1 cells with tunicamycin confirmed that all four tested target genes (*ATF3*, *GADD45A, SESN2, GDF15)* depend on ATF4 for transcriptional induction in response to ER stress (**Fig. 6I-L**). Taken together with shRNA-based knockdown of ATF4 in HCT116 cells (**Fig. 2I**), these data strongly suggest that ATF4 directly regulates the expression of our newly identified, shared p53/ISR pathway gene targets in a p53-independent fashion.

Discussion

In this study, we present a comparative analysis of the dynamic transcriptome landscape and unique and shared gene regulatory strategies downstream of two cell stress responses. We demonstrate that the p53 GRN and the ATF4-driven ISR pathway, although generally regulating distinct sets of stress response genes, converge on a set of common transcriptional targets related to metabolic control and apoptosis. We provide direct evidence that a subset of these common transcriptional targets require p53 during the cellular DNA damage response, but not as part of the Integrated Stress Response. Conversely, stress-dependent transcriptional activation of these target genes requires ATF4 during the ISR, with ATF4 being dispensable during the DDR. The genetic dependence of these common target genes on p53 and ATF4 parallels the well-studied stress-dependent stabilization (p53) and translation (ATF4) of each transcription factor (**Kastan et al, 1991 and Vattem & Wek, 2004**). Genetic loss of p53 reduces

expression of a subset of these common targets, even under ISR conditions (**Fig.5B**), but does not abrogate the ability of ATF4 to induce transcription (**Fig.6A-H**). Thus, these genes can be considered p53-dependent with regard to total mRNA abundance, but not p53-dependent for ISR-induced transcription via ATF4. The mechanisms underlying this behavior are not the focus of this study, but this observation is consistent with multiple reports of "basal" p53 activity in unstressed cells (**Wu et al., 1993 and Aylon & Oren, 2007**). Additionally, we cannot rule out an indirect role of p53 in the regulation of this subset of genes, as p53 regulates a broad network of genes that can potentially serve to feed-forward on other basal or stress response transcriptional networks (**Andrysik et al., 2017**).

p53 and ATF4 regulate a common set of target genes and likely do so through engagement with both unique and shared regulatory elements. The present study provides a molecular basis for the stress-induced activation of ATF3 via interaction of p53 and ATF4 with distinct gene regulatory elements. Using both in-vitro and in-vivo approaches, we demonstrate that a distal enhancer element approximately 13kb upstream of the TSS is required for the induction of ATF3 in response to p53 activation and DNA damage (**Fig 4A,B,D**). This enhancer, though, is not directly required for the observed ATF3 induction in response to ISR-activating stimuli, such as tunicamycin-induced ER stress. Our data suggest ISR-mediated activation is achieved via ATF4 interaction with the ATF3 promoter region. We confirm previous observations suggesting both a CARE and CRE element within the ATF3 promoter are required for a maximal transcriptional response to amino acid deprivation and ER stress (**Fig. 4C**) (**Kilberg et al., 2009**). The question remains, though, how these sites cooperate to drive ISR-mediated ATF3 transcription and how ATF4 interacts with these DNA elements. The loss of basal, unstimulated promoter activity when the CRE site is mutated and ATF4 translation is repressed suggests collaboration with other bZIP family members capable of binding to these sequences. Members of the bZIP superfamily, like ATF4, bind their DNA motifs as homo- or

heterodimers with other bZIP members (**Rodríguez-Martínez et al., 2017**) and can interact with degenerate motifs, making it difficult to assess factor occupancy solely from DNA motifs. Prior studies indicate that induction of ATF3 in response to ISR activation involves combinatorial interactions of multiple basic leucine zipper (bZIP) factors, such as ATF2 and cJUN, at the ATF3 promoter (**Fu & Kilberg, 2013**). Our CUT&RUN analysis of global ATF4 binding suggests a broad range of potential ATF4 heterodimer combinations with canonical bZIP family members, including CHOP, JunB, and Creb1 (**Fig. 3B**). Further work will be required to better understand whether different combinations of ATF4 homodimers/heterodimers drive specific gene networks within the global ATF4-dependent transcriptome. CHOP, for example, is thought to be a central regulator of ISR-induced apoptosis (**Hu et al., 2019**), and ATF4/CHOP heterodimers can both activate and repress ISR-target genes via direct promoter interactions (**Su & Kilberg, 2008**).

Our results suggest that this common, but redundant, regulation of antiproliferative gene targets by p53 and ATF4 occurs across multiple cell types, including both transformed (HCT116 and HAP1) and non-transformed (MCF10A) cell lines. Both the p53-dependent gene regulatory network and the ATF4-driven integrated stress response are antiproliferative, either through induction of apoptosis or through control of the cell cycle and CCNG1cell proliferation. p53 canonically controls cell proliferation through control of *CDKN1A/*p21 and other members of the cell cycle control network, like *CCNG1* (**Jensen et al., 2003**). Interestingly, at least four of the shared direct target genes of p53 and ATF4 (*DDIT4, GADD34, SESN2,* and *GDF15)* have been shown to inhibit proliferation via inhibition mTOR signaling (**Budanov & Karin, 2008, Aguilar-Recarte et al., 2021, Lockheart et al., 2020, and Gambardella et al., 2020, and Coronel et al., 2022**). *ATF3* is also intimately involved in the coordination of cell cycle progression via control of serine, nucleotide, and glucose metabolism (**Ku & Chang, 2020 and Marcantonio et al., 2021**). It is tempting to speculate that these genes represent a “core” set of genes that can be repurposed by numerous cell stress response pathways to enact an anti-proliferative

strategy. Indeed, investigations into the regulation of these targets in response to stress conditions regulated by other master transcription factors such as hypoxia (HIF1a), heat shock (HSF1), inflammation (IRF/STAT), xenobiotics (AHR), and infection (NF-kB), appear to be warranted. Future studies will be necessary to determine the mechanisms and extent to which parallel stress-dependent transcriptional networks may be able to redundantly control expression of these key antiproliferative metabolic and apoptotic gene targets.

Nearly half of all human malignancies harbor mutations in p53 that facilitate and promote metastasis, tumorigenesis, and resistance to apoptosis (**Zhu et al, 2015 and Mantovani et al, 2019**). These mutations most commonly lead to loss of DNA binding and an inability to transactivate canonical p53 target genes (**Bykov et al., 2018**) and promote anti-proliferative gene expression. Numerous chemotherapeutic approaches are still effective in p53 mutant tumors via induction of p53-independent apoptosis, with some showing increased efficacy in the absence of p53 (**Bykov et al., 2018**) . Given the powerful tumor suppression abilities of the p53 gene regulatory network, restoration of the p53-regulated transcriptome represents an intriguing approach for development of anticancer strategies and therapeutics in tumors harboring p53 loss-of-function mutants. Strategies to restore p53 activity via small molecule inhibition of mutant p53 or other relevant stress pathways (**Bykov et al, 2018 and Yu et al., 2012**), compound-induced degradation of mutant p53 (**Zhang et al., 2015**), and disruption of interactions between mutant p53 and other transcription factors via oligomerization inhibitor compounds (**Chowdhury et al, 2014**). Strategies employing genome-wide restoration of the p53 pathway by small molecules via p53-independent mechanisms are considered promising (**Bykov et al., 2018**). One recent investigation into restoring the p53 transcriptome in the absence of WT p53 activity examined the use of a novel compound that led to non-canonical activation of ATF4 and activation of of previously identified direct p53 transcriptional targets, such as such as death receptor 5 (*DR5/TRAIL5*) and p53 upregulated mediator of apoptosis

(*PUMA*) **(Tian et al., 2021**). Our current study provides important context for these observations. Most importantly, we observe that the canonical ATF4-dependent ISR pathway regulates expression of these p53 target genes, but in a p53-independent manner. Thus, any ISR-stimulating small molecules are likely to reactivate expression of key anti-proliferative genes like *DR5, GADD45a*, *SESN2, GADD34, GDF15,* and *DDIT4* in cancers lacking WT p53. These results suggest that the ATF4-dependent transcriptional network and the ISR pathway are potentially attractive candidates for future cancer therapeutic development, especially in cancers lacking the ability to activate these mutual gene targets via a WT p53 regulatory network.

Supplementary Figures

A

Gene Target	Forward Primer	Reverse Primer
CDKN1A/p21	AGCGATGGAACTTCGACTTTG	CGAAGTCACCCTCCAGTGGT
ATF3	CTCTGCGCTGGAATCAGTCA	CCTCGGCTTTTGTGATGGA
ATF4	CAGACGGTGAACCCAATTGG	CAACCTGGTCGGGTTTTGTT
ASNS	GGTACATCCCGACAGTGATGATATT	CCTGGACACTATGAAGTTTTGGATT
SESN2	TGCCTCCGGGACTGACA	GAATGGCAAGTAGGAGGTAATGAAA
GADD45a	AGCAGAAGACCGAAAGGATG	CAGGCACAACACCACGTTA
GDF15	ACCTGCTAACCAGGCTGCG	CGGTGTTCGAATCTTCCCAG
GAPDH	CCAGGTGGTCTCCTCTGACTTC	GTGGTCGTTGAGGGCAATG

Table A. Primers used for RT-qPCR

B

Protein Target	Vendor Information
p53	(clone DO1, BD Biosciences, #554923)
ATF3	(Abcam, #AB207434)
GAPDH	(Cell Signaling, #5174S)
ATF4	(Cell Signaling, #D4B8)

Table B. Antibodies used for Western Blotting

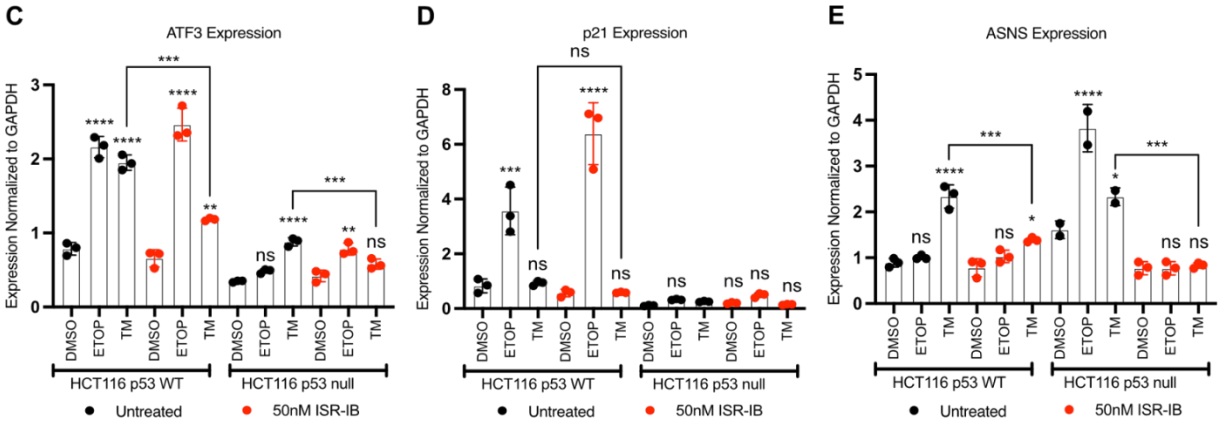


Figure S1. Supplementary Figure 1

A) Forward and reverse primer sequences used for RT-qPCR analysis. **B)** List of antibodies used for western blotting analysis. RT-qPCR analysis of the **C)** ATF3 gene, **D)** CDKN1A/p21 gene, and **E)** ASNS gene, in HCT116 p53 WT and p53 null cells following a 6 h treatment with DMSO, 100uM Etoposide (ETOP), or 2uM Tunicamycin (TM) in the presence (red) or absence (black) of 50nM ISR inhibitor (ISR-IB). All statistical comparisons were computed using a one-way ANOVA test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

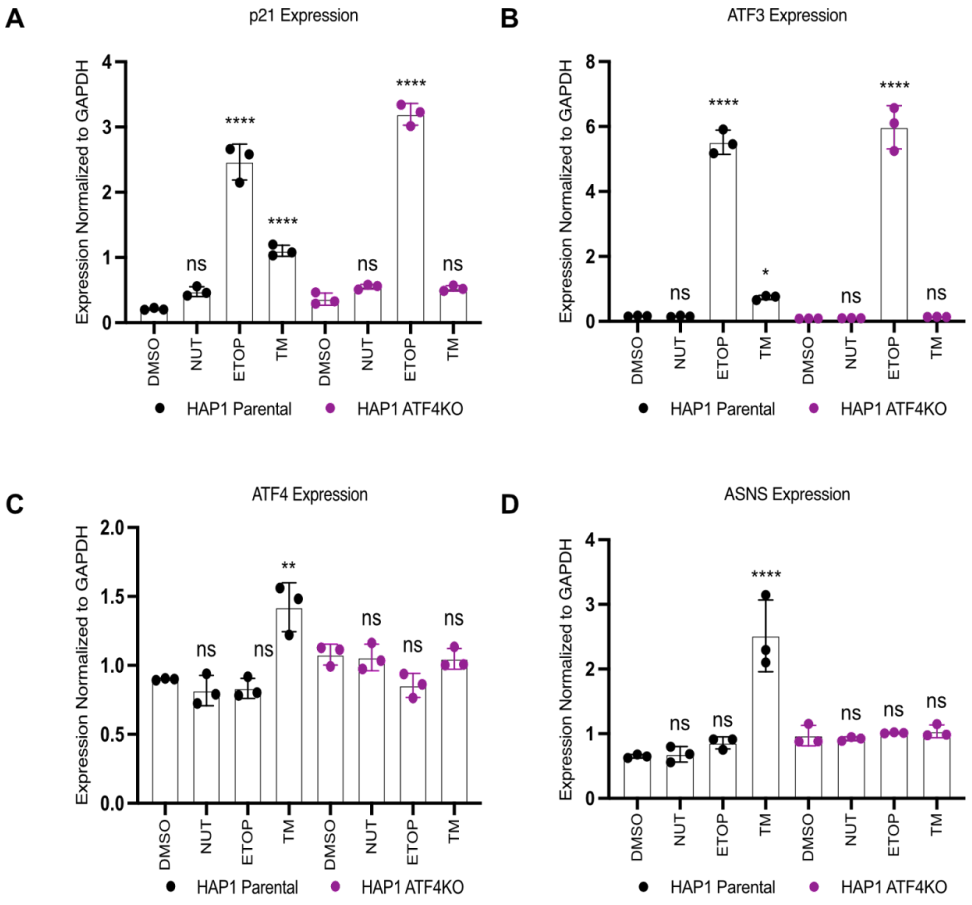


Figure S2. Supplementary Figure 2

RT-qPCR analysis of the **A)** p21 gene, **B)** ATF3 gene, **C)** ATF4 gene, and **D)** ASNS gene, in HAP1+ parental and HAP1-ATF4KO cells following a 6 hr treatment with DMSO, 5uM Nutlin-3A (NUT), 100uM etoposide (ETOP), or 2uM Tuncamycin (TM). All statistical comparisons were computed using a one-way ANOVA test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

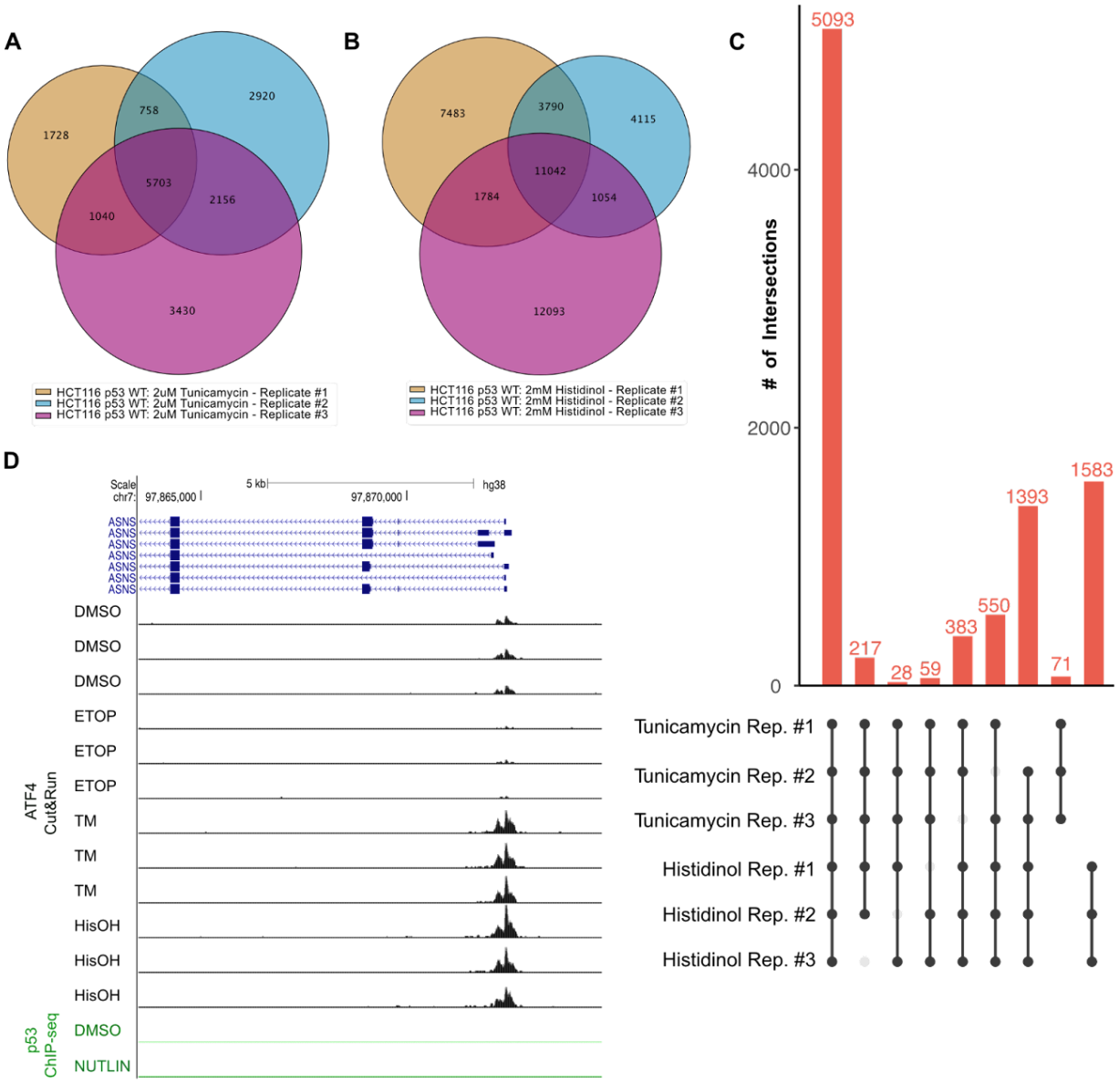
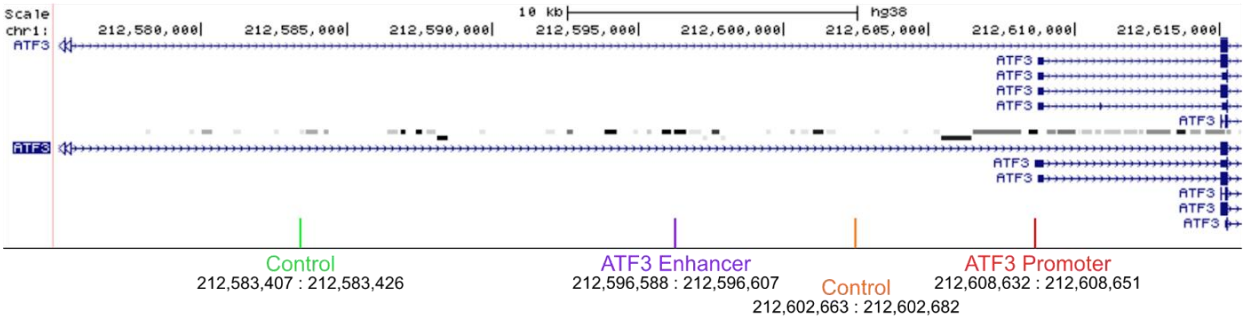


Figure S3: Supplementary Figure 3

A) Intersection of ATF4 CUT&RUN peaks for the three biological replicates of HCT116 p53 WT cells treated with 2uM tunicamycin for 6 hrs. **B)** Intersection of ATF4 CUT&RUN peaks for the three biological replicates of HCT116 p53 WT cells treated with 2mM histindinol for 6 hrs. **C)** We created a set of high-confidence ISR-activated ATF4 binding events by considering only peaks called out from 5 out of the 6 experiments with ISR-activating treatments: 2uM tuncamycin and 2mM histidinol. We observe 7,723 ATF4 binding events shared across 5/6 experimental conditions. D) Genome browser view of the *ASNS* gene locus displaying ATF4 CUT&RUN data (black) and p53 ChIP-Seq data (green) in HCT116 p53 WT cells following 6 hr treatment with various stress stimuli, including: DMSO (vehicle control), 5uM Nutlin-3A (NUTLIN), 100uM etoposide (ETOP), 2uM Tuncamycin (TM), and 2mM histidinol (HisOH).

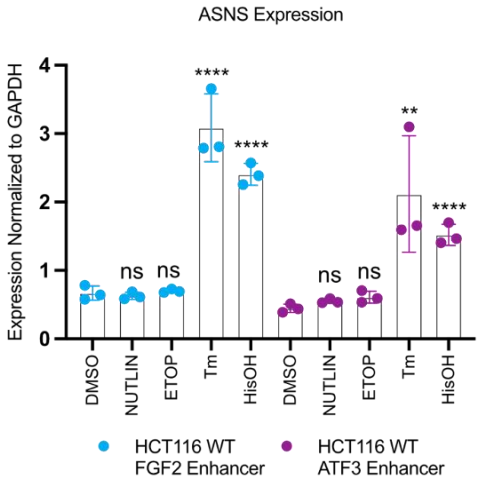
A



B

Cloning Oligo	Target Sequence	Forward Primer	Reverse Primer	Genomic Coordinates
FGF2 Enhancer Control dCas9-KRAB	TGGATGGGGGAAGTGATCTATCC	caccgTGGATGGGGGAAGTGATCTA	aaacTAGATCACTTCCCCCATCCAc	122,798,358 : 122,798,377
5' Control dCas9-KRAB	ACACACAGCAGGTTTAACTGAGG	caccgACACACAGCAGGTTTAACTG	aaacCAGTTAAACCTGCTGTGTGTc	212,583,407 : 212,583,426
3' Control dCas9-KRAB	GTTTACGGTCCATCAAAGGGTGG	caccgGTTTACGGTCCATCAAAGGG	aaacCCCTTTGATGGACCGTAAACc	212,602,663 : 212,602,682
ATF3 Enhancer dCas9-KRAB	CCAAGGCAGAAGAGTGCTGTGGG	caccgCCAAGGCAGAAGAGTGCTGT	aaacACAGCACTCTTCTGCCTTGGc	212,596,588 : 212,596,607
ATF3 Promoter dCas9-KRAB	GGAGAACCGGCCGAACGGAGCGG	caccgGGAGAACCGGCCGAACGGAG	aaacCTCCGTTCGGCCGGTTCTCCc	212,608,632 : 212,608,651

C



D

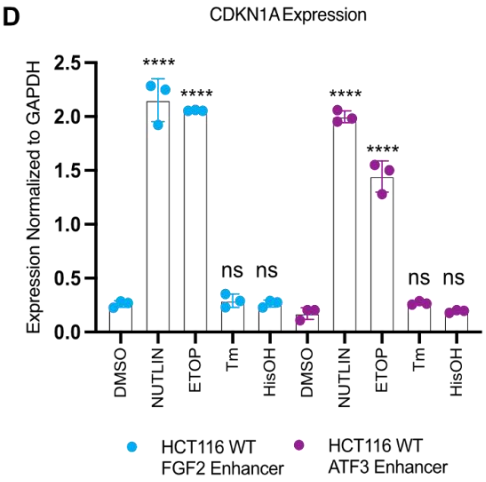


Figure S4: Supplementary Figure 4

A) Genome browser view of the *ATF3* gene locus displaying the location of dCas9-KRAB gRNA targets and the genomic coordinates spanning these targets. **B)** Table displaying the target sequence of cloning oligos used with PAM site underlined, and the respective genomic coordinates for the dCas9-KRAB target loci. RT-qPCR analysis of the **C)** ASNS gene, and **D)** CDKN1A/p21 gene, following a 6 hr treatment with DMSO (vehicle control), 5uM Nutlin-3A (NUTLIN), 100uM etoposide (ETOP), 2uM tunicamycin (Tm), or 2mM histidinol (HisOH), in HCT116 p53 WT cells where dCas9-KRAB is targeting an off-target control enhancer (blue) or the p53-bound ATF3 enhancer element (purple) for transcriptional repression. All statistical comparisons were computed using a one-way ANOVA test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

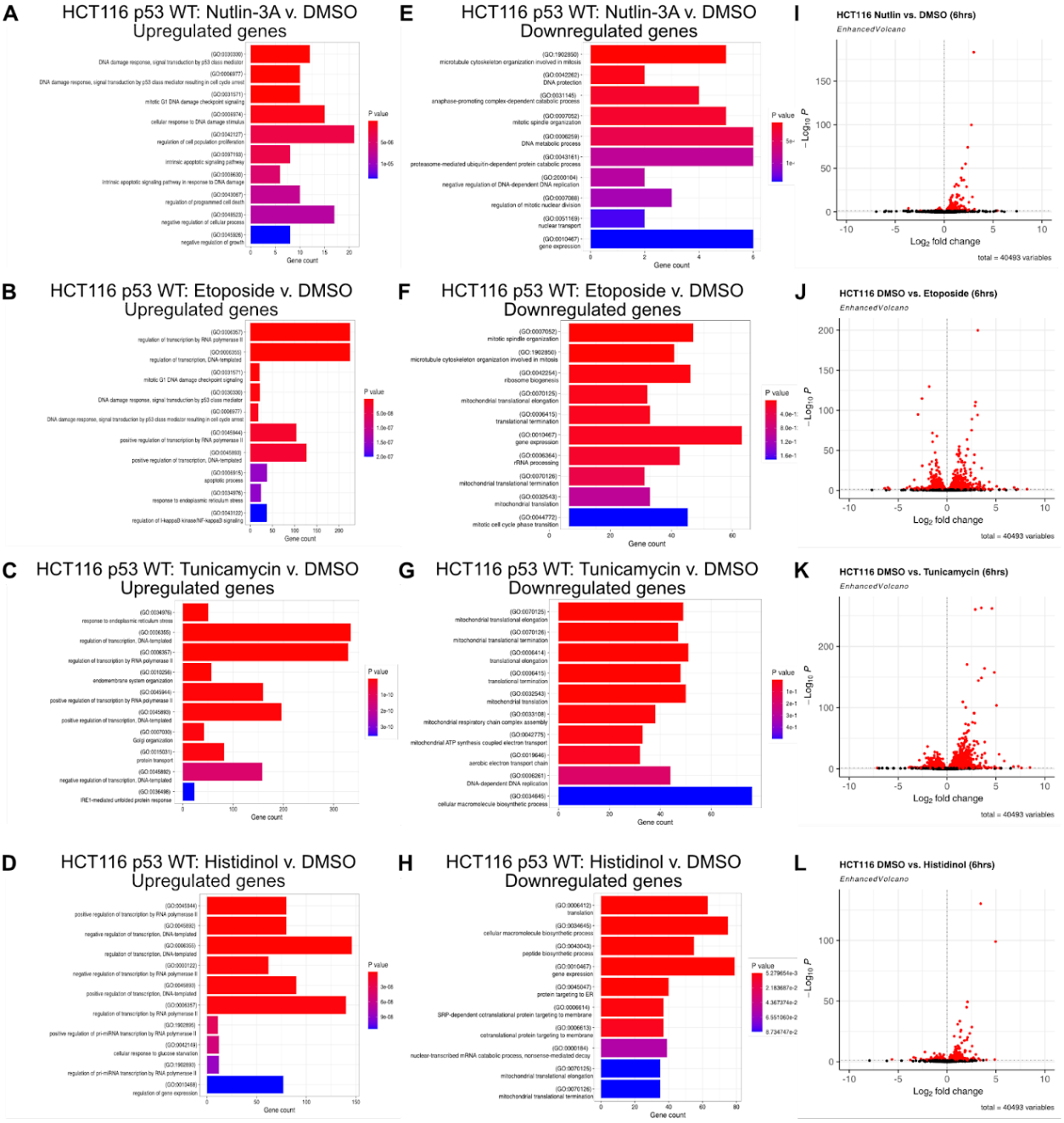


Figure S5. Supplementary Figure 5

Gene ontology analysis of the genes upregulated (A-D) and downregulated (E-H) in response to 6 hr treatments with various stress stimuli compared to vehicle control (DMSO), including: 5uM Nutlin-3A (A,E), 100uM etoposide (B,F), 2uM tunicamycin (C,G), or 2mM histidinol (D,H). Enhanced volcano plots displaying differential gene expression in HCT116 cells treated for 6 hrs with I) 5uM Nutlin-3A, J) 100uM etoposide, K) 2uM tunicamycin, and L) 2mM histidinol.

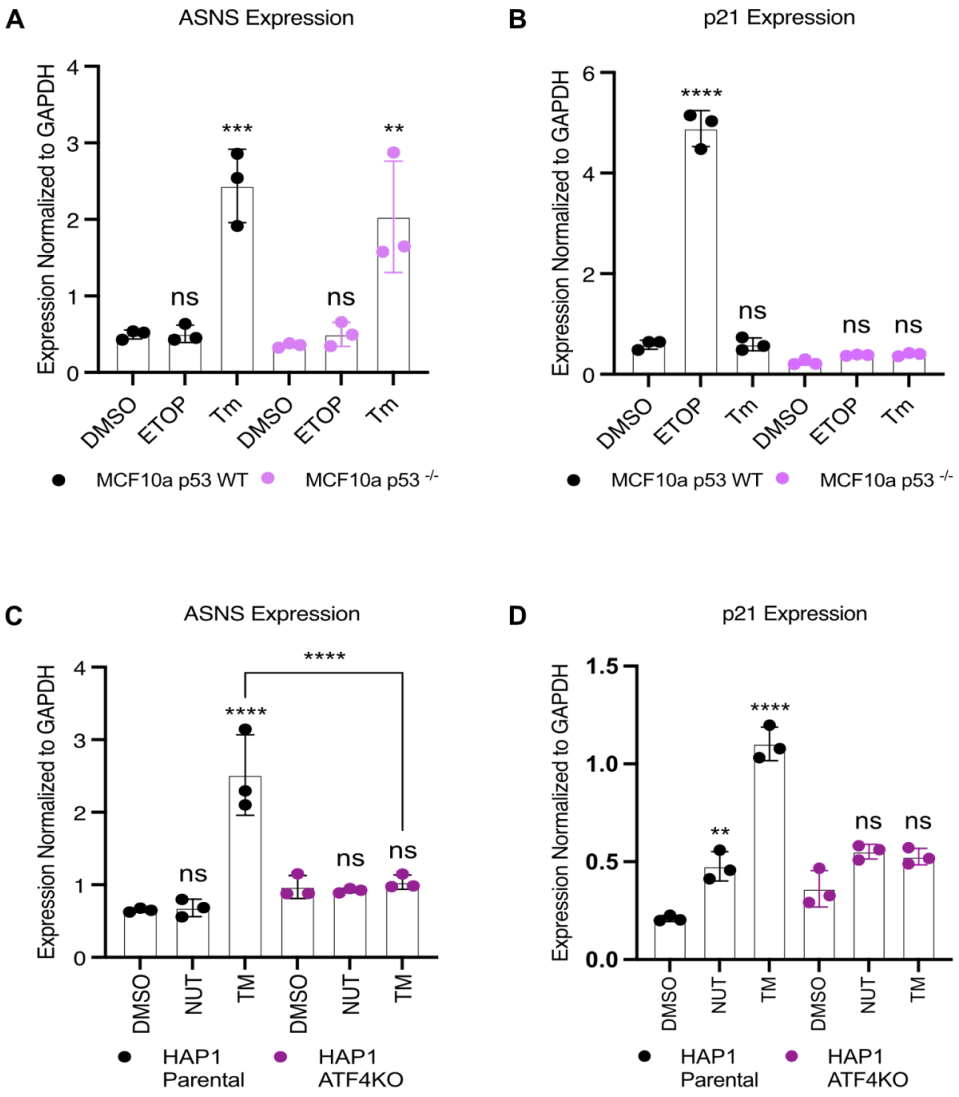


Figure S6. Supplementary Figure 6

RT-qPCR analysis of the ASNS and CDKN1A/p21 genes in **A-B**) MCF10A p53 WT and p53 null cells and **C-D**) HAP1 parental and ATF4KO cells, following a 6 hr treatment with DMSO, 5uM Nutlin-3A (NUT) or 2uM tunicamycin (TM). All statistical comparisons were computed using a one-way ANOVA test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Ch. 5 Conclusions, significance, and future directions

Conclusions

The cell employs shared gene regulatory strategies to orchestrate an appropriate cellular response to a variety of different stress conditions. It has been suggested that the transcriptional response elicited by a particular cell is dependent upon several contexts including the specific

type of stress a cell encounters and its severity. Transcriptome analysis in HCT116 p53 WT cells supports this notion, as transcriptional differences were observed resulting from different forms of stress stimuli suggesting that there seems to be stimuli-specific transcriptional regulation in response to stress. Additionally, while we have demonstrated that a conserved transcriptional response occurs across multiple different cell types, transcriptional differences were observed, presumably as a result of cell type-specific contexts. Global transcriptome analysis of HCT116 cells in response to exposure to a variety of stress stimuli results in the upregulation of a common set of target genes confirmed to be downstream targets of two parallel pathways, the p53 GRN and the ISR pathway, and hereafter referred to as "p53/ISR targets." Further work is required to determine the effects that duration and severity of stress may have upon these genes' expression.

In this study, we demonstrate that two parallel stress-activated networks, the p53 GRN and the ATF4-driven ISR, converge on a common set of target genes. These genes display both p53-dependent and p53-independent activities in response to multiple stress stimuli that activate a parallel pathway, the ISR. In addition to the ATF3 gene, highlighted throughout this paper, we have identified multiple genes that have been previously confirmed to be downstream targets of both the p53 GRN, and more recently the ISR pathway, such as: SESN2 (**Budanov & Karin, 2008 and Garaeva et al., 2016**), Growth arrest and DNA damage-inducible protein 45 alpha (GADD45a) (**Zhan et al., 1994 and Ebert et al., 2020**), Growth differentiation factor 15 (GDF15) (**Osada et al., 2007 and Li et al., 2018**), and DNA-damage-inducible transcript 4 (DDIT4) (**Ellisen et al., 2002 and Whitney et al., 2009**). While a much greater interplay exists between these two stress-dependent networks than hitherto expected, future studies will be necessary to determine how broad this manner of regulation is, and how many other stress stimuli may also lead to activation of these common target genes. Investigations into the

regulation of these targets in response to stress conditions such as hypoxia, viral infection, and mitochondrial stress are important areas for future studies.

It is generally accepted that the activation of eIF2a-dependent networks, such as the ISR, leads to a general decrease in global protein synthesis along with concomitant preferential translation of a select number of ISR transcripts, including that which encodes for ATF4 (**Pakos-Zubrucka et al., 2016**). This suggests that one of the mechanisms employed by cells that have been exposed to stress stimuli is to upregulate a subset of ATF4-dependent genes to acclimate and survive this stress condition. Using genetic loss-of-function and systems-wide approaches, we confirm that ATF4 is required for the induction of multiple p53 target genes in response to ISR activating stress conditions. Further analysis is required to determine the presence of ATF4-independent ISR target genes which may suggest putative combinatorial activity by TFs other than ATF4 that play a mediating role in the response to ISR-activating stress conditions, such as Activating Transcription Factor 6 (ATF6) or X-box binding protein 1 (XBP1). Mori and colleagues suggested that there is ATF6-dependent and -independent gene expression in response to ER stress, further supporting the premise that different combinations of TFs mediate gene expression in response to ISR-activating stimuli (**Okada et al., 2002**). Additionally, recent studies that link the DDR to the ER stress-induced unfolded protein response (UPR), suggest that both pathways are activated in response to hypoxic conditions (**Bolland et al., 2021**). Hypoxia induces expression of an RNA/DNA helicase known as Senataxin (SETX) via the PKR-like ER kinase (PERK)/ATF4 arm of the UPR (Ramachandran et al, 2021), however, ATF4 is not transcriptionally induced under hypoxic conditions. Induced expression of SETX is activated in an XBP1-dependent manner in response to ER stress (Chen et al., 2014). ATF6 and XBP1 both bind identical consensus motif sequences (Clauss et. al., 1996), therefore a comparison of differential gene expression in cells depleted of each of these

factors is necessary to elucidate the potential cooperation between these factors in regulating the expression of core ISR target genes.

Rather unexpectedly, our transcriptome analyses have revealed the presence of transcriptional differences in the genes commonly upregulated by ISR-activating stimuli in the presence or absence of p53. The identification of a set of genes commonly upregulated in response to ISR activation that is dependent upon p53 suggests that the regulation of these genes by these parallel pathways is much more complex than previously expected. While we have provided multiple pieces of evidence that the upregulation of these genes by the ISR does not directly involve p53, the possibility that p53 may indirectly affect the stress-induced rewiring of the transcriptome in response to ISR activation cannot be ruled out. Investigations into the independent and additive nature of the p53- and ATF4-mediated networks show that ATF4 can regulate expression of p53 direct p53 target genes in response to specific cellular stress conditions.

While our current results illustrate that while these stress-dependent TFs, p53 and ATF4, converge at activation of a common set of target genes, the molecular mechanisms employed by the cell to induce the expression of these targets are unique. The present study provides a detailed molecular basis for the activation of ATF3, a common target gene of the p53 and ISR pathways. Using both in-vitro and in-vivo approaches, we demonstrate that an upstream enhancer element is largely required for the induction of ATF3 in response to p53 activation and DNA damage, however it is not directly required for the induction of ATF3this gene in response to ISR-activating stress stimuli. We propose a model wherein ATF4 regulates expression of ATF3 via interaction with at least two ATF4 response elements, a CARE and CRE site, within the ATF3 promoter region in response to ISR-activating stress stimuli. We confirm previous reports in literature which suggest the majority of these CARE sites are functional ATF4-response elements regardless of which regulatory kinase is activated to phosphorylate eIF2a at

the core of the ISR, and both the CARE and CRE elements are required for maximal response to amino acid deprivation and overexpression of ATF4 (**Kilberg et al., 2009**). We add to this analysis by determining the requirement of ATF4 at these response elements by performing in-vitro reporter assays in HAP1 cells where ATF4 is not present. We conclude that ATF4 is required at these sites within the ATF3 promoter for full transcriptional activity.

Members of the bZIP superfamily can bind as homo- or heterodimers to these ATF4-responsive motifs, increasing the complexity of cis-regulatory activity by these stress-dependent TFs. Furthermore, Kilberg & Fu concluded that their results indicated induction of ATF3 in response to ISR activation involved combinatorial interactions of multiple basic leucine zipper (bZIP) factors, such as ATF2/cJUN, at this promoter region. Members of this large bZIP gene family, such as ATF4 and ATF6, play a mediating role in the ISR pathway (**Statzer et al., 2022 and Akay et al., 2012**). Future studies must be done to decipher the exact function of these proteins within the CARE and CRE sites at the ATF3 gene promoter. Our binding analysis indicates that ATF4 maintains a basal level of binding to the ATF3 gene promoter before and independently of activation by the ISR (Figure 3a). This observation is consistent with the current acceptance that the trans-acting capabilities of these stress-dependent transcription factors can be in part linked to post-transcriptional mechanisms of regulation, including phosphorylation events, histone modifications, and post-translational modifications (**Jiang et al., 2004**). Further research regarding the initial events that allow ATF4 to interact with the ATF3 gene promoter and the subsequent regulatory steps that are required to activate gene transcription as a stimulus-specific response. It has been reported that ATF4 demonstrates unique transcriptional regulation in response to prolonged or chronic ER stress when compared to its activity during acute exposure (**Guan et al., 2017**). A cell must be capable of responding to a wide variety of environmental insults and cellular perturbations, often in parallel. Therefore, the effects of concomitant treatments to induce multiple types of stress at once would be

prudent to determine any mutual antagonism, synergism, or co-regulation between these distinct stress networks.

Significance

Herein, we have demonstrated the ability of ATF4 to induce expression of canonical and direct p53 target genes in response to multiple forms of cell stress across different cell types. It has been previously reported that p53 and ATF4 can jointly induce direct p53 target genes, such as *CDKN1A/p21* and *GADD45a*, under specific cellular contexts such as during muscle immobilization resulting in atrophy (**Fox et al., 2014**). This work is significant in that it provides insight into restoring the p53 transcriptome in the absence of WT p53 functions. Nearly half of all human malignancies harbor mutations in p53 that facilitate and promote metastasis, tumorigenesis, and resistance to apoptosis (**Zhu et al, 2015 and Mantovani et al, 2019**). As such, long-term goals in the cancer research fields have been focused on developing strategies for treating cancers resulting from mutant p53 (mutp53). These cancer-promoting mutations in p53 not only impede its tumor-suppressive functions via the inability to transactivate canonical p53 target genes, but can also confer gain-of-function (GOF) properties that may contribute to tumorigenesis, metastasis, and ultimately, cancer therapy resistance (**Mantovani et al., 2019 and Zhu et al., 2015**). Current approaches aiming to restore wild-type p53 function often involve treatment with small molecules that bind to mutp53, induce its degradation, and/or disrupt protein-protein interactions between mutp53 and other TFs (**Bykov et al., 2018**). Strategies employing genome-wide restoration of the p53 pathway by small molecules via p53-independent mechanisms are considered promising therapeutics for cancers resulting from mutp53. Recent reports have demonstrated that in drug-treated cells induced ATF4 can lead to the activation of p53 transcriptional targets, including functionally important mediators such as death receptor 5 (DR5) and p53 upregulated mediator of apoptosis (PUMA) (**Tian et al., 2021**). These researchers conclude that a small molecule, PG3-Oc, partially restores p53 pathway-

signaling in tumor cells with mutant-p53, independently of p53. These researchers propose a model wherein PG3-Oc-induced upregulation of ATF4 is not through canonical ER stress, as treatment with PERK inhibitor, GSK2606414, showed no effect on the upregulation of ATF4 and downstream proapoptotic targets induced by PG3-Oc. Our current results support this conclusion, that ATF4 partially restores the expression of p53 target genes in the absence of p53, while expanding this ATF4-dependent mechanism to include the canonical ER stress response and further, the amino acid response (AAR), both of which are downstream of eIF2a at the core of the ISR.

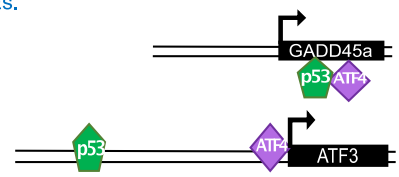
The functional analysis of various p53 mutations have revealed the need to investigate additional p53-regulated pathways that might compensate for tranactivational loss of pro-apoptotic targets. For example, p53 mutations within the TAD region result in the inability of p53 to robustly activate canonical targets including CDKN1A/p21, PUMA, and NOXA, yet these mutants retain the ability to suppress both spontaneous and oncogene-driven cancers suggesting that the robust transactivation of canonical p53 targets is dispensable for tumor suppression (**Boutelle & Attardi, 2021**). These studies do not rule out the tumor-suppressing potential of cell cycle arrest and apoptosis genes activated by other stimuli that activate and stabilize p53, such as DNA damage, oncogenic signaling, hypoxia, and nutrient deprivation. Our study emphasizes the redundancy in target gene activation by various forms of stress, some of which activate p53 (DNA DSB) and others which do not directly result in stabilization of p53 protein levels (ER stress and essential AA starvation).

Future directions

Future Directions

ChIP-Seq/ CUT&RUN Analysis

- Global trends in occupancy of p53 & ATF4 at CREs.
- Chromatin remodeling in response to stress stimuli to identify novel enhancer elements.
- Models to dissect the regulatory logic of putative stress-dependent CREs.



RNA-Seq Analysis

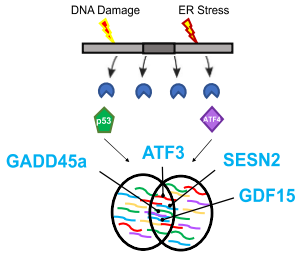
- Identify ATF4-dependent & ATF4-independent genes across cell types.
- Identify other stress-dependent TFs activated in response to these stress stimuli that may be regulating expression of these ISR target genes.
- Investigate the indirect dependence of ISR target genes upon p53.

HCT116 transcriptome analysis

- An extremely rich data set that calls for further analysis:
  - -Differences between ER stress and AA starvation response, two stimuli which both activate the ISR.
  - -Similarities between DDR and ER stress response, two stimuli which activate parallel stress-dependent networks.
  - -Down regulated targets warrant further investigation.

Further Characterization of the ISR

- Investigate the role of stress-dependent TFs that are activated in response to ISR stimulation and may be acting as effectors of the ISR to cooperate with ATF4 or compensate for the loss of ATF4 in its absence.
- Further characterization of the ISR will provide insight into the potential for small molecules that provide genome-wide restoration of the p53 transcriptome via p53-independent mechanisms.



Our transcriptome analysis of HCT116 cells has revealed a commonly upregulated set of genes that we have demonstrated are transcriptionally controlled by p53 and ATF4, two TF effectors of parallel stress-dependent networks: the p53 GRN and the ISR pathway. As p53 remains inimitable in its role as a tumor suppressor and in preventing cancer progression, we sought to identify a set of direct, p53-dependent, genes that can be induced in the absence of functional p53. Such a set of key stress-responsive genes may become attractive candidates for future anticancer therapies aimed at treating malignancies resulting from mutant p53. As such, we have limited our transcriptome analysis to bonafide p53 target genes by including Nutlin-3A in our comparison, a highly specific and non-genotoxic activator of p53. However, further

analysis of the commonly upregulated genes in response to strictly stress-activating treatments, such as etoposide (DNA DSB), tunicamycin (ER stress) and histidinol (AA starvation) would increase the number of potential therapeutic targets that may be clinically relevant for future studies. In addition to adding additional forms of clinically relevant stress conditions to study, such as hypoxia, oxidative stress, and ribonucleotide depletion, the current dataset in HCT116 we have procured is incredibly understudied at this point. As p53 acts solely as a transcriptional activator, and gene repression likely occurs via indirect p53 mechanisms, we focused our transcriptome analysis on upregulated target genes. Future investigations into the commonly downregulated gene targets in response to these stimuli, and the transcriptional differences resulting from different forms of stress that activate the same pathway, such as ER stress and AA starvation which both activate the ISR, will provide insight into the nuances of stress-induced rewiring and the influence of various cellular contexts on these gene targets.

We have highlighted one of these common target genes, *ATF3*, using this gene as a model to study enhancer promoter connections and cis-regulatory activity contributing to its expression in response to various forms of cellular stress. Our results support a model wherein the activity of multiple TFs at cis-regulatory regions influences p53-dependent enhancer-driven transcription. Further studies will be required to determine if combinations of p53 and other transcription factors, including ATF3 itself, may switch the function of p53 specific targets under certain cellular contexts. We have confirmed previous reports that ATF4 is required for the induction of *ATF3* in response to ISR-activating stress conditions, such as ER stress and AA starvation. Literature suggests that ATF3 is a metastatic factor that is induced by a variety of stress and inflammatory conditions and is overexpressed in many types of cancer cells (**Tanaka et al., 2011**). ATF3 has also been implicated in contributing to pleiotropic effects downstream of ATF4 (**Ameri & Harris, 2008**). Additionally, ATF3 can lead to either stabilization of p53 or inhibition of p53 functions, depending upon certain cellular contexts. For example, stress-

induced ATF3 binds to 40% of p53 targets to facilitate activation of pro-apoptotic genes such as DR5 and PUMA while cancer-associated ATF3 has been shown to repress these apoptotic targets (**Tanaka et al., 2011**). Whether an ATF4-ATF3-p53 cascade could be exploited in cancer therapy should be considered for future investigations. Further analysis of the role of ATF3 in regulating the common targets identified in our transcriptomic analysis may reveal an extensive network of stress-inducible transcription factors that have cell context-dependent effects on p53 target genes during the cellular response to stress and cancer development.

Finally, we have procured both binding profiles for ATF4 and p53 as well as global transcriptome data in response to both p53 and ATF4-activating stress conditions. Further analysis of the chromatin landscape resulting from the activation of these parallel pathways in response to exposure to these stresses may provide insight into novel enhancer elements and regulatory regions that may contribute to this regulatory paradigm. Additionally, further analysis of the transcriptional rewiring in response to DNA damage and ER stress across multiple cell types may elucidate cell-type and lineage-specific regulatory regions that may be important targets for future studies.

Citations

Adachi, Y., Yamamoto, K., Okada, T., Yoshida, H., Harada, A., Mori, K. (2008). ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. *Cell Struct Funct*. 33: 75–89.

Advani, VM., & Ivanov, P. (2019). Translational Control under Stress: Reshaping the Translatome. *BioEssays : news and reviews in molecular, cellular and developmental biology*, *41*(5), e1900009. https://doi.org/10.1002/bies.201900009.

Aguilar-Recarte, D., Barroso, E., Gumà, A., Pizarro-Delgado, J., Peña, L., Ruart, M., Palomer, X., Wahli, W., & Vázquez-Carrera, M. (2021). GDF15 mediates the metabolic effects of PPARβ/δ by activating AMPK. Cell Reports. 36(6): 109501. https://doi.org/10.1016/j.celrep.2021.109501.

Aitken, CE. & Lorsch, JR. (2012). A mechanistic overview of translation initiation in eukaryotes. *Nat Struct Mol Biol*. 19: 568–576.

Akay, C., Lindl, KA., Shyam, N., Nabet, B., Goenaga-Vazquez, Y., Ruzbarsky, J., Wang, Y., Kolson, DL., & Jordan-Sciutto, KL. (2012). Activation status of integrated stress response pathways in neurones and astrocytes of HIV-associated neurocognitive disorders (HAND) cortex. *Neuropathology and applied neurobiology*, *38*(2), 175–200. https://doi.org/10.1111/j.1365-2990.2011.01215.x

Amundson, SA., Bittner, M., Chen, Y., Trent, J., Meltzer, P., & Fornace, AJ., Jr (1999). Fluorescent cDNA microarray hybridization reveals complexity and heterogeneity of cellular genotoxic stress responses. *Oncogene*, *18*(24), 3666–3672. https://doi.org/10.1038/sj.onc.1202676

Anderson, ME., Woelker, B., Reed, M., Wang, P., & Tegtmeyer, P. (1997). Reciprocal interference between the sequence-specific core and nonspecific C-terminal DNA binding domains of p53: implications for regulation. *Mol. Cell. Biol.* 17: 6255-6264.

Andrysik, Z., Galbraith, MD., Guarnieri, AL., Zaccara, S., Sullivan, KD., Pandey, A., MacBeth, M., Inga, A., & Espinosa, JM. (2017). Identification of a core TP53 transcriptional program with highly distributed tumor suppressive activity. *Genome research*. 27(10): 1645–1657. https://doi.org/10.1101/gr.220533.117.

Argüello-Miranda, O., Marchand, AJ., Kennedy, T., Russo, MAX., Noh, J. (2021). Cell cycle–independent integration of stress signals by Xbp1 promotes Non-G1/G0 quiescence entry. *J Cell Biol*. 221(1): e202103171. doi: 10.1083/jcb.202103171.

Arriza, JL., Kavanaugh, MP., Fairman, WA., Wu, YN., Murdoch, GH., North, RA... (1993) Cloning and expression of a human neutral amino acid transporter with structural similarity to the glutamate transporter gene family. *J. Biol. Chem.* 268, 15329–15332.

Ashcroft, M., Kubbutat, MH., & Vousden, KH. (1999). Regulation of p53 function and stability by phosphorylation. *Molecular and cellular biology.* 19(3): 1751–1758. https://doi.org/10.1128/MCB.19.3.1751.

Ayed, A., Mulder, FA., Yi, GS., Lu, Y., Kay, LE., & Arrowsmith, CH. (2001). Latent and active p53 are identical in conformation. *Nat. Struct. Biol.* 8: 756-760.

Aylon, Y. & Oren, M. (2007). Living with p53, dying of p53. *Cell*. 130(4): 597–600. https://doi.org/10.1016/j.cell.2007.08.005.

Babu, M. M., Luscombe, N. M., Aravind, L., Gerstein, M., & Teichmann, S. A. (2004). Structure and evolution of transcriptional regulatory networks. *Current opinion in structural biology*, *14*(3), 283–291. https://doi.org/10.1016/j.sbi.2004.05.004.

Baker, SJ., Fearon, ER., & Nigro, JM. (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science. 244: 217–221.

Balachandran, S., Roberts, PC., Brown, LE., Truong, H., Pattnaik, AK., Archer, DR., & Barber, GN. (2000). Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity*. 13: 129–141.

Balagurumoorthy, P., Sakamoto, H., Lewis, MS., Zambrano, N., Clore, GM., Gronenborn, AM., Appella, E. & Harrington, RE. (1995). Four p53 DNA-binding domain peptides bind natural p53-response elements and bend the DNA. *Proc. Natl. Acad. Sci. USA*. 92: 8591-8595.

Balasubramanian, MN., Butterworth, EA. & Kilberg, MS. (2013) Asparagine synthetase: regulation by cell stress and involvement in tumor biology. *Am. J. Physiol. Endocrinol. Metab.* 304, E789–E799 doi: 10.1152/ajpendo.00015.2013.

Banerji, J., Rusconi, S., & Schaffner, W. (1981). Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences. *Cell*. 27: 299–308.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, CW., Chessa, L., Smorodinsky, NI., Prives, C., Reiss, Y., Shiloh, Y., & Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science (New York, N.Y.)*. 281(5383): 1674–1677. https://doi.org/10.1126/science.281.5383.1674.

Bargonetti, J., Manfredi, JJ., Chen, X., Marshak, DR., & Prives, C. (1993). A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein. *Genes Dev*. 7: 2565-2574.

Barrick, JE., Yu, DS., Yoon, SH., Jeong, H., Oh, TK., Schneider, D., Lenski, RE., & Kim, JF. (2009). Genome evolution and adaptation in a long-term experiment with Escherichia coli. *Nature*, *461*(7268), 1243–1247. https://doi.org/10.1038/nature08480

B'Chir, W., Maurin, A. C., Carraro, V., Averous, J., Jousse, C., Muranishi, Y... (2013). The eIF2alpha/ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic Acids Res*. 41, 7683–7699. 10.1093/nar/gkt563.

Beckman, KB. & Ames, BN. (1999) Endogenous oxidative damage of mtDNA. *Mutation Research*. 424: 51-58. doi:10.1016/S0027-5107(99)00007-X

Beer-Romero, P., Glass, S., & Rolfe, M. (1997). Antisense targeting of E6AP elevates p53 in HPV-infected cells but not in normal cells. *Oncogene*. 14(5): 595–602. https://doi.org/10.1038/sj.onc.1200872.

Belyi, VA., Ak, P., Markert, E., Wang, H., Hu, W., Puzio-Kuter, A., & Levine, AJ. (2010). The origins and evolution of the p53 family of genes. *Cold Spring Harbor perspectives in biology*. 2(6), a001198. https://doi.org/10.1101/cshperspect.a001198

Berlanga, JJ., Santoyo, J., & de Haro, C. (1999). Characterization of a mammalian homolog of the GCN2 eukaryotic initiation factor 2α kinase. *Eur. J. Biochem.* 265: 754–62.

Birben, E., Sahiner, UM., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. **World Allergy Organ J**. 1 :9-19. Doi: 10.1097/WOX.0b013e3182439613. PMID: 23268465. PMCID: PMC3488923.

Blais, JD., Filipenko, V., Bi, M., Harding, HP., Ron, D., Koumenis, C., Wouters, BG., & Bell, JC. (2004). Activating transcription factor 4 is translationally regulated by hypoxic stress. *Molecular and cellular biology.* 24(17): 7469–7482. https://doi.org/10.1128/MCB.24.17.7469-7482.2004.

Bolland, H., Ma, T. S., Ramlee, S., Ramadan, K., & Hammond, E. M. (2021). Links between the unfolded protein response and the DNA damage response in hypoxia: a systematic review. *Biochemical Society transactions*, *49*(3), 1251–1263. https://doi.org/10.1042/BST20200861

Bourdon, JC., Deguin-Chambon, V., Lelong, JC., Dessen, P., May, P., Debuire, B., & May, E. (1997). Further characterisation of the p53 responsive element--identification of new candidate genes for trans-activation by p53. *Oncogene*. 14(1): 85–94. https://doi.org/10.1038/sj.onc.1200804.

Bourdon, JC., Fernandes, K., Murray-Zmijewski, F., Liu, G., Diot, A., Xirodimas, DP., Saville, MK. & Lane, DP. (2005). p53 isoforms can regulate p53 transcriptional activity. *Genes Dev.* 19: 2122–2137.

Bourdon, JC. (2007). p53 Family isoforms. *Current pharmaceutical biotechnology*. 8(6): 332–336. https://doi.org/10.2174/138920107783018444.

Boutelle, AM. & Attardi, LD. (2021). p53 and Tumor Suppression: It Takes a Network. *Trends in cell biology*. 31(4), 298–310. https://doi.org/10.1016/j.tcb.2020.12.011.

Boyd, SD., Tsai, KY., & Jacks, T. (2000). An intact HDM2 RING-finger domain is required for nuclear exclusion of p53. *Nature Cell Biol.* 2: 563–568.

Brady, CA. & Attardi, LD. (2010). p53 at a glance. *Journal of cell science*. 123(15): 2527–2532. https://doi.org/10.1242/jcs.064501.

Brady, CA., Jiang, D., Mello, SS., Johnson, TM., Jarvis, LA., Kozak, MM., Kenzelmann Broz, D., Basak, S., Park, EJ., McLaughlin, ME., Karnezis, AN., & Attardi, LD. (2011). Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. *Cell*. 145(4): 571–583. https://doi.org/10.1016/j.cell.2011.03.035.

Bröer, A., Rahimi, F. & Bröer, S. (2016) Deletion of amino acid transporter ASCT2 (SLC1A5) reveals an essential role for transporters SNAT1 (SLC38A1) and SNAT2 (SLC38A2) to sustain glutaminolysis in cancer cells. *J. Biol. Chem.* 291, 13194–13205 doi: 10.1074/jbc.M115.700534.

Bröer, S., & Bröer, A. (2017). Amino acid homeostasis and signalling in mammalian cells and organisms. *The Biochemical journal*, *474*(12), 1935–1963. https://doi.org/10.1042/BCJ20160822.

Bröer, S. & Palacin, M. (2011) The role of amino acid transporters in inherited and acquired diseases. *Biochem. J.* 436, 193–211 doi: 10.1042/BJ20101912.

Burnett, PE., Barrow, RK., Cohen, NA., Snyder, SH., & Sabatini, DM. (1998). RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc. Natl. Acad. Sci.* 95: 1432-1437.

Brosh, R. & Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. *Nat. Rev. Cancer.* 9: 701-713.

Bulger, M. & Groudine, M. (2011). Functional and mechanistic diversity of distal transcription enhancers. *Cell*. 144: 327–339.

Budanov, AV., & Karin, M. (2008). p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. Cell, 134(3), 451–460. https://doi.org/10.1016/j.cell.2008.06.028.

Bulavin, DV., Saito, S., Hollander, MC., Sakaguchi, K., Anderson, CW., Appella, E., & Fornace, AJ., Jr. (1999). Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *The EMBO journal*, *18*(23), 6845–6854. https://doi.org/10.1093/emboj/18.23.6845

Burden, DA., & Osheroff, N. (1998). Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. *Biochimica et biophysica acta*, *1400*(1-3), 139–154. https://doi.org/10.1016/s0167-4781(98)00132-8

Bykov, VJN., Eriksson, SE., Bianchi, J., & Wiman, KG. (2018). Targeting mutant p53 for efficient cancer therapy. *Nat Rev Cancer*. 18: 89-102.

Caldecott, KW. (2008). Single-strand break repair and genetic disease. *Nat Rev Genet.* 9:619–631.

Castilho, BA., Shanmugam, R., Silva, RC., Ramesh, R., Himme, BM., Sattlegger, E. (2014) Keeping the eIF2 alpha kinase Gcn2 in check. *Biochim Biophys Acta.* 1843: 1948–1968.

Catizone, AN., Uzunbas, GK., Celadova, P., Kuang, S., Bose, D., & Sammons, MA. (2020). Locally acting transcription factors regulate p53-dependent cis-regulatory element activity. Nucleic acids research, 48(8): 4195–4213. https://doi.org/10.1093/nar/gkaa147.

Chang, J., Kim, DH., Lee, SW., Choi, KY. &Sung, YC. (1995). Transactivation ability of p53 transcriptional activation domain is directly related to the binding affinity to TATA-binding protein. *J Biol Chem.* 270: 25014–25019.

Chen, BP., Wolfgang, CD., & Hai, T. (1996). Analysis of ATF3, a transcription factor induced by physiological stresses and modulated by gadd153/Chop10. Molecular and cellular biology, 16(3), 1157–1168. https://doi.org/10.1128/MCB.16.3.1157.

Chen, JJ. (2014). Translational control by heme-regulated eIF2α kinase during erythropoiesis. *Current opinion in hematology*. 21(3): 172–178. https://doi.org/10.1097/MOH.0000000000000030.

Chen, X., Shen, J., & Prywes, R. (2002). The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. *The Journal of biological chemistry*, *277*(15), 13045–13052. https://doi.org/10.1074/jbc.M110636200.

Cheng, R., Ford, BL., O'Neal, PE., Mathews, CZ., Bradford, CS., Thongtan, T., Barnes, DW., Hendricks, JD., & Bailey, GS. (1997). Zebrafish (Danio rerio) p53 tumor suppressor gene: cDNA sequence and expression during embryogenesis. *Mol Mar Biol Biotechnol.* 6: 88–97.

Cho, Y., Gorina, S., Jeffrey, PD., & Pavletich, NP. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science*. 265: 346-355.

Chow, VT., Quek, HH. & Tock, EP. (1993). Alternative splicing of the p53 tumor suppressor gene in the Molt-4 T-lymphoblastic leukemia cell line. *Cancer letters*. 73(2-3): 141–148. https://doi.org/10.1016/0304-3835(93)90256-9.

Ciccia, A., & Elledge, SJ. (2010). The DNA damage response: making it safe to play with knives. *Molecular cell*, *40*(2), 179–204. https://doi.org/10.1016/j.molcel.2010.09.019.

Clemens, MJ. & Elia, A. (1997). The double-stranded RNA-dependent protein kinase PKR: structure and function. *J Interferon Cytokine Res.* 17: 503–524.

Concannon, CG., Orrenius, S., & Samali, A. (2001). Hsp27 inhibits cytochrome c-mediated caspase activation by sequestering both pro-caspase-3 and cytochrome c. *Gene Expression*, vol. 9, no. 4-5, pp. 195–201.

Costa-Mattioli, M., Gobert, D., Harding, H., Herdy, B., Azzi, M., Bruno, M., Bidinosti, M., Ben Mamou, C., Marcinkiewicz, E., Yoshida, M., Imataka, H., Cuello, A. C., Seidah, N., Sossin, W., Lacaille, JC., Ron, D., Nader, K., & Sonenberg, N. (2005). Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2. *Nature*. 436(7054): 1166–1173. https://doi.org/10.1038/nature03897.

Costa-Mattioli, M., & Walter, P. (2020). The integrated stress response: From mechanism to disease. *Science (New York, N.Y.)*. 368(6489): eaat5314. https://doi.org/10.1126/science.aat5314

Castro-Mondragon, JA., Riudavets-Puig, R., Rauluseviciute, I., Berhanu Lemma, R., Turchi L., Blanc-Mathieu, R., Lucas, J., Boddie, P., Khan, A., Manosalva Pérez, N., Fornes, O., Leung, TY., Aguirre, A., Hammal, F., Schmelter, D., Baranasic, D., Ballester, B., Sandelin, A., Lenhard, B., Vandepoele, K., Wasserman, WW., Parcy, F., & Mathelier, A. (2022). JASPAR 2022: the 9th release of the open-access database of transcription factor binding profiles *Nucleic Acids Res.* 50(D1):D165-D173.; doi: 10.1093/nar/gkab1113.

Chavel, CA., Dionne, HM., Birkaya, B., Joshi, J. & Cullen, PJ. (2010). Multiple signals converge on a differentiation MAPK pathway. Plos Genetics. 6(3): e1000883. https://doi.org/10.1371/journal.pgen.1000883

Chen, BP., Liang, G., Whelan, J., & Hai, T. (1994). ATF3 and ATF3 delta Zip. Transcriptional repression versus activation by alternatively spliced isoforms. *The Journal of biological chemistry*, *269*(22), 15819–15826.

Chen, BP., Wolfgang, CD., & Hai, T. (1996). Analysis of ATF3, a transcription factor induced by physiological stresses and modulated by gadd153/Chop10. *Molecular and cellular biology*, *16*(3), 1157–1168. https://doi.org/10.1128/MCB.16.3.1157.

Chen, EY., Tan, CM., Kou, Y., Duan, Q., Wang, Z., Meirelles, GV., Clark, NR., & Ma'ayan, A. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC bioinformatics*. 14: 128. https://doi.org/10.1186/1471-2105-14-128

Chen, H., Pan, YX., Dudenhausen, EE. & Kilberg, MS. (2004). Amino acid deprivation induces the transcription rate of the human asparagine synthetase gene through a timed program of expression and promoter binding of nutrient-responsive basic region/leucine zipper transcription factors as well as localized histone acetylation. *The Journal of biological chemistry.* 279(49): 50829–50839. https://doi.org/10.1074/jbc.M409173200.

Chen, X., Iliopoulos, D., Zhang, Q., Tang, Q., Greenblatt, MB., Hatziapostolou, M., Lim, E., Tam, WL., Ni, M., Chen, Y., Mai, J., Shen, H., Hu, DZ., Adoro, S., Hu, B., Song, M., Tan, C., Landis, MD., Ferrari, M., Shin, SJ., … Glimcher, LH. (2014). XBP1 promotes triple-negative breast cancer by controlling the HIF1α pathway. *Nature*, *508*(7494), 103–107. https://doi.org/10.1038/nature13119.

Chowdhury, P., Lin, GE., Liu, K., Song, Y., Lin, FT., & Lin, WC. (2014). Targeting TopBP1 at a convergent point of multiple oncogenic pathways for cancer therapy. *Nat Commun.* 5: 5476.

Coronel, L., Häckes, D., Schwab, K., Riege, K., Hoffmann, S., & Fischer, M. (2022). p53-mediated AKT and mTOR inhibition requires RFX7 and DDIT4 and depends on nutrient abundance. *Oncogene*. 41:1063–1069. https://doi.org/10.1038/s41388-021-02147-z.

Craig, EA. (1985). The heat shock response. *CRC Critical Reviews in Biochemistry*, vol. 18, no. 3, pp. 239–280.

de Nadal, E., Ammerer, G. & Posas, F. (2011). Controlling gene expression in response to stress. *Nat. Rev. Genet.* 12, 833–845 . https://doi.org/10.1038/nrg3055.

Degterev, A., Boyce, M., & Yuan, J. (2003). A decade of caspases. *Oncogene.* 22:8543–8567.

Dennis, MD., McGhee, NK., Jefferson, LS., & Kimball, SR. (2013). Regulated in DNA damage and development 1 (REDD1) promotes cell survival during serum deprivation by sustaining repression of signaling through the mechanistic target of rapamycin in complex 1 (mTORC1). *Cellular signalling.* 25(12): 2709–2716. https://doi.org/10.1016/j.cellsig.2013.08.038

Deng, J., Lu, PD., Zhang, Y., Scheuner, D., Kaufman, R. J., Sonenberg, N., Harding, HP., & Ron, D. (2004). Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2. *Molecular and cellular biology*. 24(23): 10161–10168. https://doi.org/10.1128/MCB.24.23.10161.

Deter, RL. & de Duve, C. (1967). Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes. *The Journal of cell biology*. 33(2): 437–449. https://doi.org/10.1083/jcb.33.2.437.

Dey, S., Baird, TD., Zhou, D., Palam, LR., Spandau, DF., & Wek, RC. (2010). Both transcriptional regulation and translational control of ATF4 are central to the integrated stress response. *The Journal of biological chemistry*. 285(43): 33165–33174. https://doi.org/10.1074/jbc.M110.167213.

DeYoung, MP., Horak, P., Sofer, A., Sgroi, D., & Ellisen, LW. (2008). Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes Dev.* 22(2): 239–251. doi: 10.1101/gad.1617608.

Dolgin, E. (2017). The most popular genes in the human genome. *Nature*. 551(7681): 427–431. https://doi.org/10.1038/d41586-017-07291-9.

Donehower, LA., Harvey, M., Slagle, BL., McArthur, MJ., Montgomery, CA. Jr., Butel, JS. & Bradley A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature.* 356(6366): 215-21.

Donnelly, N., Gorman, AM., Gupta, S., & Samali, A. (2013). The eIF2alpha kinases: Their structures and functions. *Cellular and Molecular Life Sciences*, 70(19), 3493–3511.

Dubois-Chevalier, J., Mazrooei, P., Lupien, M., Staels, B., Lefebvre, P., & Eeckhoute, J. (2018). Organizing combinatorial transcription factor recruitment at cis-regulatory modules. *Transcription*, *9*(4), 233–239. https://doi.org/10.1080/21541264.2017.1394424.

Earl, DJ., & Deem, MW. (2004). Evolvability is a selectable trait. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(32), 11531–11536. https://doi.org/10.1073/pnas.0404656101.

El-Deiry, WS., Kern, SE., Pietenpol, JA., Kinzler, KW. & Vogelstein, B. (1992). Definition of a consensus binding site for p53. *Nat Genet.* 1: 45–49.

El-Deiry, WS. (1998). Regulation of p53 downstream genes. *Semin. Cancer Biol.* 8: 345–357.

Elledge, SJ. (1996). Cell cycle checkpoints: preventing an identity crisis. *Science*, 274: 1664-1672.

Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O., & Oren, M. (1989). Wild-type p53 can inhibit oncogene-mediated focus formation. *Proc Natl Acad Sci USA.* 86: 8763–8767.

Ellisen, LW., Ramsayer, KD., Johannessen, CM., Yang, A., Beppu, H., Minda, K., Oliner, J. D., McKeon, F. & Haber, DA. (2002). REDD1, a developmentally regulated transcriptional target of p63 and p53, links p63 to regulation of reactive oxygen species. *Molecular cell*. 10(5): 995–1005. https://doi.org/10.1016/s1097-2765(02)00706-2.

Engeland, K. (2018). Cell cycle arrest through indirect transcriptional repression by p53: I have a DREAM. *Cell death and differentiation*. 25(1): 114–132. https://doi.org/10.1038/cdd.2017.172

Espinosa, JM., & Emerson, BM. (2001). Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. *Molecular cell.* 8(1): 57–69. https://doi.org/10.1016/s1097-2765(01)00283-0.

Fan, F., Jin, S., Amundson, SA., Tong, T., Fan, W., Zhao, H., Zhu, X., Mazzacurati, L., Li, X., Petrik, KL., Fornace, AJ., Jr, Rajasekaran, B. & Zhan, Q. (2002). ATF3 induction following DNA damage is regulated by distinct signaling pathways and over-expression of ATF3 protein suppresses cells growth. *Oncogene*. 21(49): 7488–7496. https://doi.org/10.1038/sj.onc.1205896.

Farhan, M., Wang, H., Gaur, U., Little, PJ., Xu, J. & Zheng, W. (2017). FOXO Signaling Pathways as Therapeutic Targets in Cancer. *International journal of biological sciences*. 13(7): 815–827. https://doi.org/10.7150/ijbs.20052.

Farook, JM., Shields, J., Tawfik, A., Markand, S., Sen, T., Smith, SB., Brann, D., Dhandapani, KM., & Sen, N. (2013). GADD34 induces cell death through inactivation of Akt following traumatic brain injury. *Cell Death Dis.* 4: e754.

Fawcett TW., Martindale JL., Guyton KZ., Hai T, Holbrook NJ. (1999). Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response. *Biochem J*. 339: 135–141.

Filomeni, G., De Zio, D., & Cecconi, F. (2015). Oxidative stress and autophagy: the clash between damage and metabolic needs. *Cell death and differentiation*. 22(3): 377–388. https://doi.org/10.1038/cdd.2014.150.

Fingar, DC. & Blenis, J. (2004). Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene.* 19: 3151–71.

Fink, SL. & Cookson, BT. (2005). Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infection and immunity*. 73(4): 1907–1916. https://doi.org/10.1128/IAI.73.4.1907-1916.2005.

Finlay, CA., Hinds, PW., & Levine, AJ. (1989). The p53 proto-oncogene can act as a suppressor of transformation. *Cell*. 57: 1083–1093.

Flaman, JM., Waridel, F., Estreicher, A., Vannier, A., Limacher, JM., Gilbert, D., Iggo, R. & Frebourg, T. (1996). The human tumour suppressor gene p53 is alternatively spliced in normal cells. *Oncogene*. 12(4): 813–818.

Flores, ER., Sengupta, S., Miller, JB., Newman, JJ., Bronson, R., Crowley, D., Yang, A., McKeon, F. & Jacks, T. (2005). Tumor predisposition in mice mutant for p63 and p73: Evidence for broader tumor suppressor functions for the p53 family. *Cancer Cell.* 7: 363–373.

Fu, L., & Kilberg, MS. (2013). Elevated cJUN expression and an ATF/CRE site within the ATF3 promoter contribute to activation of ATF3 transcription by the amino acid response. *Physiological genomics*. *45*(4): 127–137. https://doi.org/10.1152/physiolgenomics.00160.2012.

Fuchs, SY., Adler, V., Bushmann, T., Yin, Z., Wu, X., Jones, SN., & Ronai, Z. (1998a). JNK targets p53 ubiquitination and degradation in non-stressed cells. *Genes Develop.* 12: 2658-2663.

Fuchs, SY., Adler, V., Pincus, MR., & Ronai Z. (1998b). MEKK1/JNK signaling stabilizes and activates p53. *Proc.Natl. Acad. Sci. USA*. 95: 10541-10546.

Fulda, S., Gorman, AM., Hori, O., & Samali, A. (2010). Cellular stress responses: cell survival and cell death. *International journal of cell biology*. 214074. https://doi.org/10.1155/2010/214074.

Galehdar, Z., Swan, P., Fuerth, B., Callaghan, SM., Park, DS., & Cregan, SP. (2010). Neuronal apoptosis induced by endoplasmic reticulum stress is regulated by ATF4-CHOP-mediated induction of the Bcl-2 homology 3-only member PUMA. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 30(50): 16938–16948. https://doi.org/10.1523/JNEUROSCI.1598-10.2010.

Galluzzi, L., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Agostinis, P., Alnemri, E. S., Altucci, L., Amelio, I., Andrews, D. W., Annicchiarico-Petruzzelli, M., Antonov, A. V., Arama, E., Baehrecke, E. H., Barlev, N. A., Bazan, N. G., Bernassola, F., Bertrand, M., Bianchi, K., Blagosklonny, M. V., … Kroemer, G. (2018). Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell death and differentiation*. 25(3): 486–541. https://doi.org/10.1038/s41418-017-0012-4.

Gambardella, G., Staiano, L., Moretti, MN., de Cegli, R., Fagnocchi L., di Tullio, G., Polletti, S., Braccia, C., Armirotti, A., Zippo, A., Ballabio, A., de Matteis, MA., & di Bernardo, D. (2020). GADD34 is a modulator of autophagy during starvation. Science Advances. 6(39): eabb0205. DOI: 10.1126/sciadv.abb02.

Garaeva, AA., Kovaleva, IE., Chumakov, PM., & Evstafieva, AG. (2016). Mitochondrial dysfunction induces SESN2 gene expression through Activating Transcription Factor 4. *Cell cycle (Georgetown, Tex.)*, *15*(1): 64–71. https://doi.org/10.1080/15384101.2015.1120929.

Gardner, BM. & Walter, P. (2011). Unfolded proteins are Ire1-activating ligands that directly induce the unfolded protein response. *Science (New York, N.Y.)*. 333(6051): 1891–1894. https://doi.org/10.1126/science.1209126.

Geyer, RK., Yu, ZK., & Maki, CG. (2000). The MDM2 RING-finger domain is required to promote p53 nuclear export. *Nature Cell Biol*. 2: 569–573.

Giaccia, AJ. & Kastan, MB. (1998). The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev.* 12: 2973–2983.

Gilbert, LA., Larson, MH., Morsut, L., Liu, Z., Brar, GA., Torres, SE., Stern-Ginossar, N., Brandman, O., Whitehead, EH., Doudna, JA., Lim, WA., Weissman, JS., & Qi, LS. (2013). CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell.* 154(2): 442–451. https://doi.org/10.1016/j.cell.2013.06.044.

Gilbert, LA., Horlbeck, MA., Adamson, B., Villalta, JE., Chen, Y., Whitehead, EH., Guimaraes, C., Panning, B., Ploegh, HL., Bassik, MCl. (2014). Genome-scale CRISPR-mediated control of gene repression and activation. Cell. ;159:647–661

Graeber, TG., Peterson, JF., Tsai, M., Monica, K., Fornace, AJJ., Giaccia, AJ. (1994). Hypoxia induces accumulation of p53 protein, but activation of a G1- phase checkpoint by low-oxygen conditions is independent of p53 status. *Mol. Cell Biol.* 14: 6264–6277.

Gross, A., Yin, XM., Wang, K., Wei, MC., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P., & Korsmeyer, SJ. (1999). Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J. Biol. Chem.* 274, 1156–1163.

Guan, BJ., Krokowski, D., Majumder, M., Schmotzer, CL., Kimball, SR., Merrick, WC., Koromilas, AE., & Hatzoglou, M. (2014). Translational control during endoplasmic reticulum stress beyond phosphorylation of the translation initiation factor eIF2α. *The Journal of biological chemistry*. 289(18): 12593–12611. https://doi.org/10.1074/jbc.M113.543215.

Guo, X., Aviles, G., Liu, Y., Tian, R., Unger, BA., Lin, YT., Wiita, AP., Xu, K., Correia, MA., Kampmann, M. (2019). Mitochondrial dysfunction is signaled to the integrated stress response by OMAL DELEI and HRI. *bioRxiv*. doi: 10.1101/715896.

Hadian, K. & Stockwell, BR. (2020). SnapShot: Ferroptosis. *Cell*. 181(5): 1188–1188.e1. https://doi.org/10.1016/j.cell.2020.04.039.

Hafner, A., Lahav, G., & Stewart-Ornstein, J. (2017). Stereotyped p53 binding tuned by chromatin accessibility. *bioRxiv*. 10.1101/177667

Hager, KM. & Gu, W. (2014). Understanding the non-canonical pathways involved in p53-mediated tumor suppression. Carcinogenesis, 35(4), 740–746. https://doi.org/10.1093/carcin/bgt487.

Hai, T., Liu, F., Coukos, W., & Green, M. (1989). Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.* 3: 2083-2090.

Hai, T., Wolfgang, CD., Marsee, DK., Allen, AE. & Sivaprasad, U. (1999). ATF3 and stress responses. *Gene Expr.* 7: 321-335.

Hai, T., and Hartman, MG. (2001). The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene.* 273: 1-1.

Hai T. (2006). The ATF transcription factors in cellular adaptive responses. *Gene expression and regulation. Higher Education Press; Beijing*. 329–340.

Hai, T., Wolford, CC. & Chang, YS. (2010). ATF3, a hub of the cellular adaptive-response network, in the pathogenesis of diseases: is modulation of inflammation a unifying component? *Gene Expr.* 15: 1–11.

Han, AP., Yu, C., Lu, L., Fujiwara, Y., Browne, C., Chin, G., Fleming, M., Leboulch, P., Orkin, SH., Chen, JJ. (2001) Heme-regulated eIF2alpha kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. EMBO J 20: 6909–6918

Han, J., Back, SH., Hur, J., Lin, YH., Gildersleeve, R., Shan, J., Yuan, CL., Krokowski, D., Wang, S., Hatzoglou, M., Kilberg, MS., Sartor, MA., & Kaufman, RJ. (2013). ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nature cell biology*, *15*(5): 481–490. https://doi.org/10.1038/ncb2738.

Harding, HP., Novoa, I., Zhang, Y., Zeng, H., Wek, RC., Schapira, M, & Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell.* 6: 1099–1108.

Harding, HP., Calfon, M., Urano, F., Novoa, I. & Ron D. (2002). Transcriptional and translational control in the Mammalian unfolded protein response. *Annu Rev Cell Dev Biol*. 18: 575–99.

Harding, HP., Zhang, Y., Zeng, H., Novoa, I., Lu, PD., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, DF., Bell, JC., Hettmann, T., Leiden, JM., Ron D. (2003). An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol. Cell.* 11: 619–33.

Harding, HP., Zhang, Y., Scheuner, D., Chen, JJ., Kaufman, RJ., & Ron, D. (2009). Ppp1r15 gene knockout reveals an essential role for translation initiation factor 2 alpha (eIF2alpha) dephosphorylation in mammalian development. *Proc Natl Acad Sci USA.* 106: 1832–1837.

Harper, JW., & Elledge, SJ. (2007). The DNA damage response: ten years after. *Molecular cell.* 28(5): 739–745. https://doi.org/10.1016/j.molcel.2007.11.015.

Hart, LS., Cunningham, JT., Datta, T., Dey, S., Tameire, F., Lehman, SL., Qiu, B., Zhang, H., Cerniglia, G., Bi, M., Li, Y., Gao, Y., Liu, H., Li, C., Maity, A., Thomas-Tikhonenko, A., Perl, A. E., Koong, A., Fuchs, SY., Diehl, JA., Mills, IG., Ruggero, D., & Koumenis, C. (2012). ER stress-mediated autophagy promotes Myc-dependent transformation and tumor growth. *The Journal of clinical investigation.* 122(12): 4621–4634. https://doi.org/10.1172/JCI62973.

Hashimoto, Y., Zhang, C., Kawauchi, J., Imoto, I., Adachi, MT. & Inazawa, J. (2002). An alternatively spliced isoform of transcriptional repressor ATF3 and its induction by stress stimuli. *Nucleic Acids Res.* 30: 2398–406.

Haupt, Y., Maya, R., Kazaz, A., & Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. *Nature*. 387: 296–299.

Helton, ES., Zhu, J. & Chen, X. (2006). The unique NH2-terminally deleted (DeltaN) residues, the PXXP motif, and the PPXY motif are required for the transcriptional activity of the DeltaN variant of p63. *The Journal of biological chemistry*. 281(5): 2533–2542. https://doi.org/10.1074/jbc.M507964200.

Hightower, LE. & Hendershot, LM. (1997). Molecular chaperones and the Heat Shock Response at Cold Springs Harbor. *Cell Stress & Chaperones,* vol. 2, no.1, pp. 1-11.

Hamidi, T., Cano, CE., Grasso, D., Garcia, MN., Sandi, MJ., Calvo, EL., Dagorn, JC., Lomberk, G., Urrutia, R., Goruppi, S., Carracedo, A., Velasco, G., Iovanna, JL. (2012). Nupr1-aurora kinase A pathway provides protection against metabolic stress-mediated autophagic-associated cell death. *Clin Cancer Res.* 18: 5234–5246.

Hinnebusch, AG. (2005). Translational regulation of GCN4 and the general amino acid control of yeast. *Annu. Rev. Microbiol.* 59:407–450. 10.1146/annurev.micro.59.031805.133833

Hirai, K., Martinkova, M., Igarashi, J., Saiful, I., Yamauchi, S., El-Mashtoly, S., Kitagawa, T., Shimizu, T. (2007). Identification of Cys385 in the isolated kinase insertion domain of heme-regulated eIF2 alpha kinase (HRI) as the heme axial ligand by site-directed mutagenesis and spectral characterization. *J Inorg Biochem.* 101: 1172–1179.

Hoeijmakers, JH. (2009). DNA damage, aging, and cancer. *N Engl J Med.* 361:1475–1485.

Honda, R., Tanaka, H., & Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS letters.* 420(1): 25–27. https://doi.org/10.1016/s0014-5793(97)01480-4.

Hsu, JC., Laz, T., Mohn, KL. & Taub, R. (1991). Identification of LRF-1, a leucine-zipper protein that is rapidly and highly induced in regenerating liver. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(9): 3511–3515. https://doi.org/10.1073/pnas.88.9.3511

Hupp, TR., Meek, DW., Midgley, CA., & Lane, DP. (1992). Regulation of the specific DNA binding function of p53. *Cell*. 71: 875-886.

Hussain, SP. & Harris, CC. (1999). p53 mutation spectrum and load: the generation of hypotheses linking the exposure of endogenous or exogenous carcinogens to human cancer. *Mutat. Res.* 428: 23-32.

Inglis, AJ., Masson, GR., Shao, S., Perisic, O., McLaughlin, SH., Hegde, RS., & Williams, RL. (2019). Activation of GCN2 by the ribosomal P-stalk. *Proceedings of the National Academy of Sciences of the United States of America.* 116(11): 4946–4954. https://doi.org/10.1073/pnas.1813352116.

Ip, YT. & Davis, RJ.(1998). Signal transduction by the c-Jun N-terminal kinase (JNK)–from inflammation to development. *Curr. Opin. Cell Biol.* 10: 205–219.

Ishimura, R., Nagy, G., Dotu, I., Chuang, JH., & Ackerman, SL. (2016). Activation of GCN2 kinase by ribosome stalling links translation elongation with translation initiation. eLife. 5: e14295. https://doi.org/10.7554/eLife.14295.

Iwabuchi, K., Li, B., Bartel, P., & Fields, S. (1993). Use of the two-hybrid system to identify the domain of p53 involved in oligomerization. *Oncogene*. 8(6): 1693–1696.

Jackson, RJ., Hellen, CU., & Pestova, TV. (2010) The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol*. 11: 113–127.

Jackson, SP. (2002). Sensing and repairing DNA double-strand breaks. *Carcinogenesis*, vol. 23, no. 5, pp. 687–696.

Jayaraman, J. & Prives, C. (1995). Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus. *Cell.* 81: 1021–1029.

Jayaraman, L., & Prives, C. (1999). Covalent and noncovalent modifiers of the p53 protein. *Cellular and molecular life sciences : CMLS*. 55(1): 76–87. https://doi.org/10.1007/s000180050271.

Jeffrey, PD., Gorina, S. & Pavletich, NP. (1995). Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms. *Science.* 267: 1498–1502.

Jensen, MR., Factor, VM., Fantozzi, A., Helin, K., Huh, CG., & Thorgeirsson, SS. (2003). Reduced hepatic tumor incidence in cyclin G1-deficient mice. *Hepatology*. 37(4): 862–870. https://doi.org/10.1053/jhep.2003.50137.

Jiang, HY., Wek, SA., McGrath, BC., Lu, D., Hai, T., Harding, HP., Wang, X., Ron, D., Cavener, DR., & Wek, RC. (2004). Activating transcription factor 3 is integral to the eukaryotic initiation factor 2 kinase stress response. *Molecular and cellular biology*. *24*(3): 1365–1377. https://doi.org/10.1128/MCB.24.3.1365-1377.

Jiang, L., Kon, N., Li, T., Wang, SJ., Su, T., Hibshoosh, H., Baer, R., & Gu, W. (2015). Ferroptosis as a p53-mediated activity during tumour suppression. *Nature*. 520(7545): 57–62. https://doi.org/10.1038/nature14344.

Jiang, X., Stockwell, BR., & Conrad, M. (2021). Ferroptosis: mechanisms, biology and role in disease. *Nat. rev. Mol. cell bio*. 22(4): 266–282. https://doi.org/10.1038/s41580-020-00324-8.

Jin, HO., Seo, SK., Woo, SH., Choe, TB., Hong, SI., Kim, JI., & Park, IC. (2009). Nuclear protein 1 induced by ATF4 in response to various stressors acts as a positive regulator on the transcriptional activation of ATF4. *IUBMB Life.* 61: 1153–1158.

Jiricny, J. (2006) The multifaceted mismatch-repair system. *Nat. Rev. Mol. Cell. Biol.* 7: 335–346.

Joerger, AC. & Fersht, AR. (2008). Structural Biology of the Tumor Suppressor p53. *Annu. Rev. Biochem*. 77: 557–582. doi: 10.1146/annurev.biochem.77.060806.091238.

Kaeser, MD., & Iggo, RD. (2002). Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo. *Proceedings of the National Academy of Sciences of the United States of America*. 99(1): 95–100. https://doi.org/10.1073/pnas.012283399.

Karpinski, BA., Morle, GD., Huggenvik, J., Uhler, MD., & Leiden, JM. (1992). Molecular cloning of human CREB-2: an ATF/CREB transcription factor that can negatively regulate transcription from the cAMP response element. *Proceedings of the National Academy of Sciences of the United States of America.* 89(11): 4820–4824. https://doi.org/10.1073/pnas.89.11.4820.

Karsli-Uzunbas, G., Ahmed, F., & Sammons, MA. (2019). Control of p53-dependent transcription and enhancer activity by the p53 family member p63. *The Journal of biological chemistry*. 294(27): 10720–10736. https://doi.org/10.1074/jbc.RA119.007965.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., & Craig, RW. (1991). Participation of p53 protein in the cellular response to DNA damage, *Cancer Res*. 51: 6304–6311.

Kazemi, S., Mounir, Z., Baltzis, D., Raven, J. F., Wang, S., Krishnamoorthy, JL., Pluquet, O., Pelletier, J., & Koromilas, AE. (2007). A novel function of eIF2alpha kinases as inducers of the phosphoinositide-3 kinase signaling pathway. *Molecular biology of the cell*. 18(9): 3635–3644. https://doi.org/10.1091/mbc.e07-01-0053.

Kerr, JF., Wyllie, AH., & Currie, AR. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*. 26(4): 239–257.

Kilberg, MS., Pan, YX., Chen, H., Leung-Pineda, V. (2005). Nutritional control of gene expression: How mammalian cells respond to amino acid limitation. *Annu. Rev. Nutr.* 25: 59-85.

Kilberg, MS., Shan, J., & Su, N. (2009). ATF4-dependent transcription mediates signaling of amino acid limitation. *Trends in endocrinology and metabolism: TEM*. 20(9): 436–443. https://doi.org/10.1016/j.tem.2009.05.008.

Kim, D., Paggi, JM., Park, C., Bennett, C., & Salzberg, SL. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology*. 37: 907–915.

Kim, YW., Lee, S., Yun, J., & Kim, A. (2015). Chromatin looping and eRNA transcription precede the transcriptional activation of gene in the beta-globin locus. *Biosci. Rep.* 35: e00179. doi: 10.1042/bsr20140126.

Kimball, SR. & Jefferson, LS. (2004). Molecular mechanisms through which amino acids mediate signaling through the mammalian target of rapamycin. *Current opinion in clinical nutrition and metabolic care*, 7(1), 39–44. https://doi.org/10.1097/00075197-200401000-00008.

Kitayner, M., Rozenberg, H., Kessler, N., Rabinovich, D., Shaulov, L., Haran, TE. & Shakked, Z. (2006). Structural basis of DNA recognition by p53 tetramers. *Mol Cell.* 22: 741–753.

Kohler A. & Hurt E. (2007). "Exporting RNA from the nucleus to the cytoplasm." *Nature Reviews, Mol. Cell.* 8: 761-773. doi:10.1038/nrm2255.

Kojima, E., Takeuchi, A., Haneda, M., Yagi, A., Hasegawa, T., Yamaki, K., Takeda, K., Akira, S., Shimokata, K., Isobe, K. (2003). The function of GADD34 is a recovery from a shutoff of protein synthesis induced by ER stress: elucidation by GADD34-deficient mice. *FASEB J.* 17: 1573–1575.

Kokame, K., Kato, H., & Miyata, T. (2001). Identification of ERSE-II, a New cis—Acting Element Responsible for the ATF6-dependent Mammalian Unfolded Protein Response. *J. Biol. Chem.* 276: 9199–9205. doi: 10.1074/jbc.M010486200.

Korennykh, A. & Walter, P. (2012). Structural basis of the unfolded protein response. *Annu Rev Cell Dev Biol.* 28: 251–277.

Koutsodontis, G., Vasilaki, E., Chou, W-C., Papakosta, P., & Kardassis, D. (2005). Physical and functional interactions between members of the tumour suppressor p53 and the Sp families of transcription factors: importance for the regulation of genes involved in cell-cycle arrest and apoptosis. *Biochem. J*. 389: 443–455.

Kouwenhoven, EN., Oti, M., Niehues, H., van Heeringen, SJ., Schalkwijk, J., Stunnenberg, HG., et al. (2015) Transcription factor p63 bookmarks and regulates dynamic enhancers during epidermal differentiation. *EMBO Rep*. 16: 863–78.

Kroemer, G., Galluzzi, L., & Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiol Rev.* 87: 99–163.

Kruiswijk, F., Labuschagne, C. F., & Vousden, K. H. (2015). p53 in survival, death and metabolic health: a lifeguard with a licence to kill. Nature reviews. Molecular cell biology, 16(7), 393–405. https://doi.org/10.1038/nrm4007.

Ku, H-C. & Cheng, C-F. (2021). Master Regulator Activating Transcription Factor 3 (ATF3) in Metabolic Homeostasis and Cancer. *Front. Endocrinol.* https://doi.org/10.3389/fendo.2020.00556.

Kubbutat, MH., Jones, SN., & Vousden, KH. (1997). Regulation of p53 stability by Mdm2. *Nature,* 387(6630), 299–303. https://doi.org/10.1038/387299a0.

Kubbutat, MH., & Vousden, KH. (1997). Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. *Molecular and cellular biology*. 17(1): 460–468. https://doi.org/10.1128/MCB.17.1.460.

Kubbutat, MH. & Vousden KH. (1998). Keeping an old friend under control: regulation of p53 stability. *Mol Med Today*. 4: 250–256.

Kulaeva, OI., Nizovtseva, EV., Polikanov, YS., Ulianov, SV. & Studitsky, VM. (2012). Distant activation of transcription: mechanisms of enhancer action. *Mol. Cell. Biol.* 32: 4892–4897.

Kuleshov, MV., Jones, MR., Rouillard, AD., Fernandez, NF., Duan, Q., Wang, Z., Koplev, S., Jenkins, SL., Jagodnik, KM., Lachmann, A., McDermott, MG., Monteiro, CD., Gundersen, GW., & Ma'ayan, A. (2016). Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic acids research*, 44(W1): W90–W97. https://doi.org/10.1093/nar/gkw377.

Kumagai, A., Lee, J., Yoo, HY., & Dunphy, WG. (2006). TopBP1 activates the ATR-ATRIP complex. *Cell*. 124: 943-955.

Kumar, NM. & Gilula, NB. (1996). The gap junction communication channel. *Cell*. 84: 381-388.

Lane, DP & Crawford, LV. (1979). T antigen is bound to a host protein in SV40-transformed cells. *Nature.* 278: 261–263.

Laplante M. & Sabatini DM. (2012). mTOR signaling in growth control and disease. *Cell.* 149, 274–293. 10.1016/j.cell.2012.03.017.

Lee, AH., Iwakoshi, NN., & Glimcher, LH. (2003) XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol. Cell Biol*. 23: 7448–7459.

Lee, D., Hokinson, D., Park, S., Elvira, R., Kusuma, F., Lee, JM., Yun, M., Lee, SG., & Han, J. (2019). ER Stress Induces Cell Cycle Arrest at the G2/M Phase Through eIF2α Phosphorylation and GADD45α. *International journal of molecular sciences*. *20*(24): 6309. https://doi.org/10.3390/ijms20246309.

Lee, ES., Yoon, CH., Kim, YS., & Bae, YS. (2007). The double-strand RNA-dependent protein kinase PKR plays a significant role in a sustained ER stress-induced apoptosis. *FEBS Lett*. 581: 4325–4332.

Lee, H., & Kimelman, D. (2002). A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. *Dev Cell.* 2: 607–616.

Lee, JH. & Paull, TT. (2005). ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science*, 308: 551-554.

Lee, YY., Cevallos, RC., & Jan, E. (2009). An upstream open reading frame regulates translation of GADD34 during cellular stresses that induce eIF2alpha phosphorylation. *The Journal of biological chemistry*. 284(11): 6661–6673. https://doi.org/10.1074/jbc.M806735200.

Lenski, RE., & Travisano, M. (1994). Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proceedings of the National Academy of*

Sciences of the United States of America, *91*(15): 6808–6814. https://doi.org/10.1073/pnas.91.15.6808

Leroy B., Fournier JL., Ishioka C., Monti P., Inga A., Fronza G. & Soussi T. (2013). The TP53 website: an integrative resource centre for the TP53 mutation database and TP53 mutant analysis. *Nucleic Acids Res.* doi: 10.1093/nar/gks1033. PMID: 23161690; PMCID: PMC3531172.

Levine, AJ. (1997). p53, the cellular gatekeeper for growth and division. *Cell*. 88(3): 323–331. https://doi.org/10.1016/s0092-8674(00)81871-1.

Levine, M., Cattoglio, C., & Tjian, R. (2014). Looping back to leap forward: transcription enters a new era. *Cell*. 157: 13-25. PMID:24679523. http://doi.org/10.1016/j.cell.2014.02.009.

Levonen, AL., Hill, BG., Kansanen, E., Zhang, J., & Darley-Usmar VM. (2014). Redox regulation of antioxidants, autophagy, and the response to stress: implications for electrophile therapeutics. *Free Radical Biology and Medicine.* 71: 196–207. doi: 10.1016/j.freeradbiomed.2014.03.025.

Li, L. & Clevers, H. (2010). Coexistence of quiescent and active adult stem cells in mammals. *Science.* 327: 542–545. https://doi.org/10.1126/science.1180794.

Li, T., Kon, N., Jiang, L., Tan, M., Ludwig, T., Zhao, Y., Baer, R., & Gu, W. (2012). Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. *Cell*. 149(6): 1269–1283. https://doi.org/10.1016/j.cell.2012.04.026.

Li, X., Zhou, J., Chen, Z., & Chng, W. (2016). p53 mutations in colorectal cancer- molecular pathogenesis and pharmacological reactivation. *World J. Gastroenterol.* 21(1): 84-93.

Liang, G., Wolfgang, CD., Chen, BP., Chen, TH., & Hai, T. (1996). ATF3 gene. Genomic organization, promoter, and regulation. *The Journal of biological chemistry*, *271*(3): 1695–1701. https://doi.org/10.1074/jbc.271.3.1695.

Liedtke, C., Groger, N., Manns, MP., & Trautwein, C. (2003). The human caspase-8 promoter sustains basal activity through SP1 and ETS-like transcription factors and can be up-regulated by a p53-dependent mechanism. *J. Biol. Chem.* 278: 27593–27604.

Lill, NL., Grossman, SR., Ginsberg, D., DeCaprio, J., Livingston, DM. (1997). Binding and modulation of p53 by p300/CBP coactivators. *Nature*. 387: 823–827.

Lin, AW. & Lowe, SW. (2001). Oncogenic ras activates the ARF-p53 pathway to suppress epithelial cell transformation. *Proc. Natl. Acad. Sci. USA.* 98: 5025–5030.

Lin, J., Chen, J., Elenbaas, B. & Levine, AJ. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes Dev.* 8: 1235–1246.

Lin, YL., Sengupta, S., Gurdziel, K., Bell, GW., Jacks, T., & Flores, ER. (2009). p63 and p73 transcriptionally regulate genes involved in DNA repair. *PLoS genetics*. 5(10): e1000680. https://doi.org/10.1371/journal.pgen.1000680.

Lindahl T. & Barnes DE. (2000). Repair of endogenous DNA damage. *Cold Spring Harb Symp. Quant. Biol.* 65: 127–133.

Lindquist, S. (1986). The heat-shock response. *Annual Review of Biochemistry*. 55: 1151–1191.

Linzer, DI. & Levine, AJ. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell.* 17(1): 43-52. doi: 10.1016/0092-8674(79)90293-9. PMID: 222475.

Liu, G., Su, L., Hao, X., Zhong, N., Zhong, D., Singhal, S., & Liu, X. (2012). Salermide up-regulates death receptor 5 expression through the ATF4-ATF3-CHOP axis and leads to apoptosis in human cancer cells. *Journal of cellular and molecular medicine.* 16(7): 1618–1628. https://doi.org/10.1111/j.1582-4934.2011.01401.x

Liu, L., Ito, S., Nishio, N., Sun, Y., Chen, N., Tanaka, Y., Isobe, K. (2015). GADD34 facilitates cell death resulting from proteasome inhibition. *Anticancer Res*. 35: 5317–5324.

Liu, N. (2019). Library Prep for CUT&RUN with NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (E7645) V.2. *Protocols.* dx.doi.org/10.17504/protocols.io.bagaibse.

Livera, G., Petre-Lazar, B., Guerquin, MJ., Trautmann, E., Coffigny, H. & Habert, R. (2008) p63 null mutation protects mouse oocytes from radio-induced apoptosis. *Reproduction.* 135: 3–12. doi: 10.1530/REP-07-0054.

Ljungman, M. (2000). Dial 9-1-1 for p53: mechanisms of p53 activation by cellular stress. *Neoplasia.* 2: 208–225.

Lockhart, SM., Saudek, V., O'Rahilly, S. (2020). GDF15: A Hormone Conveying Somatic Distress to the Brain. Endocrine Reviews. 41(4): bnaa007. https://doi.org/10.1210/endrev/bnaa007.

Lomakin, IB. & Steitz, TA. (2013). The initiation of mammalian protein synthesis and mRNA scanning mechanism. *Nature*. 500: 307–311.

Los, M., Mozoluk, M., Ferrari, D., Stepczynska, A., Stroh, C., Renz, A., Herceg, Z., Wang, ZQ., Schulze-Osthoff, K. (2002). Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling *Mol. Biol. Cell*. 13: 978-988.

Lowe, SW. & Ruley, HE. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes Dev.* 7: 535–545.

Lu, D., Wolfgang, CD. & Hai, T. (2006). Activating transcription factor 3, a stress-inducible gene, suppresses Ras-stimulated tumorigenesis. *J Biol Chem.* 281:10473–81.

Lu, L., Han, AP., Chen, JJ. (2001). Translation initiation control by heme-regulated eukaryotic initiation factor 2alpha kinase in erythroid cells under cytoplasmic stresses. *Mol Cell Biol.* 21: 7971–7980.

Lu, PD., Harding, HP., & Ron, D. (2004). Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. *Journal of Cell Biology*. 167(1): 27–33.

Luo, S., Baumeister, P., Yang, S., Abcouwer, SF., & Lee, AS. (2003). Induction of Grp78/BiP by Translational Block. *J. Biol. Chem*. 278: 37375–37385. doi: 10.1074/jbc.M303619200.

Malecka, KA., Ho, WC., & Marmorstein, R. (2009). Crystal structure of a p53 core tetramer bound to DNA. *Oncogene*. 28: 325–333. doi: 10.1038/onc.2008.400.

Mantovani, F., Collavin, L., & Del Sal, G. (2019). Mutant p53 as a guardian of the cancer cell. *Cell Death Differ*. 26: 199-212.

Marcantonio, DD., Martinez, E., Kanefsky, JS., Huhn, JM., Gabbasov, R., Gupta, A., Krais, JJ., Peri, S., Tan, Y-F., Skorski, T., Dorrance, A., Garzon, R., Goldman, AR., Tang, H-Y., Johnson, N., & Sykes, SM. (2021). ATF3 coordinates serine and nucleotide metabolism to drive cell cycle progression in acute myeloid leukemia. Molecular Cell. 81(13): 2752-2764.e6. https://doi.org/10.1016/j.molcel.2021.05.008.

Marciniak, SJ., Yun, CY., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, HP., & Ron, D. (2004). CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev*. 18: 3066–3077.

Marciniak SJ. & Ron D. (2006). Endoplasmic reticulum stress signaling in disease. *Physiol. Rev.* 86:1133–1149. https://doi.org/10.1152/physrev.00015.2006.

Margolin, JF. et al., (1994). Kruppel-associated boxes are potent transcriptional repression domains. *Proc. Natl Acad. Sci. USA.* 91: 4509–4513

Marion, RM., Regev, A., Segal, E., Barash, Y., Koller, D., Friedman, N., & O'Shea, E.K. (2004). Sfp1 is a stress- and nutrient-sensitive regulator of ribosomal protein gene expression. *Proc. Natl. Acad. Sci. USA.* 101: 14315–14322. https://doi.org/10.1073/pnas.0405353101

Martinon, F., Holler, N., Richard, C., & Tschopp, J. (2000). Activation of a pro-apoptotic amplification loop through inhibition of NF-κB-dependent survival signals by caspase-mediated inactivation of RIP. *FEBS* Lett. 468: 134–136.

McLure, KG. & Lee, PW. (1998). How p53 binds as a tetramer. *EMBO J*. 17: 3342-3350.

McLure, KG. & Lee, PW. (1999). p53 DNA binding can be modulated by factors that alter the conformational equilibrium. *EMBO J.* 18: 763-770.

Meek, K., Dang, V., & Lees-Miller, SP. (2008). DNA-PK: the means to justify the ends? *Adv. Immunol.* 99: 33–58.

Menendez, D., Inga, A., & Resnick, MA. (2009). The expanding universe of p53 targets. *Nat. Rev. Cancer.* 9: 724-737.

Menendez, D., Nguyen, T-A., Freudenberg, JM., Mathew, VJ., Anderson, CW., Jothi, R., & Resnick, MA. (2013). Diverse stresses dramatically alter genome-wide p53 binding and transactivation landscape in human cancer cells. *Nucleic Acids Res.* 41: 7286–7301.

Miles, S., Li, L., Davison, J., & Breeden, LL. (2013). Xbp1 directs global repression of budding yeast transcription during the transition to quiescence and is important for the longevity and reversibility of the quiescent state. *PLoS Genet.* 9: e1003854. https://doi.org/10.1371/journal.pgen.1003854.

Mohn, KL., Laz, TM., Hsu, JC., Melby, AE., Bravo, R., & Taub, R. (1991). The immediate-early growth response in regenerating liver and insulin-stimulated H-35 cells: comparison with serum-stimulated 3T3 cells and identification of 41 novel immediate-early genes. *Molecular and cellular biology*, *11*(1), 381–390. https://doi.org/10.1128/mcb.11.1.381-390.1991

Momand, J., Zambetti, GP., Olson, DC., George, D., & Levine, AJ. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell.* 69(7): 1237–1245. https://doi.org/10.1016/0092-8674(92)90644-r.

Montes de Oca Luna, R., Wagner, D. S., & Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature.* 378(6553): 203–206. https://doi.org/10.1038/378203a0.

Moreau, P., Hen, R., Wasylyk, B., Everett, R., Gaub, MP., & Chambon, P. (1981). The SV40 72 base repair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. *Nucleic Acids Res*. 9: 6047–6068.

Morimoto, RI., Kroeger, PE., & Cotto, JJ. (1996). The transcriptional regulation of heat shock genes: a plethora of heat shock factors and regulatory conditions. *EXS*. 77: 139–163.

Nagaich, AK., Zhurkin, VB., Durell, SR., Jernigan, RL., Appella, E., & Harrington, RE. (1999). p53-induced DNA bending and twisting: p53 tetramer binds on the outer side of a DNA loop and increases DNA twisting. *Proc. Natl. Acad. Sci. USA*. 96: 1875-1880.

Nakagawa, T., Yoneda, M., Higashi, M., Ohkuma, Y., & Ito, T. (2018). Enhancer function regulated by combinations of transcription factors and cofactors. *Genes to Cells*. 23(10): 808-821. https://doi.org/10.1111/gtc.12634.

Nakatogawa, H., Suzuki, K., Kamada Y., & Ohsumi Y. (2009). Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nature Reviews Molecular Cell Biology. 10: 458–467. doi:10.1038/nrm2708.

Natarajan, K., Meyer, MR., Jackson, BM., Slade, D., Roberts, C. … (2001). Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol.Cell Biol.* 21: 4347–68.

Nemajerova, A., Amelio, I., Gebel, J., Dötsch, V., Melino, G., & Moll, UM. (2018). Non-oncogenic roles of TAp73: from multiciliogenesis to metabolism. *Cell Death Differ.* 25:144. doi: 10.1038/CDD.2017.178.

Nguyen, TT., Grimm, SA., Bushel, PR., Li, J., Li, Y., Bennett, BD., Lavender, CA., Ward, JM., Fargo, DC., Anderson, CW., Li, L., Resnick, MA., & Menendez, D. (2018). Revealing a human p53 universe. *Nucleic acids research*. 46(16): 8153–8167. https://doi.org/10.1093/nar/gky720.

Nigro, JM., Baker, SJ., Preisinger, AC. (1989). Mutations in the p53 gene occur in diverse human tumour types. *Nature.* 342: 705–708.

Nikoletopoulou, V., Markaki, M., Palikaras, K., & Tavernarakis, N. (2013). Crosstalk between apoptosis, necrosis and autophagy. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 1833(12): 3448-3459. https://doi.org/10.1016/j.bbamcr.2013.06.001.

Nikulenkov, F., Spinnler, C., Li, H., Tonelli, C., Shi, Y., Turunen, M., Kivioja, T., Ignatiev, I., Kel, A., Taipale, J. & Selivanova, G. (2012). Insights into p53 transcriptional function via genome-wide chromatin occupancy and gene expression analysis. *Cell Death Differ.* 19:1992–2002.

Nishiyama, J., Yi, X., Venkatachalam, MA., & Dong, Z. (2001). cDNA cloning and promoter analysis of rat caspase-9. *Biochem J.* 360: 49–56.

Novoa, I., Zeng, H., Harding, HP., & Ron, D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. *J Cell Biol*. 153: 1011–1022.

Oliner, JD., Pietenpol, JA., Thiagalingam, S., Gyuris, J., Kinzler, KW., & Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature*. 362(6423): 857–860. https://doi.org/10.1038/362857a0.

Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., & Taya, Y. (2000). p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell.* 102: 849–862.

Ohoka, N., Yoshii, S., Hattori, T., Onozaki, K., & Hayashi, H. (2005). TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. *The EMBO journal*, *24*(6), 1243–1255. https://doi.org/10.1038/sj.emboj.7600596

Okada T., Yoshida H., Akazawa R., Negishi M., and Mori, K. (2002). Distinct roles of activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response. *Biochem. J.* 366:585-594.

Old, LJ. (1988). Tumor necrosis factor. *Sci. Am*. 258: 59–75

Ortt, K. & Sinha, S. (2006). Derivation of the consensus DNA-binding sequence for p63 reveals unique requirements that are distinct from p53. *FEBS letters* 580: 4544–4550.

Overton, KW., Spencer, SL., Noderer, WL., Meyer, T., & Wang, CL. (2014). Basal p21 controls population heterogeneity in cycling and quiescent cell cycle states. *Proceedings of the National Academy of Sciences of the United States of America.* 111(41): E4386–E4393. https://doi.org/10.1073/pnas.1409797111.

Oyadomari, S., Takeda, K., Takiguchi, M., Gotoh, T., Matsumoto, M., Wada, I., Akira, S., Araki, E., & Mori, M. (2001). Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the

endoplasmic reticulum stress pathway. *Proceedings of the National Academy of Sciences of the United States of America.* 98(19): 10845–10850. https://doi.org/10.1073/pnas.191207498.

Pakos-Zebrucka, K., Koryga, I., Mnich, K., Ljujic, M., Samali, A., & Gorman, AM. (2016). The integrated stress response. *EMBO reports*, *17*(10), 1374–1395. https://doi.org/10.15252/embr.201642195

Palam, LR., Baird, TD., & Wek, RC. (2011). Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance CHOP translation. *The Journal of biological chemistry.* 286(13): 10939–10949. https://doi.org/10.1074/jbc.M110.216093.

Pan, H., Dung, HN., Hsu, HM., Hsiao, KM., Chen, LY. (2003). Cloning and developmental expression of p73 cDNA in zebrafish. *Biochem Biophys Res Commun.* 307: 395–400.

Pan, YX., Chen, H., Thiaville, MM., & Kilberg, MS. (2007). Activation of the ATF3 gene through a co-ordinated amino acid-sensing response programme that controls transcriptional regulation of responsive genes following amino acid limitation. *Biochem J*. 401: 299–307.

Pankow, S. & Bamberger, C. (2007). The p53 tumor suppressor-like protein nvp63 mediates selective germ cell death in the sea anemone Nematostella vectensis. *PLoS ONE.* 2: e782

Pasini, S., Corona, C., Liu, J., Greene, LA., & Shelanski, ML. (2015). Specific downregulation of hippocampal ATF4 reveals a necessary role in synaptic plasticity and memory. *Cell reports*. 11(2): 183–191. https://doi.org/10.1016/j.celrep.2015.03.025.

Perland, E. & Fredriksson, R. (2016). Classification systems of secondary active transporters. *Trends Pharmacol. Sci.* 38: 305–315 doi: 10.1016/j.tips.2016.11.008.

Pestov, DG., Strezoska, Z., Lau, LF. (2001). Evidence of p53-dependent cross-talk between ribosome biogenesis and the cell cycle: effects of nucleolar protein Bop1 on G(1)/S transition. *Mol. Cell. Biol.* 21: 4246– 4255.

Pirkkala, L., Nykänen, P., & Sistonen, L. (2001). Roles of the heat shock transcription factors in regulation of the heat shock response and beyond,. *FASEB Journal*. 15(7): 1118–1131.

Pluquet, O., & Hainaut, P. (2001). Genotoxic and non-genotoxic pathways of p53 induction. Cancer letters, 174(1), 1–15. https://doi.org/10.1016/s0304-3835(01)00698-x.

Podust, LM., Krezel, AM., & Kim, Y. (2001). Crystal structure of the CCAAT box/enhancer-binding protein beta activating transcription factor-4 basic leucine zipper heterodimer in the absence of DNA. *J Biol Chem.* 276: 505–513.

Pollard, KS., Hubisz, MJ., Rosenbloom, KR., & Siepel, A. (2010). Detection of nonneutral substitution rates on mammalian phylogenies. *Genome Res.* 20(1): 110-21. PMID: 19858363. PMC: PMC2798823.

Qi, LS., Larson, MH., Gilbert, LA., Doudna, JA., Weissman, JS., Arkin, AP. & Lim, WA. (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell*. 152: 1173–1183.

Rabouw, HH., Langereis, MA., Anand, AA., Visser, LJ., de Groot, RJ., Walter, P. (2019). Small molecule ISRIB suppresses the integrated stress response within a defined window of activation. PNAS. 116 (6) 2097-2102. https://doi.org/10.1073/pnas.1815767116

Rafie-Kolpin, M., Chefalo, PJ., Hussain, Z., Hahn, J., Uma, S., Matts, RL., Chen, JJ. (2000). Two heme-binding domains of heme-regulated eukaryotic initiation factor-2alpha kinase. N terminus and kinase insertion. *J Biol Chem.* 275: 5171–5178.

Raj, N. & Attardi, LD. (2017). The Transactivation Domains of the p53 Protein. *Cold Spring Harbor perspectives in medicine*. 7(1): a026047. https://doi.org/10.1101/cshperspect.a026047.

Rajendran, P., Nandakumar, N., Rengarajan, T., Palaniswami, R., Gnanadhas, EN., Lakshminarasaiah U., Gopas, J., Nishigaki, I. (2014). Antioxidants and human diseases. *Clinica Chimica Acta.* 436: 332–347. doi: 10.1016/j.cca.2014.06.004.

Ramachandran, S., Ma, TS., Griffin, J., Ng, N., Foskolou, IP., Hwang, MS., Victori, P., Cheng, WC., Buffa, FM., Leszczynska, KB., El-Khamisy, SF., Gromak, N., & Hammond, EM. (2021). Hypoxia-induced SETX links replication stress with the unfolded protein response. *Nature communications*, *12*(1), 3686. https://doi.org/10.1038/s41467-021-24066-z.

Ramet, M., Castren, K., Jarvinen, K., Pekkala, K., Turpeenniemi-Hujanen, T., Soini, Y., Paakko, P., & Vahakangas, K. (1995). p53 protein expression is correlated with benzo[a]pyrene-DNA adducts in carcinoma cell lines. *Carcinogenesis*. 16: 2117–2124.

Ramet, M., Castren, K., Jarvinen, K., Pekkala, K., Turpeenniemi-Hujanen, T., Soini, Y., Paakko, P., & Vahakangas, K. (1995). p53 protein expression is correlated with benzo[a]pyrene-DNA adducts in carcinoma cell lines. *Carcinogenesis.* 16: 2117–2124.

Reimold, AM., Etkin, A., Clauss, I., Perkins, A., Friend, DS., Zhang, J., Horton, HF., Scott, A., Orkin, SH., Byrne, MC., Grusby, MJ., & Glimcher, LH. (2000). An essential role in liver development for transcription factor XBP-1. *Genes & development*. 14(2): 152–157.

Reinke, V. & Lozano, G. (1997). The p53 targets mdm2 and Fas are not required as mediators of apoptosis *in vivo. Oncogene*. 15: 1527-1534.

Revel, JP. (1988). The oldest multicellular animal and its junction. In: Hertzberg EL, Johnson RL, eds. *Gap Junctions. New York*. 135-149.

Riley, T., Sontag, E., Chen, P., & Levine, A. (2008). Transcriptional control of human p53-regulated genes. *Nat Rev Mol Cell Biol.* 9: 402–412.

Ritossa, F. (1962). A new puffing pattern induced by temperature shock and DNP in drosophila. *Experientia*. 18: 571-73.

Rittershaus, ES., Baek, SH., & Sassetti, CM. (2013). The normalcy of dormancy: common themes in microbial quiescence. *Cell Host Microbe*. 13: 643–651. https://doi.org/10.1016/j.chom.2013.05.012

Rodríguez-Martínez, JA., Reinke, AW., Bhimsaria, D., Keating, AE., Ansari, AZ. (2017). Combinatorial bZIP dimers display complex DNA-binding specificity landscapes. *eLife*. 6: e19272. https://doi.org/10.7554/eLife.19272

Roeder, RG. (1996). The role of general initiation factors in transcription by RNA polymerase II. *Trends Biochem Sci*. 21: 327-35. PMID:8870495. http://doi.org/10.1016/S0968-0004(96)10050-5

Ron, D. & Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. Nature reviews. *Molecular cell biology*. 8(7): 519–529. https://doi.org/10.1038/nrm2199.

Rouschop, KM., van den Beucken, T., Dubois, L., Niessen, H., Bussink, J., Savelkouls, K., Keulers, T., Mujcic, H., Landuyt, W., Voncken, JW., Lambin, P., van der Kogel, AJ., Koritzinsky, M., & Wouters, BG. (2010). The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. *The Journal of clinical investigation*. 120(1): 127–141. https://doi.org/10.1172/JCI40027.

Rutkowski, DT., Arnold, SM., Miller, CN., Wu, J., Li, J., Gunnison, KM., Mori, K., Sadighi Akha, AA., Raden, D., & Kaufman, RJ. (2006). Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. *PLoS biology*. 4(11): e374. https://doi.org/10.1371/journal.pbio.0040374.

Rzymski, T., Milani, M., Pike, L., Buffa, F., Mellor, HR., Winchester, L., Pires, I., Hammond, E., Ragoussis, I., & Harris, AL. (2010). Regulation of autophagy by ATF4 in response to severe hypoxia. *Oncogene.* 29: 4424–4435.

Saelens, X., Kalai, M., Vandenabeele, P. (2001). Translation inhibition in apoptosis: caspase-dependent PKR activation and eIF2-alpha phosphorylation. *J Biol Chem.* 276: 41620–41628.

Sakaguchi, K., Sakamoto, H., Lewis, MS., Anderson, CW., Erickson, JW., Appella, E., & Xie, D. (1997). Phosphorylation of serine 392 stabilizes the tetramer formation of tumor suppressor protein p53. *Biochemistry*. 36(33): 10117–10124. https://doi.org/10.1021/bi970759w.

Samali, A. & Orrenius, S. (1998). Heat shock proteins: regulators of stress response and apoptosis. *Cell Stress and Chaperones*. 3(4): 228–236.

Samali, A., Robertson, JD., Peterson E. … (2001). Hsp27 protects mitochondria of thermotolerant cells against apoptotic stimuli. *Cell Stress and Chaperones*. 6(1): 49–58.

Sammons, MA., Zhu, J., Drake, AM., & Berger, SL. (2015). TP53 engagement with the genome occurs in distinct local chromatin environments via pioneer factor activity. *Genome research*. 25(2): 179–188. https://doi.org/10.1101/gr.181883.114.

Sanchez-Prieto, R., Rojas, JM., Taya, Y., & Gutkind, JS. (2000). A role for the p38 mitogen-acitvated protein kinase pathway in the transcriptional activation of p53 on genotoxic stress by chemotherapeutic agents. *Cancer research*, *60*(9), 2464–2472.

Sandelin, A., Alkema, W., Engstrom, P., Wasserman, WW., & Lenhard, B. (2004). JASPAR: an open-access database for eukaryotic transcription factor binding profiles. *Nucleic Acids Res.* 32(Database issue):D91-4. doi: 10.1093/nar/gkh012.

Schröder M. & Kaufman, RJ. (2005). The mammalian unfolded protein response. *Annual Review of Biochemistry*. 74: 739–789.

Shabtay, A. & Arad, Z. (2006). Reciprocal activation of HSF1 and HSF3 in brain and blood tissues: is redundancy developmentally related? *American Journal of Physiology*. 291(3): R566–R572.

Shamovsky, I. & Nudler, E. (2008). New insights into the mechanism of heat shock response activation. *Cellular and Molecular Life Sciences*. 65(6): 855–861.

Shen, J., Snapp, EL., Lippincott-Schwartz, J., & Prywes, R. (2005). Stable binding of ATF6 to BiP in the endoplasmic reticulum stress response. *Molecular and cellular biology*. 25(3): 921–932. https://doi.org/10.1128/MCB.25.3.921-932.2005.

Shieh, SY., Ikeda, M., Taya, Y. & Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell, 91(3), 325–334. https://doi.org/10.1016/s0092-8674(00)80416-x.

Shieh, SY., Taya, Y., & Prives, C. (1999). DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization. *The EMBO journal*. 18(7): 1815–1823. https://doi.org/10.1093/emboj/18.7.1815.

Shieh, SY., Ahn, J., Tamai, K., Taya, Y., & Prives, C. (2000). The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. *Genes & development*, *14*(3), 289–300.

Shiloh Y. (2003). ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer. Mar;3(3):155-68. doi: 10.1038/nrc1011. PMID: 12612651.

Shimazawa, M. & Hara, H. (2006). Inhibitor of double stranded RNA-dependent protein kinase protects against cell damage induced by ER stress. *Neurosci Lett.* 409: 192–195.

Shiu, RPC., Pouyssegur, J., & Pastan, I. (1977). Glucose depletion accounts for the induction of two transformation-sensitive membrane proteins in Rous sarcoma virus-transformed chick embryo fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*. 74(9): 3840–3844.

Shlyueva, D., Stampfel, G., & Stark, A. (2014). Transcriptional enhancers: from properties to genome-wide predictions. *Nature reviews. Genetics*, *15*(4), 272–286. https://doi.org/10.1038/nrg3682

Shoulders, MD., Ryno, LM., Genereux, JC., Moresco, JJ., Tu, PG., Wu, C., Yates, JR., Su, AI., Kelly, JW., & Wiseman, RL. (2013). Stress-independent activation of XBP1s and/or ATF6 reveals three functionally diverse ER proteostasis environments. *Cell reports.* 3(4): 1279–1292. https://doi.org/10.1016/j.celrep.2013.03.024.

Siepel, A., Bejerano, G., Pedersen, JS., Hinrichs, AS., Hou, M., Rosenbloom, K., Clawson, H., Spieth, J., Hillier, LW., Richards, S. (2005). Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. *Genome Res.* 15(8): 1034-50. PMID: 16024819. PMC: PMC1182216.

Sies, H. (1993). Strategies of antioxidant defense. *Eur. J. Biochem.* 215: 213-219.

Sies, H. (2015). Oxidative stress: a concept in redox biology and medicine. *Redox Biology*. 4: 180–183. https://doi.org/10.1016/j.redox.2015.01.002.

Siliciano, JD., Canman, CE., Taya, Y., Sakaguchi, K., Appella, E., & Kastan, MB. (1997). DNA damage induces phosphorylation of the amino terminus of p53. Genes & development, 11(24), 3471–3481. https://doi.org/10.1101/gad.11.24.3471.

Siu, F., Bain, PJ., LeBlanc-Chaffin, R., Chen, H., & Kilberg, MS. (2002). ATF4 is a mediator of the nutrient-sensing response pathway that activates the human asparagine synthetase gene. *J Biol Chem.* 277: 24120–24127.

Skene, PJ. & Henikoff, S. (2017). An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. *eLife*. 6: e21856. https://doi.org/10.7554/eLife.21856.

Smeenk, L., van Heeringen, SJ., Koeppel, M., van Driel, MA., Bartels, SJJ., Akkers, RC., Denissov, S., Stunnenberg, HG. & Lohrum, M. (2008). Characterization of genome-wide p53-binding sites upon stress response. *Nucl Acids Res.* 36: 3639–3654.

Soneson, C., Love, MI. & Robinson, MD. (2015). Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Research*. [version 1; peer review: 2 approved]. 4:1521 (https://doi.org/10.12688/f1000research.7563.1)

Sood, R., Porter, AC., Olsen, DA., Cavener, DR., Wek, RC. (2000). A mammalian homologue of GCN2 protein kinase important for translational control by phosphorylation of eukaryotic initiation factor 2α. *Genetics.* 154: 787–80.

Soussi, T., Dehouche, K., & Be´roud, C. (2000). p53 Website and analysis of p53 gene mutations in human cancer: forging a link between epidemiology and carcinogenesis. *Hum Mutat*. 15: 105–113.

Spitz, F., & Furlong, EE. (2012). Transcription factors: from enhancer binding to developmental control. *Nature reviews. Genetics*, *13*(9), 613–626. https://doi.org/10.1038/nrg3207

Statzer, C., Meng, J., Venz, R., Bland, M., Robida-Stubbs, S., Patel, K., Petrovic, D., Emsley, R., Liu, P., Morantte, I., Haynes, C., Mair, W. B., Longchamp, A., Filipovic, MR., Blackwell, TK., & Ewald, CY. (2022). ATF-4 and hydrogen sulfide signalling mediate longevity in response to inhibition of translation or mTORC1. *Nature communications*, *13*(1), 967. https://doi.org/10.1038/s41467-022-28599-9

Stommel, JM., Marchenko, ND., Jimenez, GS., Moll, UM., Hope, TJ., & Wahl, GM. (1999). A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *EMBO J*. 18: 1660–1672.

Suh, EK., Yang, A., Kettenbach, A., Bamberger, C., Michaelis, AH., Zhu, Z., et al. (2006). p63 protects the female germ line during meiotic arrest. *Nature*. 444: 624–8. doi: 10.1038/nature05337.

Sun, D. & Buttitta, L. (2017). States of G0 and the proliferation-quiescence decision in cells, tissues and during development. *Int. J. Dev. Biol.* 61: 357–366. https://doi.org/10.1387/ijdb.160343LB

Sun, S. & Gresham, D. (2021). Cellular quiescence in budding yeast. *Yeast.* 38: 12–29. https://doi.org/10.1002/yea.3545.

Schweichel, JU. & Merker, HJ. (1973). The morphology of various types of cell death in prenatal tissues. *Teratology*. 7: 253-266.

Szak, ST., Mays, D., & Pietenpol, JA. (2001). Kinetics of p53 binding to promoter sites in vivo. *Mol. Cell Biol.* 21: 3375–3386.

Tabula Muris Consortium, Overall coordination, Logistical coordination, Organ collection and processing, Library preparation and sequencing, Computational data analysis, Cell type annotation, Writing group, Supplemental text writing group, & Principal investigators (2018). Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. *Nature*. 562(7727): 367–372. https://doi.org/10.1038/s41586-018-0590-4.

Takahashi, N., Ogita, N., Takahashi, T., Taniguchi, S., Tanaka, M., Seki, M., & Umeda, M. (2019). A regulatory module controlling stress-induced cell cycle arrest in Arabidopsis. *eLife*. 8: e43944. https://doi.org/10.7554/eLife.43944.

Tanaka, Y., Nakamura, A., Morioka, MS., Inoue, S., Tamamori-Adachi, M., Yamada, K., Taketani, K., Kawauchi, J., Tanaka-Okamoto, M., Miyoshi, J., Tanaka, H., & Kitajima, S. (2011). Systems analysis of ATF3 in stress response and cancer reveals opposing effects on pro-apoptotic genes in p53 pathway. *PloS one.* 6(10): e26848. https://doi.org/10.1371/journal.pone.0026848.

Taniuchi, S., Miyake, M., Tsugawa, K., Oyadomari, M., Oyadomari S. (2016). Integrated stress response of vertebrates is regulated by four eIF2α kinases. *Sci Rep* 6, 32886. https://doi.org/10.1038/srep32886.

Terlikowski, SJ. (2001). Tumour necrosis factor and cancer treatment: a historical review and perspectives. *Roczniki Akademii Medycznej w Bialymstoku*. 46: 5–18.

Teske, BF., Fusakio, ME., Zhou, D., Shan, J., McClintick, JN., Kilberg, MS., & Wek, RC. (2013). CHOP induces activating transcription factor 5 (ATF5) to trigger apoptosis in response to perturbations in protein homeostasis. *Molecular biology of the cell*. 24(15): 2477–2490. https://doi.org/10.1091/mbc.E13-01-0067.

Thakore, PI., D'Ippolito, AM., Song, L., Safi, A., Shivakumar, NK., Kabadi, AM., Reddy, TE., Crawford, GE. & Gersbach, CA. (2015). Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal regulatory elements. Nature methods, 12(12), 1143–1149. https://doi.org/10.1038/nmeth.3630.

Tian X., Ahsan, N., Lulla, A., Lev, A., Abbosh, P., Dicker, DT., Zhang, S. & El-Deiry, WS. (2021). P53-independent partial restoration of the p53 pathway in tumors with mutated p53 through ATF4 transcriptional modulation by ERK1/2 and CDK9. *Neoplasia*. 23(3): 304-325. https://doi.org/10.1016/j.neo.2021.01.004.

Tissir, F., Ravni, A., Achouri, Y., Riethmacher, D., Meyer, G. & Goffinet, AM. (2009) DeltaNp73 regulates neuronal survival in vivo. *Proc Natl Acad Sci USA*. 106: 16871–6. doi: 10.1073/pnas.0903191106.

Toledo F., Wahl, GM. (2006). Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. Nat Rev Cancer. Dec;6(12):909-23. doi: 10.1038/nrc2012. PMID: 17128209.

Tozluoglu, M., Karaca, E., Haliloglu, T. & Nussinov, R. (2008). Cataloging and organizing p73 interactions in cell cycle arrest and apoptosis. *Nucl Acids Res.* 36: 5033–5049.

Trosko, JE & Inoue, T. (1997). Oxidative Stress, Signal Transduction, and Intercellular Communication in Radiation Carcinogenesis. *Stem Cells.* 15(suppl2): 59-67.

Unger, T., Juven-Gershon, T., Moallem, E., Berger, M., Vogt Sionov, R., Lozano, G., Oren, M. and Haupt, Y. (1999). Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. *EMBO J.* 18: 1805-1814.

Utsunomiya-Tate, N., Endou, H. & Kanai, Y. (1996). Cloning and functional characterization of a system ASC-like Na+-dependent neutral amino acid transporter. *J. Biol. Chem.* 271, 14883–14890 doi: 10.1074/jbc.271.25.14883.

Valente, LJ., Gray, DH., Michalak, EM., Pinon-Hofbauer, J., Egle, A., Scott, CL., Janic, A., & Strasser, A. (2013). p53 efficiently suppresses tumor development in the complete absence of its cell-cycle inhibitory and proapoptotic effectors p21, Puma, and Noxa. *Cell reports*. 3(5): 1339–1345. https://doi.org/10.1016/j.celrep.2013.04.012.

Valerie K. & Povirk LF. (2003). Regulation and mechanisms of mammalian double-strand break repair. *Oncogene*. 22(37): 5792–581.

Vallejo, M., Ron, D., Miller, CP., & Habener, JF. (1993). C/ATF, a member of the activating transcription factor family of DNA-binding proteins, dimerizes with CAAT/enhancer-binding proteins and directs their binding to cAMP response elements. *Proceedings of the National Academy of Sciences of the United States of America.* 90(10): 4679–4683. https://doi.org/10.1073/pnas.90.10.4679.

van Maanen, JM., Retèl, J., de Vries, J. & Pinedo, HM. (1988). Mechanism of action of antitumor drug etoposide: a review. Journal of the National Cancer Institute. 80(19): 1526–1533. https://doi.org/10.1093/jnci/80.19.1526.

Vassilev, LT., Vu, BT., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N., & Liu, EA. (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science (New York, N.Y.), 303(5659): 844–848. https://doi.org/10.1126/science.1092472.

Vattem, KM. & Wek, RC. (2004). Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc. Natl. Acad. Sci. USA.* 101: 11269–74.

Velasco-Miguel S, Buckbinder L, Jean P, Gelbert L, Talbott R, Laidlaw J, Seizinger B, Kley N. (1999). PA26, a novel target of the p53 tumor suppressor and member of the GADD family of DNA damage and growth arrest inducible genes. *Oncogene*. 18: 127-37. PMID:9926927.

Verfaillie, A., Svetlichnyy, D., Imrichova, H., Davie, K., Fiers, M., Atak, ZK., Hulselmans, G., Christiaens, V., & Aerts, S. (2016). Multiplex enhancer-reporter assays uncover unsophisticated TP53 enhancer logic. *Genome Res*. 26: 882–895.

Vihervaara A., Duarte FM., & Lis JT. (2018). Molecular mechanisms driving transcriptional stress responses. *Nature reviews. Genetics*, *19*(6), 385–397. https://doi.org/10.1038/s41576-018-0001-6

Vilgelm, A., El-Rifai, W. & Zaika, A. (2008). Therapeutic prospects for p73 and p63: rising from the shadow of p53. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy.* 11(4-5): 152–163. https://doi.org/10.1016/j.drup.2008.08.001.

Voellmy, R. (2004). On mechanisms that control heat shock transcription factor activity in metazoan cells. *Cell Stress and Chaperones*. 9(2): 122–133.

Vogelstein, B., Lane, D., & Levine, AJ. (2000). Surfing the p53 network. *Nature* 408: 307–310.

Vousden, KH. & Lu, X. (2002). Live or let die: the cell's response to p53. *Nature reviews. Cancer.* 2(8): 594–604. https://doi.org/10.1038/nrc864

Vurusaner, B., Poli, G. & Basaga, H. (2012). Tumor suppressor genes and ROS: complex networks of interactions. *Free Radic Biol Med.* 52: 7–18.

Wagner, A. (2013). *Robustness and Evolvability in Living Systems*. Princeton: Princeton University Press. https://doi.org/10.1515/9781400849383

Wagner, GP. & Altenberg, L. (1996). PERSPECTIVE: COMPLEX ADAPTATIONS AND THE EVOLUTION OF EVOLVABILITY. *Evolution; international journal of organic evolution*. *50*(3): 967–976. https://doi.org/10.1111/j.1558-5646.1996.tb02339.x

Watkins-Chow, DE., Cooke, J., Pidsley, R., Edwards, A., Slotkin, R., Leeds, KE., Mullen, R., Baxter, LL., Campbell, TG., Salzer, MC., Biondini, L., Gibney, G., Phan Dinh Tuy, F., Chelly,

J., Morris, HD., Riegler, J., Lythgoe, MF., Arkell, RM., Loreni, F., Flint, J., Pavan, WJ., Keays, DA. (2013). Mutation of the Diamond–Blackfan anemia gene Rps7 in mouse results in morphological and neuroanatomical phenotypes. *PLoS Genet*. 9: e1003094.

Wei, CL., Wu, Q., Vega, VB., Chiu, KP., Ng, P., Zhang, T., Shahab, A., Yong, HC., Fu, Y., Weng, Z., Liu, J., Zhao, XD., Chew, J-L., Lee, YL., Kuznetsov, VA., Sung, W-K., Miller, LD., Lim, B., Liu, ET., Yu, Q., Ng, H-H., & Ruan, Y. (2006). A Global Map of p53 Transcription-Factor Binding Sites in the Human Genome. Cell. 124(1): 207-219.

Wei, YH., Lu, CY., Wei, CY., Ma, YS., & Lee, HC. (2001). Oxidative stress in human aging and mitochondrial disease - consequences of defective mitochondrial respiration and impaired antioxidant enzyme system. *Chinese Journal of Physiology*. 44: 1-11.

Weidenfeld-Baranboim, K., Hasin, T., Darlyuk, I., Heinrich, R., Elhanani, O., Pan, J., Yokoyama, KK. & Aronheim, A. (2009). The ubiquitously expressed bZIP inhibitor, JDP2, suppresses the transcription of its homologue immediate early gene counterpart, ATF3. *Nucl Acids Res*. 37: 2194–2203.

Wek. RC. (2018). Role of eIF2α Kinases in Translational Control and Adaptation to Cellular Stress. *Cold Spring Harbor perspectives in biology*. 10(7): a032870. https://doi.org/10.1101/cshperspect.a032870.

West, SC. (2003). Molecular views of recombination proteins and their control. *Nat Rev Mol Cell Biol.* 4: 435–445.

Whitney, ML., Jefferson, LS. & Kimball, SR. (2009). ATF4 is necessary and sufficient for ER stress-induced upregulation of REDD1 expression. *Biochemical and biophysical research communications*. 379(2): 451–455. https://doi.org/10.1016/j.bbrc.2008.12.079.

Williams, BR. (1999). PKR; a sentinel kinase for cellular stress. *Oncogene.* 18: 6112–6120.

Wolfgang, CD., Chen, BP., Martindale, JL., Holbrook, NJ., Hai, T. (1997). Gadd153/Chop10, a potential target gene of the transcriptional repressor ATF3. *Mol Cell Biol.* 17: 6700–6707.

Woodstock, DL., Sammons, MA. & Fischer, M. (2021). P63 and p53: Collaborative partners or dueling rivals? *Front. Cell Dev. Biol.* 9: 701986. doi: 10.3389/fcell.2021.701986

Xie, Z., Bailey, A., Kuleshov, MV., Clarke, DBJ., Evangelista, JE., Jenkins, SL., Lachmann, A., Wojciechowicz, ML. Kropiwnicki, E., Jagodnik, KM., Jeon, M., Ma'ayan, A. (2021). Gene Set Knowledge Discovery with Enrichr. *Current Protocols*. https://doi.org/10.1002/cpz1.90.

Yamamoto, K., Yoshida, H., Kokame, K., Kaufman, RJ., Mori, K. (2004). Differential contributions of ATF6 and XBP1 to the activation of endoplasmic reticulum stress-responsive cis-acting elements ERSE, UPRE and ERSE-II. *J Biochem*. 136: 343–350.

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, MD., Dotsch, V., Andrews, NC., Caput, D. & McKeon, F. (1998). p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell.* 2: 305–316.

Yang, A., Walker, N., Bronson, R., Kaghad, M., Oosterwegel, M., Bonnin, J., Vagner, C., Bonnet, H., Dikkes, P., Sharpe, A., McKeon, F., & Caput, D. (2000). p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature*, 404(6773): 99–103. https://doi.org/10.1038/35003607.

Yang, Z. (1995). A space-time process model for the evolution of DNA sequences. *Genetics*. 139(2): 993-1005. PMID: 7713447. PMC: PMC1206396.

Ye, J., Rawson, RB., Komuro R., Chen X., Davé UP., Prywes R., Brown MS., Goldstein JL. (2000). ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol. Cell*. 6: 1355–1364. doi: 10.1016/S1097-2765(00)00133-7.

Ye, J., Kumanova, M., Hart, LS., Sloane, K., Zhang, H., De Panis, DN., Bobrovnikova-Marjon, E., Diehl, JA., Ron, D., & Koumenis, C. (2010). The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation. *The EMBO journal*. 29(12): 2082–2096. https://doi.org/10.1038/emboj.2010.81.

Yeo, NC., Chavez, A., Lance-Byrne, A., Chan, Y., Menn, D., Milanova, D., Kuo, CC., Guo, X., Sharma, S., Tung, A., Cecchi, R. J., Tuttle, M., Pradhan, S., Lim, ET., Davidsohn, N., Ebrahimkhani, MR., Collins, JJ., Lewis, NE., Kiani, S. & Church, GM. (2018). An enhanced CRISPR repressor for targeted mammalian gene regulation. *Nature methods.* 15(8): 611–616. https://doi.org/10.1038/s41592-018-0048-5.

Yin, H-M., Yan, L-F., Liu, Q., Peng, Z., Zhang, C-Y., Xia, Y., Su, D., Gu, A-H., & Zhou, Y. (2020). Activating transcription factor 3 coordinates differentiation of cardiac and hematopoietic

progenitors by regulating glucose metabolism. *Science Advances*. 6(19). DOI: 10.1126/sciadv.aay9466.

Yin, T., Sandhu, G., Wolfgang, CD., Burrier, A., Webb, RL., Rigel, DF., Hai, T., & Whelan, J. (1997). Tissue-specific pattern of stress kinase activation in ischemic/reperfused heart and kidney. *The Journal of biological chemistry*, *272*(32), 19943–19950. https://doi.org/10.1074/jbc.272.32.19943

Yin, Y., Stephen, CW., Luciani, MG. & Fåhraeus, R. (2002). p53 Stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. *Nature cell biology*. 4(6): 462–467. https://doi.org/10.1038/ncb801.

Yoshida, H., Haze, K., Yanagi, H., Yura, T., & Mori, K. (1998). Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins: involvement of basic leucine zipper transcription factors. *Journal of Biological Chemistry*. 273(50): 33741–33749.

Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., & Mori, K. (2001a). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor." *Cell* 107: 881–891.

Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M., & Mori, K. (2001b). Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6alpha and 6beta that activates the mammalian

unfolded protein response. *Molecular and cellular biology*. 21(4): 1239–1248. https://doi.org/10.1128/MCB.21.4.1239-1248.2001.

Young, SK., Palam, LR., Wu, C., Sachs, MS., & Wek, RC. (2016). Ribosome Elongation Stall Directs Gene-specific Translation in the Integrated Stress Response. *The Journal of biological chemistry*. 291(12): 6546–6558. https://doi.org/10.1074/jbc.M115.705640.

Younger, ST. & Rinn, JL. (2017). p53 regulates enhancer accessibility and activity in response to DNA damage. *Nucleic acids research*. 45(17): 9889–9900. https://doi.org/10.1093/nar/gkx577.

Zhan, Q. Bae, I., Kastan, MB., Fornace Jr., AJ. (1994). The p53-dependent gamma-ray response of GADD45. *Cancer Res.* 54: 2755–2760.

Zhang, Y., Xiong, Y., & Yarbrough, WG. (1998). A.R.F. promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell.* 92: 725–734.

Zhang, Y. & Dong, C. (2007). Regulatory mechanisms of mitogen-activated kinase signaling. *Cellular and molecular life sciences: CMLS*. 64(21): 2771–2789. https://doi.org/10.1007/s00018-007-7012-3

Zhang, Y., Liu, T., Meyer, CA., Eeckhoute, J., Johnson, DS., Bernstein, BE., Nusbaum, C., Myers, RM., Brown, M., Li W., & Liu, XS. (2008). Model-based Analysis of ChIP-Seq (MACS). *Genome Biology*. 9: R137.

Zhang, Y., Shi, J., Liu, X., Feng, L., Gong, Z., Koppula, P., Sirohi, K., Li, X., Wei, Y., Lee, H., Zhuang, L., Chen, G., Xiao, Z. D., Hung, M. C., Chen, J., Huang, P., Li, W., & Gan, B. (2018). BAP1 links metabolic regulation of ferroptosis to tumour suppression. *Nature cell biology*. 20(10): 1181–1192. https://doi.org/10.1038/s41556-018-0178-0.

Zhao, J., Li, X., Guo, M., Yu, J., & Yan, C. (2016). The common stress responsive transcription factor ATF3 binds genomic sites enriched with p300 and H3K27ac for transcriptional regulation. BMC genomics, 17, 335. https://doi.org/10.1186/s12864-016-2664-8.

Zhao, R., Gish, K., Murphy, M., Yin, Y., Notterman, D., Hoffman, WH., Tom E., Mack, DH., & Levine, AJ. (2000). Analysis of p53-regulated gene expression patterns using oligonucleotide arrays. *Genes Dev.* 14:981–993.

Zhu, J., Sammons, MA., Donahue, G., Dou, Z., Vedadi, M., Getlik, M., Barsyte-Lovejoy, D., Al-awar, R., Katona, BW., Shilatifard, A., Huang, J., Hua, X., Arrowsmith, CH., & Berger, SL. (2015). Gain-of-function p53 mutants co-opt chromatin pathways to drive cancer growth. *Nature*. 525: 206-211.

Zou, L. & Elledge, SJ. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science*. 300: 1542-1548.

Zou, W., Yue, P., Khuri, FR., & Sun, SY. (2008). Coupling of endoplasmic reticulum stress to CDDO-Me-induced up-regulation of death receptor 5 via a CHOP-dependent mechanism

involving JNK activation. *Cancer research*. 68(18): 7484–7492. https://doi.org/10.1158/0008-5472.CAN-08-1318.