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Analysis of Ahr Expression and Stability in a Recombinant Yeast Model System

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Analysis of Ahr Expression and Stability in a Recombinant Yeast Model System

by

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DEDICATION

To my ever supportive family for their unending patience and understanding. I would specifically like to dedicate this dissertation to my grandfather, Don Wilson, my father, Tom Wilson, and my husband, A.J. Cuccinello. These men have been wonderful supporters of my educational endeavors and I wish to honor and thank them.

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ABSTRACT

The aryl hydrocarbon receptor (Ahr) and the aryl hydrocarbon receptor nuclear translocator (Arnt) are well characterized bHLH-PAS transcription factors shown to regulate expression of xenobiotic metabolism genes. Extensive study has shown that upon treatment with certain aromatic hydrocarbons, mammalian cells rapidly activate the Ahr signaling pathway in order to stimulate gene expression and attempt to metabolize the xenobiotic compounds. It has been shown that after DNA-binding, the Ahr but not the Arnt protein, is quickly eliminated from the nuclear compartment thereby attenuating the dose of gene regulation administered by the Ahr•Arnt transcription factor complex. Previous studies have implicated involvement of the 26S proteasome complex in the degradation process, but the exact identity of the intermediary proteins and/or ligases remains to be defined. Identification and characterization of the protein(s) involved in degrading the receptor is essential for understanding the signaling pathway in its entirety including the mechanism for regulating the genetic response to Ahr ligands.

The model organism, *Saccharomyces cerevisiae*, was used in order to characterize the Ahr signaling pathway and degradation mechanism in a more simplified cellular setting in which the major processes required for growth and development are conserved. First, the AHR and ARNT cDNAs were stably inserted into the yeast genome such that protein expression was inducible. A time course of induction demonstrated detectable levels of Ahr and Arnt proteins via western blotting while protein function was confirmed by detection of ligand-dependent reporter activity in an

expressor strain carrying the pLXRE5-Z beta-galactosidase reporter plasmid. Additionally, a rapid reduction in protein levels was observed upon turning off the inducible *GAL1* promoter located upstream of both AHR and ARNT cDNAs.

Studies in mammalian cell culture have demonstrated that disrupting receptor chaperoning results in rapid Ahr protein turnover, as demonstrated by treatment with Hsp90 inhibitors. In order to determine if reduced Ahr protein expression in the yeast system was attributed to improper chaperoning of the exogenous protein; human heat shock proteins were constitutively expressed from yeast expression vectors in the Ahr and Arnt expressing strains, but did not confer any effect on Ahr stability when protein levels were evaluated by western blotting. Additionally, a strain of yeast was constructed such that the gene encoding the cell-wall protein, *ERG6*, was deleted from the yeast genome to allow for permeation of proteasome inhibitors. Treatment of this strain with proteasome inhibitors blocked the receptor degradation, therefore implicating the 26S proteasome in Ahr degradation when expressed exogenously in yeast.

CHAPTER ONE:

INTRODUCTION

Basic helix-loop-helix Superfamily of Transcription Factors

The basic helix-loop-helix (bHLH) family of transcription factors is composed of over 240 genes involved in the regulation of many developmental and physiological processes (reviewed by Massari and Murre 2000). Members of this family encode a basic DNA-binding region and an adjacent helix-loop-helix dimerization domain and interact with DNA subsequent to homodimerization or heterodimerization with other bHLH proteins. bHLH proteins can be divided into 3 subfamilies, the first subfamily members encode a bHLH domain, the second have a bHLH domain and an adjacent leucine zipper, and the third have a bHLH domain and a flanking PAS (PER, ARNT, SIM) domain (reviewed by Kewley *et al* 2004).

bHLH Subfamily. Members of the first bHLH subfamily include MyoD, NeuroD, and Scl and have an NH_2-terminal bHLH domain. These proteins are involved in regulation of myogenesis, neurogenesis, and hematopoiesis and are found to be constitutively expressed but restricted to particular tissues and stages in development. They are incapable of forming homodimers and instead form heterodimers with other bHLH proteins and bind to the canonical E-box element, 5'-CANNTG-3', located upstream of target genes (reviewed by Massari and Murre 2000 and Kewley *et al* 2004).

bHLH-Zip Subfamily. Similar to other bHLH proteins, bHLH-Zip members bind the canonical E-box core enhancer sequence in the promoter regions of their target genes. However, these proteins, including Myc, Max, and Mad, encode a leucine zipper dimerization domain adjacent to the bHLH region. Again, bHLH-Zip proteins are constitutively active, but are only expressed in certain tissues and developmental stages and bind at E-box enhancer sequences to initiate transcription (reviewed by Kewley *et al* 2004). The Myc/Max/Mad network of proteins functions in cell cycle control and plays a role in tumor formation (Luscher and Larsson 1999).

bHLH-PAS Subfamily. The bHLH-PAS sub-family of genes is an offshoot of the larger bHLH family and its members include: Ahr, Arnt1, Arnt2, Per, Sim1, Sim2, Clock, Hif-1α, Hif-2α, Hif-3α, Bmal1, and Bmal2 (reviewed by Kewley *et al* 2004 and Crews 1998). Members of this family function in gene expression networks that play a role in detection and adaptation to environmental changes, including xenobiotic metabolism, oxygen balance, and circadian rhythm maintenance (Gu *et al* 2000). Similar to other bHLH proteins, they possess a modular structure with specific conserved functional domains including the basic DNA-binding domain and the helix-loop-helix domain required for protein dimerization. In addition to the bHLH domain, this sub-family also shares a common PAS region, so named for the first three proteins identified as containing this homology; Per (*Drosophila* Period protein), Arnt (Aryl hydrocarbon receptor nuclear translocator protein), and Sim (*Drosophila* Single-minded protein). Additionally, most bHLH-PAS members have transcription activation domains within their C-terminus.

Classification of bHLH-PAS Members. Members of the bHLH-PAS sub-family are further divided into two classes; receptors that function in a specific pathway are

classified separately from receptors that act as generic partner proteins. Ahr, Hif, and Sim are Class I bHLH-PAS proteins that become transcriptionally active when coupled with a Class II bHLH-PAS protein, namely, Arnt and Bmal. Class I proteins are unable to form homodimers or heterodimers with other Class I proteins while Class II bHLH-PAS proteins have been isolated as homodimers and heterodimers with other members of the same class (reviewed by Kewley *et al* 2004).

Ahr-Mediated Gene Regulation

The aryl hydrocarbon receptor (Ahr) has been studied extensively in the field of toxicology for its role in transcriptional activation of drug metabolizing enzymes in response to exposure to certain persistent environmental contaminants. Each step of the signal transduction pathway has been evaluated; from its latent unliganded state, through ligand binding and nuclearization, heterodimerization with its DNA binding partner, DNA binding and transactivation of target genes, and finally its targeted degradation.

Functional Domains of Ahr. Cloning of the Ah receptor (Burbach *et al* 1992) and comparative sequence analysis revealed a basic helix-loop-helix domain located in the amino-terminus of the protein. The N-terminal basic domain consists of a short sequence of basic residues that directly interact with the major groove of DNA in the promoter region of target genes. This interaction is facilitated by the dimerization of Ahr with its DNA binding partner Arnt along the helix-loop-helix region. The HLH is comprised of two amphipathic alpha helices that are separated by a flexible loop. Upon dimerization, the HLH regions form a four-helix bundle that then stabilizes the dimer in a DNA-binding conformation (reviewed by Massari and Murre 2000).

Immunoprecipitation studies (Perdew 1988, Reyes *et al* 1992) revealed potential interactions of Ahr with the 90kDa heat shock protein (Hsp90) and the aryl hydrocarbon receptor nuclear translocator protein (Arnt), suggesting the presence of additional domains required for interaction with these proteins. In order to identify the site for interactions with these proteins and to locate additional functional domains, a series of deletion mutants were generated and expressed *in vitro* (Fukunaga 1995). This study, along with a similar experiment by Soshilov and Dension (2008) revealed precisely the amino acid sequences that compose the bHLH domain, the PAS A and PAS B domains, a ligand binding domain, the site for Hsp90 binding, as well as the C-terminal transactivation domain (TAD).

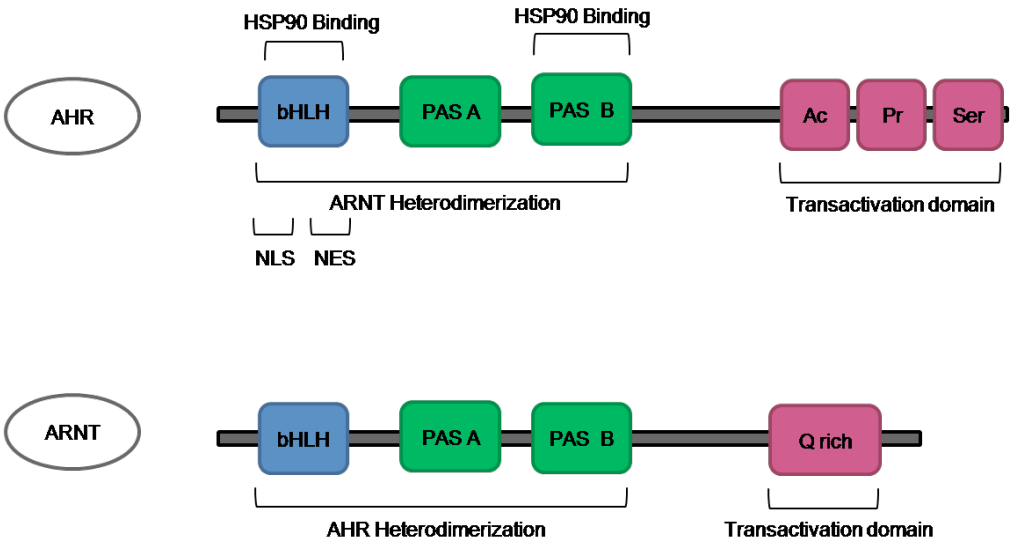


FIGURE 1.1: Ahr and Arnt Functional Domains. Ahr and Arnt share homology in their N-terminal regions, which encode conserved bHLH-PAS domains that function in DNA-binding and serve as a surface for heterodimerization of these two proteins. The bHLH of Ahr also functions in facilitating protein:protein interactions with Hsp90 and encodes a nuclear localization and nuclear export signal. Both Ahr and Arnt encode C-terminal transactivation domains that recruit transcription factors to the promoter region of target genes when Ahr and Arnt are bound together at enhancer elements of target genes.

Additional studies further characterized the TAD domain, which consists of three sub-domains shown to function synergistically (Ma *et* al 1995). Following DNA binding at enhancer sequences, certain co-activators and general transcription factors are recruited to the promoter region via the C-terminal transactivation domains of Ahr and Arnt (Hankinson 2005) to facilitate transcription initiation. Additionally, a putative nuclear localization signal (NLS) as well as a nuclear export signal (NES) was also identified in Ahr using sequence analysis (Ikuta *et* al 1998, Holmes and Pollenz 1997, Pollenz and Barbour 2000).

Cytosolic Unliganded Complex. The unliganded Ahr exists in a complex with two molecules of Hsp90 bound by the co-chaperone p23, and an immunophilin-like molecule called Xap2 as shown in Figure 1.2. Studies using the mouse Hepa-1 cell line, expressing the Ah^{b-1} isoform, revealed cytoplasmic localization of the unliganded receptor complex that is rapidly nuclearized following ligand binding (Pollenz *et al* 1993). However, analysis of other Ahr allelic variants, including Ah^{b-2} derived from mouse C_2C_{12} cells and the rat Ahr from A-7 smooth muscle cells, showed a conflicting localization pattern for the unliganded receptor complex (Pollenz and Dougherty 2005). In these cell lines, the unliganded receptor exhibits dynamic nucleocytoplasmic shuttling, however, the receptor was shown to accumulate in the nucleus following ligand binding similar to the Hepa-1 cell line.

Hsp90 is a molecular chaperone whose expression is up-regulated in response to cellular stress; however, in both stressed and unstressed cells Hsp90 plays a role in proper protein folding. Hsp90 interacts with newly synthesized proteins to facilitate folding, but also acts to stabilize and refold denatured proteins subjected to stress (Chen *et al* 2005). In this pathway, Hsp90 binds Ahr within the receptor's bHLH and PASB domains (Figure 1.1) (Soshilov and Dension 2008); it has been suggested that this

interaction maintains the receptor in an inactive state by shielding the nuclear localization signal (Ikuta *et al* 1998). Hsp90 binding has also been implicated in maintaining Ahr in a high affinity ligand binding configuration (Pongratz *et al* 1992).

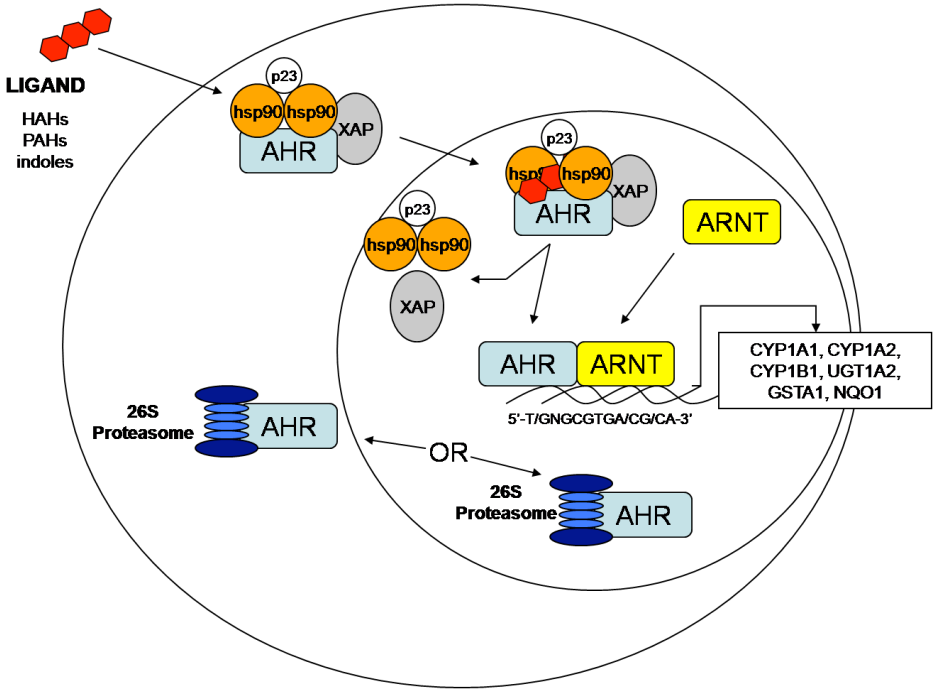


FIGURE 1.2: Canonical Ahr signaling pathway. In the absence of ligand, Ahr predominantly exists in a cytoplasmic complex with a dimer of Hsp90, p23 and Xap2. Upon ligand binding, the latent protein complex translocates to the nucleus of the cell whereby Ahr and Arnt heterodimerize, bind XREs in the 5' regulatory region of dioxin-responsive genes, and activate transcription of Phase I and Phase II xenobiotic metabolism genes. Following DNA binding, Ahr is rapidly degraded via the 26S proteasome.

The Ahr·Hsp90 interaction is maintained following ligand binding and nuclear localization, but when this complex was stabilized with the addition of sodium molybdate, Ahr was rendered unable to interact with its DNA binding partner Arnt or transactivate target genes. This result suggested that Hsp90 is dissociated from Ahr after the receptor enters the nuclear compartment. It is also likely that the constitutively nuclear Arnt protein therefore displaces Hsp90 due to a higher affinity for Ahr binding (Heid *et al* 1999). The co-chaperone protein p23 is associated with the Hsp90 dimer, as was

determined by immunoprecipitation studies (Kazlauskas *et al* 1999). This interaction occurs at the N-terminal ATP binding domain of Hsp90. It was also revealed that the dissociation of p23 from Ahr requires both ligand binding and Arnt protein in an *in vitro* expression system. This result confirmed that activation of the receptor from the latent complex to its transcriptionally active form occurs in the nuclear compartment such that Hsp90 and p23 are displaced in the presence of Arnt.

A yeast two-hybrid experiment identified an additional protein that interacts with the Ah receptor (Carver and Bradfield 1997, Ma and Whitlock 1997). Sequence analysis of the protein revealed three TPR (tetratricopeptide repeats) domains. TPR sequences are 34 amino acids long and form short α-helices to facilitate protein-protein interactions. The N-terminus of the protein contains a region of 80 amino acids with homology to the FKBP family of molecular chaperones (Carver and Bradfield 1997).

Interaction of the Xap2 protein (also referred to as Aip and Ara9) with Ahr was confirmed using immunoprecipitation experiments (Ma and Whitlock 1997, Carver *et al* 1998). The exact function of Xap2 (hepatitis B virus X-associated protein 2) in Ahr signaling is not completely understood, but it is believed to play a role in nucleocytoplasmic shuttling of the receptor (Lees *et al* 2003, Pollenz and Dougherty 2005, Pollenz *et al* 2006). Comparative analysis of Xap2 interaction with Ahr allelic variants demonstrated a relationship between Xap2 levels and nucleocytoplasmic shuttling of the unliganded receptor. The Ah^{b-1} allele, expressed in the C57BL mouse and Hepa-1 cells derived from that strain, is associated with 80-90% more Xap2 protein as compared to the Ah^{b-2} allele expressed in C_2C_{12} cells or the rat Ahr, as determined by immunoprecipitation experiments. The Ah^{b-1} allele is localized primarily in the cytoplasm, while the Ah^{b-2}, rat, and human Ahr demonstrate a dynamic shuttling between the nuclear and cytoplasmic compartment, this effect is attributed to reduced levels of Xap2 associated with the receptor (Ramadoss *et al* 2004, Pollenz *et al* 2006).

Ligand Interactions with Ahr. The Ahr signaling pathway becomes activated upon binding of ligand to the hydrophobic ligand binding pocket within the bHLH domain of the latent receptor. Ahr ligands are typically planar and hydrophobic molecules, most of which are synthetic compounds including halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons. Additionally, several naturally occurring compounds have also been identified as Ahr ligands.

Halogenated aromatic hydrocarbons (HAHs) are the most potent Ahr ligands due to their metabolic stability; HAHs include polyhalogenated dibenzo-*p*-dioxins, dibenzofurans, azobenzenes, naphthalenes, and biphenyls. Documented effects of exposure to these compounds include teratogenic, mutagenic, and carcinogenic effects. These compounds are lipophilic and are known to bioaccumulate as a result of dietary intake of fats. The most widely studied and most potent ligand of Ahr is TCDD (2,3,7,8 tetrachlorodibenzo-*p*-dioxin); it is produced as a byproduct during synthesis of 2,4,5 trichlorophenoxyacetic acid, the broad spectrum herbicide. Trace amounts of chlorinated dibenzofurans, another potent ligand, are produced as a byproduct of commercial production of the fungicide pentachlorophenol, used in paper manufacturing. Polychlorinated biphenyls (PCBs) were manufactured in the US until 1977 for commercial use as lubricants, plasticizers, and adhesives (Poland and Knutson 1982).

Polycyclic aromatic hydrocarbons (PAHs) are more metabolically labile compounds and are subsequently less potent ligands for the Ah receptor. PAH ligands include 3-methychloranthracene (3-MC), benzo(a)pyrene (BAP), benzanthracene, and benzoflavones. These compounds are produced as a byproduct of fossil fuel combustion (reviewed by Liu *et al* 2008). BAP is of particular interest as it is a carcinogen that is found in cigarette smoke. PAHs are also present in some foods

including oils, fats, and cooked meats; in this case they are produced as a byproduct of incomplete steroid metabolism (Larsen 1995).

Several endogenous compounds have been shown to bind Ahr, activate the receptor, and induce Ahr-dependent gene expression. These include indoles, tetrapyroles, arachidonic acid metabolites, and several carotinoids (Adachi *et* al 2001, Denison and Nagy 2003). Indoles, including indigo and indirubin, are tryptophan derivatives. Bilirubin is an example tetrapyrole and lipoxin A and prostaglandin G are archidonic acid metabolites that interact with Ahr.

Nuclear Import. Following ligand binding, Ahr undergoes a conformational change such that the basic nuclear localization signal (NLS) becomes exposed. The nuclear localization signal of Ahr is composed of a bipartite region spanning amino acids 12-41 (Song and Pollenz 2003). This sequence is bound by karyopherin-α, an adapter protein, that interacts with importin-β to mediate import via the nuclear pore complex (Pemberton and Paschal 2005). The intact Ahr·Hsp90·Xap2 complex is then rapidly translocated to the nuclear compartment (Pollenz *et al* 1993). Expectantly, disruption of the Ahr NLS resulted in a receptor defective for TCDD-dependent nuclear import (Song and Pollenz 2003). Studies supporting this model were carried out using the Ah^{b-1} allelic variant which has been shown to exhibit a predominantly cytoplasmic localization in the absence of ligand. Alternatively, rat Ahr, human Ahr, and mouse Ah^{b-2} alleles appear to undergo dynamic nucleocytoplasmic shuttling in the absence of ligand and exposure of each of these cell types to ligand resulted in a predominantly nuclear localization of Ahr (Pollenz and Dougherty 2005).

Heterodimization with Arnt and DNA binding. The aryl hydrocarbon receptor nuclear translocator (Arnt) protein is a bHLH-PAS protein whose expression is restricted

to the nuclear compartment of higher eukaryotic cells (Pollenz *et al* 1993). In 1992, Reyes *et al* identified Arnt as a component of the DNA binding form of the Ah receptor and sequence analysis suggested that Ahr and Arnt form a heterodimer along their bHLH-PAS domains.

Domain analysis using deletion mutants provided insight into some of the functional aspects of Arnt. The basic region of Arnt facilitates DNA binding, while the HLH and PAS domains both contribute to Ahr binding. Additionally, a C-terminal glutamine rich domain is required for transcriptional activity and can function independently of the other protein domains (Li *et al* 1994, Reisz-Porszasz *et al* 1994).

DNA binding of the activated Ahr complexed with Arnt takes place at enhancer sequences located in the promoter regions of target genes. The consensus sequence for these XREs (xenobiotic response elements) differs from the traditional E-box recognized by most members of the bHLH superfamily. The basic regions of Ahr and Arnt bind the consensus DNA sequence 5'-TNGCGTG-3' (Denison *et al* 1988) with half-site specificity such that Ahr interacts with the 5'-TNGC-3' sequence and Arnt binds the 5'-GTG-3' sequence (Swanson *et al* 1995). The CYP1A1 gene, a well studied target for Ahr mediated induction, has eight XREs located in its upstream regulatory sequence. Following Ahr·Arnt binding, the promoter region of CYP1A1 undergoes chromatin remodeling to allow adequate room for binding of transcriptional machinery.

Transcription Activation of Drug Metabolizing Enzymes. Ahr modulates the expression of a number of genes that encode phase I and phase II drug metabolizing enzymes including: cytochrome P450 1A1 (CYP1A1), CYP1A2, CYP1B1, glutathione-*S*-transferase Ya subunit, plasminogen activator inhibitor 2, interleukin 1, and UDP-glucuronsyltransferase 1A1 (Poland and Knutson 1982).

Ahr and Arnt proteins convey a signal to initiate promoter occupancy and transactivation by way of their carboxyl terminal regions (Jain *et al* 1994, Ma *et al* 1995, Ko *et* al 1997, Whitlock 1999). Within the Ahr TAD region, exists three sub-domains: an acidic region, a proline-rich region, and a serine-rich domain (Ma *et al* 1995, Ko *et al* 1997). The acidic domain spans amino acids 515-583, is rich in glutamic acid and aspartic acid residues (24%) and has the strongest transactivation potential of the three sub-domains. Amino acids 643-805 are composed of 13% proline residues and amino acids 726-805 are 16% serine residues. The latter two sub-domains exhibit weaker, but still independent abilities to activate transcription.

Ahr is phosphorylated within the C-terminal domain (Mahon and Gasiewicz 1995) likely mediated by PKC. PKC (Protein Kinase C), a serine/threonine kinase, is essential for transactivation following Ahr/Arnt binding at XREs. Subsequent to phosphorylation, Ahr is capable of recruitment and assembly of the transcription initiation complex (Chen and Tukey 1996, Long *et al* 1998).

Transcriptional adaptors called co-activators have been implicated in Ahr gene regulation (Nguyen *et al* 1999, Beischlag *et al* 2002, Hankinson 2005). In general, co-activators are recruited to DNA-bound transcription factors and communicate with proteins in the core transcription initiation complex. They also facilitate transactivation by way of their histone acetyltransferase activity which confers a more relaxed chromatin structure (Beischlag *et al* 2002). Co-activators interact with a short α-helical LXXLL motif located within the protein's transactivation domain (Flaveny *et al* 2008). Specific co-activators shown to influence Ahr-mediated gene regulation include: SRC-1(steroid receptor co-activator 1), NCoA-2 (nuclear co-activator 1), p/CIP (p300/CBP co-integrator protein), p300, and CBP (CREB binding protein) (Hankinson 2005) and it is believed that there may be some combination of co-activators involved in both chromatin remodeling as well as transcription initiation events.

Nuclear Export. As nuclear export appears to occur following Ahr-mediated transactivation of target genes, it is believed that nuclear export functions in regulation of the dose of gene induction. The Ahr nuclear export signal (NES), amino acids 63-71, is a leucine-rich sequence located within the helix 2 domain (Pollenz and Barbour 2000). The nuclear export receptor CRM-1 interacts with the NES and mediates its transfer through the nuclear pore complex (Pemberton and Paschal 2005). Expectedly, mutation of the Ahr NES resulted in accumulation of receptor in the nucleus following ligand treatment (Pollenz and Barbour 2000) that was accompanied by an increase in Ahr-mediated transcription activation.

Degradation of the Aryl Hydrocarbon Receptor

Turnover of the Ah receptor in mammalian cells can be attributed to three cellular circumstances with each playing a distinct role in the downstream gene regulatory effects of the receptor. First, the normal half-life of the latent receptor was determined via pulse-labeling Hepa1c1c7 cells grown in culture wherein the half-life was found to be approximately 28hrs (Ma and Baldwin 2000). In addition to normal protein turnover, ligand-mediated degradation of the receptor has been observed upon treatment with TCDD and similar compounds which thereby reduced the receptor half-life to approximately 3hrs (Ma and Baldwin 2000, Pollenz 1996). Finally, ligand-independent degradation of the receptor occurs following disruption of the interaction between Ahr and a dimer of Hsp90 upon treatment with benzoquinone ansamycin antibiotics (Chen et al 1997, Song and Pollenz 2002).

Ahr Half-life. The half-life of unliganded Ahr was first evaluated by Swanson and Perdew in 1993 using ligand binding to measure receptor protein levels in sucrose

density gradients of Hepa-1 cells. The results suggested that the *t* ½ for unliganded receptor was approximately 7.7 hours. When treated with the agonist βNF, the half-life of the receptor was extended to approximately 9.7 hours, while treatment with the partial antagonist αNF yielded a half-life similar to the unliganded receptor (Swanson and Perdew 1993). This result was in contrast to observed reduction in receptor levels following TCDD treatment (Prokipcak and Okey 1991). These conflicting results were attributed to variation in ligand lability such that metabolism of βNF allows receptor levels to recover more rapidly than when treated with TCDD, but is also likely due to the choice of technique.

To definitively determine the receptor half-life, Ma and Baldwin (2000) evaluated the Ahr^{b-1} *t* ½ in the presence and absence of TCDD using the more exact method of pulse-chase labeling. Immunoprecipitation following pulse-chase with [^{35}S] methionine revealed the receptor half life to be 28 hours in the absence of ligand and 3 hours following ligand treatment. Interestingly, this type of analysis has not been carried out to verify the half-life of the other Ahr mouse variants, in other species, or when expressed heterologously in other model organisms.

Ligand-mediated Ahr Degradation. Ahr degradation was first described using mouse Hepa-1 (liver hepatoma) cells following treatment with [^{3}H] TCDD (Prokipcak and Okey 1991). Nuclear and cytoplasmic fractions revealed reduction in cytoplasmic receptor in conjunction with an increase in nuclear receptor levels within two hours of ligand treatment. Subsequently nuclear receptor levels were reduced to background levels within six hours, with no reaccumulation in the cytoplasmic fraction. This result suggested that the protein was not simply being shuttled back to the cytoplasm after nuclearization, but that it was being degraded.

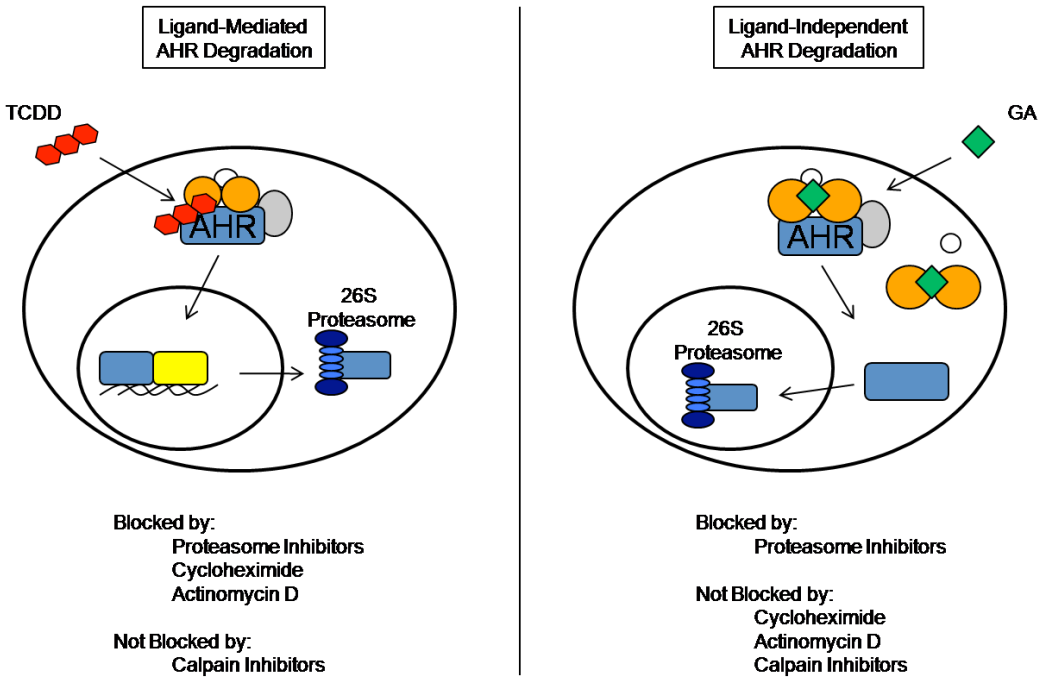


FIGURE 1.3: Mechanisms of Ahr degradation. Ahr degradation occurs via two distinct mechanisms. First, ligand-mediated degradation occurs after several events subsequent to ligand binding. The latent Ahr complex is translocated to the nuclear compartment, heterodimerizes with Arnt, and activates transcription of genes that encode xenobiotic metabolizing enzymes. Ahr is then exported to the cytoplasm where it is degraded via the 26S proteasome. This mechanism of degradation is blocked with proteasome inhibitor treatment and when active transcription and translation are blocked in the cell. An alternative degradation occurs following treatment with Hsp90 inhibitors such as geldanamycin (GA). The dimer of Hsp90 is unable to interact with Ahr which is then translocated to the nucleus. Here, Ahr does not interact with Arnt or bind DNA, but it is rapidly degraded by the 26S proteasome in the nuclear compartment. This mechanism is also blocked by proteasome inhibitors; however, treatment with transcription and translation blockers do not block Ahr turnover as is seen in the ligand-mediated system. Additionally, neither mode of degradation is blocked with calpain inhibitor treatment.

Immunofluorescence microscopy and western blotting of mouse Hepa-1 cells provided visual evidence confirming ligand-dependent receptor degradation (Pollenz 1996). TCDD treatment reduced the total Ahr pool by 85% within four hours of treatment while the amount of Arnt in the same lysates remained constant. Nuclear and cytoplasmic fractions revealed that the receptor was predominantly localized in the cytosol prior to addition of ligand and was maximally nuclear within one hour of TCDD treatment. These fractions also revealed a predominantly nuclear localization for Arnt

that was unchanged in the presence or absence of TCDD. Additionally, immunofluorescence microscopy of cells dosed with ligand for various time points mirrored the localization of the receptor that was demonstrated via western blotting (Pollenz 1996; reviewed by Pollenz 2002).

To confirm that loss of receptor was directly related to turnover of the protein as opposed to a block in transcription at the Ah locus, quantitative PCR was performed in order to evaluate the mRNA levels of Ahr over a seventy-two hour TCDD treatment. Messenger RNA levels for Ahr and Arnt were unchanged throughout the time course, while induction of P450 mRNA at one hour served as a control for the experiment (Giannone et al 1998). Certain that the observed reduction in receptor level was due to some targeted turnover of the protein itself, researchers began investigating the mechanism responsible for Ahr degradation.

Initial experiments entailed co-treatment of cells in culture with ligand and various protease inhibitors. Multiple groups demonstrated that MG-132, a compound used to inhibit the 26S proteasome, effectively prevented degradation of the ligand-activated receptor (Ma and Baldwin 2000, Roberts and Whitelaw 1999, Song and Pollenz 2002). Additionally, calpain inhibitors were tested and unable to prevent loss of the protein (Davarinos and Pollenz 1999, Roberts and Whitelaw 1999, Ma and Baldwin 2000, Pollenz 2007). Stabilization of liganded Ahr with protease inhibitors also led to an increase in cytochrome P450 expression levels (Ma and Baldwin 2000), further demonstrating the importance of understanding the degradation mechanism, as the removal of the receptor is the means in which the pathway is turned off.

Next, researchers investigated the cellular compartment in which Ahr degradation was taking place. Roberts and Whitelaw (1999) generated a constitutively nuclear Ah receptor (Ahr-NLS) found to have a very short half-life (less than one hour). The Ahr-NLS turned over rapidly in the presence or absence of ligand and when

complexed with Hsp90 or with Arnt, suggesting that the nuclear compartment is the site for receptor degradation. In contrast, a report by Davarinos and Pollenz (1999) suggests that Ahr degradation occurs in the cytoplasm following TCDD treatment, and nuclear import followed by nuclear export of the receptor. Treatment with leptomycin B (LMB), a fungal antibiotic that inhibits the function of nuclear export proteins, in combination with ligand caused an accumulation of Ahr in the nuclear compartment and as such the ligand-mediated Ahr degradation was blocked. This conclusion was further validated through the use of an Ahr with a defective nuclear export signal (AhrΔNES). Receptor turnover in the transiently expressing AhrΔNES strain was reduced when compared to wild-type Ahr turnover, as observed via western blotting and immunofluorescence microscopy (Davarinos and Pollenz 1999). The degradation site was again evaluated in AhrΔNLS mutants (Song and Pollenz 2003) whereby receptor turnover occurred with ligand treatment even when nuclearization was blocked. This study, along with several others (Pollenz and Dougherty 2006, Pollenz *et al* 2006) provided further evidence for ligand-mediated Ah receptor degradation that takes place in the cytoplasm.

Ahr degradation studies have come to focus largely on the exact manner in which Ahr is targeted by the ubiquitin-proteasome pathway for destruction. The Ahr protein has been investigated for potential sites wherein a conformational change or post-translational modification may lead to targeted degradation. Other components of the latent receptor complex as well as components of the active transcriptional activation complex have been implicated in targeting the receptor for turnover.

Sites for specific post-translational signals that may trigger ligand-mediated degradation were investigated. Treatment of the constitutively nuclear Ahr (Ahr-NLS) with phosphatase inhibitors produced a higher molecular weight protein as detected by western blotting, suggesting that the protein exists in a phosphorylated state. Ahr-NLS expressing cells were treated with combinations of ligand, MG132, and phosphatase

inihibitor and protein samples were immunoprecipitated with Ahr-specific antibody. Detection of ubiquitin by antibody staining was significant when cells were exposed to all three treatments such that Ahr was activated by ligand but could not be degraded by the 26S proteasome. It was therefore suggested that phosphorylation of the receptor following nuclearization is required for ubiquitination and subsequent degradation to occur (Roberts and Whitelaw 1999).

Pollenz *et al* (2005) evaluated the role of the transactivation domain in ligand-mediated Ahr degradation in order to determine if transcription regulation is required prior to the degradation event. Truncation mutants were generated and stably expressed in the LA-1 Hepa-1 variant line. The AHR_{500} mutant, lacking all three TAD regions, and the AHR_{640}, lacking two of the three TADs showed no CYP1A1 induction and reduced CYP1A1 induction as compared to wild-type Ahr, respectively. Though transactivation was reduced, these Ahrs were degraded following ligand treatment and degradation could be blocked with MG132 treatment suggesting that transcriptional activation is not required for Ahr turnover.

Arnt's role in Ahr turnover was investigated by Pollenz (2005) using a C4 Hepa-1 variant that expresses reduced levels of the Arnt protein with wild-type levels of Ahr. Upon TCDD treatment, Ahr levels were slightly reduced, but the majority of the receptor pool remained. In contrast, cells expressing a full complement of Arnt protein in conjunction with normal levels of Ahr, TCDD treatment greatly reduced Ahr levels as compared to vehicle-treated controls. These results show that Ahr degradation requires dimerization with Arnt and likely also requires a DNA-binding event that occurs upon dimer formation.

Giannone *et al* (1995 and 1998) demonstrated an Ahr stabilization effect in response to treatment with ligand and actinomycin D (AD), a transcription inhibitor. This effect was also observed in Hepa-1 cells treated with ligand and cycloheximide (CHX),

an inhibitor of eukaryotic translation. Blocked degradation was also observed in rat smooth muscle cells and mouse 10T1/2 embryonic fibroblasts that express the Ah^{b-2} allele (Pollenz et al 2005). These results suggest that expression of an auxiliary protein with a rapid turnover is an additional component of the degradation machinery.

Ligand-independent Ahr Degradation. Ligand-independent degradation of the receptor occurs upon administration of Hsp90 inhibitors such as geldanamycin (GA). GA, a benzoquinone ansamycin antibiotic, prevents heterodimerization of Hsp90 with the receptor and thus results in exposure of the Ahr nuclear localization signal in the amino-terminus of the receptor's protein sequence. This event subsequently induces rapid nuclear localization and degradation of the receptor in the absence of ligand (Song and Pollenz 2002). Two other Hsp90 inhibitors, herbimycin and novobiocin have been shown to reduce Ahr signaling in reporter assays by interacting with the ATPase/p23-binding site in the N-terminus of Hsp90. The unrelated compound radicicol has also been shown to disrupt ATP and p23 binding in the N-terminus of Hsp90 (Cox and Miller 2002). These studies suggest that Hsp90 plays a role in stabilizing Ahr in the latent complex.

In mammalian tissues, administration of Hsp90 inhibitors causes the Ah receptor to translocate to the nucleus where it is degraded within 3 hours of treatment, as compared to 6 hours with TCDD treatment. Interestingly, the nuclear accumulation of Ahr does not occur in conjunction with transcription activation of target genes (Song and Pollenz 2002). GA-mediated degradation was blocked when Hepa-1 cells were pre-treated with MG132, providing that turnover is still mediated by the 26S proteasome although it is occurring at a much more rapid rate.

Further experimentation in order to evaluate the mechanism of ligand-independent degradation demonstrated treatment with leptomycin B (LMB), which was

shown to block ligand-mediated degradation, does not inhibit this degradation and Ahr levels are drastically reduced in cells treated with GA in combination with LMB. This effect suggests that the degradation machinery responding to GA treatment resides in the nucleus of the cells. Importantly, cycloheximide (CHX) also did not prevent the GA-mediated degradation of the receptor (Pollenz et al 2005) suggesting that expression of an additional short-lived protein is not essential for this mechanism of degradation and thus providing additional evidence that the ligand-dependent and independent mechanism of degradation are occurring via distinct pathways.

The TAD truncation mutants generated by Pollenz (2005) were evaluated for Ahr turnover with actinomycin D (AD) and cycloheximide (CHX) treatments. While it was previously mentioned that these truncated Ahrs degraded following ligand binding, it should be noted that the proteins degraded significantly faster (2.5 X) than the wild type Ahr. Also, AD and CHX treatment did not block turnover of AHR_{500} and AHR_{640} following ligand exposure. Together, the increased rate of turnover along with the non-effect observed with transcription and translation inhibitor treatment suggests that the TAD mutants are degrading in a fashion similar to Ahr with disrupted Hsp90 chaperoning.

As demonstrated using protease inhibitors, ligand-mediated as well as ligand-independent activation and degradation of the aryl hydrocarbon receptor occurs by way of proteolysis via the 26S proteasome (Ma and Baldwin 2000, Pollenz et al 2005), but as yet, the manner in which the receptor is targeted for degradation is unclear.

Suspected Ligases involved in Ahr Degradation. Several reports have implicated Chip (Carboxyl terminus of Hsp70-interacting protein) as the particular E3 ubiquitin ligase responsible for targeting Ahr for proteasomal degradation (Lees et al 2003, Morales and Perdew 2007). Chip is a U-box dependent ubiquitin E3 ligase shown to interact with molecular chaperones and target their substrates for degradation (Jiang *et*

al 2000). With Hsp90's crucial role in receptor stability and degradation, a role for this ligase in receptor degradation seemed promising. However, several reports (Pollenz and Dougherty 2005, Morales and Perdew 2007) refuted its role as a mediator of Ahr's targeted degradation by the proteasome. While both Ahr and Hsp90 have been shown to interact with Chip via immunoprecipitation, there has been no evidence of a function in degradation in this system. Ligand-dependent Ahr degradation was observed following knock down of Chip using siRNA in Hepa-1 cells and in cells derived from a Chip-knockout mouse strain, therefore suggesting that some other E3 is responsible for targeted Ahr degradation. While, disrupting this interaction *in vivo* does not produce an effect on ligand-mediated receptor degradation, binding of Chip to Ahr or Hsp90 may plausibly play a role in degradation of misfolded receptor.

Ahr itself has been implicated as an E3 ligase when complexed with cullin 4B protein in the presence of Ahr ligands, TCDD or 3-methylcholanthracene (Wormke *et al* 2003, Ohtake *et al* 2007). This work suggested a role for Ahr in mediating the proteasomal degradation of the estrogen receptor (ER) protein when cells were treated with certain Ahr ligands. Immunoprecipitation experiments demonstrated an interaction between the Ahr·cullin4B complex and ER, while ER turnover was reduced when Ahr or cullin 4B levels were knocked down using siRNA. However, specific degradation of Ahr was unaffected when cullin 4B was knocked down using siRNA, therefore suggesting that Ahr does not likely act as an E3 ligase for its own proteolytic degradation.

Degradation Mechanisms

Ubiquitin-Proteasome Pathway. The ubiquitin-proteasome pathway includes a group of proteins involved in a highly selective mechanism of rapid protein turnover that is required in order to carry out essential regulatory processes (Ciechanover and Schwartz 1994, Jentsch 1992). Cell cycle control, transcriptional regulation, and antigen

processing in immune response are examples of cellular events in which the degradation of proteins by the ubiquitin-proteasome pathway is required (Hochstrasser 1996).

One role of the ubiquitin proteasome pathway is to function in locating, identifying, and degrading abnormal proteins in order to eliminate them from the cellular protein pool. Aberrant proteins including truncated (Kohlmann *et al* 2008), misfolded (Betting and Seufert 1996), improperly chaperoned (Hayes and Dice 1996), and improperly post-translationally modified proteins (Liu 1999) have been shown to be targeted for degradation by this mechanism.

Another role of the ubiquitin-proteasome pathway is its function in mediating the turnover of intact intracellular proteins. In general, proteins degraded by the 26S proteasome are targeted for degradation via poly-ubiquitination whereby identification and degradation of these substrates is extremely selective. Ubiquitin is a 76 amino acid peptide that is conserved among all eukaryotes and contains only three amino acid substitutions between yeast and human (Jentsch 1992).

Ubiquitin is linked to the targeted protein by way of three classes of enzymes. First, the ubiquitin molecule is conjugated to the E1 ubiquitin-activating enzyme between the ubiquitin α-carboxyl group and a cysteine residue located within the active site of the E1 enzyme (Ciechanover and Schwartz 1994). Ubiquitin E1 enzymes are encoded by 9 genes in humans and there are three known E1s in yeast. It is believed that different E1s may be expressed in different cellular compartments or may interact preferentially with various E2 ligases or substrates (Hochstrasser 1996). Subsequently, ubiquitin is transferred from the E1-ubiquitin intermediate to an E2-ubiquitin complex. The E2 enzyme is also referred to as the ubiquitin-conjugating enzyme or ubiquitin carrier protein and it becomes linked with ubiquitin at a specific cysteine residue. From here, the E2 can either donate the ubiquitin to an E3 ubiquitin ligase or the E2 can catalyze

substrate ubiquitination (Jentsch 1992, Pahl and Baeuerle 1996). Thirteen genes encode yeast E2 enzymes and there are over 30 human E2 genes identified.

E3 ubiquitin ligases have been shown to function in recognition of target proteins and recruitment of ubiquitin-E2 complexes in order to catalyze formation of the iso-peptide bond between ubiquitin and lysine residues of protein substrates (Ciechanover and Schwartz 1994). An exact method for classification and identification of E3 enzymes is still imprecise but thus far, enzymes are classified as E3s if they have been shown to stimulate substrate ubiquitination when combined with the appropriate E1 and E2 enzymes and have been shown to bind both the E2 and the substrate proteins (Hochstrasser 1996). The interaction between E3s and substrate proteins are believed to be transient, thus further complicating their identification and classification.

There are three subfamilies of E3 ligases that have been classified based on sequence homology; the HECT-domain containing E3s, the RING finger domain containing E3s, and the UBox E3s (Bernassola *et al* 2008). It is suggested that each subfamily has a specific set of substrates and within each subfamily there are further levels of classification resulting in even more precise targeting of substrates. It is the E3s that have been implicated in the extreme level of specificity in substrate recognition in turnover of intact cellular proteins by the 26S proteasome.

Lastly, E4 enzymes have more recently been shown to catalyze the addition of ubiquitin moieties to Lysine48 of the ubiquitin molecule that is substrate bound and form a multiubiquitin chain that is more efficiently targeted for degradation by the proteasome (Hochstrasser 1996, Koegl *et al* 1999).

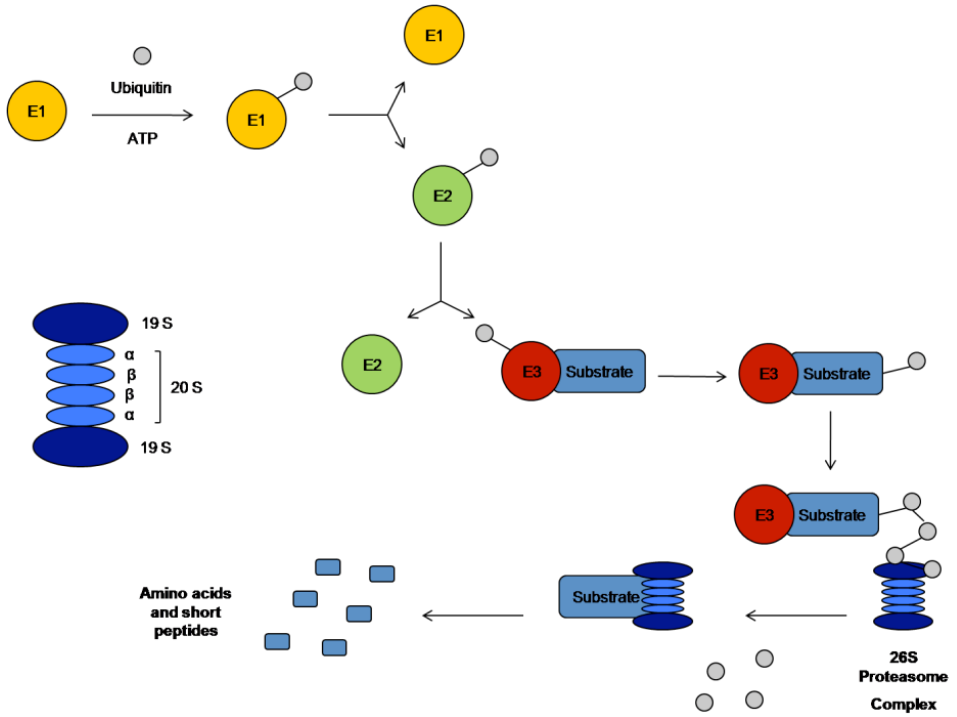


FIGURE 1.4: Ubiquitin-Proteasome Pathway. Targeted degradation of intracellular proteins via the ubiquitin-proteasome pathway is mediated by three classes of enzymes. The E1 ubiquitin-activating enzyme binds ubiquitin and facilitates its transfer to the E2 ubiquitin-conjugating enzyme. The E2 then donates ubiquitin to an E3 ubiquitin-ligase. The E3 is typically bound to the substrate for degradation and therefore transfers ubiquitin to the target protein. E4 enzymes can function in ubiquitin chain elongation and the ubiquitin chains are then recognized by the 19S regulatory subunit of the 26S proteasome complex. Lastly, the protein is proteolytically cleaved in the 20S catalytic domain of the proteasome.

The mechanism by which substrates are identified by these enzymes has been extensively investigated but still remains largely unclear. Some protein substrates contain sequence elements that serve as degradation signals. For example, the identity of the N-terminal amino acid of proteins has been shown to correlate with its *in vivo* half-life as mediated by the ubiquitin-proteasome pathway (Varshavsky 1997). This effect is termed the "N-end Rule" and suggests that arginine, lysine, histidine, phenylalanine, leucine, isoleucine, tryptophan, and tyrosine are considered destabilizing N-terminal residues in eukaryotes. The E3 ligase has binding sites for basic and bulky hydrophobic N-terminal residues such that it interacts with the substrate and triggers ubiquitination. A

9 amino acid sequence called a destruction box has been shown to be required for turnover of cyclins that mediate cell cycle progression (King et al 1996). Another sequence that has been identified is called a PEST element since these regions are rich in proline, glutamic acid, serine, and threonine. PEST regions have been implicated as targets for protein degradation (Rechsteiner and Rogers 1996). Finally, post-translational modifications including phosphorylation of particular residues have been shown to be signals for ubiquitination (Hochstrasser 1996, Kornitzer and Ciechanover 2000).

The 26S proteasome, so named for its sedimentation coefficient, resembles a dumbbell such that the catalytic 20S proteasome appears as the cylindrical handle with two regulatory 19S subunits attached to opposite ends of the 20S subunit in an ATP-dependent manner (Tanaka 1998). The 20S catalytic component of the proteasome is composed of 28 peptides, whereby fourteen dimeric subunits are arranged in rings to form a barrel structure such that the catalytic sites are located on the interior of the cylinder and are shielded from the cytoplasm (Kornitzer and Ciechanover 2000). The 19S complexes, located on either end of the 20S complex have been implicated in recognition of ubiquitinated proteins and serves as the entry point into the catalytic 20S complex (Ciechanover and Schwartz 1998). The 19S complexes contain ubiquitin chain binding proteins which bind with high affinity to ubiquitinated substrates. In addition to binding ubiquitinated proteins, the 19S complexes have the ability to unfold substrates in order to allow the proteins to enter the catalytic 20S core for degradation (Kornitzer and Ciechanover 2000, Tanaka 1998). Another component of the 19S complex consists of a deubiquitinating enzyme that serves to free ubiquitin moieties from mulit-ubiquitin chains for recycling (Tanaka 1998).

Following deubiquitination, the protein is translocated into the 20S catalytic domain for destruction. The 20S proteasome contains six protease sites within the

barrel structure; these catalytic sites mediate degradation via an amino-terminal threonine residue that undergoes a nucleophilic attack on a lysine residue of the target protein (Pahl and Baeuerle 1996). Substrates are subsequently cleaved into small peptides ranging from 4 to 24 amino acids in length. Lactacystin, epoxomicin, and MG-132 are examples of inhibitors of the 26S proteasome that covalently bind to the reacting threonine residue with carboxy-terminal aldehyde groups such that nucleophilic attack is prevented (Pahl and Baeuerle 1996).

Again, this pathway has been implicated in mediating ligand-dependent and independent Ahr degradation in all model organisms thus far examined. At this time there are still many unanswered questions regarding the exact mechanism for Ahr degradation via the proteasome, most notably, the identification of the E3 ligase and its site for interaction within the receptor are still unknown entities. Additionally, receptor half-life and mechanism of degradation have yet to be analyzed in *Saccharomyces cerevisiae* despite a great deal of work on Ahr in this model.

Calpain Family of Proteases. Calpains are ubiquitously expressed cysteine proteases that become activated in response to increased intracellular calcium levels. Specifically, μ-calpain is activated in response to micromolar influxes of Ca^{2+}, while m-calpain is activated with millimolar influxes of Ca^{2+} (reviewed by Sorimachi *et al* 1997 and Goll *et al* 2003). Each enzyme consists of two subunits that function in Ca^{2+} binding and proteolysis. There exists one calpain homologue in yeast called p83, it encodes a cysteine protease domain similar to the mammalian calpains, however it does not code for a calcium binding domain. Further analysis of this protein is needed in order to characterize its function in yeast.

One group published a report that calpains mediate Ahr turnover in a ligand-dependent manner (Dale and Eltom 2006) in contrast to all of the evidence supporting a

26S proteasome-mediated degradation event. This work was based on the hypothesis that Ahr ligand exposure causes an increase in intracellular calcium levels, thereby activating calpain proteases that subsequently degrade the receptor. These results were refuted by Pollenz (2007) who tested the effect of several calpain inhibitors on the Ahr signaling pathway showing no effect at the level of transactivation or degradation.

Endogenous and Recombinant Expression of bHLH Proteins in Yeast

Endogenous bHLH Proteins in Yeast. A number of genes encoding basic helix-loop-helix (bHLH) proteins have been identified in the yeast genome and their functions have been investigated due to their major role in gene regulation in higher eukaryotes. The yeast genome was compared to genes encoding mammalian and *Drosophila* bHLH proteins in order to identify yeast genes with potential homology (Robinson and Lopes 2000). While several genes were identified as containing bHLH domains, it should be noted that there are no homologues to any bHLH-PAS transcription regulators.

The first identified yeast gene shown to encode a bHLH protein, *PHO4*, acts as a transcription factor and activates several genes in response to phosphate starvation (Berben *et al* 1990). The Pho4 protein, which is encoded by the *PHO4* gene, binds to the core bHLH consensus sequence, 5'-CACGTG-3', which is located upstream of several genes involved in phosphate uptake. *INO2* and *INO4* are yeast bHLH genes whose protein products (Ino2 and Ino4) have been shown to form a heterodimer both *in vitro* and *in vivo*, but both are incapable of forming homodimers (Robinson and Lopes 2000). Ino2 and Ino4 bind at 5'-CATGTG-3' sequences located upstream of more than 30 genes involved in phospholipid biosynthesis (Greenberg and Lopes 1996).

Other yeast bHLH proteins have been shown to act as transcription factors in activation of genes required for glycolysis, genes involved in regulation of filamentous growth, and methionine biosynthesis. The *CBF1* gene encodes a bHLH protein and

binds a particular sequence located in centromeres. While this is atypical for bHLH proteins, the expression of *CBF1* is required to maintain chromosomal integrity (Robinson and Lopes 2000).

Expression of Heterologous Proteins in Yeast. Saccharomyces cerevisiae can be used in order to characterize genes and proteins that are not endogenously expressed. One major advantage to using yeast as opposed to mammalian tissue culture for this type of analysis is that yeast have the ability to homologously recombine similar sequences permanently into the genome. The double-stranded DNA break repair pathway is activated when the PCR product is introduced into the cells, such that the repair mechanism searches for homology in the integrating sequence. This preference allows for integration of double-stranded DNA of interest into the yeast genome in a highly directed and efficient manner (Ito *et al* 1983, Lorenz *et al* 1995). This recombination can facilitate either knocking a gene into the genome in a nonessential region or knocking a gene out simply by disrupting the target sequence or replacing it entirely.

Expression of Mammalian bHLH Proteins in Yeast. Saccharomyces cerevisiae has been used as a model organism in which non-conserved transcription factor signaling pathways have been reconstructed and subsequently dissected in order to evaluate various steps in gene regulation. Knock-in yeast strains have been generated that express members of the bHLH and bHLH-PAS family of transcription regulators including; Hif-1 (Braliou *et al* 2006), c-Myc (Amati *et al* 1992, Escamilla-Powers and Sears 2007, Hanel *et al* 1997), Ahr (Miller 1997, Miller *et al* 1998). The studies provided insight into various aspects of these signaling pathways; however, the majority of the

data analyzed consisted of reporter studies with little emphasis on analysis at the protein level.

Hif-1α is a bHLH-PAS receptor that mediates the cellular response to hypoxia. Braliou *et al* (2006) reconstituted the Hif-1α signaling pathway in yeast by transforming yeast cells with HIF-1α and ARNT galactose-inducible expression plasmids. Reporter studies confirmed Hif/Arnt heterodimerization and demonstrated transcription of a *lacZ* reporter by way of HRE (hypoxia response element) binding. The recombinant strain was also treated with Hsp90 inhibitors that resulted in reduced reporter activity; this effect can be attributed to an interaction between Hif and Hsp90 that is critical for transactivation. Finally, plasmids were constructed to express Hif with C-terminal truncations in order to identify dominant negative mutants that would function in blocking Hif-mediated transactivation (Braliou 2006). Importantly, these results were reported in terms of a series of reporter assays and did not evaluate the level of protein expression or the stability of the expressed proteins.

In the Amati *et al* (1992) study, the investigators generated a recombinant yeast strain expressing the mammalian c-Myc and Max proteins. This study showed that c-Myc requires its helix-loop-helix partner Max in order to interact with specific sequences of DNA containing the 5'-CACGTG-3' core consensus sequence to transactivate target genes. In later studies Hanel *et al* (1997) further investigated transactivation of c-Myc and Max using reporters constructed with natural promoter elements. Similar to the experiments with Hif, these studies used reporters as an output for c-Myc function in yeast and did not assess the level of protein expression or the stability of the expressed proteins. A more recent study of c-Myc and Max has evaluated the stability of the proteins in a recombinant yeast model. Escamilla-Powers *et al* (2007) used a c-Myc·Max recombinant strain to evaluate the phosphorylation state of Myc in conjunction with its stability. The results of this study suggest that Myc turnover is directly related to

the phosphorylation state of particular stabilizing and destabilizing residues confirming a similar effect has been demonstrated in mammalian cell culture. While Myc is a bHLH protein, it is not in the bHLH-PAS family that contains the Ahr and Arnt.

Ahr Studies in Yeast. Studies of the Ahr signaling pathway in yeast have been used largely to elucidate the role of molecular chaperones and co-chaperones in Ahr function. For example, studies in yeast have evaluated the auxiliary proteins found in the cytoplasmic latent receptor complex such as: Hsp90 (Carver *et al* 1994, Cox and Miller 2003, Whitelaw *et al* 1995), Xap2 (Miller 2002), and p23 (Cox and Miller 2002, Cox and Miller 2004). Reports from these studies have demonstrated that the yeast homologues for Ahr chaperones generally play a conserved role in Ah receptor signaling.

The first studies to assess Ahr signaling using a yeast model were published by Carver *et al* (1994). Human Ahr and Arnt proteins were cloned into a low copy yeast expression vector (CEN) and were constitutively expressed in an Hsp82 temperature sensitive yeast strain such that Hsp82 levels could be modulated with increasing or decreasing temperature. Cultures were grown at the permissive and restrictive temperatures and a XRE-driven *lacZ* reporter assay suggested that Hsp82 is an essential protein for Ahr signaling in yeast and similarly, Hsp90 is required for Ahr signaling in higher eukaryotes. Work by Cox and Miller (2003) and Whitelaw *et al* (1995) further confirmed that Hsp82 is an essential component of the reconstituted signaling pathway, again using the same β-galactosidase reporter assay in these studies. The level of Ahr and Arnt protein expression was never evaluated in these studies.

In 1997, Miller generated a strain of yeast expressing human Ahr and Arnt by way of a bidirectional *GAL1/GAL10* promoter. This promoter is activated with the addition of galactose to the growth medium and is turned off with the addition of glucose

to the media. The *GAL1/GAL10* promoter was utilized to allow for greater control over protein expression levels in order to prevent toxicity due to overexpression; however, the cDNAs were cloned into a 2μm high copy-number plasmid. The previous Ahr strains, expressed from plasmid encoding centromeric sequences likely contained 1-3 copies of the expression vector, while this strain carried 10-40 copies. No analysis of Ahr or Arnt protein expression was carried out in these studies. Interestingly, Miller observed constitutive reporter activity when the Ahr/Arnt expressing strain was transformed with the *lacZ* reporter plasmid. It was later suggested that the constitutive reporter activity was attributed to activation of Ahr via exogenous tryptophan in the culture medium (Miller *et al* 1998). While UV photoproducts of tryptophan have been implicated as potential ligands for Ahr (Rannug *et al* 1987), constitutive reporter activity was not observed in later studies when low copy number plasmids were used. Therefore, it is also plausible that constitutive activity resulted from gross overexpression of Ahr and Arnt proteins. Additionally, Miller (1997) grew the recombinant Ahr and Arnt strain in the presence of galactose for approximately 24 hours prior to use in the β-galactosidase reporter assay. The promoter was transcriptionally active throughout the analysis and could result in excess of Ahr and Arnt proteins (Miller 1997). Interestingly, Miller later demonstrated that stable integration of Ahr and Arnt into the yeast genome conferred less constitutive activity in the reporter assay. These analyses confirm that protein expression levels should be evaluated in order to avoid overexpression that could lead to toxic effects or even aberrant results.

In a follow-up to the 1997 study, Miller used the same recombinant strain to evaluate two yeast proteins containing tetratricopeptide repeat domains, similar to the mammalian Xap2 protein. Knock-outs of *CPR6* and *CPR7* genes caused a significant reduction in *lacZ* reporter activity while transformation of a *CPR7*, but not a *CPR6* expression vector, was able to restore Ah receptor signaling as measured by the β-

galactosidase reporter assay (Miller 2002). These results suggest that Cpr7 is required for proper Ahr signaling and is the likely yeast Xap2 homologue; however the function of Cpr7 in yeast still remains unclear. In 2002, the Miller group published a report on the co-chaperone p23, demonstrating that knock-out of the yeast homolog *SBA1* resulted in a modest decrease in reporter activity. Given that yeast *SBA1* and human p23 only have 28% sequence identity, transformation of a human p23 expressing plasmid into the *SBA1* knock out strain recovered the Ahr signaling to approximately normal levels (Cox and Miller 2002). Although these studies never evaluated the level of expression of Ahr or Arnt protein in the recombinant strain, they did suggest that the chaperones and co-chaperones that are required for proper Ahr functioning in mammalian cells, are conserved and functional in yeast.

The other type of studies that have been carried out in yeast are transactivation analyses that are useful studies for screening of potential ligands (Miller 1997, Miller 1999, Kawanishi 2003, Noguerol *et* al 2006, Ohura *et al* 2007, Sugihara *et al* 2008, Kamata *et* al 2009). Miller (1997) demonstrated a ligand-specific dose-dependent effect on transactivation as measured by the XRE-driven reporter in a strain of yeast expressing Ahr and Arnt from a bi-directional promoter. The study measured reporter activity when cultures were treated with various concentrations of known Ahr ligands including TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), βNF (β-napthoflavone), HCB (hexachlorobenzene), BAP (benzo(a)pyrene as well as several potential Ahr ligands including tryptophan, IAA (indole acetic acid), IND (indole), I3C (indole-3-carbinol), and TA (tryptamine).

In 1999, Miller demonstrated that there was an additive response when recombinant yeast were treated with a combination of aromatic and chloroaromatic hydrocarbons suggesting that there is a single ligand-binding site within the Ahr protein. Kawanishi *et al* (2003) evaluated the effect of βNF, TCDD, and BAP as was done

previously, but also evaluated several other compounds (3-methylcholanthrene and indirubin) for Ahr transactivation using a similar recombinant strain. Additionally, the reporter activity was measured in a yeast strain expressing mouse AHR and ARNT and was compared to a strain expressing the human AHR and ARNT genes to evaluate species specific variation in ligand sensitivity.

The Ahr and Arnt expressing strain originally created by Miller (1997) was used as an *in vivo* model by several other groups in order to measure reporter activity in response to many other potential Ahr ligands. Noguerol (2006) tested 21 compounds that are known pollutants and antifouling pesticides. Ohura (2007) evaluated reporter activity resulting from treatment with 18 chlorinated polycyclic aromatic hydrocarbons while Sugihara (2008) tested six known Ahr ligands for activity. Eighty-four polychlorinated biphenyls (PCBs) with varying degrees of hydroxylation were evaluated for reporter activation; the report suggests that metabolic intermediates of PCBs have higher binding affinities for Ahr and can therefore enhance Ahr transcriptional activity (Kamata 2009).

In summary, there have been a number of important studies of Ahr signaling using recombinant yeast models. These studies clearly show the utility of yeast in the evaluation of Ahr and Arnt signaling and suggest that the requisite chaperones are expressed to support Ahr-mediated signaling. There have been no studies that have taken a systematic approach to evaluate the level of Ahr and Arnt protein expression, degradation and stability in a recombinant yeast model.

Summary of System and Specific Aims

The aryl hydrocarbon receptor is a basic helix-loop-helix transcription factor shown to rapidly degrade following ligand-activated transactivation of responsive genes. Ahr requires its DNA binding partner (Arnt), another bHLH transcription factor, in order to

associate with xenobiotic response elements in the promoter regions of target genes. The current model of Ahr signaling suggests that the hydrophobic ligand, typified by 2,3,7,8 tetrachlorodibenzo-*p*-dioxin, diffuses through the cell membrane and binds to the latent cytoplasmic Ahr complex containing one molecule of Ahr, a dimer of heat shock protein 90 (Hsp90), an immunophilin-like protein (Xap2), and the co-chaperone p23 (Carver and Bradfield 1997, Kazlauskas *et al* 1999). Upon ligand binding, a conformational change exposes the nuclear localization signal such that the receptor translocates to the nuclear compartment and heterodimerizes with Arnt. Subsequently, the Ahr•Arnt complex associates with enhancer elements found upstream of responsive genes (Denison *et al*, 1988) including the gene encoding the drug metabolizing enzyme Cyp1a1 (Legraverend *et al* 1982). Finally, after DNA-binding, the Ahr but not the Arnt protein is quickly degraded by the 26S proteasome thereby attenuating the dose of gene regulation administered by the heterodimer (Pollenz 1996).

Saccharomyces cerevisiae has been used as model organism in order to further characterize signal transduction pathways in a more simplified cellular setting in which the major processes required for growth and development are conserved to mammals. The yeast model has been used to evaluate Ahr signal transduction by several groups; however the majority of the published work has focused predominantly on determining the function of the auxiliary proteins that interact with the Ahr latent receptor complex (Carver *et al* 1994, Cox and Miller 2002, Cox and Miller 2003, Cox and Miller 2004, Miller 2002, Whitelaw *et al* 1995). Across these studies, Ahr was expressed to varying degrees. The cDNA was expressed using a high-copy plasmid in some studies, a low-copy plasmid in others, and lastly the cDNA was stably integrated into the yeast genome. Additionally, various promoter sequences were used and the promoter was constitutively active in some studies while it was inducible in others.

Although these reports laid the groundwork for investigation of the Ahr signaling pathway using *S. cerevisiae* as a model organism, almost every conclusion was made based on the results of an Ahr-activated β-galactosidase reporter assay. The level of Ahr and Arnt protein expressed in the yeast strains was never quantified and the stability of the expressed proteins was never evaluated. Additionally, the ability of the Ahr to degrade in yeast was never explored.

The overall goal of this work was to generate a recombinant yeast model that could be utilized to assess the ligand-mediated degradation of the aryl hydrocarbon receptor (Ahr). The first aim was to generate novel yeast strains with mammalian AHR and ARNT cDNAs integrated into the yeast genome. The second aim was to evaluate Ahr and Arnt protein expression and evaluate the stability of these proteins when expressed heterologously in yeast. The third aim was to evaluate Ahr and Arnt function through analysis of a ligand-mediated XRE-driven reporter. Finally, the fourth aim was to characterize ligand-dependent and independent Ahr degradation.

CHAPTER TWO:

GENERATION OF AHR AND ARNT EXPRESSING YEAST STRAINS

Experimental Question and Rationale

Yeast can be used to characterize genes and proteins that they do not normally express. One major advantage to using yeast as opposed to mammalian tissue culture is that yeast have the ability to homologously recombine similar DNA sequences permanently into their genome. The double-stranded DNA break repair pathway is activated when a portion of double-stranded DNA (PCR product) is introduced into the cells, such that the repair mechanism searches for homology to the yeast genome within the integrating sequence. This preference allows for the integration of the double-stranded DNA of interest into the yeast genome in a highly directed and efficient manner (Ito *et al* 1983, Lorenz *et al* 1995). Recombination in yeast can therefore facilitate either knocking a gene into the genome in a nonessential region (gain of function) or knocking a gene out by disrupting the target sequence or replacing it entirely (loss of function).

The aim of this portion of the project was to integrate mammalian AHR and ARNT cDNAs into yeast strains in order to heterologously express these genes. A major advantage of this approach as compared to others was to utilize directed homologous recombination of the cDNAs into the yeast genome so that only one copy of each cDNA was integrated into the yeast genome. This method was chosen in order to mimic physiological levels of a given yeast protein and prevent overexpression and cellular

stress. The ultimate question asked whether or not Ahr and Arnt proteins could be detected in yeast using this type of expression system.

General Strategy. For these studies, the exogenous genes will be referenced in all capital letters (i.e. AHR, ARNT). The proteins expressed from the exogenous genes will be referenced in upper and lowercase (i.e. Ahr, Arnt). Yeast genes will be referenced using the standard yeast nomenclature (all capital letters and italics). The yeast genome does not encode AHR and ARNT homologues; therefore, yeast strains were constructed such that mouse AHR and ARNT cDNAs were stably integrated into the genome adjacent to an inducible promoter for subsequent expression analysis. Yeast strains were constructed according to a precise plan that would allow the Ahr and Arnt expressing strains to be of opposite mating types. This plan also worked to preserve the selectable markers and to prevent addition of tryptophan to the growth medium to avoid erroneous activation of Ahr (Rannug *et al* 1987).

In order to facilitate the uptake of the cDNAs, selectable marker cassettes were cloned into plasmids adjacent to AHR and ARNT cDNAs. The parental yeast strains are auxotrophic for several commonly used yeast selectable markers. The strains are therefore unable to grow in the absence of certain compounds whose production is dependent on the expression of those genes encoded by the marker cassettes. The cDNAs and their adjacent marker cassettes were PCR amplified. The resulting PCR product was transformed into yeast cells treated with lithium cations in order to facilitate uptake of the DNA into the yeast cells. This procedure is described in detail in Chapter 5. The transformed cells were spread on synthetic medium lacking the particular compound whose synthesis occurs with expression of the selectable marker. This allowed for selection of clones that have taken up the cDNAs and adjacent markers. The resulting colonies were tested for integration of cDNAs using PCR.

Construction of Ahr and Arnt Expressing Strains

Cloning of Selectable Marker Cassettes into AHR and ARNT Vectors. The stable integration of the AHR or ARNT cDNAs into the yeast genome required that a selectable marker cassette was incorporated as part of the transformed sequence. The pRS304 plasmid (Sikorski and Hieter 1989) containing the *TRP1* marker cassette encodes a gene required for tryptophan synthesis. The pRS306 plasmid (Sikorski and Hieter 1989) containing the *URA3* marker cassette encodes a gene required for the synthesis of uracil.

The AHR and ARNT expression vectors and *TRP1* and *URA3* sequences were evaluated in order to identify particular restriction enzymes that would cleave the plasmids immediately downstream of the cDNAs but would not internally cleave the marker cassettes. For the AHR expression vector, pLNcx2-AHR^{b-2} (Pollenz and Dougherty 2005), the *ClaI* site is 54 base pairs downstream of the AHR cDNA and does not cut within AHR or the *TRP* cassette. The ARNT vector, pCDNA-ARNT (Dougherty and Pollenz 2008), has an *AflII* restriction site located 72 base pairs downstream of the ARNT cDNA and this enzyme does not cleave ARNT or the *URA3* marker cassette.

The *TRP1* cassette was PCR amplified using primers designed to anneal to the cassette but also contained overhanging *ClaI* restriction sites. The pLNcx2-AHR^{b-2} vector and the *TRP1* PCR product were digested with *ClaI* restriction enzyme. Similarly, the *URA3* cassette was PCR amplified using primers with overhanging *AflII* restriction sites. The pCDNA-ARNT vector and the *URA3* PCR product were digested with *AflII* restriction enzyme. The linearized vectors were treated with alkaline phosphatase in order to prevent re-ligation of empty vector. Next, each vector and respective marker cassette was combined with DNA ligase. A schematic of the ligated plasmids is shown in Figure 2.1A, these plasmids were subsequently transformed into competent *E. coli*

and purified from clonal populations of bacterial cells and evaluated for direction of insertion.

PCR Amplification of cDNAs and Adjacent Marker Cassettes. Linking the cDNAs with the yeast selectable marker cassettes provides a means for selection of integrated clones whereby transformed yeast cells are plated on media lacking a particular compound required for growth. Therefore, only yeast that stably integrate the cDNA and its linked marker cassette into their genome will have the ability to produce that particular compound and therefore survive and grow on the media.

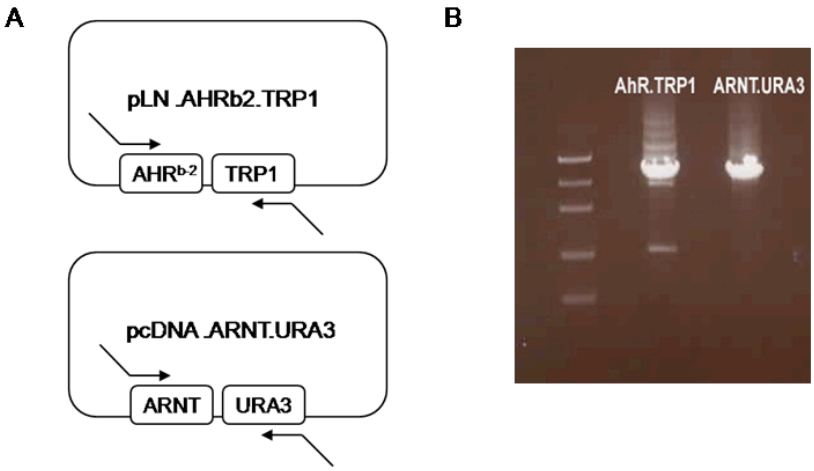


FIGURE 2.1: Plasmid maps depicting PCR primers and the result of PCR amplification. (A) Plasmid maps illustrate the ligation of selectable marker cassettes downstream of AHR and ARNT cDNAs. The arrows represent PCR primers designed to amplify the cDNA and the selectable marker cassette in order to produce one double-stranded DNA fragment. The primers also contain 50bp overhangs with homology to the integration site within the yeast genome. (B) The PCR product from amplification of the AHR.*TRP1* sequence and the ARNT.*URA3* after resolving on a 1% agarose gel. This double-stranded DNA was then transformed into parental yeast strains to generate recombinant yeast for expression analysis.

PCR primers were designed to anneal to the 5' end of the AHR or ARNT cDNA and the 3' end of the marker cassette while also containing 50 base pair overhangs with sequence identity for the target recombination locus within the yeast genome on each

primer, see Figure 2.1A. The primers were designed such that the sequences recombined to nonessential chromosomal regions within the genome of the parental strains, specifically a region of chromosome 15 for Ahr and chromosome 4 for Arnt. The cDNAs and adjacent marker cassettes were PCR amplified and this PCR product, shown in Figure 2.1B, served as a target for homologous recombination in the parental yeast strains.

Transformation Procedure. Yeast transformations were carried out according the the method of Gietz and Woods (2006) and is detailed in Chapter 5. The KHSY421 yeast strain (*MATa, ura3-52, trp1Δ63, his3Δ200, leu2Δ1*) was streaked on YPD agar media and incubated at 30°C for two days. On the third day, one colony was used to inoculate a liquid culture that was placed in a shaking incubator overnight at 30°C. The overnight culture was used to make a 25 milliliter culture at an approximate OD_{600} of 0.2. This culture was placed in the shaking incubator and grown to an OD_{600} of 0.8. The cells were pelleted and combined with lithium acetate, PEG (polyethylene glycol), the AHR.*TRP* PCR product, and boiled/snap cooled salmon sperm DNA in order to facilitate the uptake of the DNA. Following heat shock, the cells were spread on media lacking tryptophan in order to select for yeast with the integrated cDNA and *TRP* marker.

After two days, the plates shown in Figure 2.2A were evaluated for growth and each colony was streaked on another selective plate in order to obtain individual clones. These clones were grown overnight in liquid medium, genomic DNA was extracted (Invitrogen), and PCR was used to confirm proper integration of the cDNA. Specifically, one primer was designed to anneal 100bp upstream of the integration site while the second primer annealed approximately 900bp inside the AHR cDNA resulting in a 1000 base pair PCR product in strains that conferred proper integration. The PCR products were resolved on a 1% agarose gel, with the results shown in Figure 2.2B. Several

positive clones were identified, and stored for future use. Specifically, the AHR.*TRP* integrated clone called KHSY1535 (*MATa, ChrXV::AHR.TRP1, ura3-52, trp1Δ63, his3Δ200, leu2Δ1*), was used for subsequent experiments.

The ARNT strain was generated in precisely the same manner however the ARNT.*URA* PCR product was transformed into the KHSY422 parental strain (*MATα, ura3-52, trp1Δ63, his3Δ200, leu2Δ1*). Several ARNT transformants were identified and their integration was verified using PCR (Figure 2.2B); these clones were also catalogued and stored for the next set of experiments. The ARNT.*URA* integrated clone, KHSY1540 (*MATα, ChrIV::V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1*), was used in later experiments.

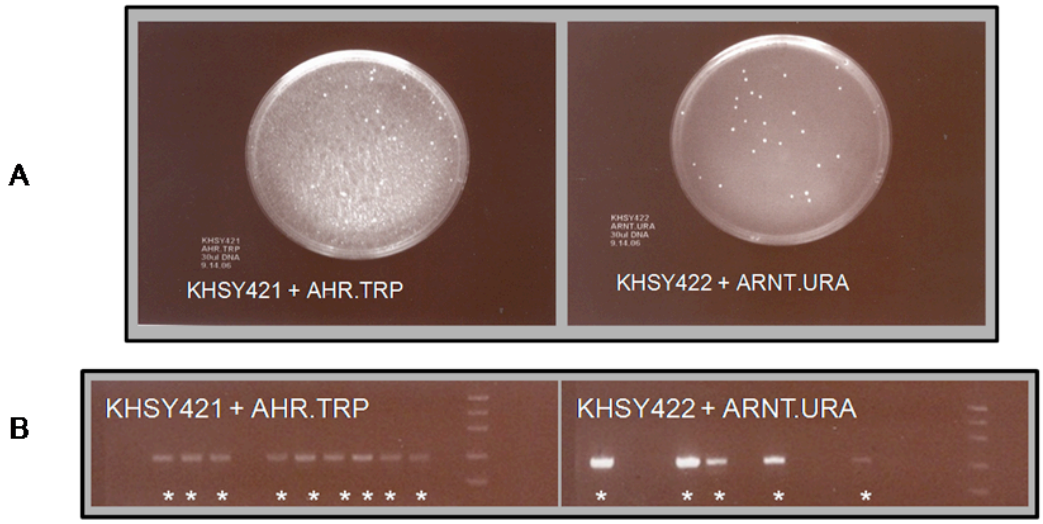


FIGURE 2.2: Transformed yeast colonies on selective media and confirmation of cDNA integration. (A) The parental strains KHSY421 and KHSY422 were transformed with the PCR amplified cDNAs/marker cassettes AHRb2.*TRP1* and ARNT.*URA3*. The transformed strains were selected for incorporation of the DNA using media lacking either tryptophan (421) or a compound required for uracil synthesis (422). (B) PCR confirmation for proper integration of AHR and ARNT genes. PCR amplification of genomic DNA prepared from twelve AHR transformants and twelve ARNT transformants revealed that 9 AHRb2.*TRP1* transformants and five ARNT.*URA3* transformants were properly integrated into the yeast genome, as indicated by asterisks. One AHRb2.*TRP1* and one ARNT.*URA3* transformant was chosen for the next step in strain construction.

Integration of the Inducible Promoter. The galactose-inducible promoter sequence was inserted in the yeast genome directly upstream of each cDNA in order to drive expression of the AHR and ARNT genes. The same experimental design was employed such that PCR amplification of the *GAL1* promoter and the adjacent G418 cassette from the pFA6a-PGAL1KanMx plasmid (Longtine et al 1998) resulted in double-stranded PCR product with 50bp overhanging sequence with identity to the yeast sequence immediately upstream of the AHR or ARNT integration site. This sequence was transformed into the AHR.*TRP* (1535) and ARNT.*URA* (1540) strains using the lithium acetate approach (Gietz and Woods 2006) and transformed yeast were spread on media selecting for G418 resistance.

Integration of the promoter was confirmed via PCR for several clones, and each positive clone was stored. The *GAL*.AHR.*TRP* clone, namely KHSY1538 (*MATa, ChrXV::KanMX-PGAL1-AHR.TRP1, ura3-52, trp1Δ63, his3Δ200, leu2Δ1*), and the *GAL*.ARNT.*URA* clone, KHSY1541 (*MATα, ChrIV::KanMX-PGAL1-V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1*), were utilized in the next step of strain construction whereby they were mated in order to generate a strain expressing both Ahr and Arnt proteins under the inducible promoter.

Next, KHSY1538 and KHSY1541 strains were streaked on medium containing glycerol as the carbon source in order to check for the petite (ρ-) mutation. Yeasts carrying the petite mutation have little or no mitochondrial DNA and form small anaerobic colonies on non-fermentable growth media containing either glycerol or ethanol (Sherman 2002). Both strains exhibited normal growth on YPG (glycerol) plates and were then used in the generation of the double knock-in strain.

Prior to mating the Ahr-expressing KHSY1538 strain with the Arnt-expressing KHSY1541 strain, both strains were further evaluated to determine if they retained a wild-type doubling time of approximately 90 minutes in YPD media (Sherman 2002).

Doubling times were measured according to the protocol described by Schmidt and Kolodner (2004).

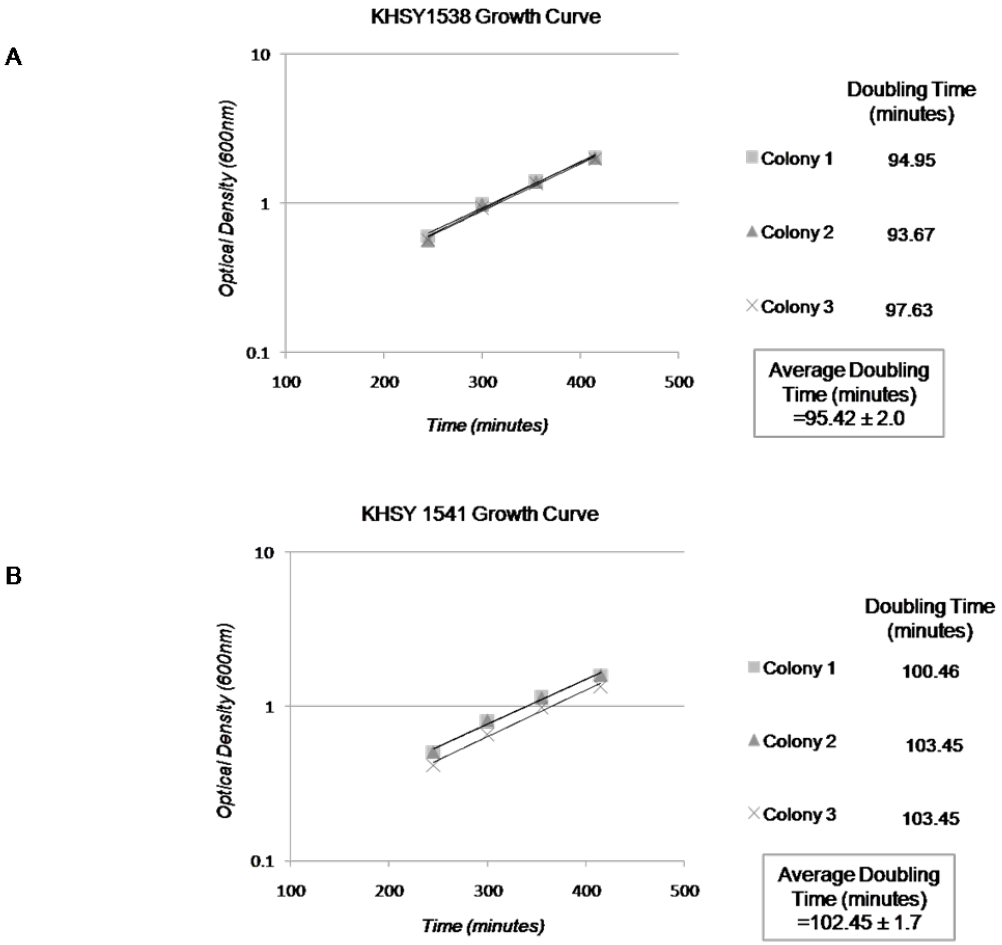


FIGURE 2.3: Growth Curves and Doubling Times for KHSY1538 and KHSY1541 strains. Ahr and Arnt knock-in strains were evaluated for doubling time. Three colonies from each strain were grown in YPD media for eight hours with a 1ml sample measured for optical density at 600nm every hour. Optical densities were plotted versus time for samples in the exponential growth phase and the equation from each linear regression was used to determine the doubling time for each yeast sample. The average of three rates was taken for each strain such that the doubling time for KHSY1538 (A) is 95 minutes and the doubling time for KHSY1541 (B) is 102 minutes.

Each strain was streaked for single colonies on YPD media and after two days, three single colonies of each strain were used to inoculate individual 2ml liquid overnight cultures. The next day, 20ml cultures were prepared at an OD_{600} of 0.2 for each of the

six overnight cultures. These cultures were placed in a 30° shaking incubator, and 1ml was measured for optical density at 600nm every hour for eight hours.

The optical densities, once the cultures reached exponential growth, were plotted as a semi-log regression using excel such that the slope of each line represented the growth rate for each colony, as shown in Figure 2.3. The doubling time for the KHSY1538 and KHSY1541 strains were then calculated using the equation of each regression line and the average of three was taken for each strain. The Ahr knock-in strain revealed an approximate doubling time of 95 minutes with a standard deviation of 2 minutes, while the Arnt knock-in strain had a doubling time of approximately 102 minutes with a standard deviation of 1.7 minutes.

Generation of the Double Knock-In Strain. The Ahr protein requires Arnt heterodimerization in order to bind XREs and therefore induce target gene expression (Reyes *et al* 1992), such that a strain needed to be constructed in which both Ahr and Arnt were expressed. The previous transformations were designed so that KHSY1538 and KHSY1541 would be of opposite mating types and crossing the strains could produce one strain expressing both Ahr and Arnt. Mating of two strains was carried out according to Sherman (2002) and required that one colony of each strain, 1538 and 1541, was mixed together in water. Ten microliters of that solution was spotted onto a YPD plate and placed in the 30°C incubator overnight. Next, the growth was streaked out for single colonies on another YPD plate and again grown overnight. The resulting colonies were genotyped to determine mating type as *MATa*, *MATα*, or diploid. Results for the mating are shown in Table 2.1.

To test for diploid strains, colonies were tested for growth on synthetic media to assess complementation of auxotrophic markers. In order to do this, a lawn of strain KHSY1435 (*MATa, thr4-*) and KHSY1436 (*MATα, thr4-*) was grown on YPD agar media

and placed in the 30°C incubator overnight. These two haploid strains are of opposite mating types and both strains are auxotrophic for the threonine selectable marker. Single colonies of the 1538/1541 mixture were spotted onto the KHSY1435 and KHSY1436 lawns. Since haploid strains (a or α) can only mate with their opposite mating type, it was expected that any 1538 (a) colonies would mate with KHSY1436 (α) and 1541 (α) would similarly mate with KHSY1435 (a) to produce a/α diploid strains. Any 1538/1541 colonies that formed diploids during the initial strain mating were therefore already (a/α) and were unable to mate with the lawn of KHSY1435 or KHSY1436.

The following day, a replica of each plate was made on minimal medium. This ensured that only 1538/1436 diploids and 1541/1435 diploids could grow on the plates while any 1538/1541 diploids formed during the initial strain mating would again be unable to grow. The KHSY1435 and KHSY1436 strains are auxotrophic for the threonine biosynthesis selectable marker, such that they are unable to grow on minimal medium unless mated to 1538 or 1541 and genetic complementation will sustain growth. While the 1538 and 1541 strains require the addition of supplemental histidine, and leucine to grow. Formation of a diploid with 1435 or 1436 allowed the strains to grow in the absence of supplemental amino acids. Therefore, all colonies that did not grow on the minimal medium were diploids generated in the original crossing of 1538 and 1541 and these colonies were next subjected to random spore isolation.

The resulting minimal plates were evaluated for cell growth. Again, growth on neither plate suggests that the sample was already heterozygous for the *MAT* locus, and was therefore diploid prior to spotting on the KHSY1435 and KHSY1436 lawns. It is those diploid strains that are useful, as they carry two sets of chromosomes and should encode both AHR and ARNT cDNAs and promoter sequences. The mating results are shown in table 2.1 below. Thirty-one diploid clones were observed and nine clones were

of the *MATa* mating type. Three of the diploid strains were stored for future use and were used for random spore isolation.

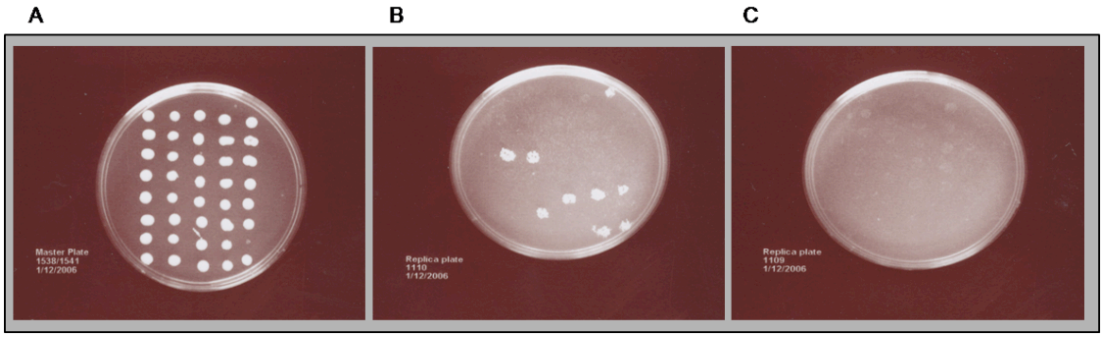


FIGURE 2.4: Mating type genotyping for AHR/ARNT Crossing. (A) Forty single colonies were picked after mating KHSY1538 with KHSY1541 strains. The colonies were spotted onto rich medium first and were subsequently spotted on a lawn of KHSY1435 and KHSY1436. (B) Growth of certain colonies on minimal media results from mating with the 1436 (*MAT α*) lawn, suggesting that those colonies must be of the opposite mating type (*MAT a*) and are therefore haploid 1538 yeast. (C) Conversely, no growth was observed on the 1435 plate and therefore no 1541 *MATα* haploid colonies were obtained. The colonies that did not exhibit growth on either plate is therefore a 1538/1541 diploid.

Lastly, meiotic progeny of the mated strains was produced such that one haploid strain encoding both AHR and ARNT was generated via random sporulation (Rockmill *et al* 1991). Three diploids were chosen for sporulation and grown in the non-fermentable carbon source 1% potassium acetate for 5 days. Growth in potassium acetate causes a shift from mitotic cell division to meiosis resulting in mature asci containing four haploid ascospores (Sherman 2002). These spores were isolated using the enzyme zymolase followed by sonication and were spread on rich medium for genotyping. The goal was to identify at least one clone containing chromosome 15 from the 1538 strain in combination with chromosome 4 of the 1541 strain such that both AHR and ARNT genes could be expressed in combination.

TABLE 2.1: Mating Type Genotyping of Crossed Strains

Clone #	MAT	Clone #	MAT	Clone #	MAT	Clone #	MAT
1	diploid	11	diploid	21	diploid	31	diploid
2	diploid	12	diploid	22	diploid	32	A
3	diploid	13	diploid	**23**	**diploid**	33	diploid
4	diploid	14	diploid	24	diploid	**34**	**diploid**
5	a	15	diploid	25	diploid	35	diploid
6	diploid	16	a	26	diploid	36	diploid
7	**diploid**	17	a	27	diploid	37	diploid
8	diploid	18	diploid	28	a	38	diploid
9	diploid	19	diploid	29	a	39	A
10	diploid	20	diploid	30	a	40	A

The Ahr and Arnt expressing strains were mated in order to generate one strain expressing both proteins. After mixing one colony of each strain, the yeast was streaked for single colonies and genotyped for mating type. Nine clones gave rise to MATa strains, while the remaining clones gave rise to diploid strains. Three diploid strains, 7, 23, and 32 were stored for future use (bolded entries).

Forty-eight haploid clones were evaluated for AHR, ARNT, and *GAL1* by way of selection for their respective auxotrophic markers. The clones were spotted on media lacking tryptophan to identify clones with AHR.*TRP*1, while media lacking uracil identified clones with ARNT.*URA3*. Additionally, the clones were tested for G418 resistance that should occur in conjunction with the heterologously integrated *GAL1* promoter. Clones that grew on all three selective plates, therefore, encoded both AHR and ARNT cDNAs and expression of these genes was inducible by way of the integrated *GAL1* promoters. Images of the selective plates are shown below in Figure 2.5.

While the random sporulation procedure generally induces spore formation in the majority of the cells, it was important to determine the mating type of the clones to ensure that a diploid is not selected for future analysis. While a diploid strain would still express both AHR and ARNT, it is not as useful because it can no longer mate with other strains. Mating type genotyping was carried out as shown previously and the plates are shown in Figure 2.6.

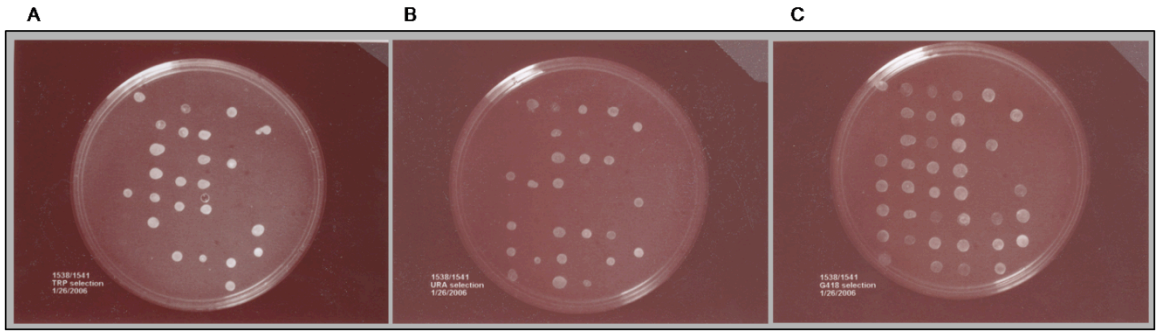


FIGURE 2.5 Genotyping of haploid spores. Forty-eight clones were plated on media lacking tryptophan (A), uracil (B), and media containing canavanine to test for G418 resistance (C). Growth on all three plates indicates expression of the selectable marker downstream of the cDNAs of interest.

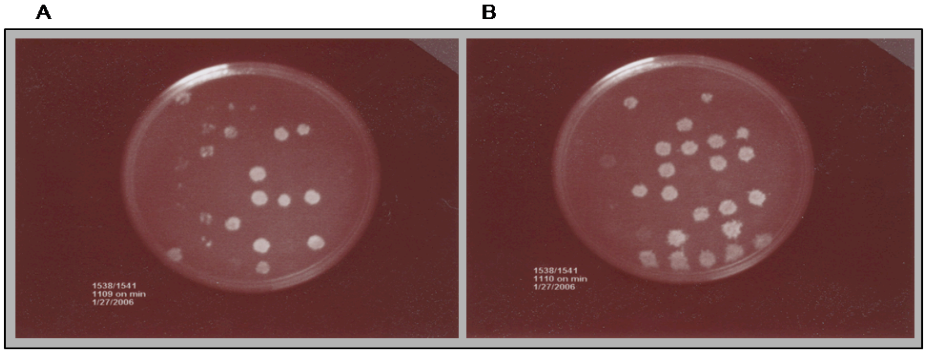


FIGURE 2.6 Mating type genotyping after sporulation of Ahr and Arnt expressing strains. Clones were spotted on a lawn of 1435 or 1436 yeast and plated on minimal media in order to determine their mating type. (A) Growth on minimal media coated with a lawn of 1435 (MATa), suggests that the colonies are of opposite mating type (MAT α) and are therefore haploids. (B) Growth on the 1436 (MATα) lawn, suggests that those colonies are of the opposite mating type (MATa) and are also haploids. Conversely, where no growth was observed on either plate, those clones were diploid.

Table 2.2 displays the genotyping results for the 48 spores that were analyzed. Several haploid clones grew on all three selective plates and were selected for future analysis; in particular clones 27, 33, and 35 were stored for use in expression analysis. Clone 33, also known as KHSY1547 (*MATa, ChrXV::KanMX6-PGAL1-AHR.TRP1, ChrIV::KanMX6-PGAL1-V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1*) is the double knock-in strain used in subsequent studies. Again, KHSY1547 was streaked on medium containing glycerol as the carbon source in order to check for the petite (ρ-)

mutation. This strain exhibited normal growth on YPG (glycerol) plates and was then used in the generation of the reporter strain.

TABLE 2.2: Genotyping and Mating type following Random Sporulation

Spore #	MAT	::G418	::URA	::TRP		Spore #	MAT	::G418	::URA	::TRP
1	alpha	x		x		25	alpha	x	x	
2	alpha					26	diploid	x		x
3	alpha					**27**	**a**	**x**	**x**	**x**
4	alpha	x	x			28	alpha	x		x
5	alpha	x		x		29	alpha	x		x
6	alpha	x	x			30	a	x	x	
7	alpha	x	x			31	alpha	x		x
8	alpha	x	x			32	a	x	x	
9	a	x	x			**33**	**a**	**x**	**x**	**x**
10	alpha	x		x		34	alpha			
11	alpha	x		x		**35**	**a**	**x**	**x**	**x**
12	alpha	x	x	x		36	a			
13	diploid	x		x		37	alpha			
14	alpha	x		x		38	diploid	x	x	
15	alpha	x	x			39	diploid	x	x	x
16	a			x		40	a	x		x
17	diploid	x	x	x		41	alpha			
18	alpha	x	x			42	alpha	x	x	x
19	a	x	x			43	a			
20	diploid	x	x	x		44	a			
21	diploid	x		x		45	alpha	x	x	
22	alpha	x	x			46	a	x		x
23	diploid	x	x	x		47	alpha	x	x	x
24	diploid	x	x			48	diploid			

This table displays the results for the 48 clones analyzed following random sporulation. Each clone was evaluated for mating type and growth on selective media in order to determine which clones express both AHR and ARNT cDNAs, express the *GAL1* promoter, and are *MATa* haploids. Clones 27, 33, and 35 grew on all three selective plates and are *MATa* haploids, and these clones were stored for future use.

The last step in strain construction required the transformation of the pLXRE5-Z reporter plasmid into the double knock-in as well as the AHR expressing strain (Cox and

Miller 2003). This plasmid has five xenobiotic response elements located upstream of the *lacZ* gene, such that binding of Ahr/Arnt heterodimers to the XREs will induce expression of *lacZ* reporter gene. The plasmid also carries a *LEU2* marker cassette for selection and a centromeric origin of replication that maintains the plasmid at a low copy number.

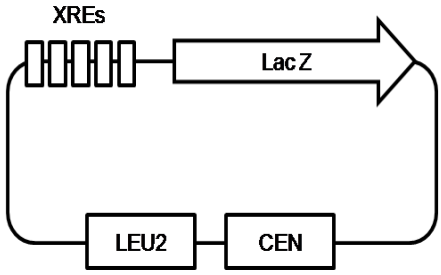


FIGURE 2.7 Plasmid map for pLXRE5-Z reporter plasmid. The plasmid was transformed into the double knock-in strain KHSY1547 and the AHR expressing strain KHSY1538. The reporter (Cox and Miller 2003) was used in these strains to evaluate transactivation of the recombinant signaling pathway.

The plasmid was transformed into the double knock-in strain as well as the AHR expressing strain using the lithium acetate method described previously; however, 100ng of plasmid DNA was substituted for double stranded DNA. Selection was carried out on medium lacking leucine and positive transformants were selected and stored as frozen stocks.

The completed strain, KHSY1566 (*MATa, ChrXV::KanMX6-PGAL1-AHR.TRP1 ChrIV::KanMX6-PGAL1-V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1, pLXRE5-Z*), therefore expresses AHR and ARNT under the inducible GAL1 promoter and contains the pLXRE5-Z reporter plasmid. Additionally, the KHSY1565 (*MATa, ChrXV::KanMX6-PGAL1-AHR.TRP1, ura3-52, trp1Δ63, his3Δ200, leu2Δ1, pLXRE5-Z*) strain expresses AHR under the inducible *GAL1* promoter and contains the reporter plasmid. While only the double knock-in strain should demonstrate XRE-binding

capability and reporter activation, the AHR knock-in strain was generated in order to confirm specific activation of the reporter as mediated by the signaling pathway as it exists in mammalian cells.

KHSY1566 was streaked on medium containing glycerol as the carbon source in order to check for the petite (ρ-) mutation, as described previously. Again, this strain exhibited normal growth on YPG (glycerol) and was stored for use in future experiments. It was also of interest to determine if this strain retained a wild-type doubling time of approximately 90 minutes in YPD media. The culture was evaluated for doubling time as described for KHSY1538 and 1541 (Schmidt and Kolodner 2004). The optical densities, once the cultures reached exponential growth, were plotted as a semi-log regression using excel such that the slope of each line represented the growth rate for each colony, as shown in Figure 2.8. The doubling time was then calculated using the equation of each regression line and the average of three was taken.

The results shown in Figure 2.8 show that the KHSY1566 strain has an approximate doubling time of 106.7 minutes with a standard deviation of 2.8 minutes. This doubling time is slightly longer than the wild type and longer than the doubling time of the previously constructed strains. It should be noted that this strain, carrying the reporter plasmid, is propagated in synthetic medium which is likely the reason for this observation.

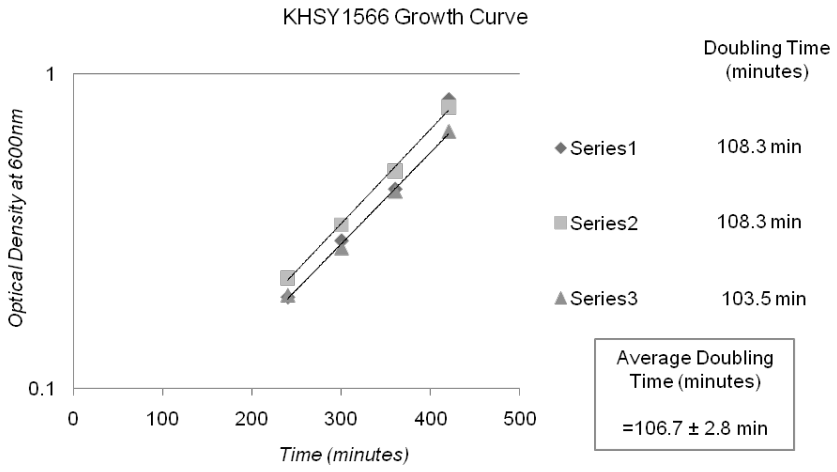


FIGURE 2.8: Growth Curve and Doubling Time for KHSY1566. The double knock-in reporter strain was evaluated for doubling time. Three colonies were grown in YPD media for eight hours with a 1ml sample measured for optical density at 600nm every hour. Optical densities were plotted versus time for samples in the exponential growth phase and the equation from each linear regression was used to determine the doubling time for each yeast sample. The average of three rates was taken and revealed that the doubling time for KHSY1566 is 106.7 minutes.

In summary, this set of studies demonstrates that several novel recombinant yeast strains have been produced that may be utilized to assess Ahr signal transduction and Ahr degradation. These strains have been validated using PCR to contain the cDNAs and reporter constructs that will recapitulate the Ahr signal transduction pathway. In addition, studies show that the integration of these constructs does not appear to impact the growth of the different strains and these results indicate that it is appropriate to pursue further analysis. The studies presented in Chapter 3 provide a comprehensive analysis of Ahr signal transduction in several of these novel strains.

CHAPTER THREE:

CHARACTERIZATION OF AHR AND ARNT PROTEIN EXPRESSION IN YEAST STRAINS AND VALIDATION OF YEAST MODEL

Experimental Question and Rationale

The central goal of these studies was to use *Saccharomyces cerevisiae* as a model to assess Ahr protein degradation. Therefore, after completing the construction of the Ahr and Arnt expressing yeast strains (Aim #1); it was essential to demonstrate that the proteins would be detectable using western blotting and antibody staining. As stated in chapter one, *Saccharomyces cerevisiae* has been used as a model for Ahr signaling however, Ahr and Arnt protein levels were not evaluated in these studies and reporter assays were utilized to measure receptor activation (Carver *et al* 1994, Cox and Miller 2002, Cox and Miller 2003, Cox and Miller 2004, Miller 2002, Whitelaw *et al* 1995). There are no studies that have evaluated recombinant Ahr and Arnt protein expression in yeast.

This chapter is broken down into several sections that encompass the various aims of the study. The first section describes the set of experiments aimed to optimize induction conditions in order to visualize the Ahr and Arnt proteins using western blotting (Aim #2). Next, the function of the Ahr and Arnt proteins was tested using the XRE reporter gene as an output (Aim #3). Once it was determined that the Ahr and Arnt could bind XREs and drive reporter expression, a series of experiments were conducted to assess Ahr and Arnt stability (Aim #2). Finally, studies aimed to assess the ligand dependent and independent degradation of the Ahr are presented (Aim #4).

General Strategy. Single knock-in strains expressing Ahr (KHSY1538) and Arnt (KHSY1541) were used in the initial western blotting experiments to confirm that the protein levels were detectable prior to mating the strains. Expression of Ahr and Arnt proteins was induced with the addition of galactose to the culture medium for various time points and protein samples were prepared from the yeast cells. These samples were then used in western blotting and antibody staining using Ahr and Arnt-specific antibodies. Upon detection of the proteins of interest in the single expression strains, the double knock-in strain (KHSY1547) was generated as described in chapter two. Finally, Ahr and Arnt protein levels were evaluated whereby both proteins could be expressed in a single yeast strain.

Reporter assays were carried out next. These studies were carried out to show that Ahr and Arnt were able to heterodimerize and bind the XRE-containing promoter of the reporter plasmid. Several strains were produced to carry out these analyses. The KHSY1566 strain expressed both Ahr and Arnt and was transformed with the XRE reporter plasmid. The KHSY1565 strain expressed only Ahr and was also transformed with the XRE reporter. Both strains were tested for reporter activation following treatment with TCDD or DMSO. Activation with TCDD was tested at a single time point to confirm that the strain expressing both Ahr and Arnt, and not the strain only expressing Ahr was able drive reporter activity. Later, the KHSY1566 strain was used to test other ligands at various doses and time points.

Finally, the Ahr and Arnt proteins were tested for stability in the presence of Ahr ligand since the results of the reporter assays provided evidence of a functioning Ahr signaling pathway in yeast. The half-life of the proteins was evaluated first to provide a baseline for the ligand treatment studies. Both the single and double knock-in strains were used to determine the half-life of the Ah receptor in yeast. Since Ahr and Arnt expression is driven by an inducible promoter, expression was induced for a time period

and subsequently turned off to evaluate the stability of the Ahr and Arnt proteins over time. The results of the half-life study led to an additional series of experiments that involved various methods of induction and sample preparation. The stability of Ahr was tested under various conditions including; reduced galactose in the growth medium, reduced induction time, and different sample preparation methods. Finally, the Ahr and Arnt expressor strains were treated with ligand under various conditions to visualize a degradation event in the yeast cells.

Detection of Ahr and Arnt protein expression in yeast strains

In the previous studies of Ahr and Arnt in yeast, the Ahr and Arnt proteins were induced with the addition of 2% galactose to the growth medium for approximately 16 hours and then the cultures were treated with ligand for an additional 8 hours to activate Ahr (Cox and Miller 2002, Miller *et al* 1998, Miller 1997). Induction of Ahr expression followed by ligand treatment allowed for heterodimerization of Ahr with Arnt and subsequent DNA-binding and reporter activation. While this experimental paradigm was effective and reporter activity was detectable for the strains tested, the level of receptor protein (having been induced for a total of 24 hours) was not evaluated at any time point. Since these studies showed that TCDD-induced β-galactosidase reporter could be detected, Ahr and Arnt protein must have been present in the samples tested. Therefore, we decided to mimic the 16 hour induction in the first set of studies.

Western blotting studies were carried out in the KHSY1538 and KHSY1541 strains. These strains are single recombinants and KHSY1538 expresses only Ahr and KHSY1541 expresses only Arnt. This experiment was completed prior to mating the strains in order to confirm the ability of each strain to express their respective genes/proteins. The rationale of this approach was to confirm expression in the individual strains before producing the double knock-in strain.

Induction of Ahr and Arnt proteins in single knock-in yeast strains. The first set of experiments was designed for detection of Ahr and Arnt protein expression by western blotting in the single knock-in strains. The inducible *GAL1* promoter was inserted upstream of the AHR and ARNT cDNAs during strain construction, as detailed in chapter two. This promoter sequence, also called UAS_G, is the binding site for the yeast Gal4 transcription factor in the presence of galactose or glycerol. When glucose is present, the UAS_G is bound by the Gal80 repressor protein which acts to block transactivation (reviewed by Lohr *et al* 1995). Therefore, the *GAL1* promoter upstream of the cDNAs is silent in the presence of glucose while Ahr and Arnt expression is activated upon addition of galactose to the growth medium. For these experiments, induction of the AHR and ARNT mRNA expression simply required the removal of glucose and the subsequent addition of galactose to liquid cultures. Expression of Ahr and Arnt proteins should begin immediately upon removal of glucose when galactose is present.

In the first set of experiments, yeast cultures were grown in the presence of galactose for 16 hours (overnight) because this was the typical time course used in previous reports using recombinant yeast strains (Cox and Miller 2002, Miller *et al* 1998, Miller 1997). Total protein was prepared from the induced cultures (2% galactose) and non-induced controls (2% glucose) using 20% trichloroacetic acid (TCA). The TCA precipitation procedure (Wright *et al* 1989) is described in detail in Chapter 5. To assure that equal levels of protein were being evaluated at each time point, the culture density was quantified at OD_{600} and the same number of cells was harvested. Briefly, each culture was centrifuged at 2000 rpm for 2 minutes and the medium was aspirated. The cell pellets, containing 1.0 ODs of cells, were combined with 100µl of 20% TCA and an equivalent volume of glass beads and were placed in a mini-bead beater for 4 minutes. Each sample was transferred to a fresh tube which was then centrifuged at 14,000 rpm

for one minute. The resulting protein pellet was resuspended in 100µl of SDS-PAGE sample loading buffer, and was then resolved on an SDS-PAGE gel for western blotting.

Once protein samples were prepared, western blotting was carried out by resolving equal volumes of the yeast protein samples on a 7% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose using a semi-dry blotting apparatus. To confirm even sample loading, nitrocellulose membranes were stained with Ponceau S dye. The detection of Ahr and Arnt was carried out using polyclonal antibodies characterized by Pollenz *et al* (1994). Ahr and Arnt induction experiments were completed several times for each induction that is presented below and representative western blots are shown in Figure 3.1.

The results show that both Ahr and Arnt could be detected in the yeast strains following overnight induction. The specificity of the Ahr and Arnt band is confirmed by the lack of reactivity in the negative control and the migration of the reactive band from the yeast samples at the same molecular mass as the positive control. To further assess the time course for the induction of Ahr and Arnt, cultures were grown overnight in glucose, spun down, the media was removed and the pellets were resuspended in galactose-containing media for 2 to 6 hours. Samples containing equivalent cell numbers (1.0 ODs) were harvested every two hours for six hours and protein was prepared using the 20% TCA precipitation method as described above.

A representative western blot for this experiment is shown in Figure 3.1B. The results show that Ahr protein is detected at the 2-hour time point. The level of Ahr protein does not appear to increase above the level observed at 2 hours, even after treatment with galactose for 6 hours. It is important to note that each lane was loaded with protein that was extracted from the same number of cells. Thus, the expression level in each lane is comparable. Since expression was easily detected at 2 hours, experiments were repeated and the level of Ahr and Arnt protein determined after 15,

30, 45, 60, 90, and 120 minutes of galactose treatment. Representative western blots for Ahr and Arnt expression are presented in Figure 3.1C.

A representative western blot for this set of experiments is shown in Figure 3.1B. The results show that Ahr protein is detected at the 2-hour time point. Importantly, the level of Ahr protein does not appear to increase above the level observed at 2 hours, even after treatment with galactose for 6 hours.

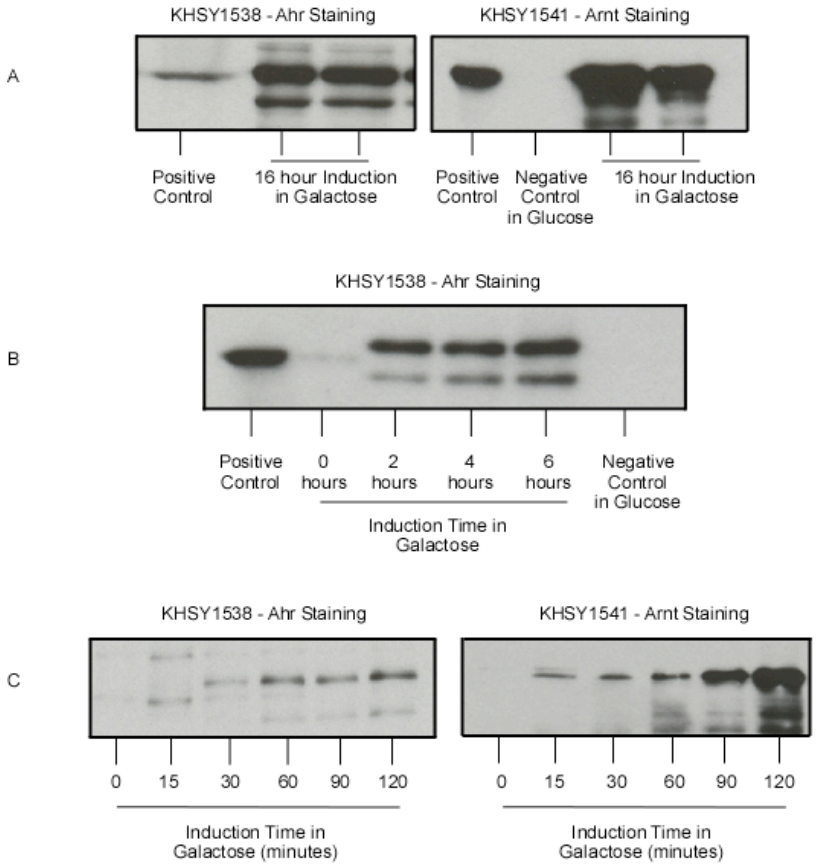


FIGURE 3.1: Western blot analysis of Ahr and Arnt protein expression in yeast. Ahr and Arnt proteins were detected in yeast cultures following induction with 2% galactose for 16 hours (A), 2 – 6 hours (B), and 15 – 120 minutes (C). Equal numbers of cells were harvested at each time point and total cellular protein prepared using 20% TCA. Fifteen microliter volumes of each sample were resolved on 7% SDS-PAGE gels and transferred to nitrocellulose for western blotting. Even sample loading was confirmed with Ponceau S staining prior to antibody detection. Ahr and Arnt protein expression was detected using 1µg/ml concentrations of polyclonal Ahr and Arnt-specific antibodies and reactivity was detected using ECL reagent.

The results show that Ahr was detected within 30 minutes of galactose treatment. In contrast, Arnt was detected at the 15-minute time point. Ahr expression appeared to reach equilibrium within 60 minutes of galactose induction, while Arnt expression continued to increase up to the 120-minute time point. The difference in these results may be related to a higher level of sensitivity of the Arnt antibody (Pollenz 1996), or differences in the turnover of the proteins. These results also highlight the fact that yeast expressing exogenous proteins from the *GAL1* promoter do not require long incubations with galactose to obtain detectable levels of protein. This is an important observation because long term induction and overexpression of exogenous proteins in yeast can lead to cell stress that might impact the interpretation of the experiment (Mattanovich 2004).

In summary, these results show for the first time that mammalian Ahr and Arnt protein can be detected in a recombinant yeast model using a western blotting approach. The ability to easily detect these proteins shows that further studies to assess Ahr degradation may be possible in the recombinant yeast model.

Induction of Ahr and Arnt proteins double knock-in yeast strain. The next set of experiments was designed to detect Ahr and Arnt proteins when co-expressed in a single yeast strain. KHSY1538 and KHSY1541 strains were mated and sporulated as described in chapter two to obtain the double knock-in strain, KHSY1547. KHSY1547 was streaked on YPD to produce single colonies and was placed in a 30°C incubator for two days. A single colony was used to inoculate an overnight culture with 2ml of liquid YPD media containing 2% glucose and was placed in a shaking 30°C incubator overnight. The following day, the optical density was measured at 600nm and a portion

of the sample was added to liquid YP medium supplemented with galactose (2% final). The induction culture was set up at an OD_{600} of 0.1 in a final volume of 10ml.

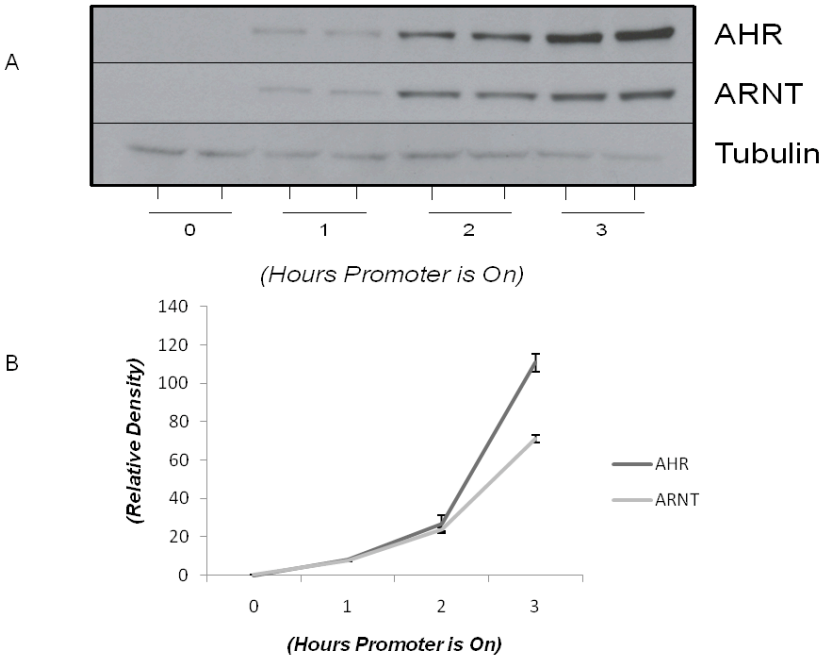


FIGURE 3.2: Induction of Ahr and Arnt proteins under the galactose-inducible promoter in yeast. Ahr and Arnt proteins were induced in a recombinant yeast strain with the addition of 2% galactose to the growth medium. 1.0 ODs of cells were harvested from the culture at 1, 2, and 3 hours following addition of galactose. Protein samples were prepared using the 20% TCA precipitation method. 15µl samples were run on 7% SDS-PAGE gels, protein was then transferred to nitrocellulose using a semi-dry blotter. Even sample loading was confirmed with Ponceau S staining prior to detection of Ahr and Arnt proteins using specific polyclonal antibodies. A 1µg/ml concentration of Ahr antibody and a 0.5µg/ml concentration of Arnt antibody was used in conjunction with 1:10000 goat anti-rabbit secondary antibody. For tubulin staining, a 1:1000 dilution of primary antibody was used with a 1:1000 dilution of goat anti-mouse secondary antibody. ECL reagent was used for detection of protein bands. Protein expression was quantified and normalized using ImageJ software. This data illustrates induction of both Ahr and Arnt proteins in the KHSY1547 recombinant yeast strain.

For the experiment shown in Figure 3.2, Ahr and Arnt protein induction was carried out for 1, 2, and 3 hours in the presence of 2% galactose. This time course was based on the studies shown in Figure 3.1. Total protein was prepared from the same

number of cells in the induced cultures using 20% trichloroacetic acid, as described. Two identical SDS-PAGE gels were run; one was stained for Ahr protein expression and the other was evaluated for Arnt protein. In addition to verifying even sample loading with Ponceau S staining, these blots were stained with α-tubulin antibody (Sigma-Aldrich) as a loading control. Protein levels were quantified and normalized using ImageJ software and were plotted using Microsoft Excel. A western blot from a representative experiment is shown in Figure 3.2A and the quantified data is shown in Figure 3.2B.

Figure 3.2 shows induction of Ahr and Arnt proteins in the KHSY1547 yeast strain that is detectable by western blotting. The time course of induction is comparable between Ahr and Arnt, and it is detectable within 1 hour of induction and strongly induced at 2 and 3 hours with slight variation in staining intensity likely due to antibody specificity (Pollenz *et al* 1994). Figure 3.2 is a representative experiment; however Ahr and Arnt inductions were carried out multiple times using the KHSY1547 strain. All experiments showed the inducible expression of both Ahr and Arnt that was easily detected within 1 hour. Based on these studies future experiments utilized a 2-3 hour induction time.

Validation of the yeast model

The previous set of experiments demonstrated detectable levels of Ahr and Arnt protein expression in the recombinant yeast strains (Aim #2). However, the study did not demonstrate the ability of the proteins to fold and function in a manner similar to endogenous Ahr and Arnt proteins. While there are several proteins belonging to the basic-helix-loop-helix (bHLH) family of proteins expressed in yeast (Berben *et al* 1990, Greenberg and Lopes 1996, reviewed by Robinson and Lopes 2000), there are no yeast bHLH/PAS homologues for Ahr and Arnt. Thus, since these proteins are not

endogenously expressed in yeast, it was possible that they may not function properly. It was crucial to confirm that Ahr and Arnt proteins were capable of heterodimerization and DNA binding, since these functions are central to the Ahr signaling pathway and ligand induced degradation (Pollenz 1996, Pollenz 2002, Song and Pollenz 2002). Therefore, our next aim was to functionally evaluate the Ahr and Arnt proteins and assess their ability to initiate transcription of a reporter gene (Aim #3).

The double knock in strain (KHSY1547) was transformed with the pLXRE5-Z reporter plasmid to generate strain KHSY1566 as described in Chapter Two. The reporter plasmid carries five xenobiotic response elements (XREs) located upstream of the *lacZ* gene (Cox and Miller 2002). For proper reporter activation to occur Ahr and Arnt protein expression must be induced and the Ahr must be activated with ligand treatment. After ligand binding, the activated Ahr dimerizes with Arnt and the resulting heterodimer interacts with the XREs to drive transcription of β-galactosidase. Several strains were constructed as detailed in Chapter Two. KHSY1538 was transformed with the reporter plasmid to produce KHSY1565. This strain only expresses the Ahr and was generated as a negative control for specific activation of the reporter. This strain should not produce ligand-inducible reporter activity, because it does not express Arnt and Arnt is required for transactivation of target genes in the Ahr signaling pathway (Reyes *et al* 1992).

Reporter Assay Method. The first set of reporter assays were carried out using a method similar to that of Miller (1997). Later studies were performed based on the protocol of Kippert (1995) that employed the use of the detergent, sodium lauroyl sarconsinate or sarcosyl. Because yeast have cell walls, Miller's protocol entailed preparation of whole cell extracts in order to release the β-galactosidase enzymes into the resulting cell lysate so that reporter activity could be detected by addition of

substrate. However, Kippert (1995) demonstrated that the results of assays performed in this manner showed a great deal of variation. Instead, Kippert suggested the use of the detergent sarcosyl to permeabilize the yeast cells to allow the substrate to bind β-galactosidase in intact cells.

The data in Figure 3.3 was generated using the Miller protocol. To test for β-galactosidase activity in intact cells, 1ml of yeast liquid culture was removed from the larger test cultures. Each sample was centrifuged for 1 minute at 14,000 rpm, the medium was aspirated, and the cell pellet was combined with 500µl of lacZ buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 1mM $MgCl_2$, 10mM KCL, and 0.4mg/ml ONPG (o-nitrophenyl-β-D-galactoside)). The pellet was resuspended in the solution and placed in a 37°C water bath and upon observation of a color change; the reactions were stopped with the addition of 1.5M sodium carbonate. The samples were centrifuged at 14,000 rpm for one minute to pellet the cells and the optical density at 420nm was measured for the supernatant. The observed optical density was normalized for protein concentration and the reaction time with the substrate using the following equation; (Abs 420nm x 1000) / [(Abs 595nm) x (reaction time in minutes)]. The resulting values, called Miller units, were plotted using Excel.

Reporter Activation Following Treatment with Ahr Ligand. To test whether the Ahr signaling pathway was functional when Ahr and Arnt were expressed exogenously in yeast, both the Ahr expressing reporter strain (KHSY1565) and the double knock-in reporter strain (KHSY1566) were assayed for β-galactosidase activity. One liquid culture of each strain was prepared from overnight cultures. Ahr expression was induced in the KHSY1565 strain with the addition of 2% galactose to the growth medium for 2 hours. Similarly, Ahr and Arnt co-expression was induced in the KHSY1566 strain with 2% galactose for 2 hours.

Whole cell extracts were prepared from both strains after induction of Ahr (1565) or Ahr and Arnt (1566) for two hours. These cell lysates were prepared prior to ligand treatment and activation of Ahr. Ahr signaling requires activation of the Ah receptor with ligand prior to Arnt heterodimerization and DNA binding (Li *et al* 1994, Whitelaw *et al* 1993). Therefore, the presence of Ahr and Arnt protein in yeast should not produce β-galactosidase activity. These samples were prepared to provide a baseline for reporter activity in these strains.

After the three hour protein induction, each culture was split in half. One sample of each strain was treated with 20nM TCDD and the other sample was treated with DMSO as a control. The dose used in these experiments was based on a study by Miller (1999) since 20nM TCDD treatment showed maximal reporter activity in similar yeast strains. The samples were placed in a shaking incubator for 3 hours to allow for Ahr activation. Whole cell extracts were prepared and the lysates were combined with the β-galactosidase substrate, ONPG. The results of the reporter study are shown in Figure 3.3.

The results of Figure 3.3 show reporter activity in the Ahr and Arnt expressing strain (KHSY1566) after 3 hours of 20nM TCDD treatment. The level of activity was approximately eight-fold higher than the time zero and DMSO (vehicle) controls. Importantly, the set of zero hour controls produced very low levels of reporter activity even after 2 hours of Ahr and Arnt expression. These results suggest that the culture conditions do not result in spurious activation of the Ahr pathway as has been noted in recombinant mammalian report assays (Miller 1997). In addition, the strains did not produce any reporter induction when incubated for 3 hours with DMSO, the vehicle used to prepare and dilute TCDD.

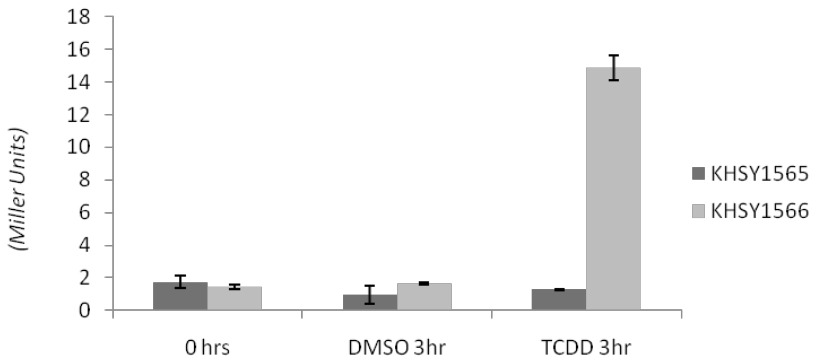


FIGURE 3.3: Analysis of β-galactosidase activity in KHSY1565 and KHSY1566 following TCDD treatment. Strains were induced with galactose for 3 hours, split into two equal aliquots and induced with 20nM TCDD or DMSO for an additional 3 hours. β-galactosidase activity was determined as detailed in the text. Note that only the KHSY1566 strain induced β-gal activity when treated with 20nM TCDD suggesting that Ahr and Arnt are capable of heterodimerizing and binding to XREs in this system.

The results in Figure 3.3 were important for several reasons. First, reporter activity was present in the KHSY1566 strain and not in KHSY1565. The reporter was activated upon binding of Ahr and Arnt heterodimers and did not show activation when Ahr was expressed alone. This is consistent with Ahr signaling in other organisms where XREs are bound by Ahr/Arnt heterodimers, but not Ahr alone (Reyes *et al* 1992). Also, we know that reporter activity is not present in samples treated with vehicle only, suggesting that Ahr and Arnt proteins are not likely heterodimerizing without first activating Ahr with ligand. These findings are consistent with the model of Ahr signaling where only the ligand-bound Ahr interacts with Arnt to form the DNA-binding species (Li *et al* 1994, Whitelaw *et al* 1993). Thus, they confirm that the basic components of the Ahr signaling pathway are intact in this yeast model. The receptor and its DNA binding partner are detectable and can function as transcription factors when activated with TCDD to drive an artificial reporter.

Interestingly, when similar studies were carried in recombinant yeast strains by other groups, a high level of reporter activity was detectable in the vehicle-treated samples. This effect was initially attributed to exogenous tryptophan present in the culture medium since tryptophan has been shown to act as an Ahr agonist and can activate the receptor (Rannug *et al* 1987, Miller 1997). However, later reports by the same group suggested that this effect was due to overexpression of Ahr and Arnt proteins (Miller 1999). In Miller's 1997 study, Ahr and Arnt proteins were expressed on a high-copy plasmid under a *GAL1*/*GAL10* bidirectional promoter in the presence of galactose for 24 hours. Our western blotting experiments provided in Figure 3.1 demonstrated that Ahr and Arnt were detectable within minutes of promoter activation and suggest that 24 hours of induction may result in extremely high protein levels, or high stress to the cells that likely had reached stationary growth. Additionally, experiments presented here were carried out using a yeast strain with a single copy of each cDNA integrated in the yeast genome, while Miller's strains carried between 10 and 40 copies of each cDNA on a high-copy plasmid (Miller 1997, 1999). This further confirms the importance of detection and analysis of exogenously expressed proteins when working in a recombinant yeast model.

Since TCDD could activate the reporter construct, it was pertinent to assess the ability of other Ahr ligands to induce β-galactosidase in our yeast model. The aryl hydrocarbon receptor signaling pathway can be activated upon exposure to halogenated aromatic hydrocarbons (HAHs), polycyclic aromatic hydrocarbons (PAHs), flavones and several naturally occurring compounds. Here the KHSY1566 strain was evaluated for reporter activity after treatment with several Ahr ligands known to have varying degrees of Ahr binding affinity and potency. TCDD, the prototypical and most potent HAH, was tested and compared to several PAHs including, benzo(*a*)pyrene (BAP) and 3-methylcholanthrene (3-MC) and the flavone, β-naphthoflavone (βNF). It was of interest

to determine which ligand produced the highest level of reporter activity for use in later western blotting experiments.

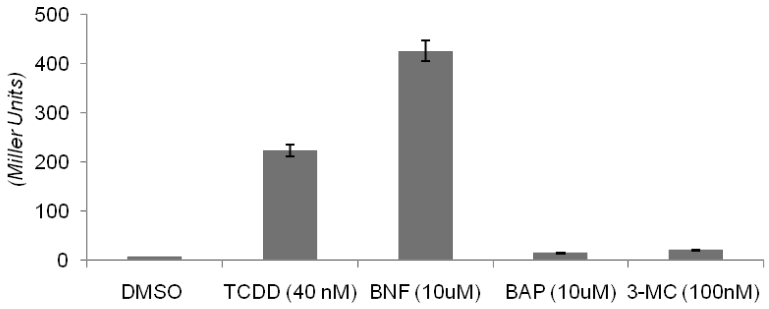


FIGURE 3.4: Analysis of β-galactosidase activity in KHSY1565 and KHSY 1566 following treatment with several known Ahr ligands. Strains were induced with galactose for 2 hours, split into two equal aliquots and induced with TCDD(40nM), βNF(10um), BAP(10uM), 3-MC(100nM), or DMSO for an additional 4 hours. β-galactosidase activity was determined as detailed in the text. Reporter activity was measured for duplicate samples and normalized values were plotted using Excel. Note that βNF and TCDD produced significant levels of β-gal activity and BAP and 3-MC produced β-gal levels only slightly above the DMSO-treated control samples.

KHSY1566 was induced with the addition of 2% galactose to the growth medium for two hours to allow for Ahr and Arnt expression. Next, the culture was split and the resulting samples were treated with either vehicle or Ahr ligands for an additional four hours. Using the cell permeabilization method (Kippert 1995), reporter activity was measured and normalized as previously described. The Miller units were plotted using Excel and are displayed in Figure 3.4.

The ligand doses tested in Figure 3.4 were selected based on previous studies (Miller 1999). Figure 3.4 shows a 25-fold induction of β-galactosidase activity with TCDD treatment and a 50-fold induction of β-gal activity with βNF treatment compared to the DMSO-treated control. This high level of reporter activity in the TCDD and βNF treated cultures is in contrast to the BAP and 3-MC treated samples. Treatment with

BAP produced a 2-fold induction of β-gal activity and 3-MC treatment caused a 3-fold induction of β-gal activity compared to the DMSO-treated control. These results were unexpected, since TCDD is known to be the most potent Ahr ligand with only pM levels required to saturate Ahr binding in mammalian cell culture (Pollenz 1996). The results show that βNF, a more labile ligand, produces higher β-galactosidase levels. One explanation for this observation is the hydrophobic nature of TCDD, making it highly insoluble in water (reviewed by Denison and Nagy 2003). Since yeast growth medium is aqueous, it is likely that the polar nature of TCDD may have caused it to come out of solution before entering the yeast cells and the effective dose may actually be be lower than 40nM. Testing this hypothesis would require radio labeled-TCDD and since these experiments generate mixed-waste, they are not approved at USF.

To further evaluate the Ahr signaling pathway and better refine the ligand activation the next set of experiments assessed whether increasing amounts of ligand produced dose-dependent transactivation of the reporter in the recombinant yeast model. In tissue culture cell lines, the degree of transactivation of CYP1A1 is dependent on the potency of the ligand as well as the dose (Song and Pollenz 2002). Therefore, the reporter strain was used to determine whether a dose-dependent increase in reporter activity could be detected. This experiment would also provide information regarding TCDD solubility since it would be expected that if the TCDD remained in solution, activation of the β-gal reporter would reach saturation at high doses.

TCDD and βNF were chosen for these studies since they induced the highest levels of β-galactosidase activity in the previous experiment (Figure 3.4). The KHSY1566 strain was induced for Ahr and Arnt protein expression for 2 hours, split into several cultures, and individual cultures were treated with increasing amounts of TCDD, βNF, or vehicle for an additional 4 hours. All samples were harvested at the same time and processed for β-galactosidase activity as previously described.

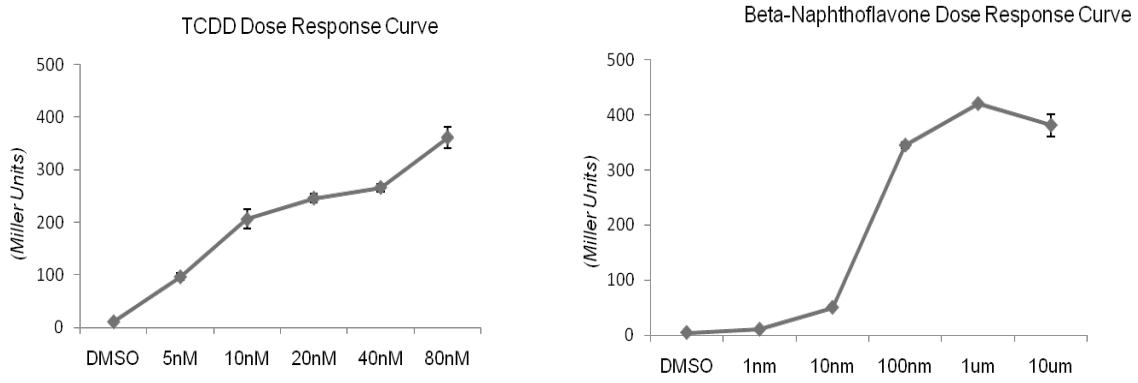


FIGURE 3.5: Dose-response analysis of KHSY1566 with TCDD and βNF. Strains were induced with galactose for 2 hours, split into two equal aliquots and induced with increasing doses of TCDD or βNF for an additional 4 hours. β-galactosidase activity was determined as detailed in the text. Reporter activity was measured for duplicate samples and normalized values were plotted using Excel. Note that reporter activation is evident for both treatments in a dose-dependent manner.

The results in Figure 3.5 are consistent with data published by other groups in yeast (Miller 1999, Sugihara *et al* 2008). Ahr ligand treatment induced expression of the Ahr-specific reporter plasmid in a dose-dependent manner. For βNF, the results show a sigmoidal dose response curve with saturation at ~1μM. The EC_{50} for βNF was approximately 60nM. TCDD was tested using a range of 5nM to 80nM, and higher doses were not tested due to the insolubility of the chemical. The resulting TCDD dose response curve did not appear as a sigmoidal curve; instead the curve is linear and does not reach saturation, even at 80nM. A TCDD dose of 80nM is 1.5 orders of magnitude above the EC_{50} shown in mammalian cell culture models (Poland and Knutson 1982, Bradfield and Poland 1988, Song and Pollenz 2002). This result suggests that TCDD did not saturate the Ah receptors present in the yeast cells and that the hydrophobicity of TCDD was causing to the compound to come out of solution or become sequestered by

protein or other components of the culture medium. Therefore, the effective dose of TCDD could not be calculated.

Generation of a Functional Strain. Based on the data shown in the previous figures, it is suggested that the KHSY1566 yeast strain expresses a functioning Ahr signaling pathway. The results that support this statement include:

- Ahr and its DNA-binding partner Arnt are detectable by Western blotting (Figures 3.1 and 3.2).
- Ahr-specific activation of the XRE driven reporter can be detected after treatment with known Ahr ligands (Figure 3.3).

These results suggest that at least some fraction of the expressed Ahr and Arnt proteins are properly folded, can dimerize with each other and can associate with specific DNA elements in the yeast cells. These observations also suggested that the key components of the Ahr signaling pathway are functional when expressed in our yeast model. This information is crucial because there are several other important proteins in the Ahr signaling pathway that were not exogenously expressed in our strains. Functional Ahr signaling also requires Hsp90, Xap2, p23 and other transcriptional activators (Pongratz *et al* 1992, Carver and Bradfield 1997, Ma and Whitlock 1997, Kazlauskas *et al* 1999). Yeast homologues to the mammalian Hsp90, Xap2, p23, several co-activators and other general transcription factors have been identified and appear to perform the functions of their mammalian counterparts in order for the pathway to function (Cox and Miller 2002, Cox and Miller 2003). The final step in the Ahr signaling pathway is the proteasome-mediated degradation of Ahr and this will be evaluated extensively in later experiments. From this point on, the KHSY1566 strain was used to further analyze the signaling pathway when expressed in yeast.

Assessment of Ahr Protein Turnover in Yeast

Ahr degradation, as studied in mammalian cells, can be considered in three different scenarios. First, the normal half-life of the latent receptor has been reported to be approximately 28 hours (Ma and Baldwin 2000). This is based on the normal synthesis and destruction of the protein to maintain homeostasis. Second, many reports show that the Ahr is degraded in a ligand-dependent manner (reviewed by Pollenz 2002, 2010). The receptor half-life in the presence of ligand is reduced to approximately 3 hours (Ma and Baldwin 2000, Pollenz 1996). Finally, degradation of the receptor has been observed in a ligand-independent manner following treatment with benzoquinone ansamycin antibiotics (Chen et al 1997, Song and Pollenz 2002, Pollenz *et al* 2005). While many groups have published data on Ahr degradation in higher eukaryotes, there have been no reports on Ahr protein expression or degradation in a yeast model. Such a model is advantageous since it would allow genetic dissection of the various proteins involved in the process.

Stability of the Ahr in the Absence of Ligand. The recombinant yeast strains were created so that the different aspects of Ahr degradation could be evaluated in a model that was amenable to genetic dissection. However, initial studies suggested that ligand-mediated degradation of the Ahr was not detectable in the recombinant strain when the Ahr was continually induced (see later sections of this chapter). Therefore, it was crutial to better understand the stability of the Ahr in the absence of ligand in the yeast model and that would allow for the use of other experimental approaches to assess the Ahr degradation. Therefore, it was essential to assess what was happening to the level of Ahr protein after the inducible expression of the Ahr was turned off. It was

hypothesized that if the expression of the Ahr is turned off it may be possible to see changes in the level of Ahr expression upon ligand treatment.

The stability of the Ah receptor was evaluated in the Ahr-expressing recombinant yeast strain (KHSY1538). Pulse-labeling has been used to determine the Ahr half-life in mammalian cell culture studies (Ma and Baldwin 2000); however, we did not have the ability to use a radioactive method and a simpler approach was employed here. Since Ahr and Arnt were expressed downstream of an inducible promoter, it was possible to induce protein expression for a time period and subsequently turn off the promoter to evaluate the Ahr and Arnt protein levels using western blotting. Importantly, this technique was used by others to test the half-life of exogenously expressed proteins in yeast (Ke *et al* 2003).

The results presented in the Ke *et al* (2003) study provided an additional consideration for the promoter off approach. In the Ke study (2003), levels of thymidine kinase (Thk) proteins expressed under the *GAL1* promoter were evaluated via western blotting using the promoter off scheme described throughout this chapter. The results of this study showed that the Thk protein was reduced in the cultures over time once the inducible promoter was turned off. This effect was attributed to the rate of yeast cell division (approximately 90 minutes for wild type *S. cerevisiae* (Sherman 2002)) that will cause the recombinant protein levels to be “diluted” from the culture over time as the yeast bud and continue to propagate in the absence of new recombinant protein expression. The quantified data from this study revealed a direct correlation between the density of the yeast culture and the level of Thk protein detected on the western blot.

Therefore, it was hypothesized that a similar effect would be observed with Ahr and Arnt protein levels when the *GAL1* promoter was turned off in the Ahr/Arnt recombinant strain. *S. cerevisiae* divide every 90 minutes (wild type) during exponential growth, and according to this theory the number of cells that contain Ahr and Arnt protein

would be reduced by half after the first doubling (~90 minutes). After 180 minutes, the protein levels would be expected to be further diluted to 25%. This model assumes that the recombinant protein is essentially stable (~24 hr half-life) and is not significantly degraded during the short time line of these studies. A schematic that illustrates this process is presented in Figure 3.6. Note that for this system to be amenable to the study of Ahr degradation, all results would fall to the left of the predicted trend line. It is also important to note that the cultures utilized in the following experiments were asynchronous and the cells appeared to persist in the lag growth phase for the initial time points of each experiment before reaching exponential growth. Therefore, the time line of the reduction in the level of recombinant proteins may not exactly mirror the theoretical prediction (it would likely shift the predicted trend line to the right).

It is possible that the effect of yeast cell division on the level of Ahr and Arnt proteins in the recombinant strain may confound the ability to detect a ligand-mediated effect on Ahr protein levels in the promoter off paradigm. Western blotting and antibody staining of Ahr and Arnt should reveal reduced levels of protein over time due to active cell division. Because we are looking for a ligand-mediated effect on Ahr protein levels, a more rapid reduction in Ahr protein would have to be observed than the anticipated dilution would cause (a left shift of the predicted curve). Using the promoter off method, it was hypothesized that a loss of Ahr and Arnt proteins would be observed over time and an enhanced loss of only Ahr in the presence of ligand.

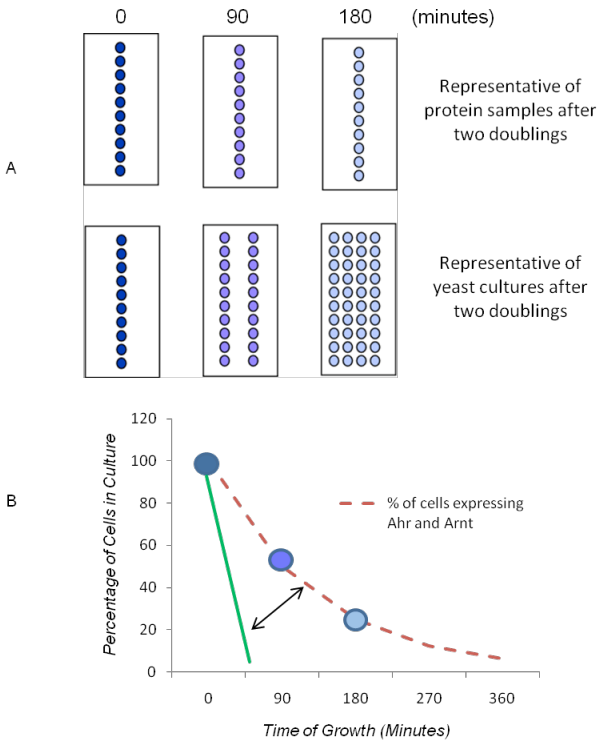
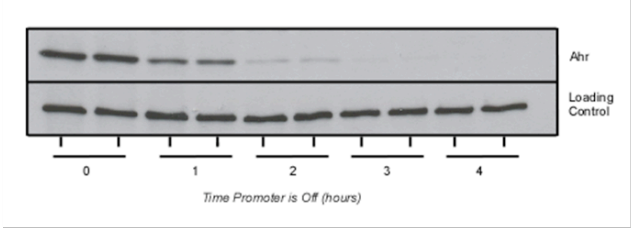


FIGURE 3.6: Predicted pattern of protein detection when an inducible promoter is turned off and the recombinant protein is stable. This figure represents the effect of cell division on the detection of exogenously expressed proteins in the promoter off experiment. This model is based on a protein that would have a normal half-life of 24 hrs (highly stable). A. In this example, the inducible expression of the proteins was activated. All cells in this culture express the given proteins (dark blue). The inducible promoter is turned off. The cells continue to divide over time and double after ~90 minutes. This culture at 90 minutes then contains double the number of cells but the same level of protein. After 180 minutes the culture doubles in size again with the same level of exogenous protein that was present at time zero. For the analysis, equal number of cells are harvested at the 0, 90 and 180 minutes time points to produce equal sample loading on the SDS-PAGE gels for comparison of protein levels. The prepared protein samples will contain decreasing amounts of the target protein. B. Theoretical quantification of the dilution effect. Note that a shift to the left (green line) would indicate that the recombinant protein is being degraded during the time course and to observe enhanced degradation of the recombinant protein that could be quantified, it would be essential to observe additional shifts to the left.

For the studies detailed below, the double knock-in strain, KHSY1547 was utilized since both Ahr and Arnt proteins are expressed at the same time. Additional results from the single knock-in strains are presented in Appendix A. Ahr and Arnt proteins were induced with galactose for two hours and transcription of AHR and ARNT mRNAs was blocked with the addition of glucose to the growth medium. Duplicate sets

A



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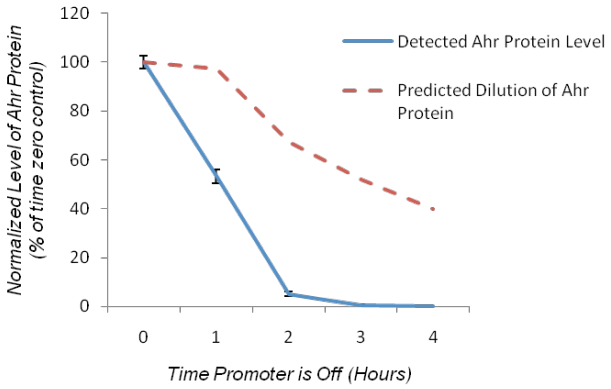


C

Time (Hours)	Optical Density 600nm	Predicted amount of Ahr
0	2.43	100
1	2.5	97
2	3.6	67
3	4.68	52
4	6.09	40

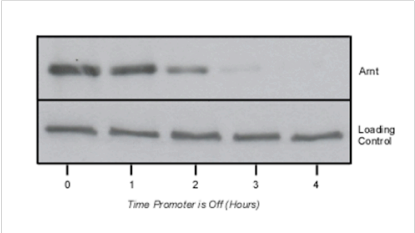
FIGURE 3.7: Detection of Ahr protein degradation in yeast. The KHSY1547 strain was induced with 2% galactose for two hours followed by a change to growth media containing 2% glucose. Glucose was added to the medium in order to transcriptionally repress the *GAL1* promoter. Protein samples from an equal number of cells were prepared using 20% TCA at various time points after the promoter was turned off. A. Western blotting and antibody staining revealed a rapid loss of Ahr protein over time. The loading control shows that equal amounts of protein were loaded in each lane. B. Ahr protein bands were quantified using ImageJ software and normalized to the loading control. The normalized data was plotted using Excel. Note the addition of the red dashed line that represents the predicted level of Ahr that would be present in the cells based on the culture densities shown in part C. C. Optical density of the yeast culture at the time each sample was prepared. Since the OD represents the number of cells in the culture, this can be used to produce a trend line that would show the theoretical loss of the Ahr if the reduction was solely based on dilution through cell division. Note that the Ahr protein level decreased over time and the line of the graph is shifted to the left of the predicted trend line. This observation suggests that Ahr is being degraded from the cells when the promoter is turned off and the loss of protein is not entirely due to dilution of proteins as the cells divide.

of protein samples were prepared at time zero and then equal numbers of cells harvested at one hour intervals. Protein samples were prepared using the TCA methods. The results for Ahr protein expression are shown in Figure 3.7A. The level of Ahr protein was quantified using ImageJ software and normalized to the loading control (Figure 3.7B).

The western blotting results in Figure 3.7A show that the Ahr protein levels are rapidly reduced after the *GAL1* promoter is turned off. Importantly, the plot of Ahr protein is shifted to the left of the predicted trend line (dashed line in Figure 3.7B) and suggests that the estimated $t_{1/2}$ of the Ahr in this model is ~1 hour. This half-life is significantly shorter than what has been determined in mammalian cell culture models (Ma and Baldwin 2000). This result implies that Ahr protein levels are not simply being diluted from the yeast culture, but instead, the protein is being actively degraded in a non-liganded state and may be unstable when expressed in yeast. It is important to note that the reduction of the Ahr protein does not appear to be related to premature initiation of the Ahr pathway by endogenous ligands present in the yeast or yeast media since minimal levels of β-gal induction are observed in the absence of exogenous ligand in this model (Figures 3.3-3.5).

Based on the results presented in figure 3.7, it was pertinent to assess the stability of Arnt in this system. Arnt is predicted to be a stable protein with a half-life of >20 hrs in mammalian cells (Pollenz 2002, 2010). The samples prepared in the experiment detailed in 3.7 were evaluated for Arnt and the results are presented in Figure 3.8. The results show that Arnt protein levels are also rapidly reduced after the *GAL1* promoter is turned off and the plot of Arnt protein is shifted to the left of the predicted trend line. The estimated $t_{1/2}$ for Arnt was ~2 hours in this experiment. Thus, both Ahr and Arnt proteins appear to be less stable when expressed in yeast compared to mammalian cells. The rapid loss of the Ahr in the absence of ligand complicated the

A



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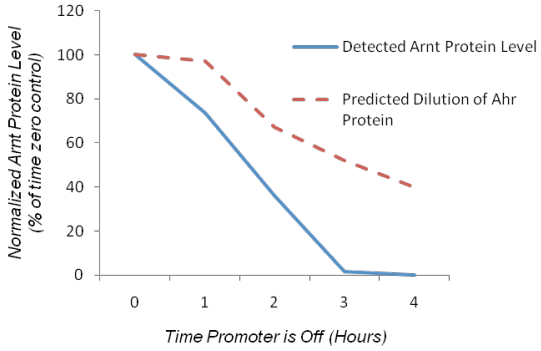

C

Time (Hours)	Optical Density 600nm	Predicted amount of Ahr
0	2.43	100
1	2.5	97
2	3.6	67
3	4.68	52
4	6.09	40

FIGURE 3.8: Detection of Arnt protein degradation in yeast. The KHSY1547 strain was induced with 2% galactose for two hours followed by a change to growth media containing 2% glucose. Glucose was added to the medium in order to transcriptionally repress the *GAL1* promoter. Protein samples from an equal number of cells were prepared using 20% TCA at various time points after the promoter was turned off. A. Western blotting and antibody staining revealed a rapid loss of Arnt protein over time. The loading control shows that equal amounts of protein were loaded in each lane. B. Arnt protein bands were quantified using ImageJ software and normalized to the loading control. The normalized data was plotted using Excel. Note the addition of the red dashed line that represents the predicted level of Arnt that would be present in the cells based on the culture densities shown in part C. C. Optical density of the yeast culture at the time each sample was prepared. Since the OD represents the number of cells in the culture, this can be used to produce a trend line that would show the theoretical loss of the Arnt if the reduction was solely based on dilution through cell division. Note that the Arnt protein level decreased over time and the line of the graph is shifted to the left of the predicted trend line. This observation suggests that Arnt is being degraded from the cells when the promoter is turned off and the loss of protein is not entirely due to dilution of proteins as the cells divide.

use of this model for assessment of Ahr degradation. The original aim of this project was to produce a model to genetically evaluate Ahr degradation in yeast in a ligand-dependent manner. Thus, experiments designed to evaluate the level of Ahr protein in the presence of ligand using the promoter off model would require the Ahr to be reduced within a matter of minutes in order to see changes from the control. Thus, additional experiments were performed to determine the impact of cell growth, dilution and protein overexpression in an effort to determine if conditions could be identified that would result in a more stable Ahr.

Since the Ahr was being rapidly lost from cultures when the *GAL1* promoter was was transcriptionally repressed, it is possible that this was due to overexpression in the recombinant yeast strains. Importantly, similar results were also observed for the single knock-in strains as shown in Appendix B. Overexpression of exogenous proteins correlates with an increase in expression of chaperone proteins in the cell in order to facilitate proper protein folding. In cases of extreme overexpression, the cells may be unable to produce enough chaperones to properly fold the newly synthesized proteins (Mattanovich *et al* 2004). Unfolded proteins in the cytoplasm of *Saccharomyces cerevisiae* have been shown to cause the unfolded protein response (UPR). UPR then detects the misfolded proteins in the cytoplasm and conjugates the substrates with ubiquitin in order to degrade the unfolded and misfolded proteins via the 26S proteasome (Eisele et al 2008, Mattanovich *et al* 2004). Therefore, we chose to use the galactose-inducible promoter as opposed to a constitutively active promoter upstream of the AHR and ARNT cDNAs to avoid overexpression, protein turnover, and cell stress. With the inducible promoter, could be carefully modulate the amount of protein produced in order to avoid gross overexpression that could occur when using a strong, constitutively active promoter. However, without a true baseline to directy assess the number of protein molecules in each yeast cell, it was unclear whether this system was

expressing a level of protein that was comparable to that found in mammalian cells. We hypothesized that inducing the promoter with less galactose, might illustrate whether overexpression was contributing to the rapid loss of Ahr.

Next, it was hypothesized that the chaperone proteins (i.e. Hsp90) required for Ahr folding may not be functioning efficiently in yeast and resulting in rapid loss of Ahr. This hypothesis will be explored in later sections of this chapter, but did not appear to impact the stability of the Ahr. Finally, it was proposed that Ahr proteins may be degrading in the yeast strains as a result of an intrinsic yeast specific degradation signal within the protein. It is possible that a signal or particular sequence within the proteins is recognized by the yeast cell's degradation machinery that targets the proteins for degradation. However, there is no database of degradation signals in yeast that can be used to assess this question without carrying out mutagenesis of the Ahr. In addition, the sequence involved in Ahr degradation in mammals is also currently unknown. Because Ahr and Arnt have some sequence in common, there may be a degradation signal within the bHLH-PAS sequence since they share a high identity within this region.

Optimization of Sample Preparation for Ahr Stability. Addition of 2% galactose to the growth medium is standard protocol for maximum activation of the *GAL1* promoter in yeast (Sherman 2002), and initial studies were carried out under these conditions. It was hypothesized that reducing the amount of galactose added to the culture medium would lead to a reduction in the expression of Ahr and Arnt proteins. Lowering the amount of sugar, should reduce the degree of *GAL1* promoter activation and produce a lower level of Ahr and Arnt expression. It was then hypothesized that lowered expression of the exogenous proteins may lead to production of more stable proteins. Due to the high sensitivity of the Ahr and Arnt antibodies, it was believed that the protein could still be detected even when expressed at much lower levels.

In order to test this hypothesis, a series of 20ml cultures were prepared containing decreasing amounts of galactose. The percentage and volumes of galactose used to prepare the cultures are shown in Table 3.0. Each culture was prepared such that the starting cell density at OD_{600} equaled 0.1. All volumes of galactose were brought up to 1ml with sterile water prior to addition to the culture media. The cultures were then placed in a 30°C shaking incubator for 3 hours to allow for protein induction.

While the purpose of adding galactose to the growth medium was to activate the promoter for these experiments, it also functions as the sole carbon source for the yeast to sustain cell growth. For this reason, each culture tested in Figure 3.9 was supplemented with an additional 1% sucrose. Sucrose does not affect the *GAL1* promoter; it does not activate or inhibit the promoter in the way galactose and glucose do (reviewed by Lohr *et al* 1995, Platt and Reece 1998). Addition of sucrose to the culture medium, therefore, allowed the cultures with the minutest amounts of galactose to continue to grow without exhausting their carbon source.

% Galactose	Volume (ml) in 20ml
0.02	0.01
0.05	0.025
0.1	0.05
0.2	0.1
2.0	1.0
2.0 Glucose (- control)	1.0

TABLE 3.0: Percentage of galactose added to yeast cultures to minimized induction of Ahr and Arnt protein levels and reduce cellular stress. KHSY1547 was used in five separate cultures containing decreasing amounts of galactose. One additional culture was prepared as a negative control and contained only glucose as a carbon source. Each volume of galactose was brought up to one milliliter with sterile water before it was added to the culture medium.

Protein samples were prepared as previously described using 20% TCA. Two SDS-PAGE gels were prepared; one was loaded with 5µl of each sample and the other was loaded with 15µl of each sample. The negative control, which was grown in presence of 2% glucose, was loaded in the first lane of each gel. Samples prepared from cultures grown in the presence of increasing amounts of galactose were loaded next, and a sample of protein prepared from mouse Hepa-1 cells was loaded as a positive control in the last lane. The mouse cells express the Ah^{b-1} allele of the receptor and it migrates at a lower molecular mass than the Ah^{b-2} that is expressed in the yeast cells. The representative experiment is presented in Figure 3.9.

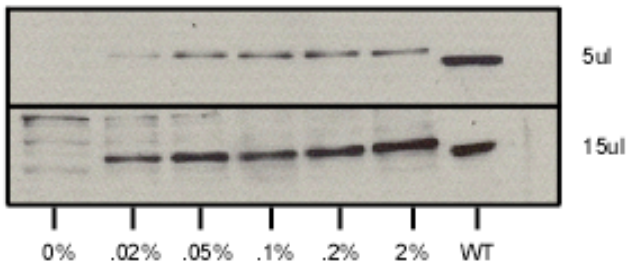

FIGURE 3.9: Ahr protein expression is reduced with decreased levels of galactose. Six 20ml cultures were prepared using the KHSY1547 strain. The culture medium was prepared using the amounts of sugar listed in Table 3.0. After three hours of induction in a 30°C shaking incubator, protein samples were prepared using 20% trichloroacetic acid. SDS-PAGE gels were run with either 5 or 15µl of lysate and stained with anti-Ahr antibodies. The western blotting results show that Ahr protein induction can be reduced by adding less galactose to the culture medium.

Figure 3.9 shows Ahr expression is detectable in all cultures grown in galactose. The samples grown in 2%, 0.2%, 0.1%, and 0.05% galactose all showed Ahr staining when 15µl of the sample was run on the SDS-PAGE gel. Even the induction with 0.02% galactose, the equivalent of 10µl in a 20ml culture, was enough to induce detectable expression of the proteins by way of the *GAL1* promoter. Overall levels of Ahr

expression induced by 0.05% galactose were reduced approximately by half compared to 2.0% galactose. This result suggests that the addition of 2% galactose to the growth medium is not required in order to produce detectable levels of Ahr. Therefore, the strains could be tested for protein stability following induction with less galactose added to the culture medium and determine whether reduced levels of Ahr induction can improve the protein stability and reduce the rapid loss that was observed in Figure 3.10.

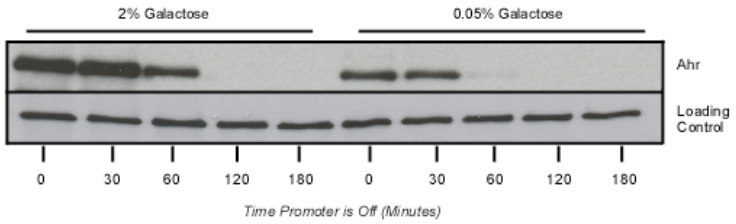

FIGURE 3.10: Activation of the *GAL* promoter with 2% or 0.05% galactose and subsequently turning off the promoter with glucose causes rapid Ahr turnover in yeast. Two separate cultures of equal cell density were prepared using the KHSY1547 strain. One culture was prepared with 2% galactose while the other was prepared with 0.05% galactose. The western blotting results show that 2% galactose leads to a stronger activation of the promoter than 0.05% galactose. Removal of galactose and addition of glucose to the growth medium causes rapid degradation of Ahr after induction with 2% or 0.05% galactose, suggesting that minimizing the activation of the promoter does not produce a more stable Ah receptor protein.

The KHSY1547 strain was propagated and split into two aliquots. One culture was induced with the standard 2% galactose and the other was induced with 40-fold less galactose at 0.05%. Both cultures were also supplemented with an additional 1% sucrose in order to maintain cell viability and division, particularly in the 0.05% galactose culture. The cultures were placed in a 30°C shaking incubator and induced for 3 hours to allow for Ahr and Arnt protein induction. After three hours, the cultures were centrifuged at 2000 rpm for 2 minutes and the culture medium was removed. The cell pellets were washed with 1 ml of sterile water and centrifuged again. The cells were then resuspended in medium containing 2% glucose in order to turn off the *GAL1*

promoter. Samples from both cultures were harvested at 0, 30, 60, 120, and 180 minute time points after the promoter was turned off. Protein was prepared using the TCA method from an equal numbers of cells, as described. Protein samples were resolved on a 7% SDS-PAGE gel and stained with anti-Ahr antibodies. The resulting western blot is shown in Figure 3.10. The results show that the level of Ahr protein induced with 0.05% galactose is approximately half of the level induced with 2% galactose at time zero. However, in both experiments, the Ahr is rapidly lost from the cells once the promoter is turned off This result suggests that while reducing the induction with minimal galactose in the culture medium does result in less induction of the Ahr protein, it is does not appear to enhance Ah receptor stability in the recombinant yeast model.

The next experiment was carried out to test Ahr signaling when reduced amounts of galactose are used for *GAL1* induction. This experiment was important because the results would demonstrate if there was a correlation between the level of protein observed using western blotting and the proportion of that protein pool that was functional and could activate the XRE reporter. The KHSY1566 strain was tested for reporter activation after induction in 1.0%, 0.1%, and 0.01% galactose. Again, these cultures were supplemented with 1% sucrose in order prevent depletion of the yeast resources.

The results of the β-galactosidase assay shown in Figure 3.11 demonstrate that induction of the *GAL1*-inducible promoter with galactose is dose-dependent. Induction with 1% galactose produced a 5-fold induction of β-gal activity over the uninduced and vehicle-treated controls. Importantly, induction with ten-fold less galactose at 0.1% revealed β-gal levels that were only slightly above the negative controls. This result suggests that these low amounts of galactose cannot be used in the reporter assays and higher amounts are required in order to induce expression of enough protein. When these results are compared to Figure 3.9 it is clear that there is some correlation

between the level of protein detectable in the western blots and the portion that is functional as measured by the reporter assay.

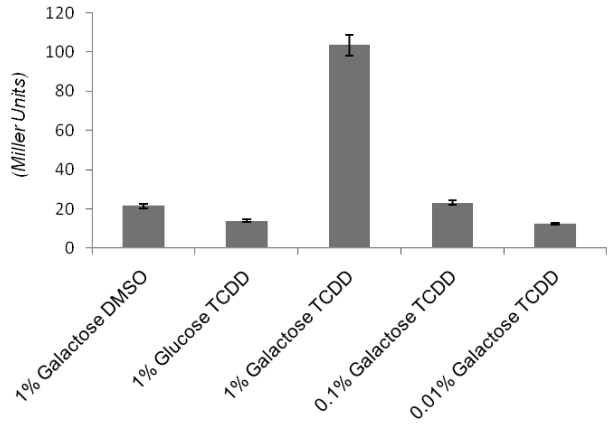

FIGURE 3.11: Analysis of β-galactosidase activity in KHSY1566 following activation of the *GAL1* promoter with decreasing amounts of galactose. The reporter strain was induced with 1%, 0.1%, or 0.01% galactose for 2 hours prior to dosing with 20nM TCDD for an additional 3 hours. Two controls were also evaluated. An uninduced strain was treated with 20nM TCDD and an induced strain was treated with vehicle for the same time course. β-galactosidase activity was determined as detailed in the text. Significant levels of β-gal activity were observed when the strain induced with 1% galactose was treated with 20nM TCDD. The culture induced with 0.1% galactose still showed some level of induction, but it was significantly reduced compared to the 1% induction. Also, the 0.01% induction was below background levels. This result suggests that there is a correlation between the level of Ahr observed on the western blots and the functional protein that is being produced.

Preparation of Soluble Protein Fractions. The previous hypothesis suggested that Ahr turnover in yeast may be due to overexpression of the AHR and ARNT mRNA by the *GAL1* promoter. However, the theory that addition of less galactose and the subsequent reduction of protein induction did not appear to produce a more stable protein. Another possibility arose after questioning our protein preparation technique. The procedure employed in the previous western blotting experiments used 20% trichloracetic acid to precipitate protein from the yeast cell pellets. This procedure was useful for its simplicity and because it produced samples with a high concentration of

protein in a small sample volume. Ahr and Arnt proteins were detectable in samples prepared using this method, but we asked whether this sample preparation method may have contributed in some way to the instability of our proteins in yeast.

We asked if the AHR and ARNT mRNAs were being overexpressed such that the Ahr and Arnt proteins were being shuttled to inclusion bodies within the cells and subsequently undergoing degradation. If this was the case, and the TCA procedure precipitated all proteins within the cell, it was possible that insoluble and/or nonfunctional proteins were precipitated along with a functional pool of protein. With that, we wondered if there may be a more stable Ahr present in the soluble protein fraction. If there is a stable form of the receptor in the cells, could the soluble proteins be isolated away from the insoluble proteins.

In the next several experiments, soluble protein fractions were prepared from the double knock-in strain using a phosphate buffer extraction method described in Chapter 5. One large culture of KHSY1547 was grown for several hours in glucose and was split in half so that one half was induced with 2% galactose and the other remained uninduced with glucose. The cultures were placed in a shaking incubator for 3 hours to allow for Ahr protein induction. After the induction period, a sample of each culture was used to determine the cell density at OD_{600}. 1.5 ODs of each culture was removed and protein was extracted using the standard 20% TCA protocol used previously. Previous attempts to prepare whole cell lysates produced samples that were too dilute for detection of our proteins using western blotting. Therefore, the cell number was increased 10-fold and 15.0 ODs of culture was used for the soluble protein extraction procedure.

The 15.0 ODs of yeast cells were spun at 2000 rpm for 2 minutes and the pellet was suspended in 1ml of cold phosphate buffer (20mM NaPi pH 7.7, 300mM NaoAc, 10% glycerol, protease inhibitor cocktail). The samples were then spun at 5000 rpm in a

refrigerated centrifuge at 4°C for five minutes. The pellet was resuspended in 100µl of cold phosphate buffer, an equal volume of glass beads were added, and the tubes were placed in a mini-bead beater for 4 times for one minute each, and placed on ice for two minutes in between each. The resulting lysate was spun again at 14,000 rpm for 30 minutes at 4°C. The resulting supernatant, containing presumed soluble proteins was then combined with an equal volume of 20% TCA in order to precipitate our proteins and later resuspend them using a smaller volume of SDS-PAGE sample buffer.

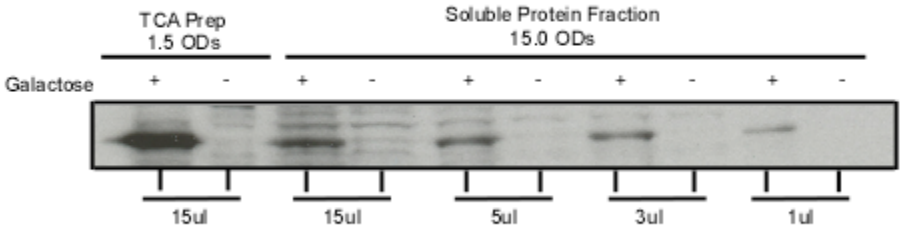

FIGURE 3.12: Detection of Ahr protein in soluble protein fractions. Protein samples from galactose induced KHSY1547 cultures reveal Ahr staining following TCA precipitation or soluble protein extraction methods. Intensity of Ahr staining is much lower in samples prepared using the soluble extraction procedure, especially considering that the sample contained ten-times more cells to start with. Decreasing amounts of sample were loaded to determine the minimum sample volume required for detection.

Figure 3.12 shows detectable levels of Ahr protein in both the trichloroacetic acid precipitation and the soluble protein extraction methods. Interestingly, 1.5 ODs of cells prepared with TCA shows much more intense staining than 15 ODs of cells prepared for soluble protein. This observation may suggest that there are two separate Ahr protein pools within the yeast cells, one soluble fraction containing the functional receptor, and one insoluble fraction containing improperly chaperoned receptor. If this is the case, the results of Figure 3.12 imply that the soluble protein pool is indeed a very small fraction of the total Ahr when expressed in our yeast model. However, this effect could simply be due to the efficacy of the protein preparation procedure.

The stability of Ahr and Arnt was tested again using protein extracts prepared using phosphate buffer for the protein preparation procedure to obtain what was believed to be the soluble protein fraction. The double knock-in strain was induced using 2% galactose for three hours before the promoter was turned off, as previously described. Protein samples were then prepared via TCA precipitation or using phosphate buffer to prepare the soluble fraction. Samples were obtained at 0, 1, 2, and 3 hour time points after the promoter was turned off with the removal of galactose and addition of glucose to the growth medium. Figure 3.13 shows two SDS-PAGE gels; one stained with anti-Ahr antibody and the other stained with anti-Arnt antibody.

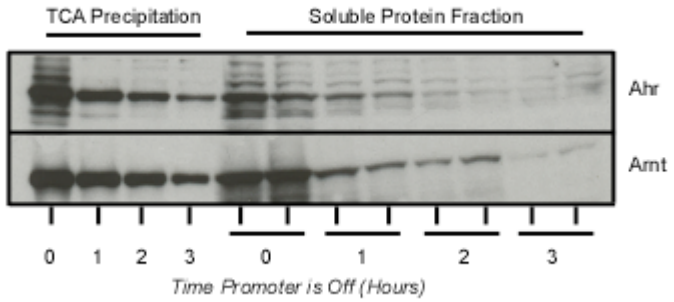

FIGURE 3.13: Turnover of Ahr and Arnt in samples prepared using trichloroacetic acid or the soluble protein extraction method when the inducible promoter is turned off. Samples were prepared using both methods to evaluate whether the proteins were being overexpressed and forming an insoluble pool. The TCA extraction method precipitated all proteins in the yeast cells, whereas the alternative protein extraction purified the soluble protein pool only. A culture of KHSY1547 was grown in the presence of galactose for three hours to induce expression of Ahr and Arnt. The promoter was turned off with the addition of glucose to the culture medium, and portions of the culture were removed at 0, 1, 2, and 3 hours after the promoter was turned off. Protein samples were prepared from 1.0 ODs of cells using 20% trichloroacetic acid and soluble protein extractions were prepared from 15.0 ODs of cells. Western blotting and antibody staining revealed rapid turnover of Ahr and Arnt proteins when the promoter was turned off for samples prepared via both methods.

Similar to what was observed in Figure 3.12, the protein level detectable after TCA precipitation of 1.0 ODs of cells was significantly higher than the protein detected in the soluble protein extractions from 15 times more starting material. Also consistent with

previous experiments, Ahr and Arnt proteins rapidly turned over in the samples prepared via TCA precipitation. However, Figure 3.13 also shows the Ahr and Arnt proteins are lost in the soluble fraction when the promoter was turned off. This result implied that there may not be two separate Ahr protein pools and the TCA precipitation procedure was simply more effective at bringing down the proteins than the soluble protein extractions. Importantly, the results from the whole cell lysates show that the Ahr is still reduced in the cells and exhibits a $t_{1/2}$ of approximately 1 hr, while Arnt shows a longer $t_{1/2}$ that is consistent with the results presented in Figure 3.8. It is apparent from these results that the Ahr protein is rapidly turning over in the samples prepared by either TCA precipitation or soluble protein extraction. Thus, these results suggest that the preparation of soluble protein extracts is not required. If Ahr does indeed degrade in a ligand-dependent manner in these cells, observation of a measurable effect may not be possible. Thus, the next set of studies aimed to determine if the rapid reduction of Ahr was related to improper chaperoning of the receptor by the yeast Hsps.

Analysis of Ligand-Independent Ahr Degradation in Yeast. Heat shock protein 90 (Hsp90) comprises approximately 1-2% of the cellular protein pool of eukaryotes (reviewed by Sreedhar *et al* 2004). There are two Hsp90 isoforms in mammals, termed Hsp90α and Hsp90β. Hsp90α is expressed at low levels but is highly inducible under conditions of cell stress while Hsp90β is known to be constitutively expressed and less inducible than its counterpart (reviewed by Sreedhar *et al* 2004). Similarly, the *HSP82* and *HSC82* genes in yeast, encode two isoforms of Hsp90 whose sequences are highly conserved (Borkovich *et al* 1989). It has been shown that the yeast *HSP82* shows 61% amino acid identity and exhibits similar function to the human Hsp90α while the yeast *HSC82* shows 49% identity to the human Hsp90β (Cox and Miller 2003).

Several groups have utilized *S. cerevisiae* to evaluate the role of Hsp90 in Ahr signaling (Carver *et al* 1994, Cox and Miller 2003, Whitelaw *et al* 1995). First, Carver (1994) used a yeast strain in which both *HSP82* and *HSC82* genes were disrupted and cell viability was maintained by transformation with a plasmid expressing *HSP82* at approximately 5% of the wild type level. Plasmids carrying the AHR and ARNT cDNAs were transformed into the Hsp90 deficient yeast strain. Ahr signaling was evaluated using a reporter plasmid similar to the pLXRE5-Z used in our experiments. These investigators demonstrated that Ahr signaling was not detectable in their yeast strain. However, increased expression of *HSP82* from 5% to wild type levels rescued a functioning Ahr signaling pathway in the strain. This experiment confirmed the hypothesis that Hsp90 is an essential component of the Ahr signaling pathway but it also suggested that the yeast Hsp90 homologues are able to function in place of human Hsp90 in the reconstituted system. This information was critical to our experiments. These results suggested that there is enough conserved sequence identity, most likely within the protein's functional domains, for yeast Hsp82 and Hsc82 to function in place of mammalian Hsp90α and Hsp90β in Ahr signaling. It is noted however, that in these studies the level of Ahr or Arnt expression was never evaluated.

While previous studies suggested that the yeast Hsp90 homologues, the *HSP82* isoform in particular (Cox and Miller 2003), function in Ahr signaling in yeast, we asked if the expression level was sufficient to properly chaperone the Ahr and Arnt in our yeast model. If expression of yeast Hsps was inadequate in our strains, the unfolded proteins could be targeted for rapid degradation or sequestered to inclusion bodies (Kamasawa 1999). Experiments in mammalian cell culture have shown that Ahr is rapidly degraded upon disruption of the Ahr·Hsp90 interaction in the unliganded cytoplasmic complex (Chen 1997, Song and Pollenz 2002). Treatment of mammalian cells with certain chemicals, such as geldanamycin, inhibit and disrupt Ahr association with Hsp90 and

result in rapid turnover of Ah receptor protein in the absence of ligand treatment. This type of degradation is referred to as ligand-independent degradation (Song and Pollenz 2002 and others) and the time course of degradation looks very similar to the results shown in Figures 3.6-3.8.

We investigated whether Ahr loss could be accelerated in the presence of Hsp90 inhibitors treatment. This effect may be detectable in the yeast strains if the Ah receptor is indeed turning over because of an improper interaction with Hsp90. Cox and Miller (2003) reported that treatment of a yeast strain expressing a recombinant Ahr signaling pathway with Hsp90 inhibitors caused a reduction in Ahr signaling as measured by reporter activity. Based on the observed degradation of Ahr with Hsp90 inhibitor treatment in cell culture (Song and Pollenz 2002), one could hypothesize that reporter activity could be reduced in yeast strains with Hsp90 inhibitor treatment because the Ah receptor is degraded. But as stated previously, the mechanism to this reduction could not be correlated to reductions in Ahr levels because protein levels were never evaluated. The study by Cox and Miller (2003) referenced above showed that geldanamycin, a particular Hsp90 inhibitor often used to induce ligand-dependent Ahr degradation, did not produce a statistically significant reduction in β-galactose reporter activity. However, they did show that several other compounds with similar function were effective at ablating Ahr signaling as measured by the β-galactosidase reporter. Specifically, novobiocin and radicicol treatment caused a dose-dependent decrease in β-gal activity.

In order to test the effects of Hsp90 disruption in the yeast strains, cultures of KHSY1547 were treated with DMSO, 33μM novobiocin, and 5μM radicicol. The effective doses for these drugs were determined in the above referenced study. The cultures were pretreated for one hour prior to induction of Ahr and Arnt proteins with 2% galactose. Treatment with Hsp90 inhibitors or DMSO continued during the three hour

induction time and protein samples were harvested in triplicate using 20% TCA. Western blotting and antibody detection was carried out as previously described and even sample loading was confirmed with Ponceau S staining. A representative western blot is presented in Figure 3.14.

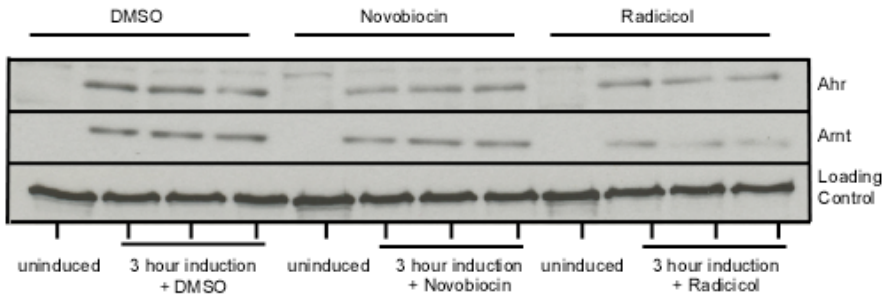

FIGURE 3.14: Effect of Hsp90 inhibitors on Ahr and Arnt expressing yeast strain. The double knock-in strain was evaluated for Hsp90 inhibitor- mediated ligand-independent degradation. A culture of KHSY1547 was split into three smaller cultures. Each was pretreated for one hour with DMSO (control), 33μM novobiocin, or 5μM radicicol. After one hour of pre-treatment, the cells were spun down and subsequently brought up in medium containing 2% galactose for protein induction and the respective treatment. After three hours, protein samples were prepared in triplicate for each culture. In the western blots shown above, it appears that Ahr and Arnt protein levels are slightly reduced after treatment with novobiocin or radicicol as compared to the DMSO control.

The results show that treatment of cells with novobiocin and radicicol had minimal impact on the level of Ahr or Arnt protein levels at any of the time points evaluated. In mammalian cell culture, treatment with these compounds results in rapid and nearly complete degradation all Ahr protein within a three hour time course (Song and Pollenz 2002). It should be noted that treatment with these Hsp90 inhibitors was concurrent with activation of the *GAL1* promoter, and thus expression was continuous throughout the study.

Impact of human Hsp90 expression on Ahr stability in yeast. While the effect of Hsp90 inhibitor treatment on Ahr levels was minimal, it was still possible that

chaperoning was involved in turnover of Ahr and Arnt proteins expressed in yeast. Increased turnover may have been due to the level of expression of the yeast Hsp90 homologues. Therefore, we obtained human Hsp90 expression vectors from Dr. Jill Johnson at the University of Idaho and transformed these into the double knock-in strain KHSY1547. We hypothesized that increased levels of mammalian Hsp90 might result in increased stability of Ahr. These plasmids allowed for expression of Xpress-tagged human Hsp90α and Hsp90β isoforms, driven by the yeast constitutive *GPD* (glyceraldehydes-3-phosphate dehydrogenase) promoter. Maps of the Hsp vectors are shown in Figure 3.15A.

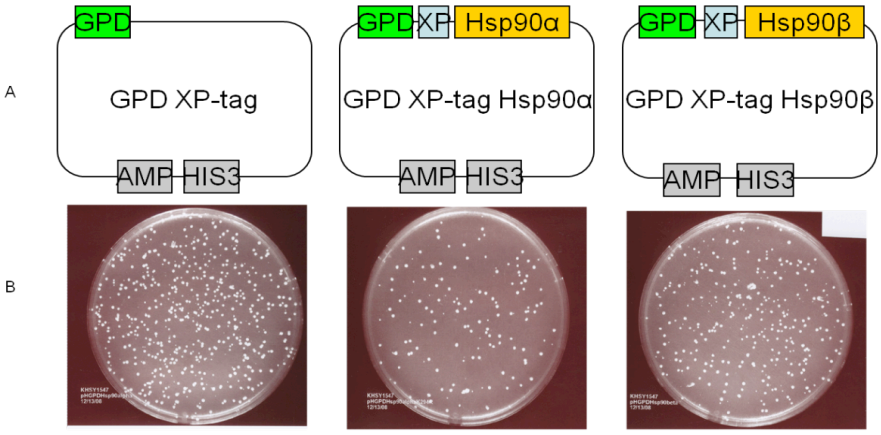

FIGURE 3.15: Human Hsp90 vector maps and transformation of KHSY1547 with each plasmid. Human Hsp90 expression vectors were obtained from Dr. Jill Johnson at the University of Idaho. Expression of Xpress-tagged Hsp90 α and β isoforms is driven by the constitutive GPD promoter. The AHR and ARNT expressing strain was transformed with empty vector, Hsp90α, and Hsp90β plasmids in three separate experiments. The transformed yeast were selected on HIS dropout media and the resulting plates are shown above. A single clone of each was used in subsequent experiments to evaluate AHR protein stability when Hsp90 is overexpressed.

Three individual cultures of KHSY1547 were prepared and transformed with Hsp90α, Hsp90β, and an empty vector to produce three new strains called KSHY2228, KHSY2229, and KHSY2230 respectively. The transformed yeast were selected on media lacking histidine and the resulting plates are shown in Figure 3.14B. One clone

from each transformation was used in subsequent experiments to evaluate Ahr protein stability in cells expressing high levels of human Hsp90.

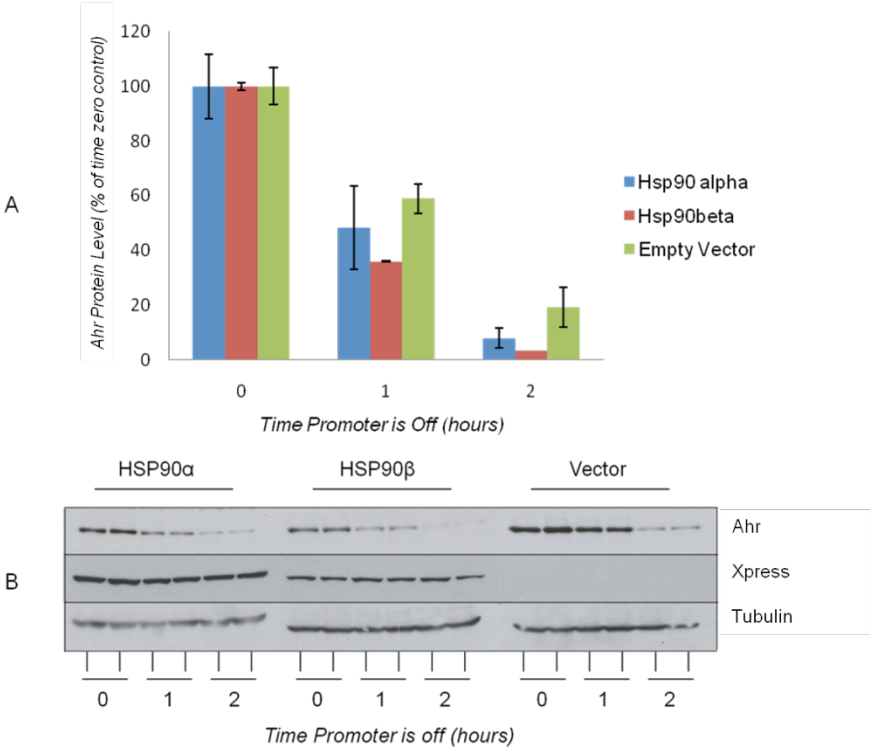

FIGURE 3.16: Ahr turnover is evident in strains overexpressing human heat shock proteins. Two yeast strains, KHSY2228 and 2229 express human Hsp90α and β isoforms from a constitutive reporter. The third yeast strain carries the empty vector. Ahr protein was induced with the addition of 2% galactose to the growth medium for three hours and was then turned off with the addition of glucose. Protein samples were prepared at 0, 1, and 2 hour time points after the promoter was turned off. Staining with Xpress antibody revealed high levels of expression of the human heat shock proteins, with no staining detectable in the control strain. Staining with antibody specific to Ahr showed rapid turnover of the proteins over time, similar to the degradation observed in previous experiments. This result suggests that enhanced expression of the Hsp90 chaperone proteins does not stabilize Ahr in our yeast expression system.

Upon generation of these Hsp90 expressing strains, we proceeded to test Ahr stability again. Ahr and Arnt proteins were induced with the addition of galactose for four hours in the various Hsp90 expressing yeast strains. After four hours, the inducible *GAL1* promoter was turned off to block transcription of Ahr and Arnt and to assess the

stability of these proteins. Samples were collected at 0, 60, and 120 minutes after the promoter was shut off and protein was prepared using 20% TCA as previously described. Results for Ahr are shown in Figure 3.16.

Three identical SDS-PAGE gels were prepared using equal amounts of each protein sample. Western blotting and staining with antibodies specific to the Xpress tag (Invitrogen) revealed intense bands at approximately 90kDa in the KHSY2228 and KHSY2229 strains. Staining for the Xpress tag did not show reactivity in the cells containing the empty vector. It is noted that the level of expression for the Hsp90 protein is consistent at all time points and this was expected based on expression from a constitutive promoter. The same samples were also probed with Ahr-specific antibodies and the resulting western blot shows a rapid loss of Ahr with a $t_{1/2}$ of ~1 hour. None of the strains showed a significant change in the loss of Ahr when compared to the strain with the empty vector. The loading of equal levels of protein across all samples is validated by the staining for tubulin.

In summary, the results from these experiments show that although Hsp90 can be expressed, there is no apparent change to the stability of the Ahr protein. Given the number of different experiments that were carried out to generate a more stable receptor in our inducible model we concluded that we should move forward and test the strain for ligand-mediated degradation of the Ahr while continuing to hypothesize about other solutions to the problem.

Effect of Ahr Ligand Treatment in Yeast Strains. Extensive research in cell culture models has demonstrated that Ahr ligand exposure results in activation of the Ahr signaling pathway that terminates in degradation of the receptor (Prokipcak and Okey 1991, Pollenz 1996, reviewed by Pollenz 2002). Degradation of Ahr is mediated by the 26S proteasome (Ma and Baldwin 2000, Roberts and Whitelaw 1999, Song and

Pollenz 2002) and is a crucial step in the signaling pathway as it attenuates the dosage of Ahr and Arnt-directed transactivation of regulated genes (Ma and Baldwin 2000; reviewed by Pollenz 2002, 2010). While many studies clearly show that the ligand-mediated Ahr turnover occurs via the 26S-proteasome, the exact residues that are modified on the Ahr to target it for degradation remain unknown.

As stated previously, the Ahr and Arnt expressing yeast strains were generated in order to screen for proteins involved in mediating Ahr turnover. The goal was to determine if Ahr degraded in yeast in a ligand-dependent manner. Next, we would knock out genes from the yeast genome that could be responsible for targeting the receptor for proteasomal degradation. A candidate approach would be employed initially, focusing on E3 ligases as they are the suggested mediators between the protein targeted for degradation and the 26S-proteasome. It could then be determined if a particular E3 was responsible for targeting Ahr by assessing Ahr protein levels after ligand treatment. If the knocked out ligase was responsible for Ahr turnover, we would observe high levels of receptor after ligand treatment compared to the control strain that would still express the ligase in question.

Unfortunately, the half-life experiments shown in Figures 3.8 and 3.13 revealed degradation of the receptor in the absence of Ahr ligand and these results proved to complicate this approach. To assess ligand-mediated degradation it was essential to have a model that was amenable to observing a quantifiable reduction in Ah receptor levels after ligand treatment in order to test for blocked turnover in the appropriate E3 knock-out. Since attempts at stabilizing the receptor expression were unsuccessful, it was still pertinent to determine if ligand treatment would result in an enhanced rate of degradation than was observed in the absence of ligand.

Ligand-mediated degradation of the Ahr was evaluated under several induction conditions. First, the KSHY1566 strain was treated with TCDD and induced with

galactose simultaneously. The nature of our inducible system allowed for such an approach and it was hypothesized that the ability of the ligand to immediately bind a newly synthesized Ahr might result in immediate degradation. We would then observe a lack of accumulation of the synthesized Ahr compared to controls that did not receive ligand treatment.

The KHSY1566 strain was used in the following experiments. This strain expresses both Ahr and Arnt under the *GAL1* promoter but also carried the pLXRE5-Z reporter plasmid. We used this strain for the following experiments because we know that DNA binding is required for Ahr degradation to occur (Pollenz *et al* 2005). An overnight culture of KHSY1566 was split in half; one portion of the culture was treated with 20nM TCDD and 2% galactose and the other half of the culture was treated with DMSO and 2% galactose. Protein samples were prepared using 20% TCA at various time points after treatment/induction. All samples contained the same cell numbers. The resulting western blot, stained with anti-Ahr antibodies, is shown in Figure 3.17.

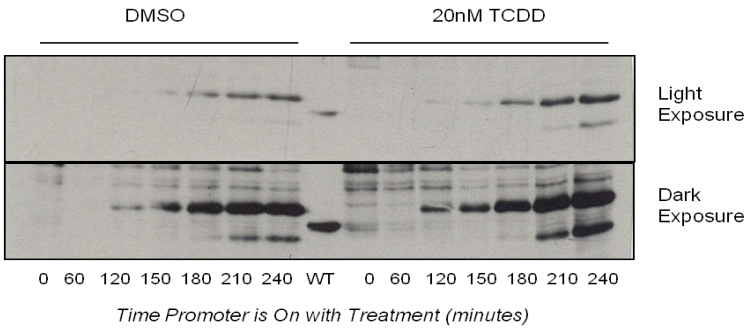

FIGURE 3.17: Induction of Ahr protein with galactose and simultaneous treatment with TCDD revealed equal levels of Ahr protein. KHSY1566 was treated with 20nM TCDD and 2% galactose or with DMSO and 2% galactose. Protein samples were prepared using 20% trichloroacetic acid at 0, 60, 120, 150, 180, 210, and 240 minutes after treatment with TCDD and galactose induction. Western blotting and antibody detection revealed equivalent levels of Ahr protein induction after treatment with TCDD or DMSO.

The results revealed that Ahr protein expression levels increased over time in the yeast cells in the presence or absence of the known Ahr ligand, TCDD. This observation was in contrast to our anticipated result since it was hypothesized that reduced levels of Ahr protein would be observed in the TCDD treated culture compared to the DMSO-treated control. In spite of this result, we still wanted to explore the effect of ligand on Ahr protein under different induction conditions.

In the next experiment, yeast cultures were treated with TCDD or DMSO after a three hour induction in galactose. These studies would result in a strain that expressed an Ahr protein pool prior to ligand treatment. The presence of Ahr protein in the yeast cells prior to treatment represents a more physiological scenario since endogenous levels of Ahr protein are present in mammalian cell culture prior to ligand treatment. In a culture of the KHSY1566 strain, the *GAL1* promoter was activated with galactose for three hours to induce Ahr and Arnt protein expression. After three hours, the culture was split in half; one half was treated with DMSO and the other with 20nM TCDD. The promoter was left on during the three hour treatment time in order to prevent the rapid ligand-independent loss of Ahr reported throughout the previous sections. It was reasoned that we may observe some measurable difference in Ahr protein levels between treated and control cultures, even if the Ahr protein was not completely degraded due to the continual induction by galactose.

The results of this experiment are shown in Figure 3.18. Ahr and Arnt protein were detectable via western blotting after the 3 hour induction with galactose. However, subsequent treatment with TCDD for 3 hours did not result in reductions in the level of Ahr. This result is in contrast to experiments performed in mammalian cell culture where the Ah^{b-2} allele expressed in C_2C_{12} cells (mouse skeletal muscle myoblasts) was reduced by 90% after 2 - 3 hour exposure to TCDD (Pollenz 1996).

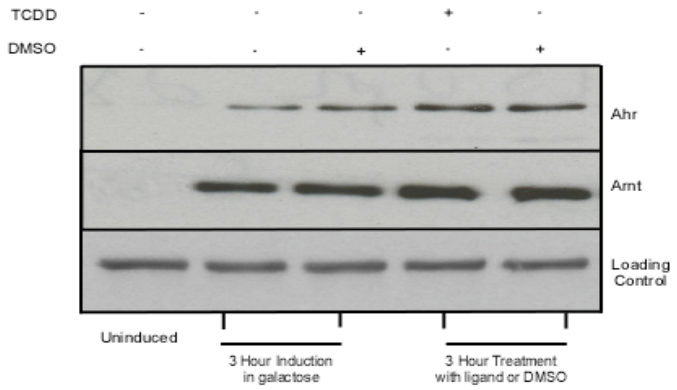

FIGURE 3.18: Effect of ligand treatment on Ahr protein in induced cultures. Ahr and Arnt proteins were induced with the addition of 2% galactose to the culture medium for three hours prior to treatment with 20nM TCDD or DMSO. TCDD or DMSO treatment was carried out for an additional three hours in the presence of galactose. Yeast protein samples were prepared using 20% TCA. Western blotting and antibody staining revealed expression of Ahr and Arnt proteins after three hours of induction and the expression levels increased after three additional hours in the presence or absence of TCDD.

Undaunted by the previous results, the KHSY1566 strain was tested for the effect of TCDD on Ahr protein levels from whole cell extracts. As discussed for Figure 3.13, it was a concern that the TCA precipitation method for protein extraction may have been bringing down functional Ahr proteins along with misfolded Ahr as a result of overexpression from the *GAL1* promoter. If this was the case, the TCA sample preparation method may have impaired the ability to visualize ligand-mediated degradation of the receptor in the previous experiments.

For this experiment, a culture of KHSY1566 was grown in the presence of galactose for 3 hours and was then split in half. Half of the culture was treated with 20nM TCDD and the other was treated with DMSO as a control still in the presence of galactose. Protein samples were prepared from these cultures by both TCA precipitation and whole cell extraction as previously described. Figure 3.19 shows the western blotting results of the TCA precipitated and soluble fractions of yeast protein after treatment with TCDD or DMSO for 0, 2, and 4 hours.

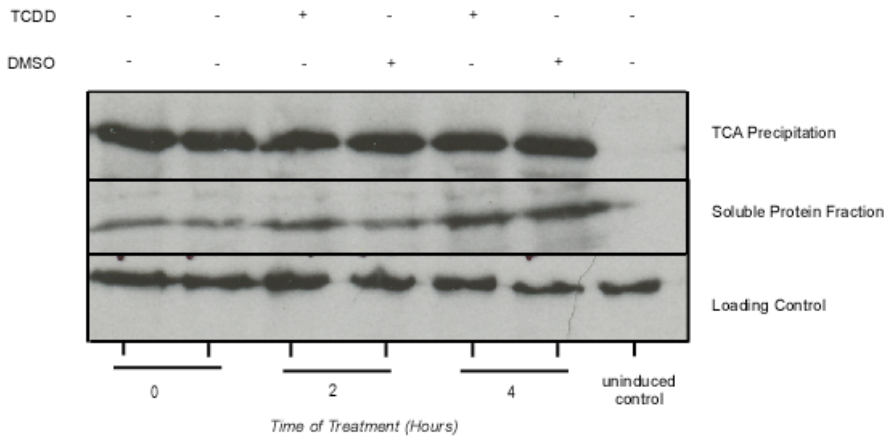

FIGURE 3.19: Ahr protein levels after TCDD treatment in TCA precipitated and soluble protein fraction samples. Induction of Ahr and Arnt proteins was carried out for 3 hours with the addition of 2% galactose to the growth medium. After 3 hours, the culture was split in half and treated with either 20nM TCDD or DMSO. The cultures were quantified and 1.0 ODs of cells were prepared for protein using the TCA precipitation method. Additionally, 15.0 ODs of cells from the identical cultures were used to prepare soluble protein fractions. Samples were prepared before exposure to ligand and 2 and 4 hours after ligand treatment. Western blotting of the samples revealed Ahr protein levels remained consistent over time with TCDD treatment in both sample preparation methods.

The level of Ahr protein detected in the TCA precipitated samples after the initial three hour induction in galactose remained essentially constant throughout the experiment. An identical affect was observed in the whole cell extracts even though the overall level of Ahr protein loaded from the two different preparations was different. The inability to observe a ligand-mediated degradation event is in contrast to published reports (Pollenz 1996) and suggests that the sample preparation method is not a variable in our inability to detect Ahr protein degradation.

As a final attempt to observe ligand-mediated Ahr degradation, the issue of promoter activation was revisited. As stated before, the strength of the promoter directly impacts the level of protein that is accumulated in a cell (in conjunction with the protein half-life) and in this system it could cause rapid production of Ahr protein that masks the effect of the ligand treatment. In this way, a decrease in Ahr protein levels by ligand

treatment is overcome by a rapid expression and synthesis. Even in stable mammalian cell culture models, the expression of the Ahr from the strong CMV promoter results in reduced magnitude of Ahr degradation of the recombinant proteins because the expression is much stronger than from the endogenous Ahr promoter (Song and Pollenz 2002, Pollenz and Dougherty 2005). In order to prevent synthesis of new Ahr protein once the ligand was added, the Ahr and Arnt proteins were first induced with galactose for several hours and then expression was turned off with the addition of glucose to the medium. TCDD or DMSO was added directly to the media at the time the promoter was turned off. It was hypothesized that a more rapid $t_{1/2}$ for the degradation of the Ahr in the presence of ligand would be observed compared to cultures treated with DMSO. It was also of interest to assess levels of Arnt protein for this experiment. Since Ahr, but not Arnt, undergoes ligand-dependent degradation (Pollenz 1996), the rate of Ahr turnover could be compared to Arnt and should therefore turnover more quickly if the degradation is specific to a ligand-mediated event. Figure 3.20 shows a representative experiment.

The results show that the Ahr and Arnt protein are reduced at 2 and 4 hours. Importantly, treatment with TCDD had no impact on the level of either Ahr or Arnt proteins. Although these results were disappointing, they were not unexpected based on the previous results. For the ligand to have an impact on the level of Ahr in this system, the $t_{1/2}$ would have needed to be reduced to less than 30 minutes to distinguish the result from the level of reduction that was observed under control conditions.

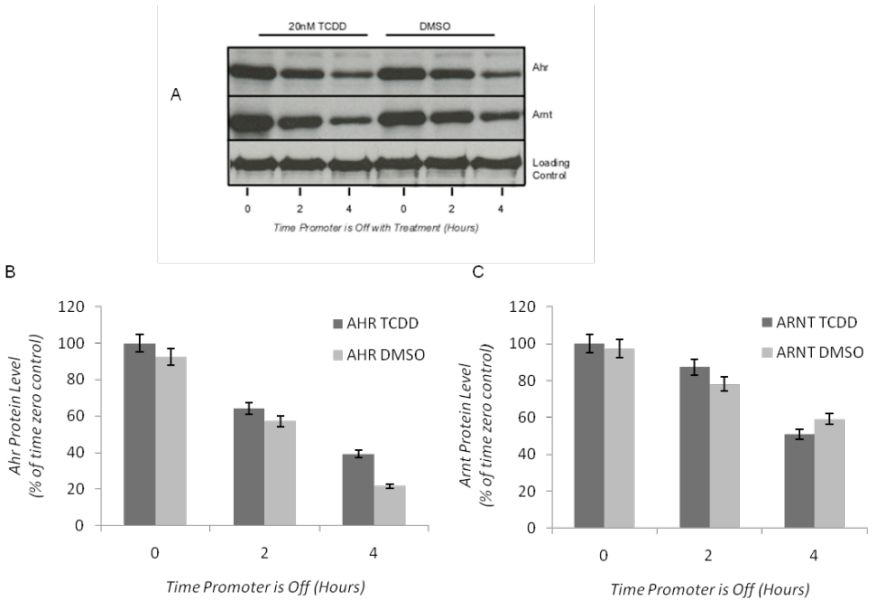

FIGURE 3.20: Effect of TCDD on Ahr and Arnt protein after the promoter is turned off. A culture of KHSY1566 was grown in the presence of 2% galactose for 3 hours. That culture was then split in half, and each resulting culture was spun down to remove the galactose from the growth media. The cell pellets were resuspended in growth medium containing 2% glucose to block to the promoter from producing more Ahr and Arnt. The cultures were immediately treated with 20nM TCDD or DMSO and placed in a shaking incubator. Yeast protein samples were prepared from each culture at 0, 2, and 4 hours of treatment after the promoter was turned off.

Mechanism of Ahr Degradation in Yeast

All of the studies presented thus far clearly suggest that the Ahr is rapidly lost from the yeast cells when expression is turned off. The results also suggest that the ability to evaluate ligand-mediated degradation is currently not supported by this model. However, the study of Ahr degradation has multiple components that include 1) normal homeostatic turnover, 2) ligand-mediated turnover and 3) ligand-independent turnover. Since the majority of studies that have evaluated both the ligand-dependent and independent degradation of the Ahr have shown that the degradation is mediated by the 26S proteasome, the next series of experiments were carried out to determine whether the Ahr degradation in our recombinant yeast model was also mediated by the 26S proteasome pathway. This series of experiments was critical, because it would

demonstrate that the loss of Ahr that is observed when the promoter was turned off was in fact related to degradation and not dilution. In addition, the ability to show that the Ahr was being degraded via the 26S proteasome in this model would allow future genetic analysis of the proteins involved in the process. Such findings might identify E3 ligases that are also functioning in mammalian cells.

There are two distinct protein degradation pathways in yeast. Proteins can be degraded by way of the vacuolar system which is similar in function to the mammalian lysosome (Baba *et al* 1997) or by the substrate-specific ubiquitin-proteasome pathway (Eisele and Wolf 2008). Studies in yeast have shown that the 26S proteasome pathway mediates degradation of unfolded cytoplasmic proteins (Lee and Goldberg 1997, Eisele and Wolf 2008). Therefore, the following experiments tested the effect of proteasome inhibition on Ahr and Arnt stability in the recombinant yeast strain.

Generation of a Permeable Yeast Strain. Saccharomyces cerevisiae is resistant to proteasome inhibitor treatment due to the inability of these chemicals to permeate the yeast cell wall. Previous groups have generated mutant yeast strains in which a nonessential gene encoding the cell wall protein, *ERG6*, was disrupted (Lee and Goldberg 1996, Lee and Goldberg 1998). Strains with the Δ*erg6* mutation demonstrated enhanced permeability to certain compounds. Therefore, we needed to disrupt the *ERG6* gene in the Ahr and Arnt expressor strain so they could be treated with the appropriate inhibitors. The double knock-in strain, KHSY1547, was used to generate a permeable yeast strain by knocking out *ERG6* using homologous recombination.

The *ERG6* gene was knocked out of the KHSY1547 strain using homologous recombination as described in chapter two. Primers were designed to amplify the *HIS3* auxotrophic marker cassette from the pRS303 plasmid (Sikorski and Hieter 1989). These primers amplified *HIS3* with overhanging sequence homology to the yeast *ERG6*

gene in order to direct recombination of the *HIS3* marker with the *ERG6* gene. The double knock-in strain, KHSY1547, was transformed with the resulting PCR product using lithium cations as described in chapter two. The resulting clones were selected for the Δ*erg6* mutation by plating the transformed cells on medium lacking histidine. Figure 3.21A is a photo of the transformed clones that grew on the selective medium after two days in a 30°C incubator. Several clones were selected for analysis and proper integration of the *HIS3* cassette was confirmed via PCR.

The growth rate of the *ERG6* knock-out was compared to the parental strain as an additional test for the transformed clones. Previous reports have shown that the growth rate of the Δ*erg6* strain is about 50% lower than the growth rate of wild-type strains (Lee and Goldberg 1998). Therefore, several clones were chosen and streaked on YPD medium to look for slowed growth in comparison to the parental strain.

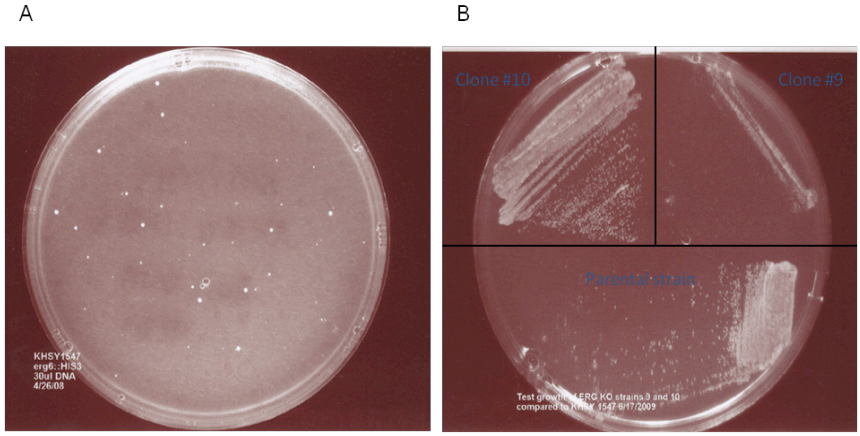

FIGURE 3.21: Selection of *Δerg6* Clones for use in Proteasome Inhibitor Studies. The *HIS3* auxotrophic marker cassette was PCR amplified using primers with sequence homology to the yeast *ERG6* gene. PCR product was combined with cells of the KHSY1547 strain for homologous recombination of the *HIS3* cassette to the *ERG6* locus. (A) Transformed cells were spread on medium lacking histidine to select for *HIS3::ERG6* clones. (B) Several clones were chosen and streaked on YPD medium to test for growth compared to the parental strain. Clone #10, in the upper left quadrant, demonstrated growth comparable to the parental strain on the lower half of the plate. Clone #9 appears to have the Δ*erg6* mutation as it shows less growth on the plate compared to the parental strain.

Figure 3.21B shows the parental strain streaked on the bottom half of the plate. Two clones that grew on the plate shown in Figure 3.21A were streaked on quadrants of the top of the plate and it was placed in a 30°C incubator for two days. The photo shows denser growth of the parental strain that is comparable to the growth observed for clone #10. In contrast, significantly reduced growth was observed for clone #9 as compared to the parental strain. The obvious reduction in growth suggests that clone #9 may carry the Δ*erg6* mutation.

To further confirm the *Δerg6* mutation in clone #9, the doubling time was calculated in the presence or absence of MG132, a compound that inhibits the activity of the 26S proteasome. A study by Lee and Goldberg (1998) demonstrated that a strain with the Δ*erg6* mutation had a doubling time that was longer than wild type and importantly, Fujimuro (1998) showed that a similar yeast strain had an even longer doubling time with proteasome inhibitor treatment. These articles suggested that the mutation in the yeast cell wall impaired the ability of the yeast to grow at a normal rate. They also suggested that proteasome inhibitors were capable of entering the otherwise impermeable cells and showed evidence that the chemicals affected the turnover of key proteins involved in cell cycle control. The observed reduction in growth rate due to stalled cell division also translates to an increased doubling time for the yeast. In the following experiment, the doubling time for the parental strain (KHSY1547) was compared to the *Δerg6* clone that exhibited slowed growth in Figure 3.21.

The double knock-in strain served as the parental strain for the *Δerg6* mutant strain generation. The doubling time for this strain was tested in the presence and absence of 50μM MG132. This dose was chosen based on a dose-response experiment reported by Lee and Goldberg (1998) with 50μM MG132 reported as the effective dose. The doubling time of the permeable yeast strain was also tested in the presence or absence of 50μM MG132. Triplicate cultures of 1547 and the *Δerg6* mutant

were prepared at a starting optical density$_{600nm}$ of 0.1. One milliliter of each culture was removed and quantified every hour for 8 hours. Data points were plotted in Excel for the samples after the yeast strains reached exponential growth. Using the equation of each exponential line, the time required for the cells to double was calculated. The average was taken for each replicate and the standard deviation was calculated. The results are provided in a graph shown in Figure 3.22.

The results of Figure 3.22 show that KHSY1547 had a doubling time of 88.89 minutes with a standard deviation of 1.99 minutes. This strain demonstrated a doubling time comparable to wild type *Saccharomyces cerevisiae* that has a doubling time of approximately 90 minutes in rich medium (Sherman 2002). Treatment of the 1547 strain with MG132 did not affect the growth rate of the cells. The doubling time for KHSY1547 with MG132 was 88.12 minutes with a standard deviation of 1.28 minutes. This result is consistent with previous reports that MG132 is unable to penetrate the yeast cell wall.

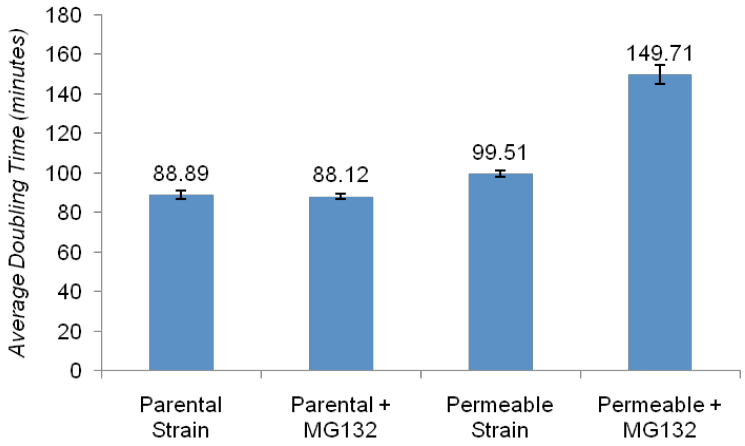

FIGURE 3.22: Effect of MG132 on the doubling time of the parental and permeable strains. The doubling time of KHSY1547 was tested in the presence or absence of a 26S-Proteasome inhibitor, MG132. The *Δerg6* strain was also evaluated under the same conditions. A dose of 50μM MG132 was added to the treatment cultures at the beginning of the experiment. Cultures were prepared in triplicate with a starting optical density (600nm) of 0.1. The density of the culture was measure every hour for 8 hours. The data points after the exponential growth phase was reached were plotted in Excel to calculate the average doubling time and standard deviation.

Next, the permeable yeast strain had a doubling time of 99.51 minutes with a standard deviation of 1.63 minutes. This result is significantly different than the 1547 strain, but is not identical to the reports by other groups. The growth rate of a *Δerg6* mutant strain evaluated by Lee and Goldberg (1998) was approximately 50% lower than wild type. This would suggest a doubling time of approximately 135 minutes in the permeable strain. Although the observed growth rate was not identical to published reports, the doubling time for the *Δerg6* strain was markedly longer than the parental strains and was measured in the presence of MG132. The doubling time was 149.71 minutes with a standard deviation of 4.89 minutes in the permeable strain treated with 50μM MG132. This data is consistent with a report by Fujimuro (1998), where the doubling time for a similar permeable strain was increased in the presence of MG132. The effect of MG132 on growth rate is notably only present in the permeable strain, as shown in Figure 3.21, suggesting that the compound is indeed able to enter the cells in this strain. Therefore, the *Δerg6* strain was used in the following studies to evaluate the mechanism of Ahr protein degradation in the recombinant expression model.

Ahr Protein Stability with Proteasome Inhibitor Treatment. The following experiments were carried out using the permeable strain to assess Ahr and Arnt turnover in the presence of proteasome inhibitors. Figure 3.6-3.8 demonstrated rapid degradation of the exogenously expressed proteins when the inducible promoter was turned off. Using a similar experimental strategy, we evaluated Ahr and Arnt levels when the promoter was turned off in the presence or absence of MG132. Since MG132 is known to block the activity of the 26S proteasome, the results of this experiment would determine whether ligand-independent turnover of Ahr and Arnt in yeast is proteasome – mediated. It was hypothesized that treatment with MG132 would block degradation of

Ahr and Arnt if the turnover was specifically mediated by the proteasome. Alternatively, if the MG132 has no effect on Ahr and Arnt protein levels, a dilution effect caused by rapid division of yeast cells may be responsible for their degradation.

The experimental paradigm for these studies was similar to that described for Figures 3.6-3.8. However, the *Δerg6* mutant strain was used due to its increased permeability to proteasome inhibitors. An overnight culture of this strain was used to prepare an induction culture with a starting OD_{600} of 0.1 with 2% galactose. The culture was placed in a 30°C shaking incubator to induce expression of Ahr and Arnt proteins for three hours. After three hours, the culture was split in half. For both resulting cultures, the promoter was turned off with the removal of galactose and addition of 2% glucose to the medium, as previously described. A schematic for the induction and treatment time course is shown in Figure 3.23A. In this experiment, one culture was treated with 50μM MG132 and the other was treated with DMSO. Again, the effective dose of MG132 treatment in yeast was determined by Lee and Goldberg (1998). At the zero time point, the inducible promoter was turned off together with MG132 or DMSO treatment. Duplicate 1.0 OD protein samples were prepared from each culture at the zero time point. The cultures were returned to the 30°C shaking incubator and protein samples were prepared again after 1, 2, 3, and 4 hours.

The western blotting results shown in Figure 3.23B reveal equal levels of Ahr protein detected at the zero time point between both cultures. When the inducible promoter is turned off, Ahr is rapidly lost in the DMSO-treated culture with a $t_{1/2}$ of approximately 1 hour. This result is consistent similar with experiments carried out in KHSY1547, shown in Figures 3.6-3.8. However, treatment of the permeable strain with MG132 shifted the $t_{1/2}$ of Ahr to approximately 3.5 hours. While the level of Ahr protein in

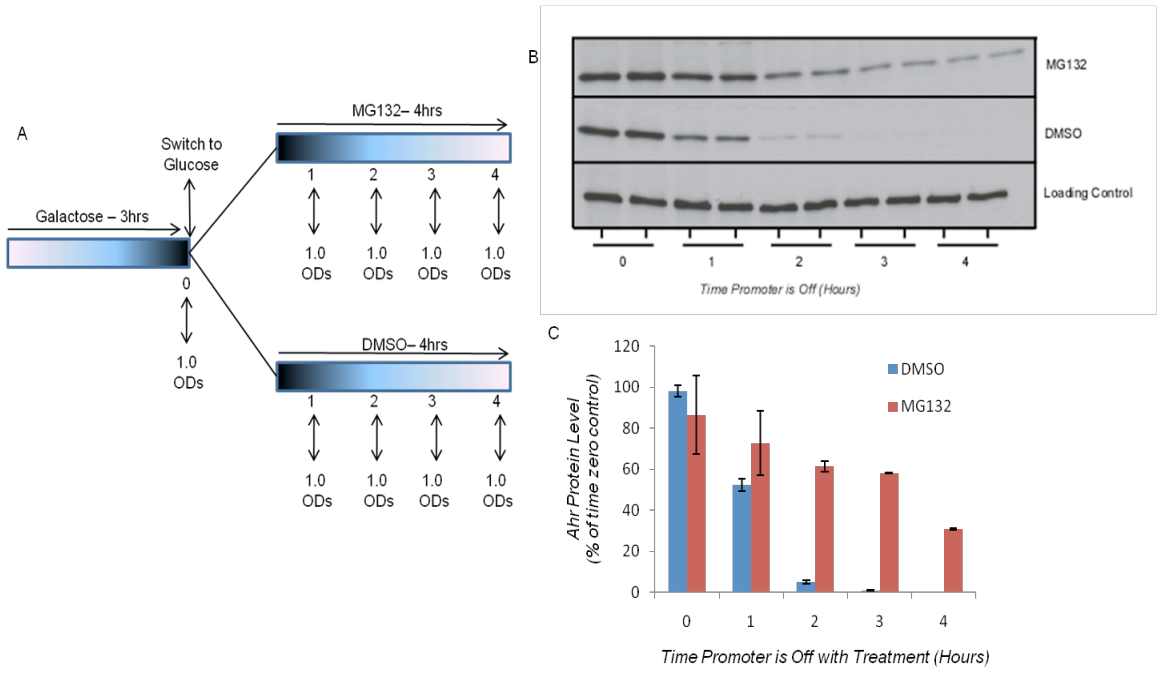

FIGURE 3.23: Effect of MG132 on Ahr Degradation in Yeast. (A) A schematic for the induction and treatment of the permeable recombinant expression strain. A culture was prepared in 2% galactose for a 3 hour induction of Ahr and Arnt proteins. Then, the galactose-inducible promoter was turned off and the culture was split into two equal samples, dosed with 50μM MG132 or DMSO. Duplicate samples of 1.0 ODs were removed from each culture at 0, 1, 2, 3, and 4 hours following treatment and protein samples were prepared using 20% TCA. (B) Protein samples prepared from equal numbers of cells were resolved on 7% SDS-PAGE gels. Western blotting was carried out using a semi-dry blotting apparatus. The membrane was stained with 1μg/ml anti-Ahr polyclonal antibodies and ECL reagent was used for detection. (C) Ahr protein expression was quantified using ImageJ software and was plotted such that Ahr expression at the zero time point is equivalent to 100%. Note the rapid Ahr protein turnover that was undetectable after three hours when the culture was treated with DMSO. However, Ahr protein levels were markedly higher than the control when treated with 50uM MG132. This suggests that the observed turnover of Ahr and Arnt in Figures 3.6-3.8 was not a dilution effect caused by the yeast cell division, but is evidence of degradation mediated by the 26S proteasome.

the MG132 treated culture is still reduced over time, this loss of protein is consistent with the dilution of the protein from the expanding cultures. This finding is illustrated in Figure 3.24 and shows that the level of Ahr in the presence of MG132 trends toward the predicted loss based on dilution within the budding cells. It is important to note that this experiment was carried out several times and the same result was consistently observed with MG132 treatment.

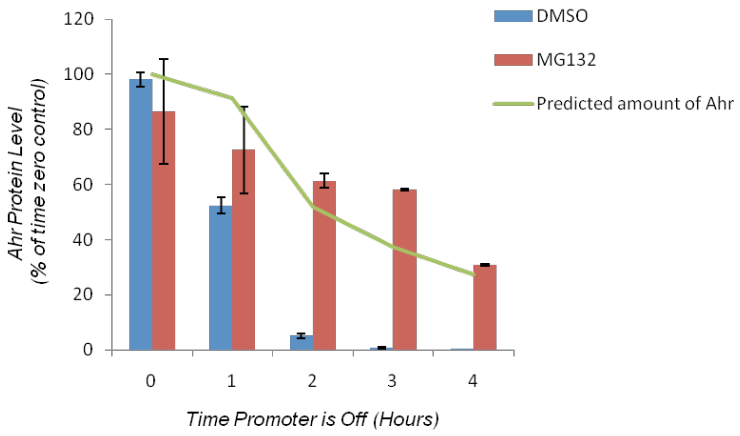

FIGURE 3.24: Ahr protein levels shift towards predicted levels with MG132 treatment. The histogram from Figure 3.22 is shown above with the addition of the green line that represents the predicted level of Ahr that would be present in the cells based on the culture densities. Note that the Ahr protein level decreased over time and the trend is shifted to the right toward the predicted trend line with MG132 treatment. This observation suggests that the turnover of Ahr presented in this and previous figures was not a dilution effect caused by the yeast cell division, but is evidence of degradation mediated by the 26S proteasome.

The ability to detect higher levels of Ahr protein in the presence of MG132 demonstrates that Ahr degradation in yeast terminates through a proteaosme mediated mechanism. However, it is unclear whether this is specific to the Ahr. To determine the effect of MG132 treatment on Arnt protein degradation in yeast, the same protein samples that were evaluated in the Ahr studies (Figure 3.23-3.24) were used in western blotting with antibodies specific for the Arnt protein. The results of this experiment are shown in Figure 3.25.

Similar to the results observed with the Ahr, the Arnt protein levels were stabilized with proteasome inhibitor treatment. The western blots in Figure 3.25B reveal steady turnover of Arnt in the DMSO-treated culture. In contrast, Arnt protein is maintained at high levels when the promoter is turned off in the presence of MG132. Quantification of the western blots is shown in Figure 3.25C. Importantly, the trend for

Arnt protein loss in the MG132 treated cultures is consistent with the expected dilution of the protein from the yeast cultures shown in Figure 3.26.

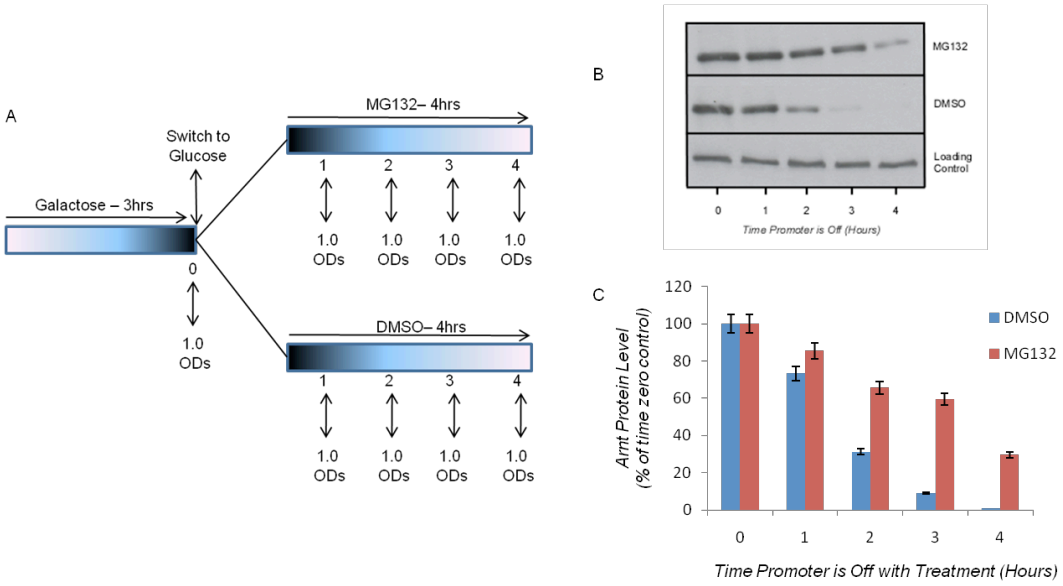

FIGURE 3.25: Effect of MG132 on Arnt Degradation in Yeast. (A) A schematic for the induction and treatment of the permeable recombinant expression strain. A culture was prepared in 2% galactose for a 3 hour induction of Ahr and Arnt proteins. Then, the galactose-inducible promoter was turned off and the culture was split into two equal samples, dosed with 50μM MG132 or DMSO. Duplicate samples of 1.0 ODs were removed from each culture at 0, 1, 2, 3, and 4 hours following treatment and protein samples were prepared using 20% TCA. (B) Protein samples prepared from equal numbers of cells were resolved on 7% SDS-PAGE gels. Western blotting was carried out using a semi-dry blotting apparatus. The membrane was stained with 0.5μg/ml anti-Arnt polyclonal antibodies and ECL reagent was used for detection. (C) Arnt protein expression was quantified using ImageJ software and was plotted such that Arnt expression at the zero time point is equivalent to 100%. Note the rapid Arnt protein turnover that was undetectable after three hours when the culture was treated with DMSO. However, Arnt protein levels were markedly higher than the control when treated with 50uM MG132. This suggests that the observed turnover of Ahr and Arnt in Figures 3.6-3.8 was not a dilution effect caused by the yeast cell division, but is evidence of degradation mediated by the 26S proteasome.

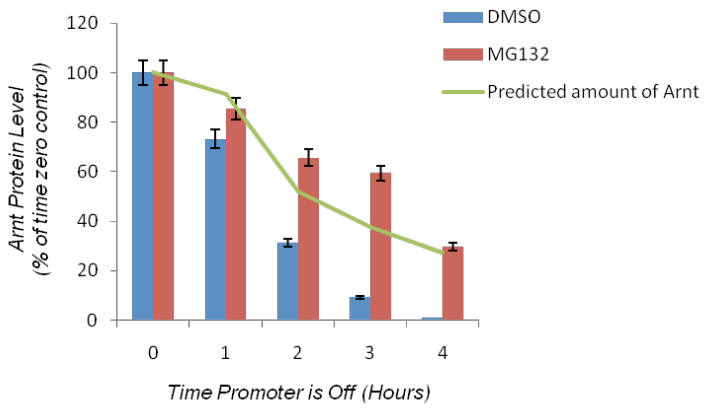

FIGURE 3.26: Arnt protein levels shift towards predicted levels with MG132 treatment. The histogram from Figure 3.24 is shown above with the addition of the green line that represents the predicted level of Arnt that would be present in the cells based on the culture densities. Note that the Arnt protein level decreased over time and the trend is shifted to the right toward the predicted trend line with MG132 treatment. This observation suggests that the turnover of Arnt shown in this and previous figures was not a dilution effect caused by the yeast cell division, but is evidence of degradation mediated by the 26S proteasome.

Collectively, the results presented in Figures 3.23 - 3.26 suggest that the reduction in Ahr and Arnt proteins over time is not due to dilution of the proteins from the yeast cultures. Instead, the results show that Ahr and Arnt proteins are being degraded via the proteasome pathway when expressed exogenously in the yeast expression model. Based on these data, it appears that Ahr and Arnt proteins are degraded via the 26S proteasome when expressed in yeast strains. The implications of this result will be expanded and discussed further in chapter 4.

Summary of Experimental Results.

- AHR and ARNT cDNAs were stably integrated into the genome of strains of *S. cerevisiae.*
- Expression of Ahr and Arnt proteins was induced upon activation of the *GAL1* promoter.

- The level of induction of proteins could be modulated with varying levels of galactose.
- Ahr and Arnt form functional heterodimers capable of binding XREs and driving transcription of a synthetic reporter.
- Several ligands at varying doses were shown to activate the recombinant Ahr signaling pathway.
- Ahr and Arnt protein levels were reduced when the inducible promoter was turned off.
- Modulation of induction conditions and sample preparation did not produce stable levels of Ahr and Arnt.
- Degradation was not impacted with increased expression of mammalian chaperone proteins.
- Ligand treatment does not illicit an observable degradation event in our yeast model.
- The loss of Ahr and Arnt proteins is reversed when the 26S proteasome pathway is blocked.

CHAPTER 4:

CONCLUSIONS, IMPLICATIONS, AND FUTURE DIRECTIONS

Conclusions and Implications

The data presented throughout this manuscript demonstrates an in depth investigation into the expression and stability of murine Ahr and Arnt proteins when expressed in *Saccharomyces cerevisiae.* The first aim of this research was to construct yeast strains expressing Ahr and Arnt under control of the galactose-inducible *GAL1* promoter. Generation of the various strains is detailed in chapter 2. However, it is important to note that careful consideration went into the construction of these strains. The expressor strains were created so that a single copy of each cDNA was stably integrated downstream of an inducible promoter. The strains were produced in this manner to allow for modulation of the expression of the protein levels and to avoid overexpression that is likely occurring when proteins are expressed from a constitutive promoter. The ability to detect and control Ahr and Arnt protein expression was highlighted in chapter 3.

Expression of Ahr and Arnt under the GAL1 promoter is Detectable in Yeast. The results of these analyses showed for the first time that mammalian Ahr and Arnt protein could be detected in a recombinant yeast model using a western blotting approach. Importantly, the Ahr and Arnt proteins were detectable within minutes of

galactose induction. These results demonstrated that extensive incubation with galactose is not required for *GAL1* promoter–driven expression to obtain detectable levels of protein. This was an important observation because these strains were constructed with the goal of generating a functional signaling pathway. It was crucial to have a regulated system that could be used to assess overexpression of Ahr and Arnt so that the integrity of the pathway is maintained. The use of the *GAL1* promoter allowed the levels of expressed proteins to be carefully modulated and this is demonstrated in Figures 3.9-3.11. The results show that reducing the amount of galactose to the growth cultures coincided with induction of lower amounts of Ahr and Arnt proteins; however the modification of those induction conditions did not produce stable levels of Ahr and Arnt. Importantly, this could not have been evaluated if a constitutively active promoter had been used in these studies.

Mammalian Ahr and Arnt Proteins are Functional when Expressed in Yeast. A series of reporter studies revealed that the Ahr signaling pathway could be activated in a ligand-dependent manner in the yeast model. Reporter assays showed that Ahr and Arnt proteins can function as transcription factors when activated with TCDD and other ligands to drive transcription of an artificial reporter. Importantly, the lack of induction of reporter activity in the absence of ligand showed that only the ligand-bound Ahr interacted with Arnt to form the DNA-binding species when expressed in yeast. This observation was critical as it confirmed that the basic components of the Ahr signaling pathway were intact in the yeast model. Additionally, these reporter assays suggested that the Ahr pathway was not falsely activated by any component of the yeast culture medium. It also showed that the level of protein induction in our yeast model was perhaps more “physiological” since other groups observed reporter activity in the absence of ligand due to overexpression of Ahr and Arnt.

Ahr and Arnt Protein Levels are reduced in Yeast Cells over Time. A major finding in this study was that Ahr and Arnt protein levels were reduced upon transcriptional repression of the *GAL1* promoter. The reduction of the Ahr protein was not a result of premature initiation of the Ahr pathway by endogenous ligands present in the yeast or yeast media, as evidenced by baseline levels of β-galactosidase induction in vehicle-treated controls. It was anticipated that since yeast protein samples were prepared during a time course when the promoter was turned off, that the recombinant proteins would be "diluted" from the culture over time as the yeast propagated in the absence of galactose. Importantly, the loss of Ahr and Arnt protein levels from the protein samples was more rapid than could be explained by dilution through yeast cell division (budding).

This result implied that Ahr and Arnt proteins were being actively degraded and may be unstable when expressed in yeast. This result was not completely unexpected, since Ahr and Arnt are foreign proteins and yeast do not express bHLH/PAS orthologs, but the findings clearly complicated the goal of a model that could be evaluated for ligand-mediated degradation.

Overexpression of Chaperone Proteins does not Impact the Loss of Ahr and Arnt. It was possible that Ahr and Arnt turnover was the result of overexpression in our recombinant yeast strains. Thus, it was hypothesized that the chaperone proteins required for Ahr folding may not be functioning efficiently in yeast. This was the most logical hypothesis because of the critical role Hsp90 plays in the stability of Ahr in higher eukaryotes.

It has been shown that treatment of mammalian cells with Hsp90 inhibitors that disrupt the Ahr·Hsp90 interaction, causes a rapid turnover of Ah receptor protein in the

absence of ligand treatment. It was hypothesized that the interaction between the recombinant Ahr and Hsp90 may be compromised in yeast due to higher levels of Ahr than the yeast Hsp90 homologues. This imbalance could then lead to rapid Ahr degradation in the absence of ligand. Interestingly, the results showed that overexpression of mammalian Hsp90 did not increase the stability of the proteins.

It has also been shown that overexpression of exogenous proteins correlates with an increase in expression of chaperone proteins to facilitate protein folding. However, in cases of gross protein overexpression, the cells may be unable to produce enough chaperones to properly fold the newly synthesized proteins. Although we did not feel that the Ahr and Arnt proteins were being overexpressed to that extreme, it was possible that the rate of Ahr and Arnt synthesis was exceeding correlate expression of the yeast chaperones. Unfolded proteins in the cytoplasm of *Saccharomyces cerevisiae* are then targeted for degradation via the 26S proteasome. Again, the results of this study suggest that overexpression of mammalian Hsp90 did not impact Ahr or Arnt stability in these yeast strains. It should also be noted that this theory does not align with the ability to detect reporter activity. Specific activation of the Ahr through ligand binding implies that some population of the expressed Ahr pool is folded properly since unfolded Ahr will not be activated by ligand.

Ligand-Mediated Degradation of Ahr was not detected in Yeast. Ligand-mediated degradation of the Ahr could not be detected under any of the conditions tested during the course of these studies. Ahr protein levels were unchanged with ligand treatment when the promoter was active or repressed. Even when cells were treated with ligand at the same time as induction, no significant loss of Ahr was detected. These observations were in contrast to our anticipated result and complicated the use of this model for assessment of Ahr degradation. The original aim of the research project was

to produce a model to genetically evaluate Ahr degradation in yeast in a ligand-dependent manner, but a ligand-dependent effect could not be observed.

Ahr and Arnt Protein Degradation is Reversed with Proteasome Inhibitor Treatment. As stated in Chapter 3, yeast protein degradation occurs via the substrate-specific 26S proteasome pathway or by way of the non-specific vacuolar degradation pathway. It was of interest to determine which mechanism was responsible for the degradation of Ahr and Arnt. This information could indicate whether these proteins were being shuttled nonspecifically to the vacuole, or if the proteins were being specifically targeted to the proteasome for degradation. The results of this study revealed steady turnover of Ahr and Arnt in the DMSO-treated culture but these proteins were maintained at high levels when the promoter was turned off in the presence of MG132, a chemical known to block the activity of the 26S proteasome. The ability to detect higher levels of Ahr and Arnt protein in the presence of MG132 demonstrates that their degradation in yeast terminates through a proteasome-mediated mechanism. This is a positive finding because it does suggest that the study of Ahr degradation via the proteasome is still possible in this model and may be able to yield results that are translatable to a mammalian system (see Future Studies).

Hypothesis for Ahr and Arnt turnover in Yeast. These data provide evidence that the mouse Ahr and Arnt proteins are being degraded through the 26S proteasome in the yeast strains due to high levels of expression from an inducible promoter. The mechanism of degradation may occur through a degradation signal that is not recognized in mammals and is specific to yeast. Importantly, Arnt is not degraded in a ligand specific manner and has a much longer half-life in mammals than in these studies. The observation that Arnt is also turning over rapidly in the yeast via the 26S

proteasome, suggests that it may also have a putative sequence recognized by the yeast enzymes. Unfortunately, there is no database of putative degradation signals in yeast (or in mammals for that matter) that can be used to assess this question through amino acid analysis. To address this question directly would require mutagenesis of the Ahr and Arnt (although the yeast is amenable to selection schemes for such purposes).

Future Directions

Further Analysis of Ahr and Arnt in Yeast. There are additional experiments that would be of interest to provide further confirmation of the conclusions presented in this text. In particular, with regards to ligand treatment, it would be of interest to observe the effect of other known Ahr agonists on Ahr protein in these strains. Several ligands were tested and produced varying degrees of β-galatosidase reporter activity as shown in Figure 3.4. It would be of interest to evaluate Ahr and Arnt protein levels via western blotting with various ligand treatments at a range of doses. However, it is unlikely that a ligand-dependent degradation event would be detected, regardless of the ligand or dose used. This hypothesis is based on the fact that the ligand and dose used for the western blotting studies produced significant levels of β-galactosidase reporter activity in parallel experiments. The β-gal results suggest that the Ah receptor was activated under the conditions tested and degradation would have been detectable according to the mammalian model.

Another experimental approach that was not explored in this work would address the rate of yeast cell division. It has been shown that the yeast cell cycle can be arrested in S phase with the addition of hydroxyurea to the growth medium (Ke *et al* 2003). In this work, the galactose inducible promoter was repressed together with hydroxyurea treatment. This, in effect, allowed for the detection of exogenous protein turnover in the absence of cell division. With this method, we could evaluate the stability

of Ahr and Arnt proteins without having to consider the dilution of the exogenous proteins due to cell division. It should be noted however, that Ahr is a transcription factor that has been shown to play a role in cell cycle progression (Puga *et al* 2002). Since Ahr plays a role in cell cycle progression, arresting the cell cycle during the course of the experiments could influence the integrity of the signaling pathway.

Analysis of Ahr and Arnt Stability in E3 Knock-out Strains. While this yeast model does not appear to be amenable for analysis of ligand-mediated degradation of Ahr, these strains may be useful for the analysis of other aspects of Ahr signaling. Since these strains exhibit ligand-independent degradation of Ahr and Arnt, it would be of interest to further explore this occurrence. One way to identify proteins involved in mediating the turnover of Ahr and Arnt in yeast would be to knock out candidate genes. A marker cassette is used to disrupt the open reading frame of evolutionarily conserved genes in the ubiquitin-proteasome pathway in the Ahr and Arnt expressing strains. The goal would be to create a strain of yeast that does not degrade Ahr and Arnt proteins indicating that the knocked-out gene is involved in the degradation process. A list of sixty yeast genes involved in the ubiquitin-proteasome pathway was generated using the *Saccharomyces cerevisiae* genome database and is provided in Appendix C.

One particular E3 ligase was knocked out of the Ahr and Arnt expressor strain. The details concerning the generation of the *Δubr1* knockout strain is provided in Appendix C. Additionally, western blots stained for Ahr protein in the *Δubr1* knockout strain are included in Figure C.1. The preliminary results of this work do not suggest that *UBR1* is a mediator of Ahr degradation in yeast. However, this was an important E3 ligase to evaluate because of its known role in degradation of unfolded cytoplasmic proteins (Eisele and Wolf 2008).

Overexpression of Mammalian E3 Ligases in the Recombinant Yeast Strain. Since ligand-mediated degradation of Ahr was not observed under the conditions evaluated, it was hypothesized that the particular E3 ligase that targets Ahr for degradation is not conserved from mammals to yeast. Therefore, it would be interesting to observe Ahr protein levels in the recombinant strain with overexpression of mammalian E3s. Using a candidate approach, particular E3s implicated in degradation of Ahr and other bHLH proteins could be cloned into yeast expression vectors. Transformation of these expression vectors and subsequent overexpression of the E3s in the Ahr and Arnt yeast strains could reveal a potential target that degrades the receptor. With this method, one could evaluate the effect of individual candidate E3s in a systematic way and in a simpler cellular context.

As discussed in chapter 1, several reports have implicated Chip (Carboxyl terminus of Hsp70-interacting protein) as the E3 ligase responsible for targeting Ahr for degradation via the ubiquitin-proteasome pathway (Lees et al 2003, Morales and Perdew 2007). Although several reports have refuted the role of Chip in Ahr degradation using cell culture models, it would be of interest to test the effect of Chip overexpression in the recombinant yeast strain since yeast do not express a homologue. It has also been suggested that cullin4B interacts with ligand-bound Ahr (Wormke *et al* 2003, Ohtake *et al* 2007). These studies provided evidence of an interaction between these proteins, but did not offer direct evidence that Ahr degradation is mediated through cullin 4B. Again, one could evaluate the effect of overexpression of each individual candidate E3 in a systematic way in the absence of other complicating factors.

Analysis of Ahr and Arnt Isoforms using a Yeast Model. While there are many ways to further analyze Ahr and Arnt protein degradation in these strains, it is important to note that these strains could be used to analyze other aspects of the Ahr signaling

pathway. For example, the construction of a strain expressing Ah^{b-1} allele (that is naturally more stable than the Ah^{b-2} allele used in the recombinant strain) would allow for a direct comparison of the signaling of the two different proteins. These two alleles of the receptor have been compared *in vitro* and *in vivo*, but have yet to be expressed comparatively in yeast. A similar comparison could be made between the Arnt 1 and Arnt 2 isoforms to determine how they directly influence Ahr signaling in this model. In this case, the Ahr signaling pathway could be constructed using Arnt 2 in place of Arnt 1. This new strain could be used to evaluate the ability of Ahr and Arnt 2 to form heterodimers and bind the β-galactosidase reporter in the absence of Arnt1. This would be a very useful experiment, as an Arnt 1 knockout is lethal in mammals (Kozak *et al* 1997) thus complicating the ability to assess Ahr·Arnt 2 interactions.

CHAPTER FIVE:

MATERIALS AND METHODS

Materials

Buffers. Phosphate buffer is 20mM NaPi pH 7.7, 300mM NaoAc, 10% glycerol, protease inhibitor cocktail. LacZ buffer is 60mM Na_2HPO_4, 40mM NaH_2PO_4, 1mM $MgCl_2$, and 10mM KCl. SDS-PAGE sample buffer is 125 mM Tris, pH 6.8, 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM dithiothreitol, 0.005% bromophenol blue. Tris-buffered saline is 50 mM Tris and 150 mM NaCl, pH 7.5. TTBS is 50 mM Tris, 0.2% Tween 20, 300 mM NaCl, pH 7.5. TTBS+ is 50 mM Tris, 0.5% Tween 20, 300 mM NaCl, pH 7.5. Blocking milk is 5% dry milk in TTBS.

Reagents and Chemicals. Restriction enzymes and buffers, *AflII and ClaI,* were purchased from Promega (Madison, WI). Alkaline phosphatase was purchased from New England Biolabs (Ipswich, MA). 2,3,7,8-tetracholorodibenzo-*p*-dioxin (TCDD) was obtained from Radian Corporation (Austin, TX) and was solubilized in dimethyl sulfoxide (Me_2SO) obtained from Research Organics. Benzo[a]pyrene (BAP), 3-methylcholanthrene (3-MC) and β-naphthoflavone (βNF) were purchased from Sigma Aldrich (St. Louis, MO) and solubilized in dimethyl sulfoxide (Me_2SO). MG132, Radiciol, and Novobiocin were purchased in solution from CalBiochem. G418 was purchased from Axxora LLC (San Diego, CA).

Yeast Media. YPD (yeast extract-peptone-dextrose) consists of 10 g/l yeast extract (Fisher Scientific), 20 g/l Bacto-peptone (BD Diagnostic Systems), and 2% glucose (Fisher Scientific). YPD plates consisted of the above components with the addition of bacto-agar (BD Diagnostic Systems) at a concentration of 20 g/l. YPG consists of 10 g/l yeast extract (Fisher Scientific), 20 g/l Bacto-peptone (BD Diagnostic Systems), 20g/L agar, and 3% (v/v) glycerol (Sigma Aldrich). Synthetic medium consists of 6.7 g/l bacto-yeast nitrogen base without amino acids (Fisher Scientific), 10g/l dropout amino acid mix minus tryptophan, uracil, or leucine (US Biologicals), and 2% glucose (Fisher Scientific) or 2% galactose (Acros Organics).

Plasmids. Various constructs were obtained from the sources listed below.

TABLE 5.1: Sources For Plasmids

Plasmid Name	Use in Experiments	Source
pRS303	*HIS* cassette	Sikorski and Hieter 1989
pRS304	*TRP* cassette	Sikorski and Hieter 1989
pRS306	*URA* cassette	Sikorski and Hieter 1989
pFA6a-PGAL1KanMx	*GAL* promoter	Longtine et al 1998
pLXRE-5Z	Reporter construct	Cox and Miller 2003
pLNcx2-AHR^{b-2}	Ahr cDNA	Pollenz and Dougherty 2005
pCDNA-ARNT	Arnt cDNA	Dougherty and Pollenz 2008

Antibodies. Specific antibodies against Ahr and Arnt were identical to those described previously (Holmes and Pollenz, 1997; Pollenz et al., 1994). Antibody to the Xpress tag was purchased from Invitrogen and α-tubulin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Peroxidase-conjugated goat anti-mouse antibody and peroxidase-conjugated goat anti-rabbit antibody was purchased from Jackson

Immunoresearch. Antibodies were used at various concentrations that are included in the appropriate figure legends.

Methods

Yeast Transformations. Yeast transformations were carried out according the the method of Gietz and Woods (2006) using lithium cations, single-stranded carrier DNA, and polyethylene glycol. Parental yeast strains were streaked on pre-warmed YPD agar plates and placed in a 30°C incubator for two days. A single colony of the parental strain was used to inoculate 2 ml of YPD liquid media that was placed in a 30°C shaking incubator overnight. This culture was used to seed a 25 ml culture with an OD_{600} = 0.2. When the OD_{600} of the culture reached 0.8 (+/- 0.04), the culture was transferred to a conical tube and centrifuged at 2000 rpm for 2 minutes. The supernatant was removed, rinsed with water, and centrifuged again. The cell pellet was resuspended with 1 ml of sterile 100mM LiAc (pH 7.4) and transferred to a sterile 1.5 ml Eppendorf tube. After centrifugation at 14000 rpm for 30 seconds, the supernatant was removed and the pellet was resuspended in the equivalent of 1% of the final culture volume of 100mM LiAc (pH7.4). The cell suspension was divided equally between four Eppendorf tubes and each was centrifuged at 14000 rpm for 1 minute. The supernatant was removed and each pellet was combined with the following transformation reagents without disturbing the cell pellet; 240µl of 50% (w/v) PEG-3350, 36µl of 1M LiAc (pH7.4), 75µl PCR product or plasmid DNA in water, and 5µl of boiled/snap-cooled salmon sperm DNA (2mg/ml). Each tube was vortexed for one minute to resuspend the cell pellet in the transformation reagents. The tubes were placed in a 30°C incubator for 30 minutes without shaking and were heat shocked in a 45°C water bath for 15 minutes. The tubes were centrifuged at 7000 rpm for 1 minute and the supernatant was removed. The cell

pellet was combined with 100µl of sterile water and was spread on selective agar media. After two days, transformants were streaked again on selective media to obtain single colonies for further analysis.

Random Sporulation. Meiotic progeny was generated via random sporulation (Rockmill *et al* 1991). Diploids heterozygous for the desired cDNA integrations were grown overnight at 30°C in YPD, washed, transferred to 0.1% potassium acetate (Fisher Scientific), and incubated for 5 days in a 30°C shaking incubator. Asci were incubated in the presence of 500 µg/ml zymolase (MP Biomedicals) in 1 M sorbitol (Fisher Scientific) for 20 min at 30°C and enriched for haploid spores. Spores were plated on YPD, incubated at 30°C, and genotyped by spotting on synthetic drop-out media (US Biological) to detect the presence of *TRP1* and *URA3* marker cassettes linked to the AHR and ARNT cDNAs. The presence of the *GAL1* promoter linked to the kanMX6 cassette was detected by the ability of haploids to grow on YPD supplemented with 200 µg/ml G418 (Axxora LLC, San Diego, CA).

Activation and Repression of the Galactose-inducible Promoter. Yeast strains with integrated *GAL1* promoter sequences were streaked on agar media and placed in a 30°C incubator for two days. A single colony was used to inoculate liquid YPD medium and was placed in a 30°C shaking incubator overnight. The following day, the culture was centrifuged at 2000 rpm for 2 minutes. The YPD medium was removed, the pellet was rinsed with water, and the sample was centrifuged again. The supernatant was discarded and the cell pellet was resuspended in growth medium supplemented with 2% galactose (Acros Organics). The culture was returned to the 30°C shaking incubator for various time courses to allow for protein induction. Repression of the inducible promoter occurred upon addition of 2% glucose to the culture medium.

Preparation of Soluble Protein Fractions. Soluble protein fractions were prepared using a cold phosphate buffer extraction method. A liquid culture of the desired strain was propagated under conditions required for the given experiment. After the induction/treatment period, a sample of each culture was used to determine the cell density at OD_{600}. To obtain samples of even cell number, 15 ODs were removed from each culture. The optical density was converted to OD units by dividing 15 ODs by the optical density to determine the volume required. Each culture was centrifuged at 2000 rpm for 2 minutes and the pellet was suspended in 1ml of cold phosphate buffer (20mM NaPi pH 7.7, 300mM NaoAc, 10% glycerol, protease inhibitor cocktail). Each sample was centrifuged at 5000 rpm in a refrigerated centrifuge at 4°C for five minutes. The pellet was resuspended in 100µl of cold phosphate buffer and an equal volume of glass beads, and the tubes were placed in a mini-bead beater for 4 times for one minute each, and placed on ice for two minutes in between each. The resulting lysate was spun again at 14,000 rpm for 30 minutes at 4°C. The supernatant was then combined with an equal volume of 20% trichloroacetic acid (TCA) followed by centrifugation at 14,000 rpm for one minute. The resulting protein pellet was resuspended in 100µl of SDS-PAGE sample loading buffer and the pH of the sample buffer was adjusted with the addition of 2 M Tris. Each sample was centrifuged at 14000 rpm for one minute to remove cell debris and the supernatant was used in western blotting.

Preparation of TCA Precipitated Protein Samples. Total protein was prepared from the yeast cultures according to the procedure of Wright *et al* (1989) using 20% TCA to precipitate proteins. The yeast culture density was quantified at OD_{600}. To obtain samples of even cell number, 1.5 ODs were removed from each culture. The optical density was converted to OD units by dividing 1.5 ODs by the optical density to

determine the volume required. Each culture was centrifuged at 2000 rpm for 2 minutes and the medium was aspirated. The cell pellets, containing 1.5 ODs of cells, were combined with 100µl of 20% TCA and an equivalent volume of glass beads and were placed in a mini-bead beater for 4 minutes. Each sample was transferred to a fresh tube which was then centrifuged at 14,000 rpm for one minute. The resulting protein pellet was resuspended in 100µl of SDS-PAGE sample loading buffer and the pH of the sample buffer was adjusted with the addition of 2 M Tris. Each sample was centrifuged at 14000 rpm for one minute to remove cell debris and the supernatant was used in western blotting.

Western blotting. Western blotting and antibody detection were carried out as previously described (Pollenz, 1996) Protein samples were resolved by denaturing electrophoresis on 7% SDS-PAGE gels and were electrophoretically transferred to nitrocellulose. Protein detection was carried out with varying concentrations of primary antibody in blocking milk for 1 hour. The nitrocellulose membranes were washed with three changes of TTBS or TTBS+ solution for a total of 45 min. The blot was then incubated in blocking milk containing a 1:10,000 dilution of goat anti-rabbit or goat anti-mouse-HRP secondary antibodies for 1 hour and again washed in TTBS or TTBS+ as above. Prior to detection, the blots were washed with PBS for 5 minutes. Protein bands were visualized with the enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ). The relative concentration of target proteins was determined by computer analysis of the autoradiographs. Blots were quantified first by scanning images using a Cannon Canoscan 8800F scanner. Raw densitometric values were quantified using ImageJ software and were divided by α-Tubulin levels in order to normalize the target protein values in each sample.

Reporter Analysis. β-galactosidase activity was tested according to Miller (1997). One milliliter of yeast liquid culture was removed from larger test cultures. Each sample was centrifuged for 1 minute at 14,000 rpm, the medium was aspirated, and the cell pellet was combined with 500µl of lacZ buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 1mM $MgCl_2$, 10mM KCL, and ONPG (4mg/ml in lacZ buffer)). The pellet was resuspended in the solution and placed in a 37°C water bath and upon observation of a color change; the reactions were stopped with the addition of 1.5M sodium carbonate. The samples were centrifuged at 14,000 rpm for one minute to pellet the cells and the optical density at 420nm was measured for the supernatant. The observed optical density was normalized using the following equation: (Absorption @ 420nm x 1000) / (Absorbtion @ 595nm) X (ml of cell suspension added) X (minutes of reaction time).

LITERATURE CITED

Adachi, J., Y. Mori, S. Matsui, H. Kitagawa, C.A. Miller, III, T. Kato, K. Saeki, and T. Matsuda. 2001. Indirubin and Indigo are Potent Aryl Hydrocarbon Receptor Ligands Present in Human Urine. *The Journal of Biological Chemistry*. 276:31475-31478.

Amati, B., S. Dalton, M.W. Brooks, T.D. Littlewood, G.I. Evan, and H. Land. 1992. Transcriptional activation by the human c-MYC oncoprotein in yeast requires interaction with Max. *Nature*. 359:423-426.

Baba, M., M. Osumi, S.V. Scott, D.J. Klionsky, and Y. Ohsumi. 1997. Two Distinct Pathways for Targeting Proteins from the Cytoplasm to the Vacuole/Lysosome. *The Journal of Cell Biology*. 139:1687-1695.

Beischlag, T.V., S. Wang, D.W. Rose, J. Torchia, S. Reisz-Porszasz, K. Muhammad, W.E. Nelson, M.R. Probst, M.G. Rosenfeld, and O. Hankinson. 2002. Recruitment of the NCoA/SRC-1/p160 Family of Transcriptional Coactivators by the Aryl Hydrocarbon Receptor/Aryl Hydrocarbon Receptor Nuclear Translocator Complex. *Molecular and Cellular Biology*:4319-4333.

Berben, G., M. Legrain, V. Gilliquet, and F. Hilger. 1990. The Yeast Regulator Gene PHO4 Encodes and Helix-Loop-Helix Motif. *Yeast*. 6:451-454.

Bernassola, F., M. Karin, A. Ciechanover, and G. Melino. 2008. The HECT Family of E3 Ubiquitin Ligases: Multiple Players in Cancer Development. *Cancer Cell*. 14:10-21.

Betting, J., and W. Seufert. 1996. A Yeast Ubc9 Mutant Protein with Temperature-sensitive *in Vivo* Function is Subject to Conditional Proteolysis by a Ubiquitin-and Proteasome-dependent Pathway. *The Journal of Biological Chemistry*. 271:25790-25796.

Borkovich, K.A., F.W. Farrellu, D.B. Finkelstein, J. Taulien, and S. Lindquist. 1989. Hsp82 Is an Essential Protein That Is Required in Higher Concentrations for

Growth of Cells at Higher Temperatures. *Molecular and Cellular Biology*. 9:3919-3930.

Bradfield, C.A., and A. Poland. 1988. A Competitive Binding Assay for 2.3.7.8-tetrachlorodibenzo-*p*-dioxin and Related Ligands of the Ah Receptor. *Molecular Pharmacology*. 34:682-688.

Braliou, G.G., E. Venieris, A. Kalousi, and G. Simos. 2006. Reconstitution of Human Hypoxia Factor HIF-1 in Yeast : A Simple *In Vivo* System to Identify and Characterize HIF-1α Effectors. *Biochemical and Biophysical Research Communications*. 346:1289-1296.

Burbach, K.M., A. Poland, and C.A. Bradfield. 1992. Cloning of the Ah-Receptor cDNA Reveals a Distinctive Ligand-Activated Transcription Factor. *Biochemistry*. 89:8185-8189.

Carver, L.A., and C.A. Bradfield. 1997. Ligand-dependent Interaction of the Aryl Hydrocarbon Receptor with a Novel Immunophilin Homolog *In Vivo*. *The Journal of Biological Chemistry*. 272:11452-11456.

Carver, L.A., V. Jackiw, and C.A. Bradfield. 1994. The 90-kDa Heat Shock Protein Is Essential for Ah Receptor Signaling in a Yeast Expression System. *The Journal of Biological Chemistry*. 269:30109-30112.

Carver, L.A., J.J. LaPres, S. Jain, E.E. Dunham, and C.A. Bradfield. 1998. Characterization of the Ah-Receptor-associated Protein, ARA9. *The Journal of Biological Chemistry*. 273:33580-33587.

Chen, B., W.H. Piel, L. Gui, E. Bruford, and A. Monteiro. 2005. The HSP90 Family of Genes in the Human Genome: Insights into their Divergence and Evolution. *Genomics*. 86:627-637.

Chen, H.-S., S.S. Singh, and G.H. Perdew. 1997. The Ah Receptor is a Sensitive Target of Geldanamycin-Induced Protein Turnover. *Archives of Biochemistry and Biophysics*. 348:190-198.

Chen, Y.-H., and R.H. Tukey. 1996. Protein Kinase C Modulates Regulation of the CYP1A1 Gene by the Aryl Hydrocarbon Receptor. *The Journal of Biological Chemistry*. 271:26261-26266.

Ciechanover, A., and A.L. Schwartz. 1994. The Ubiquitin-Mediated Proteolytic Pathway: Mechanisms of Recognition of the Proteolytic Substrate and Involvement in the Degradation of Native Cellular Proteins. *FASEB*. 8:182-191.

Ciechanover, A., and A.L. Schwartz. 1998. The Ubiquitin-Proteasome Pathway: The Complexity and Myriad Functions of Proteins Death. *Proc. Natl. Acad. Sci.* 95:2727-2730.

Cox, M.B., and C.A. Miller, III. 2002. The p23 Co-chaperone Facilitates Dioxin Receptor Signaling in a Yeast Model System. *Toxicology Letters*. 129:13-21.

Cox, M.B., and C.A. Miller, III. 2003. Pharmacological and Genetic Analysis of 90-kDa Heat Shock Isoprotein-Aryl Hydrocarbon Receptor Complexes. *Molecular Pharmacology*. 64:1549-1556

Cox, M.B., and C.A. Miller. 2004. The Cooperation of heat shock protein 90 and p23 in Aryl Hydrocarbon Receptor Signaling. *Cell Stress and Chaperones*. 9:4-20.

Crews, S.T. 1998. Control of Cell-Lineage Specific Development and Transcription by bHLH-PAS Proteins. *Genes and Development*. 12:607-620.

Dale, Y.R., and S.E. Eltom. 2006. Calpain Mediated the Dioxin-Induced Activation and Down-Regulation of the Aryl Hydrocarbon Receptor. *Molecular Pharmacology*. 70:1481-1487.

Davarinos, N.A., and R.S. Pollenz. 1999. Aryl Hydrocarbon Receptor Imported into the Nucleus following Ligand Binding is Rapidly Degraded via the Cytoplasmic Proteasome following Nuclear Export. *The Journal of Biological Chemistry*. 274:28708-28715.

Denison, M.S., J.M. Fisher, and J.P. Whitlock, Jr. 1988. The DNA Recognition Site for the Dioxin-Ah Receptor Complex. *The Journal of Biological Chemistry*. 263:17221-17224

Denison, M.S., and S.R. Nagy. 2003. Activation of the Aryl Hydrocarbon Receptor by Structurally Diverse Exogenous and Endogenous Chemicals. *Annual Review of Pharmocological Toxicology*. 43:309-334.

Dougherty, E.J., and R.S. Pollenz. 2008. Analysis of Ah Receptor-ARNT and Ah Receptor-ARNT2 Complexes *In Vitro* and in Cell Culture. *Toxicological Sciences*. 103:191-206.

Eisele, F., and D.H. Wolf. 2008. Degradation of Misfolded Protein in the Cytoplasm is Mediated by the Ubiquitin Ligase Ubr1. *FEBS Letters*. 582:4143-4146.

Ema, M., N. Ohe, M. Suzuki, J. Mimura, K. Sogawa, S. Ikawa, and Y. Fuji-Kuriyama. 1994. Dioxin Binding Activities of Polymorphic Forms of Mouse and Human Aryl hydrocarbon Receptors. *The Journal of Biological Chemistry*. 269:27337-27343.

Escamilla-Powers, J.R., and R.C. Sears. 2007. A conserved pathway that controls c-Myc protein stability through opposing phosphorylation events occurs in yeast. *Journal of Biological Chemistry*. 282:5432-5442.

Flaveny, C., R.K. Reen, A. Kusnadi, and G.H. Perdew. 2008. The Mouse and Human Ah Receptor Differ in Recognition of LXXLL Motifs. *Archives of Biochemistry and Biophysics*. 471:215-233.

Fujimuro, M., H. Takada, Y. Saeki, A. Toh-e, K. Tanaka, and H. Yokosawa. 1998. Growth-Dependent Change of the 26S Proteasome in Budding Yeast. *Biochemical and Biophysical Research Communications*. 251:818-823.

Fukunaga, B.N., M.R. Probst, S. Reisz-Porszasz, and O. Hankinson. 1995. Identification of Functional Domains of the Aryl Hydrocarbon Receptor. *The Journal of Biological Chemistry*. 270:29270-29278.

Giannone, J.V., W. Li, M. Probst, and A.B. Okey. 1998. Prolonged Depletion of AH Receptor Without Alteration of Receptor mRNA Levels After Treatment of Cells in Culture with 2,3,7,8,-Tetrachlorodibenzo-*p*-dioxin. *Biochemical Pharmacology*. 55:489-497.

Giannone, J.V., A.B. Okey, and P.A. Harper. 1995. Characterization of Polyclonal Antibodies to the Aromatic Hydrocarbon Receptor. *Can J Physiol Pharmacol*. 73:7-17.

Gietz, D., and R.A. Woods. 2006. Yeast Transformation by the LiAc/SS Carrier DNA/PEG Method. *Methods in Molecular Biology*. 313:107-120.

Goll, D.E., V.F. Thompson, H. Li, W. Wei, and J. Cong. 2003. The Calpain System. *Physiol Rev*. 83:731-801.

Greenberg, M.L., and J.M. Lopes. 1996. Genetic Regulation of Phospholipid Biosynthesis in *Saccharomyces cerevisiae*. *Microbiology Review*. 60:1-20.

Gu, Y.Z., J.B. Hogenesch, and C.A. Bradfield. 2000. The PAS Superfamily: Sensors of Environmental and Developmental Signals. *Annual Review of Pharmocological Toxicology*. 40:519-561.

Hanel, F., K. Peukert, and T. Munder. 1996. Control of Myc-Max Mediated Transactivation in yeast by Natural Promoter Elements. *Journal of Basic Microbiology*. 37:23-28.

Hankinson, O. 2005. Role of Coactivators in Transcriptional Activation by the Aryl Hydrocarbon Receptor. *Archives of Biochemistry and Biophysics*. 433:379-386.

Hayes, S.A., and J.F. Dice. 1996. Roles of Molecular Chaperones in Protein Degradation. *The Journal of Cell Biology*. 132:255-258.

Heath-Pagliuso, S., W.J. Rogers, K. Tullis, S.D. Seidel, P.H. Cenijn, A. Brouwer, and M.S. Denison. 1998. Activation of the Ah Receptor by Tryptophan and Tryptophan Metabolites. *Biochemistry*. 37:11508-11515.

Heid, S.E., R.S. Pollenz, and H.I. Swanson. 1999. Role of Heat Shock Protein 90 Dissociation in Mediating Agonist-Induced Activation of Aryl Hydrocarbon Receptor. *Molecular Pharmacology*. 57:82-92.

Hochstrasser, M. 1996. Ubiquitin-Dependent Protein Degradation. *Annual Review of Genetics*. 30:405-439.

Holmes, J.L., and R.S. Pollenz. 1997. Determination of Aryl Hydrocarbon Receptor Nuclear Translocator Protein Concentration and Subcellular Localization in Hepatic and Nonhepatic Cell Cultures Lines: Development of Quantitative Western Blotting Protocols for Calculation of Aryl Hydrocarbon Receptor and Aryl Hydrocarbon Receptor Nuclear Translocator Protein in Total Cell Lysates. *Molecular Pharmacology*. 52:202-211.

Ikuta, T., H. Eguchi, T. Tachibana, Y. Yoneda, and K. Kawajiri. 1998. Nuclear Localization and Export Signals of the Human Aryl Hydrocarbon Receptor. *The Journal of Biological Chemistry*. 273:2895-2904.

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of Intact Yeast Cells Treated with Alkali Cations. *Journal of Bacteriology*. 153:163-168.

Jain, S., K.M. Dolwick, J.V. Schmidt, and C.A. Bradfield. 1994. Potent Transactivation Domains of the Ah Receptor and the Ah Receptor Nuclear Translocator Map to their Carboxyl Termini. *The Journal of Biological Chemistry*. 269:31518-31524.

Jentsch, S. 1992. The Ubiquitin-Conjugation System. *Annual Review of Genetics*. 26:179-207.

Jiang, J., C.A. Ballinger, Y. Wu, Q. Dai, D.M. Cyr, J. Hohfeld, and C. Patterson. 2001. CHIP is a U-box-dependent E3 Ubiquitin Ligase. *The Journal of Biological Chemistry*. 276:42938-42944.

Kamata, R., F. Shiraishi, D. Nakajima, H. Takigami, and H. Shiraishi. 2009. Mono-hydroxylated Polychlorinated Biphenyls are Potent Aryl Hydrocarbon Receptor Ligands in Recombinant Yeast Assays. *Toxicology in Vitro*. 23:736-743.

Kawanishi, M., M. Sakamoto, A. Ito, K. Kishi, and T. Yagi. 2003. Construction of Reporter Yeasts for Mouse Aryl Hydrocarbon Receptor Ligand Activity. *Mutation Research*. 540:99-105.

Kazlauskas, A., L. Poellinger, and I. Pongratz. 1999. Evidence that the Co-chaperone p23 Regulates Ligand Responsiveness of the Dioxin (Aryl Hydrocarbon) Receptor. *The Journal of Biological Chemistry*. 274:13519-13524.

Ke, P.-Y., C.-C. Yang, I.-C. Tsai, and Z.-F. Chang. 2003. Degradation of Human Thymidine Kinase is Dependent on Serine-13 Phosphorylation: Involvement of the SCF-Mediated Pathway. *Biochemical Society*. 370:265-273.

Kewley, R.J., M.L. Whitelaw, and A. Chapman-Smith. 2004. The Mammalian Basic Helix-Loop-Helix/PAS Family of Transcriptional Regulators. *The International Journal of Biochemistry & Cell Biology*. 36:189-204.

King, R.W., M. Glotzer, and M.W. Kirschner. 1996. Mutagenic Analysis of the Destruction Signal of Mitotic Cyclins and Structural Characterization of Ubiquitinated Intermediates. *Molecular Biology of the Cell*. 7:1343-1357.

Kippert, F. 1995. A Rapid Permeabilization Procedure for Accurate Quantitative Determination of β-Galactosidase Activity in Yeast Cells. *Microbiology Letters*. 128:201-206.

Ko, H.P., S.T. Okino, Q. Ma, and J.P. Whitlock, Jr. 1997. Transactivation Domains Facilitate Promoter Occupancy for the Dioxin-Inducible CYP1A1 Gene In Vivo. *Molecular and Cellular Biology*. 17:3497-3507.

Koegl, M., T. Hoppe, S. Schlenker, H.D. Ulrich, T.U. Mayer, and S. Jentsch. 1999. A Novel Ubiquitination Factor, E4, is Involved in Multiubiquitin Chain Assembly. *Cell*. 96:635-644.

Kohlmann, S., A. Schafer, and D.H. Wolf. 2008. Ubiquitin Ligase Hul5 is Required for Fragment-Specific Substrate Degradation in Endoplasmic Reticulum-Associated Degradation. *The Journal of Biological Chemistry*. 283:16374-16383.

Kornitzer, D., and A. Ciechanover. 2000. Modes of Regulation of Ubiquitin-Mediated Protein Degradation. *Journal of Cellular Physiology*. 182:1-11.

Kozak, K.R., B. Abbott, and O. Hankinson. 1997. ARNT-Deficient Mice and Placental Differentiation. *Developmental Biology*. 191:297-305.

Larsen, J.C. 1995. Levels of Pollutants and their Metabolites: Exposures to Organic Substances. *Toxicology*. 101:11-27.

Lee, D.H., and A.L. Goldberg. 1996. Selective Inhibitors of the Proteasome-dependent and Vacuolar Pathways of Protein Degradation in *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry*. 271:27280-27284.

Lee, D.H., and A.L. Goldberg. 1998. Proteasome Inhibitors Cause Induction of Heat Shock Proteins and Trehalose, which together confer Thermotolerance in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*. 18:30-38.

Lees, M.J., D.J. Peet, and M.L. Whitelaw. 2003. Defining the Role for XAP2 in Stabilization of the Dioxin Receptor. *The Journal of Biological Chemistry*. 278:35878-35888.

Legraverend, C., R.H. Hannah, H.J. Eiden, I.S. Owens, D.W. Nebert, and O. Hankinson. 1982. Regulatory Gene Product of the Ah Locus. *The Journal of Biological Chemistry*. 257:6402-6407.

Li, H., L. Dong, and J.P. Whitlock, Jr. 1994. Transcriptional Activation Function of the Mouse Ah Receptor Nuclear Translocator. *The Journal of Biological Chemistry*. 269:28098-28105.

Liu, G., Z. Niu, D.V. Nierkerk, J. Xue, and L. Zheng. 2008. Polycyclic Aromatic Hydrocarbons (PAHs) from Coal Combustion: Emission, Analysis, and Toxicology. *Reviews of Environmental Contamination and Toxicology*. 192:1-28.

Liu, Y., P. Choudhury, C.M. Cabral, and R.N. Sifers. 1999. Oligosaccharide Modification in the Early Secretory Pathway Directs the Selection of a Misfolded Glycoprotein for Degradation by the Proteasome. *The Journal of Biological Chemistry*. 274:5861-5867.

Lohr, D., P. Venkov, and J. Zlatanova. 1995. Transcriptional Regulation in the Yeast GAL Gene Family: a Complex Genetic Network. *FASEB*. 9:777-787.

Long, W.P., M. Pray-Grant, J.C. Tsai, and G.H. Perdew. 1998. Protein Kinase C Activity is Required for Aryl Hydrocarbon Receptor Pathway-Mediated Signal Transduction. *Molecular Pharmacology*. 53:691-700.

Longtine, M.S., A. McKenzie, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Phillipsen, and J.R. Pringle. 1998. Additional Modules for Versatile and Economical PCR-Based Gene Deletion and Modification in *Saccharomyces cerevisiae*. *Yeast*. 14:953-961.

Lorenz, M.C., R.S. Muir, E. Lim, J. McElver, S.C. Weber, and J. Heitman. 1995. Gene Disruption with PCR Products in *Saccharomyces cerevisiae*. *Gene*. 158:113-117.

Luscher, B., and L.G. Larsson. 1999. The Basic Region/ Helix-Loop-Helix/ Leucine Zipper Domain of Myc Proto-oncoproteins: Function and Regulation. *Oncogene*. 18:2955-2966.

Ma, Q., and K.T. Baldwin. 2000. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced Degradation of Aryl Hydrocarbon Receptor (AhR) by the Ubiquitin-Proteasome Pathway. *The Journal of Biological Chemistry*. 275:8432-8438.

Ma, Q., L. Dong, and J.P. Whitlock. 1995. Transcriptional Activation by the Mouse Ah Receptor. *The Journal of Biological Chemistry*. 270:12697-12703.

Ma, Q., and J.P. Whitlock. 1997. A Novel Cytoplasmic Protein That Interacts with the Ah Receptor, Contains Tetratricopeptide Repeat Motifs, and Augments the Transcriptional Response to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin. *The Journal of Biological Chemistry*. 272:8878-8884.

Mahon, M.J., and T.A. Gasiewicz. 1995. Ah Receptor Phosphorylation: Localization of Phosphorylation Sites to the C-Terminal Half of the Protein. *Archives of Biochemistry and Biophysics*. 318:166-174.

Massari, M.E., and C. Murre. 2000. Helix-Loop-Helix Proteins: Regulators of Transcription and Eucaryotic Organisms. *Molecular and Cellular Biology*. 20:429-440.

Miller, C.A., III. 1997. Expression of the Human Aryl Hydrocarbon Receptor Complex in Yeast. *The Journal of Biological Chemistry*. 272:32824-32829.

Miller, C.A., III. 1999. A Human Aryl Hydrocarbon Receptor Signaling Pathway Constructed in Yeast Displays Additive Responses to Ligand Mixtures. *Toxicology and Applied Pharmacology*. 160:297-303.

Miller, C.A. 2002. Two Tetratricopeptide Repeat Proteins Facilitate Human Aryl Hydrocarbon Receptor Signaling in Yeast. *Cellular Signaling*. 14:615-623.

Miller, C.A., III, M.A. Martinat, and L.E. Hyman. 1998. Assessment of Aryl Hydrocarbon Receptor Complex Interactions using pBEVY plasmids: Expression Vectors with Bi-directional Promoters for use in *Saccharomyces cerevisiae*. *Nucleic Acids Research*. 26:3577-3583.

Morales, J.L., and G.H. Perdew. 2007. Carboxyl Terminus of hsc70-Interacting Protein (CHIP) Can Remodel Mature Aryl Hydrocarbon Receptor (AhR) Complexes and Mediate Ubiquitination of Both the AhR and the 90 kDa Heat-Shock Protein (Hsp90) *in vitro*. *Biochemistry*. 46:610-621.

Nguyen, T.A., D. Hoivik, J.-E. Lee, and S. Safe. 1999. Interactions of Nuclear Receptor Coactivator/Corepressor Proteins with the Aryl Hydrocarbon Receptor Complex. *Archives of Biochemistry and Biophysics*. 367:250-257.

Noguerol, T.-N., S. Boronat, M. Casado, D. Raldua, D. Barcelo, and B. Pina. 2006. Evaluating the Interactions of Vertebrate Receptors with Persistant Pollutants and Antifouling Pesticides using Recombinant Yeast Assays. *Analytical and Bioanalytical Chemistry*. 385:1012-1019.

Ohtake, F., A. Baba, I. Takada, M. Okada, K. Iwasaki, H. Miki, S. Takahashi, A. Kouzmenko, K. Nohara, T. Chiba, Y. Fuji-Kuriyama, and S. Kato. 2007. Dioxin Receptor is a Ligand-Dependent E3 Uniquitin Ligase. *Nature Letters*. 446:562-566.

Ohura, T., M. Morita, M. Makino, T. Amagai, and K. Shimoi. 2007. Aryl Hydrocarbon Receptor-Mediated Effects of Chlorinated Poylcyclic Aromatic Hydrocarbons. *Chemical Research in Toxicology*. 20:1237-1241.

Pahl, H.L., and P.A. Baeuerle. 1996. Control of Gene Expression by Proteolysis. *Current Opinion in Cell Biology*. 8:340-347.

Pemberton, L.F., and B.M. Paschal. 2005. Mechanisms of Receptor-Mediated Nuclear Import and Nuclear Export. *Traffic*. 6:187-198.

Perdew, G.H. 1988. Associated of the *Ah* Receptor with the 90-kDa Heat Shock Protein. *The Journal of Biological Chemistry*. 263:13802-13805.

Platt, A., and R.J. Reece. 1998. The Yeast Galactose Genetic Switch is Mediated by the Formation of a Gal4p-Gal80p-Gal3p Complex. *EMBO Journal*. 17:4086-4091.

Poland, A., and J.C. Knutson. 1982. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and Related Halogenated Aromatic Hydrocarbons: Examination of the Mechanism of Toxicity. *Annual Review of Pharmocological Toxicology*. 22:517-554.

Pollenz, R.S. 1996. The Aryl-Hydrocarbon Receptor, but not the Aryl-Hydrocarbon Receptor Nuclear Translocator Protein, is Rapidly Depleted in Hepatic and Nonhepatic Culture Cells Exposed to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin. *Molecular Pharmacology*. 49:391-398.

Pollenz, R.S. 2002. The Mechanism of AH Receptor Protein Down-Regulation (Degradation) and its Impact on AH Receptor-Mediated Gene Regulation. *Chemico-Biological Interactions*. 141:41-61.

Pollenz, R.S. 2007. Specific Blockage of Ligand-Induced Degradation of the Ah Receptor by Proteasome but not Calpain Inhibitors in Cell Culture Lines from Different Species. *Biochemical Pharmacology*. 74:131-143.

Pollenz, R.S., and E.R. Barbour. 2000. Analysis of the Complex Relationship between Nuclear Export and Aryl Hydrocarbon Receptor-Mediated Gene Regulation. *Molecular and Cellular Biology*. 20:6095-6104.

Pollenz, R.S., and E.J. Dougherty. 2005. Redefining the Role of the Endogenous XAP2 and C-terminal Hsp70-interacting Protein on the Endogenous Ah Receptors Expressed in Mouse and Rat Cell Lines. *The Journal of Biological Chemistry*. 280:33346-33356.

Pollenz, R.S., J. Popat, and E.J. Dougherty. 2005. Role of Carboxyl-terminal Transactivation Domain and Active Transcription in the Ligand-Induced and Ligand-Independent Degradation of the Mouse Ah^{b-1} Receptor. *Biochemical Pharmacology*. 70:1623-1633.

Pollenz, R.S., C.A. Sattler, and A. Poland. 1993. The Aryl Hydrocarbon Receptor and Aryl Hydrocarbon Receptor Nuclear Translocator Protein Show Distinct Subcellular Localizations in Hepa 1c1c7 Cells by Immunofluorescence Microscopy. *Molecular Pharmacology*. 45:428-438.

Pollenz, R.S., S.E. Wilson, and E.J. Dougherty. 2006. Role of Endogenous XAP2 Protein in the Localization and Nucleocytoplasmic Shuttling of the Endogenous Mouse Ahb-1 Receptor in the Presence and Absence of Ligand. *Molecular Pharmacology*. 70:1369-1379.

Pongratz, I., G.G.F. Mason, and L. Poellinger. 1992. Dual Roles of the 90kDa Heat Shock Protein Hsp90 in Modulating Functional Activities of the Dioxin Receptor. *The Journal of Biological Chemistry*. 267:13728-13734.

Prokipcak, R.D., and A.B. Okey. 1991. Downregulation of the Ah Receptor in Mouse Hepatoma Cells Treated in Culture with 2,3,7,8-tetrachlorodibenxo-*p*-dioxin. *Can J Physiol Pharmacol*. 69:1204-1210.

Puga, A., Y. Xia, and C. Elferink. 2002. Role of Aryl Hydrocarbon Receptor in Cell Cycle Regulation. *Chemico-Biological Interactions*. 141:117-130.

Ramadoss, P., J.R. Petrulis, B.D. Hollingshead, A. Kusnadi, and G.H. Perdew. 2004. Divergent Roles of Hepatitis B Virus X-Associated Protein 2 (XAP2) in Human versus Mouse Ah Receptor Complexes. *Biochemistry*. 43:700-709.

Rannug, A., U. Rannug, H.S. Rosenkranz, L. Winqvist, R. Westerholm, E. Agurell, and A.K. Grafstrom. 1987. Certain photooxidized derivatives of tryptophan bind with very high affinity to the Ah receptor and are likely to be endogenous signal substances. *The Journal of Biological Chemistry*. 262:15422-15427.

Rechsteiner, M., and S.W. Rogers. 1996. PEST Sequences and Regulation of Proteolysis. *Trends in Biochemical Science*. 21:267-271.

Reisz-Porszasz, S., M.R. Probst, B.N. Fukunaga, and O. Hankinson. 1994. Identification of Functional Domains of the Aryl Hydrocarbon Receptor Nuclear Translocator Protein (ARNT). *Molecular and Cellular Biology*. 14:6075-6086.

Reyes, H., S. Reisz-Porszasz, and O. Hankinson. 1992. Identification of the Ah Receptor Nuclear Translocator Protein (Arnt) as a Component of the DNA Binding Form of the Ah Receptor. *Science*. 256:1193-1195.

Rockmill, B., E.J. Lambie, and G.S. Roeder. 1991. Spore Enrichment. *Methods in Enzymology*. 194:146-149.

Roberts, B.J., and M.L. Whitelaw. 1999. Degradation of the Basic Helix-Loop-Helix/Per-ARNT-Sim Homology Domain Dioxin Receptor Via the Ubiquitin/Proteasome Pathway. *The Journal of Biological Chemistry*. 274:36351-36356.

Robinson, K.A., and J.M. Lopes. 2000. *Saccharomyces cerevisiae* Basic Helix-Loop-Helix Proteins Regulate Diverse Biological Processes. *Nucleic Acids Research*. 28:1499-1505.

Schmidt, K.H., and R.D. Kolodner. 2004. Requirement of Rrm3 Helicase for Repair of Spontaneous DNA Lesions in Cells Lacking Srs2 or Sgs1 Helicase. *Molecular and Cellular Biology*. 24:3213-3226.

Sherman, F. 2002. Getting Started with Yeast. *Methods in Enzymology*. 350:3-41.

Sikorski, R.S., and P. Hieter. 1989. A System of Shuttle Vectors and Yeast Host Strains Designed for Efficient Manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*. 122:19-27.

Song, Z., and R.S. Pollenz. 2002. Ligand-Dependent and Independent Modulation of Aryl Hydrocarbon Receptor Localization, Degradation, and Gene Regulation. *Molecular Pharmacology*. 62:806-816.

Song, Z., and R.S. Pollenz. 2003. Functional Analysis of Murine Aryl Hydrocarbon (AH) Receptors Defective in Nuclear Import: Impact on AH Receptor Degradation and Gene Regulation. *Molecular Pharmacology*. 63:597-606.

Sorimachi, H., S. Ishiura, and K. Suzuki. 1997. Structure and Physiological Function of Calpains. *Journal of Biochemistry*. 328:721-732.

Soshilov, A., and M.S. Denison. 2008. Role of the PER/ARNT/SIM Domains in Ligand-Dependent Transformation of the Aryl Hydrocarbon Receptor. *The Journal of Biological Chemistry*. 283:32995-33005.

Sreedhar, A.S., E. Kalmar, P. Csermely, and Y.-F. Shen. 2004. Hsp90 Isoforms: Functions, Expression, and Clinical Importance. *FEBS Letters*. 562:11-15.

Sugihara, K., T. Okayama, S. Kitamura, K. Yamashita, M. Yasuda, S. Miyairi, Y. Minobe, and S. Ohta. 2008. Comparative Study of Aryl Hydrocarbon Receptor Ligand Activities of Six Chemicals *In Vitro* and *In Vivo*. *Toxicokinetics and Metabolism*. 82:5-11.

Swanson, H.I., W.K. Chan, and C.A. Bradfield. 1995. DNA Binding Specificities and Pairing Rules of the Ah Receptor, ARNT, and SIM Proteins. *The Journal of Biological Chemistry*. 270:26292-26302.

Swanson, H.I., and G.H. Perdew. 1993. Half-Life of Aryl Hydrocarbon Receptor in Hepa 1 Cells: Evidence for Ligand-Dependent Alterations in Cytosolic Receptor Levels. *Archives of Biochemistry and Biophysics*. 302:167-174.

Tanaka, K. 1998. Proteasomes: Structure and Biology. *Journal of Biochemistry*. 123:195-204.

Varshavsky, A. 1997. The N-end Rule Pathway of Protein Degradation. *Genes to Cells*. 2:13-28.

Whitelaw, M., I. Pongratz, A. Wilhelmsson, J.-A. Gustafsson, and L. Poellinger. 1993. Ligand-Dependent Rectruitment of the Arnt Coregulator Determines DNA Recognition by the Dioxin Receptor. *Molecular and Cellular Biology*. 13:2504-2514.

Whitelaw, M.L., J. McGuire, D. Picard, J.-A. Gustafsson, and L. Poellinger. 1995. Heat Shock Protein Hsp90 Regulates Dioxin Receptor Function *In Vivo*. *Proc. Natl. Acad. Sci.* 92:4437-4441.

Whitlock, J.P., Jr. 1999. Induction of Cytochrome P4501A1. *Annual Review of Pharmocological Toxicology*. 39:103-125.

Wormke, M., M. Stoner, B. Saville, K. Walker, M. Abdelrahim, R. Burghardt, and S. Safe. 2003. The Aryl Hydrocarbon Receptor Mediates Degradation of Estrogen Receptor alpha through Activation of Proteasomes. *Molecular and Cellular Biology*. 23:1843-1855.

Wright, A.P., M. Bruns, and B.S. Hertley. 1989. Extraction and rapid inactivation of proteins from *Saccharomyces cerevisiae* by trichloroacetic acid precipitation. *Yeast*. 5:51-53.

APPENDICES

Appendix A: Table of Strains Generated

TABLE A.1: List of Strains Generated

Strain	Genotype	Use in Experiments
KHSY421	*MATa, ura3-52, trp1Δ63, his3Δ200, leu2Δ1*	Parental strain for AHR expression
KHSY422	*MATα, ura3-52, trp1Δ63, his3Δ200, leu2Δ1*	Parental strain for ARNT expression
KHSY1538	*MATa, ChrXV::HIS3MX6-PGAL1-AHR.TRP1, ura3-52, trp1Δ63, his3Δ200, leu2Δ1*	AHR expressor strain
KHSY1541	*MATα, ChrIV::HIS3MX6-PGAL1-V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1*	ARNT expressor strain
KHSY1547	*MATa, ChrXV::HIS3MX6-PGAL1-AHR.TRP1, ChrIV::HIS3MX6-PGAL1-V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1*	Double knock-in strain expressing both AHR and ARNT
KHSY1565	*MATa, ChrXV::HIS3MX6-PGAL1-AHR.TRP1, ura3-52, trp1Δ63, his3Δ200, leu2Δ1, pLXRE5-Z*	AHR expressor strain with AHR activated reporter plasmid
KHSY1566	*MATa, ChrXV::HIS3MX6-PGAL1-AHR.TRP1 ChrIV::HIS3MX6-PGAL1-V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1, pLXRE5-Z*	Double knock-in strain with AHR activated reporter plasmid
KHSY2228	*MATa, ChrXV::HIS3MX6-PGAL1-AHR.TRP1, ChrIV::HIS3MX6-PGAL1-V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1, pHGPD*	Double knock-in strain with empty vector used in HSP90 studies
KHSY2229	*MATa, ChrXV::HIS3MX6-PGAL1-AHR.TRP1, ChrIV::HIS3MX6-PGAL1-V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1, pHGPDHsp90alpha*	Double knock-in strain over expressing human HSP90 alpha from a transformed plasmid
KHSY2230	*MATa, ChrXV::HIS3MX6-PGAL1-AHR.TRP1, ChrIV::HIS3MX6-PGAL1-V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1, prHGPDHsp90beta*	Double knock-in strain over expressing human HSP90 beta from a transformed plasmid
KHSY2501	*MATa, ChrXV::HIS3MX6-PGAL1-AHR.TRP1, ChrIV::HIS3MX6-PGAL1-V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1, erg6*	Permeable double knock-in strain for protease studies
KHSY2502	*MATa, ChrXV::HIS3MX6-PGAL1-AHR.TRP1, ChrIV::HIS3MX6-PGAL1-V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1, hul5*	Double knock-in strain with HUL5 E3 ligase knocked out
KHSY2502	*MATa, ChrXV::HIS3MX6-PGAL1-AHR.TRP1, ChrIV::HIS3MX6-PGAL1-V5-ARNT.URA3, ura3-52, trp1Δ63, his3Δ200, leu2Δ1, ubr1*	Double knock-in strain with UBR1 E3 ligase knocked out

Appendix B: Additional Figures

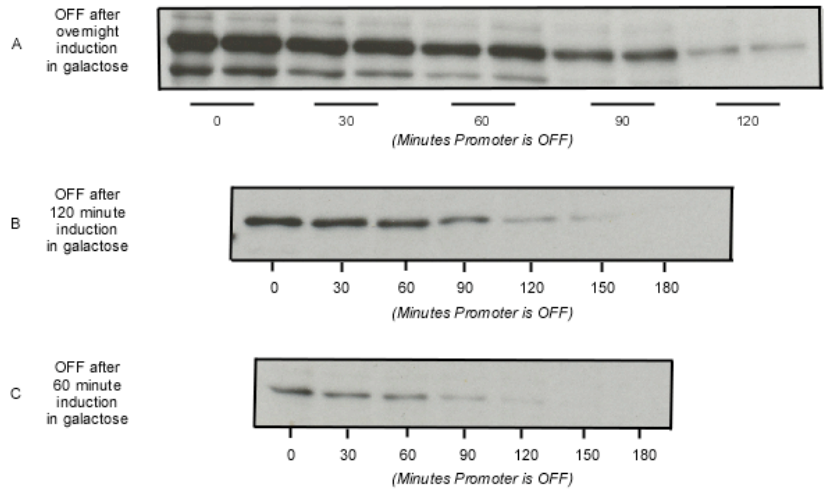

FIGURE A.1: Reduction of Ahr protein levels in yeast upon addition of glucose to the growth medium. The KHSY1538 strain was induced for Ahr protein expression with the addition of 2% galactose to the growth medium overnight (A), for 2 hours (B), or for 1 hour (C). Subsequently, the galactose-containing media was removed and replaced with glucose-containing media. The glucose acted to shut down the *GAL* promoter located upstream of the AHR cDNA. Upon addition of glucose to the culture medium, the Ah receptor is degraded over time, regardless of the length of the initial induction of the protein expression.

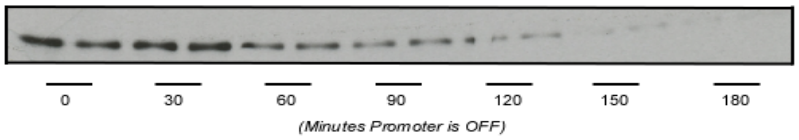

FIGURE A.2: Levels of Arnt protein expressed in the KHSY1541 strain are reduced following addition of glucose to the growth medium. A two hour induction in 2% galactose followed by a change to growth media containing 2% glucose caused the *GAL1* promoter to stop transcription of the ARNT mRNA. Protein samples prepared from samples of the culture taken over time show a rapid degradation of the Arnt protein that is comparable to the Ahr degradation pattern previously described.

Appendix C: E3 Knockout Preliminary Results

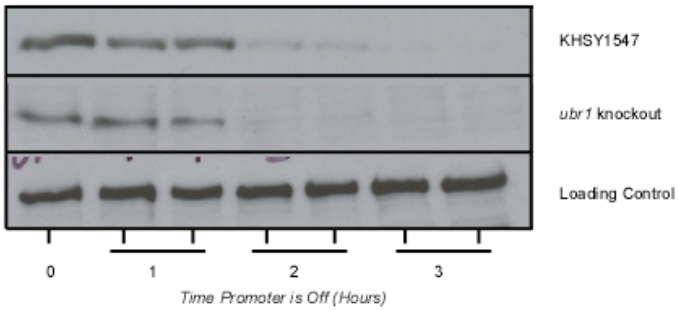

FIGURE A.3: Ahr protein is degraded in the *Δubr1* strain. The Ahr and Arnt expressor strain was used to knock out the yeast E3 ligase, UBR1. This strain was tested for Ahr stability as previously described. Ahr and Arnt protein expression was induced in the double knock in strain and the knock out strain with 2% galactose for two hours. The *GAL1* promoter was turned off and protein samples were obtained 0, 1, 2, and 3 hours after the promoter was turned off. Note that Ahr is degraded over time in both the double knock-in strain and the E3 mutant strain over time. The rate of degradation in both strains appears comparable suggesting that UBR1 is not likely the intermediate E3 ligase that targets Ahr for proteasomal degradation.

TABLE A.2: Yeast Genes Involved In Proteasome-Mediated Degradation

Gene ID	Name	Δ
APC1	Largest subunit of the Anaphase-Promoting Complex Cyclosome (APC/C), which is a ubiquitin-protein ligase (E3)	I
APG7, CVT2, ATG7	Autophagy-related protein and dual specificity member of the E1 family of ubiquitin-activating enzymes	V
CDC16	metal-binding nucleic acid-binding protein, interacts with Cdc23p and Cdc27p to catalyze the conjugation of ubiquitin to cyclin B (putative)	I
CDC4	F-box protein which acts as ubiquitin-protein ligase (E3)	I
DSK2	Nuclear-enriched ubiquitin-like polyubiquitin-binding protein	V
HSE1	Subunit of the endosomal Vps27p-Hse1p complex required for sorting of ubiquitinated membrane proteins into intralumenal	V

	vesicles prior to vacuolar degradation	
HUB1	Ubiquitin-like protein modifier	V
HRT1, HRT2 RBX1, ROC1	Skp1-Cullin-F-box ubiquitin protein ligase (SCF) subunit	I
HUL4	Protein with similarity to hect domain E3 ubiquitin-protein ligases (E3)	V
HUL5	Protein with similarity to hect domain E3 ubiquitin-protein ligases (E3)	V
LEO1	Component of the Paf1 complex	V
PAF1	RNA polymerase II-associated protein	V
RAD23	Protein with ubiquitin-like N terminus	V
RUB1	Ubiquitin-like protein with similarity to mammalian NEDD8	V
STP22, VPS23	Component of the ESCRT-I complex, which is involved in ubiquitin-dependent sorting of proteins into the endosome	V
TUL1	Golgi-localized RING-finger ubiquitin ligase (E3)	V
UBA1	Ubiquitin activating enzyme (E1), involved in ubiquitin-mediated protein degradation	I
UBC1	Ubiquitin-conjugating enzyme (E2) that mediates selective degradation of short-lived and abnormal proteins	I
UBC2, RAD6	Ubiquitin-conjugating enzyme (E2)	V
UBC3, CDC34	Ubiquitin-conjugating enzyme or E2 (cell cycle) woks with SKP1, RBX1, CDC53	V
UBC4	Ubiquitin-conjugating enzyme (E2) that mediates degradation of short-lived and abnormal proteins	V
UBC5	Ubiquitin-conjugating enzyme (E2) that mediates selective degradation of short-lived and abnormal proteins	V
UBC6,	Ubiquitin-conjugating enzyme (E2) involved in ER-associated	V

DOA2	protein degradation	
UBC7, QRI8	Ubiquitin conjugating enzyme (E2), involved in the ER-associated protein degradation pathway	V
UBC8	Ubiquitin-conjugating enzyme (E2) that negatively regulates gluconeogenesis	V
UBC9	SUMO-conjugating enzyme involved in the Smt3p conjugation pathway	I
UBC10, PAS2, PEX4	Peroxisomal ubiquitin conjugating enzyme (E2)	V
UBC11	Ubiquitin-conjugating enzyme (E2) most similar in sequence to Xenopus ubiquitin-conjugating enzyme E2-C	V
UBC12	Enzyme that mediates the conjugation of Rub1p, a ubiquitin-like protein, to other proteins; related to E2 ubiquitin-conjugating enzymes	V
UBC13	Ubiquitin-conjugating enzyme (E2) involved in the error-free DNA postreplication repair pathway	V
UBI1, RPL40A	Fusion protein, identical to Rpl40Bp, that is cleaved to yield ubiquitin	V
UBI2, RPL40B	Fusion protein, identical to Rpl40Ap, that is cleaved to yield ubiquitin	V
UBI3, RPS37	Fusion protein that is cleaved to yield a ribosomal protein of the small (40S) subunit and ubiquitin	V
UBI4, SCD2	Ubiquitin gene	V
UBP12	Ubiquitin-specific protease present in the nucleus and cytoplasm that cleaves ubiquitin from ubiquitinated proteins	V
UBP1	Ubiquitin-specific protease that removes ubiquitin from ubiquitinated proteins	V
UBP2	Ubiquitin-specific protease that removes ubiquitin from ubiquitinated proteins	V
UBP3	Ubiquitin-specific protease that interacts with Bre5p	V

UBP4, DOA4, DOS1, MUT4 NPI2, SSV7,	Ubiquitin hydrolase, required for recycling ubiquitin from proteasome-bound ubiquitinated intermediates	V
UBP5	Putative ubiquitin-specific protease that does not associate with the proteasome	V
UBP6	Ubiquitin-specific protease situated in the base subcomplex of the 26S proteasome	V
UBP7	Ubiquitin-specific protease that cleaves ubiquitin-protein fusions	V
UBP8	Ubiquitin-specific protease that is a component of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) acetylation complex	V
UBP9	Ubiquitin-specific protease that cleaves ubiquitin-protein fusions	V
UBP10, DOT4	ubiquitin-specific protease that deubiquitinates ubiquitin-protein moieties	V
UBP11	Ubiquitin-specific protease that cleaves ubiquitin from ubiquitinated proteins	V
UBP12	Ubiquitin-specific protease present in the nucleus and cytoplasm that cleaves ubiquitin from ubiquitinated proteins	V
UBP13	Putative ubiquitin-specific protease	V
UBP14	Ubiquitin-specific protease that specifically disassembles unanchored ubiquitin chains	V
UBP15	ubiquitin-specific protease that may play a role in ubiquitin precursor processing	V
UBP16	Putative ubiquitin-specific protease	V
UBR1, PTR1	Ubiquitin-protein ligase (E3) that interacts with Rad6p/Ubc2p to ubiquitinate substrates of the N-end rule pathway	V
UBR2,	Cytoplasmic ubiquitin-protein ligase (E3)	V
UFD1	Protein that interacts with Cdc48p and Npl4p, involved in recognition of polyubiquitinated proteins and their presentation to the 26S proteasome for degradation	I

UFD2	Ubiquitin chain assembly factor (E4) also functions as an E3	V
UFD3, DOA1, ZZZ4	WD repeat protein required for ubiquitin-mediated protein degradation	V
UFD4	Ubiquitin-protein ligase (E3) that interacts with Rpt4p and Rpt6p	V
UFD5, RPN4, SON1	Transcription factor that stimulates expression of proteasome genes; Rpn4p levels are in turn regulated by the 26S proteasome in a negative feedback control mechanism	V
URM1	Ubiquitin-like protein with only weak sequence similarity to ubiquitin; depends on the E1-like activating enzyme Uba4p	V
YUH1	Ubiquitin C-terminal hydrolase that cleaves ubiquitin-protein fusions to generate monomeric ubiquitin	V