2021

POST-TRANSLATIONAL MODIFICATION AND DEGRADATION MECHANISMS OF THE ARYL HYDROCARBON RECEPTOR

Yujie Yang
University of the Pacific

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POST-TRANSLATIONAL MODIFICATION AND DEGRADATION MECHANISMS OF
THE ARYL HYDROCARBON RECEPTOR

By

Yujie Yang

A Dissertation Submitted to the
Graduate School
In Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Thomas J. Long School of Pharmacy
Pharmaceutical and Chemical Sciences

University of the Pacific
Stockton, California

2021
POST-TRANSLATIONAL MODIFICATION AND DEGRADATION MECHANISMS OF THE ARYL HYDROCARBON RECEPTOR

By

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POST-TRANSLATIONAL MODIFICATION AND DEGRADATION MECHANISMS OF THE ARYL HYDROCARBON RECEPTOR

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By

Yujie Yang
ACKNOWLEDGEMENTS

My gratitude goes to my advisor Dr. William Chan for his continuous support and instruction. He is a very responsible advisor and principal investigator. I learned a lot in Chan lab. His attitude and enthusiasm for research keep inspiring me to be a better scientist.

I would also like to thank Dr. Vierra, Dr. Russu, Dr. Livesey and Dr. Alhamadsheh for being my committee members. Their suggestions are very helpful and appreciated. Finally, I thank Dr. Barney Jordan and all my friends for helping me and keeping me accompanied through this long journey.
The aryl hydrocarbon receptor (AHR) is a transcription factor first discovered to be activated by exogenous ligands, such as dioxins, and helps promote downstream gene (e.g. CYP1A1) transcription to metabolize the toxicants. With the reports of various AHR targets genes, the expression levels and activities of AHR have been implicated in many physiological and pathological situations. Understanding how AHR protein level is regulated would provide more information to target AHR. AHR stays in the cytosol in the absence of ligand in a complex with HSP90, p23 and XAP2. After ligand activation, AHR translocates into the nucleus, fulfilling its transactivation function and then is finally degraded by proteasomes. Here, we discovered a new mechanism that controls basal AHR protein level: the selective autophagy. Loss of AHR co-chaperone p23 leads to increased protein degradation of AHR through autophagy in HeLa cells. Inhibition of autophagy using several inhibitors (chloroquine, bafilomycin A1 or 3-methyladenine) increased AHR protein levels. Knocking down of key macroautophagy protein LC3B increases AHR protein levels and decreases the responsiveness of AHR to CQ treatment. The interaction between AHR and LC3B as well as AHR and autophagy receptor p62 were confirmed in vitro and in situ. AHR is found to be lysine (K) 63-ubiquitinated in HeLa cells, which is a common signal for the autophagy-lysosomal degradation.
We also discovered that AHR is controlled by glycogen synthase kinase 3β (GSK3β) phosphorylation. Inhibition of GSK3β activity or its expression level increased AHR protein levels while expression of HA tagged-GSK3β lowers AHR protein levels. AHR protein level is regulated through autophagy. We confirmed the GSK3β-mediated phosphorylation of AHR by phos-tag gel electrophoresis couples with Western blot analysis and identified three putative phosphorylation sites of AHR in the C-terminal half of AHR sequence. Moreover, phosphorylated AHR constitutes the active pool for transactivation and phosphorylation tagged AHR for the autophagy-lysosomal degradation, which may act as way to limit its function.
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<tr>
<td>3MA</td>
<td>3-methyladenine</td>
</tr>
<tr>
<td>3MC</td>
<td>3-methylcholanthrene</td>
</tr>
<tr>
<td>6-AN</td>
<td>6-aminonicotinamide</td>
</tr>
<tr>
<td>ActD</td>
<td>actinomycin D</td>
</tr>
<tr>
<td>AHR</td>
<td>aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AHRR</td>
<td>AHR repressor</td>
</tr>
<tr>
<td>alkyl-PCDFs</td>
<td>alkyl substituted chlorinated dibenzofurans</td>
</tr>
<tr>
<td>ALPS</td>
<td>amphiphatic lipid packing sensor</td>
</tr>
<tr>
<td>AP</td>
<td>ammonium persulfate</td>
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<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ARNT</td>
<td>AHR nuclear translocator</td>
</tr>
<tr>
<td>Baf A1</td>
<td>bafilomycin A1</td>
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<tr>
<td>BaP</td>
<td>benzo[a]pyrene</td>
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<tr>
<td>BATS</td>
<td>Barkor autophagosome targeting sequence</td>
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<tr>
<td>BCA</td>
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<tr>
<td>bHLH-PAS</td>
<td>basic helix-loop-helix-PER-ARNT-SIM</td>
</tr>
<tr>
<td>CHIP</td>
<td>C-terminal hsp70-interacting protein</td>
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<td>CHX</td>
<td>cycloheximide</td>
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<td>CK1</td>
<td>casein kinase 1</td>
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<td>CMA</td>
<td>chaperone-mediated autophagy</td>
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<tr>
<td>CQ</td>
<td>chloroquine</td>
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</table>
cyp1a1  cytochrome P450 1a1
DC       dendritic cells
DFCP1    double FYVE-containing protein 1
DIM      diindolylmethane
DMEM     Dulbecco's modified eagle medium
DMSO     dimethyl sulfoxide
DRE      dioxin response element
Dvl      Dishevelled
EAE      experimental autoimmune encephalomyelitis
EDTA     ethylenediaminetetraacetic acid
Erk kinase extracellular signal-regulated kinase
FICZ     6-formylindolo[3,2-b]carbazole
Fz       Frizzled
GAPDH    glyceraldehyde 3-phosphate dehydrogenase
GFP      green fluorescent protein
GSK3     glycogen synthase kinase-3
HA-GSK3β HA fusion of GSK3β
HBSS     Hank’s balanced salt solution
HEPES    4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1α    hypoxia-inducible factor 1-alpha
HSC70    heat shock cognate protein of 70KDa
HSP90    heat shock protein 90
ITE      2-(1'H-indole-3-carbonyl)-thiazole-4-carboxylic acid
          methyl ester
K48      lysine 48
<table>
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<th>Description</th>
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<td>lysine 63</td>
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<td>kDa</td>
<td>kilodalton</td>
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<td>lysosomal associated membrane protein 2A</td>
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<td>LBD</td>
<td>ligand binding domain</td>
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<td>LC3-interacting region</td>
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<td>microtubule associated protein 1 light chain 3</td>
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<td>sodium chloride</td>
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<td>NEM</td>
<td>N-ethylmaleimide</td>
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<td>NES</td>
<td>nuclear export signal</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
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<td>NP</td>
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<tr>
<td>NT</td>
<td>no treatment</td>
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<td>p23KD</td>
<td>p23 knockdown</td>
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<td>Phox and Bem1</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
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<td>PER</td>
<td>periodic</td>
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<td>PI3KC1</td>
<td>class I phosphatidylinositol 3-kinase</td>
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<td>PI3KC2</td>
<td>class II phosphatidylinositol 3-kinase</td>
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<td>PI3KC3</td>
<td>class III phosphatidylinositol 3-kinase</td>
</tr>
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<td>Acronym</td>
<td>Description</td>
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<td>-----------</td>
<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>PLA</td>
<td>proximity ligation assay</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonylefluoride</td>
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<tr>
<td>PtdIns3P</td>
<td>phosphatidylinositol 3-phosphate</td>
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<tr>
<td>SAhRM</td>
<td>selective AHR modulators</td>
</tr>
<tr>
<td>SAR</td>
<td>selective autophagy receptor</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SIM</td>
<td>single minded</td>
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<tr>
<td>SKP1</td>
<td>S-phase-kinase-associated-protein 1</td>
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<td>TAD</td>
<td>transactivation domain</td>
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<tr>
<td>TAM</td>
<td>tumor associated macrophages</td>
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<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
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<tr>
<td>TCF/LEF</td>
<td>T cell factor/lymphoid enhancer factor</td>
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<tr>
<td>TDG</td>
<td>tideglusib</td>
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<td>TDO</td>
<td>tryptophan-2,3-dioxygenase</td>
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<td>TEMED</td>
<td>tetramethylethylenediamine</td>
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<td>Tris-glycine-SDS</td>
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<td>TIP60</td>
<td>HIV Tat-interactive protein 60 kDa</td>
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<td>target of rapamycin complex 1</td>
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<td>Treg</td>
<td>regulatory T cells</td>
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<tr>
<td>TUBE</td>
<td>specific tandem ubiquitin binding entity</td>
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<tr>
<td>UBA</td>
<td>ubiquitin-associated domain</td>
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<tr>
<td>ULK1</td>
<td>Unc-51 like kinase-1</td>
</tr>
<tr>
<td>WCL</td>
<td>whole cell lysate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>XAP2</td>
<td>hepatitis B virus-associated protein</td>
</tr>
<tr>
<td>β-TrCP</td>
<td>β-transducin repeat containing proteins</td>
</tr>
<tr>
<td>βNF</td>
<td>beta-naphthoflavone</td>
</tr>
<tr>
<td>λ-PP</td>
<td>lambda phosphatase</td>
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INTRODUCTION

The Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AHR) was first identified by Alan Poland’s laboratory in 1976 (Poland, Glover, & Kende, 1976) and is originally recognized as a receptor/transcription factor in response to environmental toxicants such as dioxins and polycyclic aromatic hydrocarbons. Since then, more roles of AHR in physiological and pathological situations such as organ development, immune responses, nervous system homeostasis, and carcinogenesis have been uncovered. Not surprisingly, AHR has become a promising target for the treatment of diseases such as autoimmune diseases, inflammation, and cancers. However, the AHR function is often ligand-, cell context-, and tissue-specific, confounding the feasibility of targeting AHR for rational drug design. Understanding the basic biology of AHR would provide much-needed information for effective modulation of the AHR function.

AHR Complex and Classical Signaling Pathway

AHR belongs to the basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) family, which contains dimeric transcription factors that share similar N-terminal sequence to AHR nuclear translocator (ARNT), and two Drosophila proteins “single-minded” (SIM; involved in neuronal development) and “periodic” (PER; a circadian regulator), hence named as PAS domain in 1991 (Crews, Thomas, & Goodman, 1988; Hoffman et al., 1991; Konopka & Benzer, 1971; Reyes, Reisz-Porszasz, & Hankinson, 1992). Figure 1.1 below showed the domain structure of AHR protein (Faber, Soshilov, Giani Tagliabue, Bonati, & Denison, 2018; C. A. Flaveny & Perdew, 2009; Fukunaga, Probst, Reisz-Porszasz, & Hankinson, 1995; Ikuta,
Eguchi, Tachibana, Yoneda, & Kawajiri, 1998; Schulte, Green, Wilz, Platten, & Daumke, 2017). The N-terminal bHLH domain is responsible for DNA binding, followed by two tandem PAS domains, which contain two reverted repeats of 51-amino acids in length conserved in PAS family proteins (PAS-A and PAS-B), contributing to dimerization and ligand binding. The C-terminal half of AHR contains a transactivation domain (TAD) consisting of three separable regions, namely acidic, glutamine-rich, and proline-serine/threonine-rich motifs (Fukunaga et al., 1995). The ligand binding domain (LBD) of AHR locates in PAS-B domain (Burbach, Poland, & Bradfield, 1992). However, soluble AHR constructs containing PAS-B domain are hard to purify in large amounts and the crystal structure has not been reported yet. Knowing the structural data of LBD may provide better understanding of the ligand-specific responses of AHR.

Figure 1.1. Structure of AHR domains. Q, glutamine; P, proline; S, serine; T, threonine; TAD, transactivation domain.

AHR-ARNT heterodimer forms via three interfaces located in bHLH and PAS-A domains. Although PAS-B domain is reported to be involved in dimerization of other PAS proteins, AHR: ARNT is stable without PAS-B domain (Schulte et al., 2017). AHR construct lacking the PAS-B domain is constitutively active in mice (Andersson et al., 2002), suggesting that ligand binding is important for the
localization of AHR but not for the transactivation function and PAS-B domain is not required for dimerization. It is more like that PAS-B functions as a regulation domain for dimerization. Residues 13-39 act as the nuclear localization signal (NLS), which contains two cluster of basic amino acids connected by a spacer of 17 amino acids in length. Leucine-rich sequence (amino acids 55-75) is reported to function as the nuclear export signal (NES) (Ikuta et al., 1998). Differences in structures of AHR may explain the species- and strain- differences of AHR responsiveness. The C57BL/6J mice expressing the mAHRb allele showed a ~10-fold higher affinity for AHR ligands comparing to DBA mice mAHRd allele strain and human AHR. The N-terminal sequence of AHR in different strains and species are highly conserved while the C-terminal transactivation domains (TAD) share less similarity (C. A. Flaveny & Perdew, 2009). It is reported that the variable TAD can result in different co-activators recruited and thus may regulate different downstream gene expression (C. Flaveny, Reen, Kusnadi, & Perdew, 2008).

AHR stays in the cytoplasm in the absence of ligand treatment in a complex with two molecules of HSP90, one molecule of its co-chaperone p23 and one molecule of XAP2. HSP90 is required for the correct folding of AHR and stabilize AHR in an inactive form in the cytoplasm. Binding with HSP90 also maintains AHR in an accessible conformation for ligand binding (Antonsson, Whitelaw, McGuire, Gustafsson, & Poellinger, 1995; Coumailleau, Poellinger, Gustafsson, & Whitelaw, 1995; Pongratz, Mason, & Poellinger, 1992; Whitelaw, McGuire, Picard, Gustafsson, & Poellinger, 1995). The roles of p23 in AHR complex are not clearly defined. It is reported that p23 can stabilize the AHR complex and help XAP2 in AHR cytoplasmic retention (A Kazlauskas, Poellinger, & Pongratz, 1999; A Kazlauskas, Sundström, Poellinger, & Pongratz, 2001; Arunas Kazlauskas, Poellinger, & Pongratz, 2000). As
the co-chaperone protein of HSP90, p23 can interact with the ATP-bound HSP90 and stabilize the conformation of HSP90 in complex with its client proteins. Whether this is also true for AHR-HSP90 complex has not been confirmed. Studies in yeast using yeast p23 homolog Sba1 support this ATP-dependent HSP90 stabilization (Cox & Miller 3rd, 2004). Our lab previously showed that AHR protein level is decreased when p23 is knocked down in multiple cell lines and this protection role of p23 is HSP90-binding independent (P. M. Nguyen et al., 2012a; Pappas et al., 2018). p23 is also reported to help with nuclear import, HSP90 release from AHR complex and the formation of AHR/ARNT/DRE complex in ligand activation (A Kazlauskas et al., 1999; Shetty, Bhagwat, & Chan, 2003). However, the effects of p23 on AHR signaling were not observed in the p23-null mouse embryo (C. Flaveny, Perdew, & Miller 3rd, 2009). XAP2 can stabilize AHR complex and retain AHR in the cytoplasm (Arunas Kazlauskas et al., 2000; LaPres, Glover, Dunham, Bunger, & Bradfield, 2000; Meyer, Petrulis, & Perdew, 2000; Petrulis, Hord, & Perdew, 2000). It has been reported that XAP2 may protect AHR from ubiquitination and sequential proteasomal degradation by inhibition of E3 ligase C-terminal hsp70-interacting protein (CHIP) in constitutively nuclear localized AHR expressing stable 293T cell lines (Michael J Lees, Peet, & Whitelaw, 2003) and in vitro studies, even though AHR protein levels were not altered after CHIP knockdown in Hepa1c1c7 cells (Morales & Perdew, 2007). There are conflicting data on whether XAP2 can influence AHR signaling function.

Ligand binding triggers the conformational change and exposes the nuclear localization signal (NLS) in the N-terminus of AHR, leading the AHR complex to translocate into the nucleus. Once in the nucleus, the AHR complex dissociates and AHR heterodimerizes with AHR nuclear translocator (ARNT). Recently, Denison’s
group reported multi-step events and ligand-dependent transitional states that attach more mechanistic details between the ligand binding and transactivation (A. A. Soshilov, Motta, Bonati, & Denison, 2020). Since there is no structural information available for AHR complex, e.g. AHR: HSP90 binding structure, they proposed HSP90 “closed” and “open” conformation based on the reported Cryo-EM structure of HSP90: CDK4 (HSP90 client protein) (Verba et al., 2016). Binding of HSP90 in the PAS-B domain stabilizes AHR in an inactive state and the dimer of HSP90 is in a closed conformation clipping AHR. Upon ligand binding, the PAS-B domain conformation changes and the HSP90 dimer is in an open mode, in which NLS is exposed, leading to AHR translocation. In the meantime, PAS-A domain is more exposed (A. Soshilov & Denison, 2008), which may facilitate the binding of ARNT in the nucleus. ARNT binds to the AHR complex in the nucleus and forms a transitional state, which can only be captured in the presence of HSP90 binding stabilizer sodium molybdate. Then ARNT heterodimerization with AHR will quickly displaces HSP90 and dissociates AHR complex. The heterodimer then binds to the dioxin response element (DRE) in the enhancer region of the downstream target genes and promotes transcription. The minimal recognized sequence for DRE is 5’-NGCGTG-3’ (N stands for any nucleotide), where AHR binds to the 5’ part (GC) and ARNT recognizes the 3’-half GTG (Swanson, Chan, & Bradfield, 1995; Yao & Denison, 1992).

One of the classical gene products and also the prototype target of AHR transactivation is CYP1A1, which is widely used as a biomarker for AHR function. It is widely expressed in various tissues not at a constitutively high level but is highly inducible (up to ~100 fold) (Mescher & Haarmann-Stemmann, 2018). Other metabolism enzymes such as CYP1A2, CYP1B1, UGT and NQO1 are also reported
AHR repressor (AHRR) is a negative regulator of AHR function, and its expression is transactivated by AHR, which is a key mechanism for limiting AHR excessive activation. AHRR also belongs to the bHLH-PAS protein family. The sequence of bHLH and PAS-A domains in AHR shares about 55% similarity to that in AHRR (Sakurai, Shimizu, & Ohto, 2017; Schulte et al., 2017) while AHRR does not have PAS-B domain (Mimura, Ema, Sogawa, & Fujii-Kuriyama, 1999). The mechanism of AHRR repression is not clearly defined. There are several models proposed, of which the most widely accepted one is the competitive mechanism for ARNT and DRE binding (Mimura et al., 1999). The residues involved in the interaction to DRE region and ARNT are mostly conserved between AHR and AHRR. The crystal structure of human AHRR bHLH-PAS-A: bovine ARNT bHLH-PAS-A-PAS-B and human AHR bHLH-PAS-A: mouse ARNT bHLH-PAS-A showed that domain conformation and interaction interface were rather comparable between those two complexes (Sakurai et al., 2017; Schulte et al., 2017). These data support that AHRR can repress AHR function via competitive binding to ARNT and DRE DNA. Another competition mechanism presented by Evans et al. proposed that AHRR can also compete with AHR for binding some unknown transcription factors (Evans et al., 2008). Repression is also reported to be mediated through SUMOylation of the C-terminal AHRR, which recruits co-repressors to the promoter region of target genes (Oshima, Mimura, Sekine, Okawa, & Fujii-Kuriyama, 2009). This SUMOylation is not observed in AHR proteins. The crystal structure analysis revealed an interaction interface between AHRR PAS-A and ARNT PAS-B, unique in PAS protein heterodimer (others reported are PAS-B to PAS-B homotypic...
interaction) and the residues involved and the corresponding secondary structures in AHRR are not found in AHR (Sakurai et al., 2017), which might play roles for the unique SUMO modification.

Another mechanism to limit AHR transactivation is its quick degradation after ligand activation. Ligand binding shortens AHR half-life and promotes AHR ubiquitination, which tagged AHR for proteasomal degradation (Q. Ma & Baldwin, 2000; Roberts & Whitelaw, 1999b). It is reported that this degradation happens following nuclear export (Davarinos & Pollenz, 1999). Dissociation of HSP90 from AHR that mimics the transformation status of AHR, while having a different conformation as ligand bound-AHR complex, also promotes AHR for proteasomal degradation (M J Lees & Whitelaw, 1999). This degradation is reported to happen both in the nucleus and cytoplasm (Song & Pollenz, 2002, 2003).

Roles of AHR in Diseases

The development of AHR deficient mice models identify various physiological and pathological roles for AHR. Of all the important roles of AHR, its influence in autoimmune diseases, gut inflammation and carcinogenesis show promising druggability.

AHR and immune responses. Within all T cell subsets, T\textsubscript{H}17 cells and regulatory T (T\textsubscript{reg}) cells show the highest AHR expression levels when comparing to others (Rothhammer & Quintana, 2019). AHR expression levels increase during T\textsubscript{H}17 development (Quintana et al., 2008) and AHR activation influences the differentiation and conversion between pathogenic (pro-inflammatory) and non-pathogenic (anti-inflammatory) T\textsubscript{H}17 cells, depending on specific stimuli, such as IL-23. IL-23 exposure induces pathogenic T\textsubscript{H}17 differentiation, signatured by production of TGF-β3, while differentiation in response to IL-6 and TGFβ still
possess some IL-10 producing anti-inflammatory activities (Y. Lee et al., 2012; McGeachy et al., 2007). It has been reported that AHR endogenous ligand 6-formylindolo[3,2-b]carbazole (FICZ) can promote the pathogenic T\textsubscript{H}17 cells generation, which significantly worsens the mice model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) (Quintana et al., 2008). Another AHR endogenous ligand 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) is reported to inhibit the T\textsubscript{H}17 differentiation and favoring the IL-10 production by T\textsubscript{reg} cells and dendritic cells in EAE and allergic rhinitis (Quintana et al., 2008; Wei et al., 2014). Together with TGFβ signaling, AHR also mediates the transdifferentiation of T\textsubscript{H}17 cells into anti-inflammatory T\textsubscript{reg} cells (T\textsubscript{R}1 cells) (Gagliani et al., 2015). AHR activation by TCDD and ITE can affect the expression of cytokines such as IL-10 and promote the differentiation of immune suppressive T\textsubscript{R}1 cells and FOXP3\textsuperscript{+} T\textsubscript{reg} cells (Gandhi et al., 2010; Quintana et al., 2008). AHR activation can also affect dendritic cells (DC), mostly contributing to anti-inflammatory activities in differentiated DCs. AHR signaling can induce tolerogenic DCs, promoting the generation of T\textsubscript{reg} cells and inhibiting pathogenic T\textsubscript{H}17 differentiation (N. Baba et al., 2012; Benson & Shepherd, 2011; Hauben et al., 2008; N. T. Nguyen et al., 2010).

The effects of AHR signaling on immune cells explain the reported AHR roles in mucosal/surface sites (lung, skin, and gut) inflammation and autoimmune diseases, neurodegenerative disorders, transplantation tolerance and neoplastic diseases. The gut homeostasis involves a complex network of epithelial cells and stromal cells, establishing healthy structural barriers and local immune system. Other than AHR roles in intraepithelial lymphocytes, intestinal DCs and innate lymphoid cells, AHR also controls intestinal epithelial cell regeneration and helps
maintain the intestinal barrier integrity (Metidji et al., 2018). Together with AHR agonists from diet and host metabolism, microbiome metabolism also provides AHR ligands or AHR ligand precursors that can be further metabolized by the host, playing important roles in gut inflammation, as well as anatomically distant tissues such as the gut-brain axis. AHR activation by agonists derived from microbiome metabolites is reported to suppress the pro-inflammatory activities of microglia and astrocytes, improving central nervous system inflammation and neurodegeneration (Rothhammer et al., 2018, 2016). Moreover, impaired AHR agonists production by microbiome are reported in inflammatory bowel diseases (Lamas et al., 2016), metabolic syndrome (Natividad et al., 2018) and multiple sclerosis (Rothhammer et al., 2017).

**AHR in cancer.** Alteration of AHR expression levels and activities were observed in various cancer types (Kolluri, Jin, & Safe, 2017; Murray, Patterson, & Perdew, 2014). Both oncogenic and tumor suppressor roles of AHR were reported, largely dependent on cell context, ligands encountered and tumor stages. Parallel with the protective roles of AHR in immune response mentioned above, especially in the gut-brain axis, AHR was reported to show tumor suppressor effects in intestinal and colon cancer. AHR mice were reported to be more prone to cecal cancer due to abnormal accumulation of β-catenin (Ikuta et al., 2013; Kawajiri et al., 2009). Feeding the APCmin/+ AHR+/− mice with AHR ligands IAA, I3C of natural-AHR ligands containing diet significantly decreases the incidence of intestinal tumors as well as tumor numbers in the small intestine. Ahr null mice showed increased incidence of colitis-associated colorectal tumor compared to wild type mice. AHR ligand I3C diet can significantly decrease the tumor numbers in wild type but not ahr null mice
(Díaz-Díaz et al., 2016), confirming the protective role of AHR in intestinal and colon tumorigenesis.

A contrasting role of AHR in brain tumor was proposed. Gramatzki et al. reported that AHR is expressed in glioma in vivo. Antagonism of AHR by CH223191 reduced glioma tumor growth and invasiveness through TGFβ/Smad signaling (Gramatzki et al., 2009). AHR is activated by its endogenous ligand kynurenine, produced by highly expressed tryptophan-2,3-dioxygenase (TDO) in glioma cells and acts in an autocrine manner. The TDO-AHR signaling promotes glioma cell motility and clonogenic survival (Opitz et al., 2011). TDO-kynurenine-AHR axis can also promote stage 4 glioma (glioblastoma) progression through recruitment of macrophages. AHR signaling in tumor associated macrophages (TAM) promotes its polarization to immunosuppressive M2 macrophages, which can impair CD8+ tumor-infiltrating T cell function. The kynurenine expression level, AHR-related gene expression levels as well as TAM infiltration are higher in glioblastoma. Higher mortality is correlated with higher AHR expression level (Takenaka et al., 2019). However, Jin et al. reported that the inhibitory effect of CH223191 is AHR-independent. Based on their data in established and patient-derived glioma cells, kynurenine effect is cell context- and response-dependent and AHR plays a tumor-suppressive role in glioblastoma (Jin et al., 2019).

Most of the studies on AHR roles in breast cancer support that AHR is protective against tumor growth as well as invasion and metastasis (Kolluri et al., 2017). Higher AHR expression level is associated with improved survival rate and decreased distant metastasis (O’Donnell, Koch, Bisson, Jang, & Kolluri, 2014). AHR is reported to show high expression level in pancreatic cancer while activation of AHR by alternate alkyl substituted chlorinated dibenzofurans (alkyl-PCDFs) and
diindolylmethane (DIM) can inhibit pancreatic cancer cell growth (Koliopanos et al., 2002). In prostate cancer, the roles of AHR are dependent on androgen receptor (AR). Upregulation of AHR protein level upon E3 ligase subunit β-transducin repeat containing proteins (β-TrCP) inhibition reduced tumor growth in AR-positive prostate cancer cells (Gluschnaider et al., 2010) while it shows pro-oncogenic effects in AR-negative or AR signaling-independent prostate cancer cells (Haque, Francis, & Sehgal, 2005; Tran, Richmond, Aaron, & Powell, 2013). The effects of AHR expression and/or activity in lung and liver cancer cells have mixed results of pro- and anti-cancer roles (Kolluri et al., 2017). The underlying mechanisms of AHR in various hallmarks of tumorigenesis, including roles in tumor microenvironment, cancer stem cell development, cancer cell proliferation and cell cycle, metastasis and immunity, were well summarized in several review papers (Feng, Cao, & Wang, 2013; Murray et al., 2014). Given the complexed, but important roles of AHR in cancer, Stephen Safe lab first proposed the concept of non-toxic selective AHR modulators (SAhRM) to target AHR in a tissue-specific manner (Safe, Lee, & Jin, 2013), which needs more comprehensive and extensive understanding of regulation of AHR expression and its subsequent signaling.

**Autophagy**

Autophagy is a highly conserved pathway to maintain cellular homeostasis. By degrading cellular contents, including aggregated proteins, lipid droplets, glycogen, organelles, pathogens and etc., cells can recycle nutrients and respond to different stress situations. Therefore, autophagy plays important roles in human diseases, such as neurodegeneration (Nixon, 2013), multiple cancers (Levy, Towers, & Thorburn, 2017), inflammation and infections (Levine, Mizushima, & Virgin, 2011). There are three major types of autophagy: macroautophagy,
microautophagy and chaperone-mediated autophagy. Chaperone-mediated autophagy (CMA) is a selective degradation pathway for proteins with a KFERQ-like motif, which can be recognized by Hsc70 and delivered to lysosomes via LAMP-2A. Microautophagy is less studied than the other two types of autophagy. Substrates are directly sequestered in manners of lysosomal protrusion/invagination or endosomal invagination for degradation in the lysosome (Oku & Sakai, 2018). Macroautophagy involves the formation of a double-membrane structure, autophagosome, and its fusion with the lysosome. My projects mostly studied the involvement of macroautophagy in the regulation of AHR.

**Macroautophagy**

Since Christian de Duve discovered lysosomes in rat liver and defined autophagy in 1960s (DE DUVE, PRESSMAN, GIANETTO, WATTIAUX, & APPELMANS, 1955; Klionsky, 2008), there have been many important findings of autophagy reported in many different species, especially the research performed in yeast. Macroautophagy was initially viewed as a non-selective, self-eating process triggered by stresses such as starvation. In the recent two decades, there have been more and more types of selective autophagy identified, as means of selective degradation for protein aggregates (aggrephagy), fragments of ER (ER-phagy), mitochondria (mitophagy), ribosome (ribophagy), glycogen (glycophagy), lipid droplets (lipophagy), and other organelles or cell components (Kirkin, 2020). The most critical and typical event in macroautophagy (referred to as autophagy thereafter) is the formation of autophagosome for the sequestration of cellular contents. It is initiated from the nucleation of autophagy-related proteins and cargo proteins. The cup-shaped structure then elongates and seals to form the double-membrane autophagosome. Upon fusion with lysosomes, the inner membrane and
the engulfed contents of the autophagosome are degraded by lysosomal hydrolases. Autophagy is a well-organized hierarchical process. Depending on how autophagy happens, as a basal event or induced by different stimuli, the autophagy-related gene (Atg) proteins employed, as well as the sequence of events, can be slightly different. But they share the similar machinery.

**Key modules of macroautophagy machinery.** There are five key modules for the macroautophagy machinery (table x): (1) Atg1 or ULK1 complex; (2) PI3K complex; (3) Atg2-Atg18 or PI3P-binding complex; (4) the Atg9 cycling system; (5) Atg12-Atg5-Atg16(L) and Atg8 (or LC3B) conjugation system. The diagram of the involvement of those modules in selective autophagy is shown under the selective autophagy section (Figure 1.2).

**Atg1/ULK1 kinase complex.** The Atg1/ULK1 (the homolog of Atg1 in mammals) complex is the farthest upstream module of core autophagy machinery. The Atg1 complex in yeast is mainly comprised of Atg1, Atg11, Atg13, Atg17, Atg29 and Atg31; ULK1 (or ULK2), ATG13, FIP200 and ATG101 form the ULK1 complex in mammals (Mizushima, 2010). Initiation of autophagy starts from the activation of Atg1 complex. Atg1 (or ULK1/2 in mammalian cells) is a Ser/Thr kinase, which can be triggered to be activated through autophosphorylation. Starvation-induced autophagy activates Atg1 by target of rapamycin complex 1 (TORC1), which is responsible for upstream signal sensing, including amino acid, glucose, growth factors (e.g. insulin) as well as oxygen (Sengupta, Peterson, & Sabatini, 2010). Energy-sensing AMPK pathway is also reported to regulate autophagy independent of mTORC1 (Shang & Wang, 2011). When cells are under stress conditions, such as starvation, TORC1 is inhibited, leading to the hypophosphorylation of its downstream substrate Atg13. Dephosphorylation of Atg13 promotes its interaction
with Atg1 and Atg17-Atg29-Atg31 complex. In mammalian cells, ULK1/2, ATG13, FIP200 and Atg101 form stable complexes independent of nutrient status and this complex interacts directly with mTORC1. Additional proteins are then recruited and trigger downstream steps. Cargo-induced selective autophagy, basal events that happen in nutrient-rich condition, bypass mTORC1 regulation. Cargo proteins are required for activation of the Atg1 complex. Cells lacking either cargo protein, autophagy receptor, or Atg11, reduced the level of autophosphorylated Atg1 (Kamber, Shoemaker, & Denic, 2015). Atg11 is a key scaffold protein in cargo-induced selective autophagy by bridging the cargo-autophagy receptor and Atg1 complex. FIP200 is proposed to be the mammalian counterpart for Atg17 and Atg11 (Hara et al., 2008). The ULK1 kinase complex triggers downstream autophagic events at least in part by phosphorylation. Numerous autophagy-related proteins are reported to be ULK1 substrates, including ULK1 itself, class III PI3K complex subunits (PI3KC3-C1), and Atg9 (Papinski et al., 2014). It is reported that following activation, ULK1 autophosphorylation promotes its ubiquitination and proteasomal degradation via Cul3-KLHL20 ubiquitin ligase, helping in autophagy termination to maintain homeostasis (Liu et al., 2016).

**The class III Phosphatidylinositol 3-kinase (PI3KC3) complex.** The PI3KC3 is downstream of ULK1 complex activation. The net result of PI3KC3 activity is the production of phosphatidylinositol 3-phosphate (PtdIns3P), a signaling lipid on phagophore membranes. There are three classes of PI3K complexes (I, II and III). The class I PI3K complex (PI3KC1) is reported to inhibit autophagy while PI3KC3 activates autophagy (Petiot, Ogier-Denis, Blommaart, Meijer, & Codogno, 2000). VPS34-VPS15 is the core of the complex, of which VPS34 being the catalytic subunit of the kinase. Together with Beclin1 (or Atg6/Vps30), ATG14 and NRBF2
(or Atg38), they form complex I (PI3KC3-C1), which is more studied and reported to be involved in autophagosome initiation, while VPS34-VPS15 and Beclin1 can also assembly with UVRAG (or Vps38) instead of ATG14, forming complex II (PI3KC3-C2). The conformation of the PI3KC3 complex is highly dynamic, of which the “dislodged” conformation of VPS34 being the catalytically active form. This is reported to be negatively regulated by VPS15 via binding to the lipid kinase domain of VPS34 (Stjepanovic, Baskaran, Lin, & Hurley, 2017). Vps15 also serves as a “bridge”, connecting the regulatory subcomplexes, such as Beclin1-ATG14, to the catalytic subunit, communicating the regulatory signals to control PI3KC3 activity. The VPS34 catalytic domain is not directly contacted to the Beclin1-ATG14 regulatory sequences. Thus, the assembly of the complex is dependent on VPS15. Beclin1 is an important regulatory subunit of PI3KC3 complex. It can be phosphorylated and dephosphorylated by multiple kinases, including ULK1 (activated phosphorylation), and phosphatases (Hurley & Young, 2017). Another important regulatory sequence in Beclin1 is the binding sequence for Bcl-2, which inhibits the activity of the PI3KC3 complex. ATG14 is exclusively contained in PI3KC3-C1 and is important for its localization to the phagophore membrane via the amphipathic lipid packing sensor (ALPS) motif in the Barkor autophagosome targeting sequence (BATS) domain (Brier et al., 2019). The ALPS motif helps PI3KC3-C1 sensing the highly curved lipid structure on the phagophore membrane (Antonny, 2011; Brier et al., 2019). The PI3KC3-C2 is reported to be less active in targeting the highly curved structure, which might be the reason that PI3KC3-C2 is not responsible for production of PtdIns3P and the stimulation of LC3 lipidation (Brier et al., 2019). UVRAG acts as a metabolic switch for PI3KC3-C1. Its phosphorylation suppresses the complex activity while dephosphorylation promotes the complex
assembly (X. Ma et al., 2017). The PI3KC3-C2 is defined by the replacement of UVRAG instead of ATG14 (no NRBF2 either). The exact roles of PI3KC3-C2 are not clear, but it is reported to be involved in the later stages of autophagosome maturation and endocytic trafficking (Liang et al., 2008).

**The PI3P effectors and ATG9 trafficking system.** Double FYVE-containing protein 1 (DFCP1) and WIPI proteins (WIPI 1/2/4) are PI3P effectors, helping promote the elongation of isolation membrane. WIPIs (WIPI2 particularly) localize to the highly curved opening edge of the isolation membrane (Graef, Friedman, Graham, Babu, & Nunnari, 2013; Suzuki, Akioka, Kondo-Kakuta, Yamamoto, & Ohsumi, 2013), adjacent to DFCP1, through binding to PI3P. WIPIs proper recruitment and functions are dependent on ATG9. ATG9 is the only transmembrane protein recruited to the phagophore, together with WIPIs (Atg2-Atg18 complex as yeast homolog) the ATG9 vesicles provide membrane sources for autophagosome formation (Carlsson & Simonsen, 2015). This elongation of phagophore membrane provides support for LC3 lipid conjugation (Brier et al., 2019). WIPI2 also interacts with ATG16L, recruiting the ATG12-ATG5-ATG16L complex, thus acting as a scaffold protein for LC3 lipidation (Proikas-Cezanne, Takacs, Dönnes, & Kohlbacher, 2015).

**ATG12-ATG5-ATG16L and LC3-PE conjugation systems.** These two conjugation systems are important for the expansion and closure of the isolation membrane, functioning at the late steps of autophagosome formation. Microtubule associated protein 1 light chain 3 (MAP1LC3 or LC3) is synthesized as a precursor protein. It is then processed by a cysteine protease ATG4 and the resulting C-terminal glycine-exposed form is termed as LC3-I. LC3-I is first activated by Atg7 (E1-like) and then transferred to Atg3 (E2-like). The ATG12-ATG5-ATG16L complex
acts as the E3 ligase for LC3, linking it to an amino group of phosphatidylethanolamine (PE), a major phospholipid on the (isolation) membrane (Ichimura et al., 2000), referred as LC3-II. There are three members of LC3 family: LC3A, LC3B and LC3C. Among them, LC3B is the most studied and is a well-accepted marker for autophagy activity. LC3A and LC3C are reported to share high sequence similarity with LC3B, as well as some location and functional similarities and differences (Koukourakis et al., 2015; Schaaf, Keulers, Vooijs, & Rouschop, 2016). ATG4 deconjugates LC3-PE on the outer membrane, while the conjugated LC3 on the inner membrane, are degraded together with cargos in the lysosome. Deficiency in Atg4 deconjugation activity will lead to incomplete, elongated autophagosomes in yeast (U. Nair et al., 2012). ATG12 is synthesized as a C-terminal glycine-exposed form. It is activated by ATG7 (E1-like), then transferred to ATG10 (E2-like) and finally conjugated with ATG5. This ATG12-ATG5 conjugate then form a complex with ATG16L in a 2:2:2 stoichiometric manner (Fujioka, Noda, Nakatogawa, Ohsumi, & Inagaki, 2010). This complex helps LC3 lipidation as an E3 ligase. ATG16L in this complex promotes its localization to the isolation membrane, via WIPI2 binding, and determines the site of LC3 lipidation. Once being conjugated to the isolation membrane, LC3-II maintains involvement in the whole autophagy process, making it a good indicator of autophagosome formation and autophagic flux (Mizushima, Yoshimori, & Levine, 2010).

Selective autophagy and aggrephagy. In the early 2000s, the concept that cargos can be degraded in a highly selective way started to gain more attention. Autophagic degradation is not triggered by stimuli, such as starvation, but induced by the cargo itself. For example, deletion of the cargo protein prApe1 in S. cerevisiae impaired the organization of phagophore under growing conditions, but it did not
affect the formation of autophagosome during starvation (Shintani & Klionsky, 2004). The machinery involved is similar to the stimuli-induced autophagy, as described above, but the sequential processes are not the same. It is reported that LC3 recruitment requires the intact lipid conjugation system but is independent of other core ATG proteins in *Salmonella* xenophagy (Kageyama et al., 2011). The LC3-interacting region (LIR) motif was found in ULK1 (Alemu et al., 2012) and all key components of PI3KC3 (Birgisdottir et al., 2019). Their anchoring to the phagophore require the recruitment of LC3. It is reported recently by Marten’s group that the cargo receptor p62 can recruit both LC3 and ULK1 complexes, via direct binding with FIP200 in a mutually exclusive way (Turco et al., 2019), promoting the formation of autophagosome. Therefore, the proposed cargo-induced model for selective autophagy is as follows (Figure 1.2), though more information is needed to clarify the details of hierarchy and ATG proteins involved: (1) The aggregation of cargo proteins with or without modification (e.g. ubiquitination); (2) The binding of cargo receptor to cargos; (3) Recruitment of LC3-PE and other ATG proteins; (4) Formation and expansion of local isolation membrane; (5) Autophagosome formation and fusion with lysosome. The size of the autophagosome is in the range of 0.1-1.5 μm, depending on the cell type and inducing signal (Rogov, Dötsch, Johansen, & Kirkin, 2014). The size of autophagosomes in selective autophagy is reported to be smaller than that in starvation-induced autophagy. The close apposition between cargos and autophagosome membrane helps to exclude the non-specific materials during sequestration whereas in starvation-induced autophagy, random materials could be introduced into the autophagosome (M. Baba, Takeshige, Baba, & Ohsumi, 1994; Sawa-Makarska et al., 2014). The degradation of cargo proteins by selective macroautophagy is termed as aggrephagy. The cargo proteins and/or the cargo
receptors need to be aggregated/oligomeric to be delivered into the autolysosome. The aggregated or oligomerized proteins can provide larger surface for multiple binding of cargo receptors and/or ATG proteins, which offer higher avidity and may concentrate ubiquitin like proteins (UBL) for further autophagic events amplification.

Selectivity is mainly conveyed by autophagy cargo receptors or selective autophagy receptors (SAR). They specifically bind to autophagy cargos and tether autophagy machinery to the cargos via the simultaneous binding to LC3B. The binding of cargo receptor to LC3B is dependent on the LC3-interacting region (LIR) of the receptor. The canonical LIR motif shares the common sequence W/F/Y-X-X-L/I/V, binding to the hydrophobic pockets of LC3. There are usually acidic residues or serine/threonine phosphorylation upstream of the LIR in near proximity, contributing to strengthen the interaction. The interaction between cargo and cargo

*Figure 1.2.* Phagophore assembly and isolation membrane expansion in selective autophagy.
receptor, in most cases reported, is ubiquitin dependent. Ubiquitin-independent cargo-receptor direct bindings are also reported (see details in review (Kirkin, 2020)). Those reported proteins are either not mammalian proteins or not involved in aggrephagy, but other subtypes, such as mitophagy or ER-phagy. Some proteins containing LIR motifs can be degraded in a SAR-independent way via direct binding to LC3.

Protein ubiquitination codes for degradation or downstream signaling. Different linkages of ubiquitin direct the ubiquitinated proteins to different degradation pathways: K63 ubiquitination is usually recognized by cargo receptors to undergo lysosomal degradation whereas proteins with K48 ubiquitination are specifically delivered to 26S proteasome for degradation. p62 is the first reported, but also common mammalian ubiquitin-dependent cargo receptor. Hereafter, I use p62 as a representative receptor to illustrate the selective nature of cargo receptors. The ubiquitin-associated (UBA) domain, which mediates the interaction with the ubiquitin chain, is located at the C-terminal of p62. The interaction between ubiquitin and UBA domain of cargo receptors are relatively weak, with a high micromolar range affinity (Long et al., 2010). Oligomerization of p62 increases the avidity for cargo binding and is required for effective cargo degradation (Bjørkøy et al., 2005). The dimerization and oligomerization of p62, mediated via UBA and its N-terminal Phox and Bem1 (PB1) domain, controls the activity of p62. The UBA domain can form a stable dimer, which is a biologically inactive form (Long et al., 2010; Sims, Haririnia, Dickinson, Fushman, & Cohen, 2009). This dimerization partially accounts for the low affinity of mono-ubiquitin binding. The oligomerization driven by the PB-1 domain aligns p62 proteins into a long helical form with the appearance of filaments. In the oligomerized forms, UBA domains will be in close proximity without forming
dimers and this array of UBA domains provides the selectivity for K63-linkage ubiquitin chains (Sims et al., 2009; Wurzer et al., 2015). The binding affinity of p62 and other SARs to LC3 is also low in micromolar range (Wirth et al., 2019; Zaffagnini & Martens, 2016). Oligomerization of p62 renders high avidity for the LC3 cluster while the affinity of LIR to free LC3 is not changed. Therefore, high avidity-driven interaction promotes the binding of the cargo-p62 complex to LC3-concentrated membrane (Wurzer et al., 2015).

Various post-translational modifications are found to regulate the activities of ATG proteins. As described above, the binding affinity of LIR:LC3 and UBA:ubiquitin is relatively low. Phosphorylation of serine residues adjacent to the LIR motif provides a negatively charged environment, thus increasing the binding affinity to LC3 (see details in review (Zaffagnini & Martens, 2016)). The binding affinity of UBA:ubiquitin can also be increased by phosphorylation of Ser403 within the UBA domain of p62 (Matsumoto, Wada, Okuno, Kurosawa, & Nukina, 2011). This interaction is also reported to be enhanced by mono-ubiquitination of p62 (Peng et al., 2017). Phosphorylation on LC3 is also observed and is reported to be essential for the fusion of autophagosome to the lysosome (Wilkinson et al., 2015). Acetylation of LC3 is reported to control the nuclear-cytoplasm shuttling (Huang et al., 2015).

**GSK3β Phosphorylation**

**GSK3β Biology**

Glycogen synthase kinase-3 (GSK3) was first identified as one of the key enzymes that controls glycogen synthesis by phosphorylation and inhibition of its first reported substrate glycogen synthase (Embi, Rylatt, & Cohen, 1980). There are more than a hundred proteins proposed to be GSK3 substrates, which define the
wide range of cellular processes that GSK3 is involved, including glucose metabolism and nutrient sensing, cell growth and development, and pathological situations, such as diabetes, cancers, neurodegenerative diseases, and psychiatric diseases. GSK3 is ubiquitously expressed with two isoforms or paralogs of GSK3: GSK3α and GSK3β, encoded by two different genes. They share a largely identical catalytic domain sequence while GSK3α has an additional glycine-rich region at the N-terminus as well as some C-terminal differences compared to GSK3β (Kaidanovich-Beilin & Woodgett, 2011). Despite the similarity in the catalytic region of the two isoforms, they exhibit differences in terms of tissue-specific expression and some isoform-specific effects. Deletion of GSK3β lead to embryonic lethality (Hoeflich et al., 2000), while GSK3α knockout mice are viable, but show increased sensitivity to glucose and insulin (MacAulay et al., 2007), indicating that these two isoforms are not interchangeable.

Compared to most conventional kinases, GSK3 shows some special characteristics: (a) the phosphorylation of GSK3 requires pre-phosphorylation at 4 amino acids C-terminal to GSK3 phosphorylation sites, termed as priming, by other kinases. This pre-phosphorylation creates a binding site for GSK3, thus significantly enhancing GSK3 phosphorylation (Robertson, Hayes, & Sutherland, 2018). Hence, the consensus GSK3 recognition sequence is Ser/Thr-X₃ or 4-(P) Ser/Thr (X is any amino acid) and usually there are 1-3 Ser/Thr in a row with 3 or 4 amino acids in between. GSK3 recognizes the C-terminal phosphorylated Ser/Thr and sequentially phosphorylates the substrate in the C- to N-terminus direction. However, there are also reports for priming sites that are more than 4 amino acids adjacent to the GSK3 target sites, at much further sites. There are also examples that do not require priming (Robertson et al., 2018; Calum Sutherland, 2011). (b) GSK3 is activated by
phosphorylation at Tyr 279 (GSK3α) or Tyr 216 (GSK3β) and it is phosphorylated during synthesis of GSK3 and constitutively remains phosphorylated (Cole, Frame, & Cohen, 2004). (c) GSK3 is inhibited, instead of activated, upon stimulation such as insulin, growth factors and Wnt signaling, by phosphorylation at Ser 21 (GSK3α) or Ser 9 (GSK3β) (C. Sutherland, Leighton, & Cohen, 1993). This N-terminal Ser phosphorylation acts as a pseudo-primed site for GSK3 recognition and this inhibition can be overcome by competition of higher concentration of substrates (Frame, Cohen, & Biondi, 2001). Since this phosphorylation is mimicking the primed substrates, this inhibition may not affect the phosphorylation of substrates that do not need priming.

**The GSK3β-Regulated Protein Degradation and β-catenin Destruction Complex**

GSK3 phosphorylation can alter the protein activity, protein-protein interaction, protein localization as well as protein half-life of its substrates. There are different pools of GSK3β, incorporated into various protein complexes, responding to different stimuli and primed by specific kinases. Phosphorylation by GSK3 can promote substrates for ubiquitination and degradation, but not all the proposed GSK3β substrates go through this process (Calum Sutherland, 2011). In addition to the GSK3 phosphorylation, which generates a “phosphodegron” for their specific E3 ligases, there are other components that help with E3 recognition and ubiquitination. Here, I use the well-studied GSK3β substrate, β-catenin, as an example to illustrate the classical degradation process after GSK3β phosphorylation.

**Wnt/β-catenin signaling off mode.** As a transactivation coactivator, β-catenin signaling controls Wnt responsive gene expression, whose abnormal regulation are indicated in diseases, such as cancers and metabolic diseases (MacDonald, Tamai, & He, 2009). In the absence of Wnt signal, β-catenin is
continuously degraded in the cytoplasm with the help of its destruction complex.

This complex is composed of two scaffold protein Axin and the adenomatous polyposis coli (APC), two kinases casein kinase 1 (CK1) and GSK3β. The roles of these components are not fully clear. In general, Axin interacts with β-catenin and two kinases and is the limiting step for the complex assembly. It places β-catenin to close proximity of CK1 for priming, after which β-catenin can be recognized by GSK3β and thus phosphorylated. Axin and APC can also be phosphorylated by CK1 and GSK3β, which enhance their interaction with β-catenin. There are two ser/thr phosphatase involved in this destruction complex: PP1 and PP2A. PP1 can dephosphorylate Axin and cause disassembly of this complex (Luo et al., 2007). PP2A dephosphorylates β-catenin, which can be protected by APC (Su et al., 2008).

It should be noted that APC and PP2A are multifunction proteins whose roles are not fully understood. The phosphorylated β-catenin can then be recognized by β-transducin repeat containing proteins (β-TrCP), a subunit of β-catenin E3 ligase. It is the SCF containing complex of RING E3 ligase family that helps with the β-catenin ubiquitination. The SCF containing complex is composed of four proteins in the binding order of: RBX1, cullin-1, S-phase-kinase-associated-protein 1 (SKP1) and F-box protein (Robertson et al., 2018). β-TrCP is a phosphorylation-dependent F-box protein, which recognizes and binds to phosphorylated substrates. Cullin-1 and SKP1 are two scaffold proteins which connect β-TrCP and RBX1, which transfers ubiquitin from E2 conjugating enzyme to substrate proteins. β-TrCP usually targets proteins that contain a destruction motif or phosphodegron: D-S-G-X-X-S, of which the two serine are phosphorylated by GSK3β. There are also examples of GSK3β/β-TrCP substrates that only contain a minimal S/T-X-X-X- (P) S/T stretch. The ubiquitinated β-catenin is then degraded by the proteasome.
**Wnt/ β-catenin signaling on mode.** When Wnt proteins bind to transmembrane receptor Frizzled (Fz) and low-density lipoprotein receptor-related protein 6 (LRP6), with help of scaffold protein Dishevelled (Dvl), the Wnt-Fz-LRP5/6 complex recruits Axin, which leads to LRP6 protein phosphorylation by GSK3β, resulting in the inhibition of β-catenin phosphorylation and dissociation of the destruction complex. β-catenin is hence free and translocates into the nucleus, where it replaces the transcriptional repressor Groucho (Roose et al., 1998) from the Wnt responsive gene DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) (Behrens et al., 1996; Molenaar et al., 1996). This association of β-catenin with TCF converts TCF from repressor to activator, turning on the Wnt downstream gene transcription.
CHAPTER 2: SELECTIVE AUTOPHAGY MAINTAINS THE ARYL HYDROCARBON RECEPTOR LEVELS IN HELA CELLS: A MECHANISM THAT IS DEPENDENT ON THE P23 CO-CHAPERONE

Abstract

The aryl hydrocarbon receptor (AHR) is an environmental sensing molecule which impacts diverse cellular functions such as immune responses, cell growth, respiratory function, and hematopoietic stem cell differentiation. It is widely accepted that the degradation of AHR by 26S proteasome occurs after ligand activation. Recently, we discovered that HeLa cells can modulate the AHR levels via protein degradation without exogenous treatment with a ligand, and this degradation is particularly apparent when the p23 content is down-regulated. Inhibition of autophagy by a chemical agent (such as chloroquine, bafilomycin A1, or 3-methyladenine) increases the AHR protein levels in HeLa cells whereas activation of autophagy by short-term nutrition deprivation reduces its levels. Treatment with chloroquine retards the degradation of AHR and triggers physical interaction between AHR and LC3B. Knockdown of LC3B suppresses the chloroquine-mediated increase of AHR. Down-regulation of p23 promotes AHR degradation via autophagy with no change of the autophagy-related gene expression. Although most data in this study were derived from HeLa cells, human lung (A549), liver (Hep3B), and breast (T-47D and MDA-MB-468) cancer cells also exhibit AHR levels sensitive to chloroquine treatment and AHR–p62/LC3 interactions. Here we provide evidence supporting that AHR undergoes the p62/LC3-mediated selective autophagy in HeLa cells.
The aryl hydrocarbon receptor (AHR) is originally recognized as a transcription factor in response to environmental toxicants such as dioxins and polycyclic aromatic hydrocarbons (Poland et al., 1976). Since then, more roles of AHR such as its involvement in organ development (Schmidt, Su, Reddy, Simon, & Bradfield, 1996), immune responses (Quintana et al., 2008; Ye et al., 2017), nervous system homeostasis (Juricek & Coumoul, 2018), and carcinogenesis (Kolluri et al., 2017; Opitz et al., 2011) have been reported. Not surprisingly, AHR has become a promising target for the treatment of diseases such as autoimmune diseases, inflammation, and cancers. However, the AHR function is often ligand-, cell context-, and tissue-specific, confounding the feasibility of targeting AHR for rational drug design. Understanding the basic biology of AHR will provide much-needed information for effective modulation of the AHR function.

AHR resides in the cytoplasm as a complex with HSP90, XAP2, and p23. The p23 co-chaperone is in a variety of HSP90 client protein complexes (Holt et al., 1999; Johnson & Toft, 1995; S. C. Nair et al., 1996; Roland & J., 1999). It has been reported that p23 stabilizes the ATP-induced conformation of the HSP90-AHR complex (Cox & Miller 3rd, 2004; Young, Moarefi, & Ulrich Hartl, 2001). AHR function can also be enhanced by p23 in vitro (Jinyun Chen, Yakkundi, & Chan, 2019; Cox & Miller 3rd, 2004; P. M. Nguyen et al., 2012a; Shetty et al., 2003), but surprisingly this enhancement is not observed in the p23-null mouse embryo (C. Flaveny et al., 2009), possibly due to yet unidentified compensatory mechanisms. The effect of p23 on AHR is largely uncharacterized at present.

Autophagy is essential in maintaining cell homeostasis by delivering cytosolic cargos to lysosomes for degradation. There are three types of autophagy:
macroautophagy which involves the formation of autophagosome, followed by its fusion with lysosome. Microautophagy, which involves substrates that are directly sequestered via lysosomal invagination; and lastly, chaperone-mediated autophagy, a process that involves the HSC70-mediated delivery of client proteins into lysosome via interaction with LAMP-2A (for general review, see (Mizushima, Levine, Cuervo, & Klionsky, 2008)). Although macroautophagy is generally known to deliver bulk cargos to lysosomes in a non-selective manner, it can also be selective in delivering cargo proteins for lysosomal degradation. Recognition of client proteins by cargo receptors (such as p62) provides the selectivity of macroautophagy. This recognition, in most cases, is mediated through the ubiquitination of client proteins. Different linkages of ubiquitin destine the ubiquitinated proteins to different degradation pathways; for example, K63 ubiquitination is usually recognized by cargo receptors to undergo lysosomal degradation whereas proteins with K48 ubiquitination are specifically delivered to 26S proteasome for degradation (Korolchuk, Menzies, & Rubinsztein, 2010). After K63 ubiquitination of a client protein, LC3B-II, a phosphatidylethanolamine-conjugated, membrane-bound form of cleaved LC3B-I (Y. K. Lee & Lee, 2016), forms the autophagosome with the client protein–cargo receptor complex, leading to lysosomal degradation of the client protein (Y. K. Lee & Lee, 2016; Zaffagnini & Martens, 2016).

Upon binding of a ligand, AHR translocates into the nucleus and activates gene transcription. The liganded AHR is subsequently degraded by the ubiquitin–proteasome system to limit its transactivation function (Q. Ma & Baldwin, 2000). However, how AHR is regulated in the absence of exogenous ligand treatment remained unclear. We previously discovered that down-regulation of just half of the p23 cellular content is sufficient to cause a lower cellular content of AHR in various
cancer and untransformed cell types in vitro (Jinyun Chen et al., 2019; P. M. Nguyen et al., 2012a; Pappas et al., 2018). AHR is degraded significantly faster in the p23 knockdown Hepa1c1c7 cells; however, inhibition of proteasome activity by MG132 cannot restore the AHR protein levels, suggesting that AHR is not degraded by the 26S proteasome when p23 is down-regulated (P. M. Nguyen et al., 2012a).

Treatment with MG132, surprisingly, decreases the AHR protein levels in both wild type and p23 knockdown Hepa1c1c7 cells, suggesting that mechanisms in degrading the unliganded AHR protein could be enhanced by proteasomal inhibition. It has been reported that crosstalk occurs between the ubiquitin–proteasome system (UPS) and autophagy (Korolchuk, Mansilla, Menzies, & Rubinsztein, 2009a; Nam, Han, Devkota, & Lee, 2017). Blockade of UPS leads to protein aggregation, which in turn triggers the autophagy-mediated protein degradation (Zhu, Dunner, & McConkey, 2010). In addition, reduced amino acids in cells as a consequence of MG132 treatment can activate autophagy (Harhouri et al., 2017). Thus, we explored whether the autophagy-lysosomal system is responsible for the degradation of AHR without exogenous ligand treatment. Here we provide evidence to support that the basal AHR protein levels in HeLa cells are controlled by selective autophagy. Knockdown of p23 reduces AHR protein levels by promoting the K63-linked ubiquitination of AHR, followed by the p62/LC3-mediated AHR degradation.

**Results**

**Autophagy Activity in Wild Type and p23 Stable Knockdown HeLa Cells Governs the Levels of AHR**

We previously observed that suppression of the AHR levels after down-regulation of p23 in Hepa1c1c7 mouse hepatoma cells was caused by increased AHR degradation, which could not be reversed by the treatment of a proteasome
inhibitor MG132 (P. M. Nguyen et al., 2012b). In this study, we observed that MG132 similarly reduced the AHR content in wild type and p23 knockdown (human cervical) HeLa cells in a statistically significant manner (Figure 2.1A). We then explored whether this AHR degradation could be mediated through autophagy-mediated lysosomal degradation. We treated the HeLa cells with an autophagy inhibitor CQ at 20 or 40 μM for six hours. We observed that the AHR protein levels in both wild type and p23 stable knockdown HeLa cells were increased in the presence of 40 μM CQ (Figure 2.1B, C). The cellular AHR protein level in p23 stable knockdown HeLa cells was about 54% when compared to that of wild type HeLa cells (Figure 2.1C, “WT” versus “NT”). However, the AHR levels in p23 stable knockdown cells became even higher than the levels in wild type cells after treatment with 40 μM CQ for six hours, suggesting that mechanism of the p23-mediated AHR degradation can be reversed by CQ. Next, we performed the cycloheximide experiment to examine whether degradation of AHR is mediated through autophagy. Basically, when protein synthesis in HeLa cells is blocked by cycloheximide, any reduction of the AHR content is the consequence of protein degradation. If this degradation is mediated through autophagy, CQ should, in principle, reverse the AHR reduction in the presence of cycloheximide. Indeed, we observed that degradation of the AHR protein in p23 stable knockdown and wild type HeLa cells was inhibited in the presence of CQ (Figure 2.1D, E, “CHX” versus “CHX+CQ”). Two other autophagy inhibitors, namely Baf A1 and 3MA, also increased the AHR protein levels in p23 stable knockdown HeLa cells (Figure 2.1F, G). However, only 5 mM 3MA, but not 6 nM Baf A1, increased the AHR levels in wild type HeLa cells. An autophagy inducer metformin decreased the AHR protein levels in p23 knockdown but not in wild type HeLa cells at 1 mM concentration
(Figure 2.1H). We cannot rule out the possibility that a higher concentration of Baf A1 and metformin is necessary for eliciting changes of the AHR levels in wild type HeLa cells. Nonetheless, these data supported that the basal AHR undergoes the autophagy-mediated degradation in HeLa cells and this degradation is more apparent when p23 is down-regulated.
Using an autophagy inhibitor or activator to reveal that the aryl hydrocarbon receptor (AHR) undergoes autophagy in wild type (WT) and p23 stable knockdown (p23KD) HeLa cells. (A) MG132 (10 μM for 6 h) decreased AHR protein levels more in HeLa cells transiently transfected with p23 shRNA (2.5 μg for 48 h) than in WT HeLa cells. EndoFectin transfection reagent (2:1) was used. The below images are representative of the replicate data (means ± S.D, n = 4). Conditions with no MG132 treatment were arbitrarily set as one (with no error bar) for data normalization. Multiple t-tests corrected with the Hold-Sidak method for multiple comparisons were performed to determine statistical significance. (B, C) WT and (C) p23KD HeLa cells were treated with 20 μM or 40 μM chloroquine (CQ) dissolved in water for 6 h. Western analysis results showed a higher increase of AHR in p23KD than in WT HeLa cells after 40 µM CQ treatment. For (B, C), the below images are representative of the replicate data of one experiment (means ± SD, n = 3). Conditions with no addition as no treatment (NT) were arbitrarily set as one for comparison. This experiment was repeated once with similar results. One-way ANOVA with Sidak’s multiple comparisons test was performed to determine statistical significance. (D) WT and (E) p23KD HeLa cells were treated with 40 μg/mL of cycloheximide (CHX) for 6 h in the presence or absence of 40 μM CQ for
(Figure 2.1 Continued)

12 h (6 h pre-treatment and then co-treated with CHX for another 6 h). The degradation of AHR in both cell lines was inhibited by CQ. For (D, E), the below images are representative of the replicate data (means ± SD, n = 3). Conditions with no addition as no treatment (NT) were arbitrarily set as one (with no error bar) for data normalization. Multiple t-tests corrected with the Hold-Sidak method for multiple comparisons were performed to determine statistical significance. (F) Treatment with 6 nM Bafilomycin A1 (Baf A1) dissolved in DMSO for 3 h increased AHR protein levels only in p23KD cells but not WT HeLa cells. (G) Treatment with 5 mM 3-methyladenine (3MA) dissolved in DMEM for 24 h increased AHR protein levels in WT and p23KD HeLa cells. For (F, G), the below images are representative of the replicate data (means ± SD, n = 3 for (F), n = 4 for (G)). Conditions with DMSO treatment and no addition as no treatment (NT) were arbitrarily set as one (with no error bar) for data normalization. Multiple t-tests corrected with the Hold-Sidak method for multiple comparisons were performed to determine statistical significance. (H) Treatment with 1 mM metformin (Met) for 4 h decreased AHR protein levels in p23KD HeLa cells but not in WT HeLa cells. The above images represent the replicate data of one experiment (means ± SD, n = 3). Conditions with no treatment (NT) were arbitrarily set as one for comparison. This experiment was repeated once with similar results. Multiple t-tests corrected with the Hold-Sidak method for multiple comparisons were performed to determine statistical significance. For (A–H), each Western lane contained 30 μg of whole-cell lysate. Data in (D, F) were normalized by β-actin (as shown) whereas the rest of the data were normalized by total protein stain.

**Short-Term Nutrient Deprivation Triggers Degradation of AHR in HeLa Cells When Either p23 or HSP90 Is Down-Regulated**

To further investigate whether autophagy is involved in the degradation of AHR, we treated the HeLa cells with HBSS to mimic nutrient deprivation, which is known to cause autophagy. We observed that the AHR protein levels were reduced after 15 min of HBSS treatment and were then gradually increased up to four hours in p23 knockdown HeLa cells (Figure 2.2A). In contrast, the AHR protein levels of wild type HeLa cells were steadily increased up to four hours of HBSS treatment. Pre-treatment with CQ hampered the reduction of AHR in p23 knockdown HeLa cells, suggesting that this decrease of AHR protein levels is caused by autophagy (Figure 2.2B). We hypothesized that some compensatory mechanism may be
involved in protecting AHR from degradation under nutrition deprivation in HeLa cells. Given that HSP90 stabilizes the cytosolic AHR complex, we examined whether HSP90 would protect AHR from degradation caused by nutrient deprivation in HeLa cells. We down-regulated HSP90 in HeLa cells with shRNA via lentiviral infection and then treated these HSP90 knockdown cells with HBSS. We observed that these cells exhibited similar characteristics as the p23 knockdown cells but not as the wild type cells, suggesting that this decrease of AHR could be protected by HSP90 (Figure 2.2A). qPCR data showed that 4 h HBSS treatment significantly increased the amount of the ahr message in wild type HeLa cells (Figure 2.2C). Co-treatment with a transcription inhibitor actinomycin D with HBSS in wild type HeLa cells abolished the increase of AHR protein levels caused by HBSS (Figure 2.2D). Collectively, these data supported that although nutrient deprivation caused degradation of AHR via autophagy initially, it increased the synthesis of the AHR protein, which led to the steady rise of the AHR protein levels up to four hours of nutrient deprivation.
Figure 2.2. Short-term nutrient deprivation triggers the degradation of AHR in HeLa cells when either p23 or HSP90 is down-regulated. (A) Zero to four hours' treatment of HBSS (nutrient deprivation) in wild type (WT), p23 stable knockdown (p23KD), and HSP90 stable knockdown (HSP90KD) HeLa cells. Fifteen minutes of HBSS treatment decreased AHR protein levels in p23KD and HSP90 HeLa cells but not in WT HeLa cells. Longer nutrient deprivation of up to 4 h increased AHR protein levels in all three cell lines. The graph represents replicate data of means ± SD (upper error bars shown), n = 3 for all, except n = 4 for HSP90KD data from 0 to 1 h. Zero timepoints in each cell line were arbitrarily set as one for comparison. Data were analyzed by unpaired two-tailed t-test for comparisons between zero and 15 min timepoints in each individual cell line (significant data points marked with # symbol). Data were analyzed by two-way ANOVA (mixed-model) analysis corrected with the Dunnett test for multiple comparisons to determine the statistical significance between WT and p23KD or WT and HSP90 KD at each time point (significant data points marked with * symbol). WT HeLa data were set as a control group at each timepoint for comparison. The images above represent the replicate data. Note that the timepoints are intentionally presented not in chronological order.
to preserve the integrity of the original immunoblots. (B) A 40 μM amount of CQ pre-
treatment for 6 h blocked the AHR decrease in p23KD HeLa cells treated with HBSS
for 0–1 h. Zero time points in each condition were arbitrarily set as one for
comparison. The graph represents replicate data of means ± SD (upper error bars
shown), n = 3. Data were analyzed by multiple t-tests corrected with the Hold-Sidak
method for multiple comparisons to determine the statistical significance. The
images above represent the replicate data. (C) A 4 h HBSS treatment significantly
increased the ahr message levels in WT HeLa cells. The graph represents replicate
data of means ± SD, n = 3 of one experiment. This experiment was repeated once
with similar results. Data were analyzed by unpaired t-test to determine statistical
significance. (D) Treatment with 5 μg/mL of actinomycin D (ActD) for 4 h abolished
the increase of AHR protein levels induced by HBSS (nutrient deprivation) in WT
HeLa cells. The graph represents replicate data of means ± SD, n = 3 of one
experiment. This experiment was repeated once with similar results. Data were
analyzed by one-way ANOVA with Tukey’s multiple comparisons test to determine
statistical significance. For A to D, each Western lane contained 30 μg of whole-cell
lysate. The intensity of all Western bands was normalized by total protein stain.

**Down-Regulation of p23 in HeLa Cells Exhibits Higher Autophagic Flux**

Next, we examined whether knockdown of p23 in HeLa cells stimulates
autophagy, which in turn accelerates the degradation of the AHR protein. It is well
accepted that LC3B-II plays a key role in macroautophagy (Jiang & Mizushima,
2015). Its turnover can be used as a marker for autophagic flux, which reflects the
frequency of events from autophagosome formation to substrate degradation during
macroautophagy. In other words, the amount of LC3B-II in the autophagosomes and
lysosomes is directly proportional to the autophagy activity, which is referred to as
the autophagic flux. We measured the LC3B-II protein levels and observed that the
LC3B-II band was more intense in p23 knockdown HeLa cells than in wild type cells
(Figure 2.3A, “WT, 0 h” versus “p23KD, 0 h”). Since CQ inhibits the LC3B-II
degradation by acidic proteases in the lysosomes, the LC3B-II content can be more
easily detected in the presence of CQ. The autophagic flux, which was determined
by the slope of LC3B-II levels over time with CQ treatment, was also higher in p23
Figure 2.3. Down-regulation of p23 in HeLa cells exhibits higher autophagic flux. (A) Western blot analysis showing that p23 stable knockdown (p23KD) HeLa cells have higher basal LC3B-II protein levels as well as higher autophagic flux than wild type (WT) HeLa cells in the presence of 40 μM CQ. The graph in A represents replicate data of means ± SD (upper error bars shown), n = 3 of one experiment. This experiment was repeated once with similar results. Data were analyzed by multiple t-tests corrected with the Hold-Sidak method for multiple comparisons to determine the statistical significance. The above images are representative of the replicate data. Note that the hour under WT is intentionally presented as “6 h, 0 h, 12 h, 24 h” to preserve the integrity of the original immunoblot. Each Western lane contained 30 μg of whole-cell lysate. The intensity of Western bands was normalized by the total protein stain. (B) Qiagen RT2 Profiler PCR array analysis of 84 autophagy-related gene expression showing that knockdown of p23 did not alter the autophagy-related gene transcription when compared to wild type (WT). The scatter plot compares WT HeLa (x-axis) to p23 knockdown (p23KD) HeLa (y-axis) with a fold regulation setting as 2.
knockdown than in wild type cells (Figure 2.3A), suggesting that down-regulation of p23 in HeLa cells sensitized macroautophagy. Next, we performed a PCR array to test whether knockdown of p23 would change the expression of autophagy-related genes. The expression of 84 autophagy-related genes (Table 5 in APPENDIX C) was measured using the Qiagen RT² Profiler PCR array kit. However, the gene expression related to autophagy was not significantly different between wild type and p23 knockdown HeLa cells (Figure 2.3B), suggesting that the down-regulation of p23 leads to higher autophagic activity without affecting the autophagy-related gene expression.

**AHR Undergoes Selective Autophagy in p23 Knockdown and Wild Type HeLa Cells**

Thus far, our data suggested possibly an LC3B-II-dependent lysosomal degradation of AHR in HeLa cells—this type of degradation has been described as selective (macro)autophagy. The selectivity of autophagy is mainly conveyed by the specific binding of cargo proteins to cargo receptors (e.g., p62) and the binding of LC3B-II to the cargo protein–cargo receptor complex (Danieli & Martens, 2018). We first examined the role of LC3B in the degradation of AHR in HeLa cells. Stable knockdown of LC3B gene (*MAP1LC3B*) in HeLa cells to 12.5% of the wild type LCB-I content showed an increase of the AHR protein levels when compared to the wild type HeLa cells (Figure 2.4A, left graph). In addition, knockdown of LC3B led to less LC3B-II formed from LC3B-I lipidation and suppressed the CQ effect on AHR, consistent with our suspicion that CQ might block the LC3B-mediated degradation of the AHR protein (Figure 2.4A,B, right graphs). Next, we transiently down-regulated 40% of the LC3B-I content in p23 knockdown HeLa cells and observed a higher increase of AHR when compared to the LC3B knockdown HeLa cells (Figure 2.4A,B,
The response of AHR to CQ treatment was also significantly suppressed by the LC3B knockdown (Figure 2.4B, right graph). Next, we examined whether AHR physically interacts with LC3B-II, leading to selective autophagy of the AHR protein. We observed that LC3B-II interacted with AHR in our coimmunoprecipitation experiment and this interaction can be significantly enhanced by CQ in both wild type and p23 knockdown HeLa cells (Figure 2.4C). There was also a significant amount of p62 co-immunoprecipitated with AHR (Figure 2.4D).
Figure 2.4. AHR interacts with LC3B-II and p62 in p23 stable knockdown (p23KD) and wild type (WT) HeLa cells. (A) Stable knockdown of LC3B in HeLa cells increased AHR protein levels and reduced the extent of the CQ-mediated increase of AHR. The images above show LC3B-I, LC3B-II, and AHR protein levels in WT and LC3B stable knockdown (KD) HeLa cells with or without treatment with 40 μM CQ for 6 h of triplicate samples. The graphs represent replicate data of means ± SD of one experiment, n = 3. WT (left graph) and NT (right graph) were arbitrarily set as 1 for data normalization. This experiment was repeated once with similar results. Data of the left graph were analyzed by multiple t-tests corrected with the Hold-Sidak method for multiple comparisons to determine statistical significance whereas data of the right graph were analyzed by one-way ANOVA with Sidak’s multiple comparisons test to determine statistical significance. (B) Transient knockdown of LC3B in p23 stable knockdown (p23KD) HeLa cells showed a higher increase of AHR protein levels when compared to that in WT HeLa cells (see A) and reduced the extent of CQ-mediated increase of AHR. The graphs represent replicate data of means ± SD of one experiment, n = 3. p23KD (left graph), and no addition as no treatment (NT, right graph) were arbitrarily set as 1 for data normalization. This experiment was repeated once with similar results. Data of the left graph were analyzed by multiple t-tests corrected with the Hold-Sidak method for multiple comparisons to determine statistical significance whereas data of the right graph were analyzed by one-way ANOVA with Sidak’s multiple comparisons test to determine statistical significance. (C) LC3B-II was co-immunoprecipitated by AHR polyclonal antibody SA210 in both cell lines after treatment with 40 μM CQ for 6 h. Data were presented from three independent experiments as means ± SD, n = 3. WT HeLa NT group was arbitrarily set as one for comparison (no error bar). Data were analyzed by multiple t-tests corrected with the Hold-Sidak method for multiple comparisons to determine statistical significance. The images below are representative of the replicate data. (D) p62 was co-immunoprecipitated by AHR monoclonal antibody A-3x after treatment with 40 μM CQ for 6 h in WT HeLa cells. This experiment was repeated once with similar results. For A-B, each Western lane contained 30 μg of whole-cell lysate or the whole immunoprecipitation content from 1 to 2 mg of whole-cell lysate starting material (C and D). Data were normalized by total protein stain.
Proximity ligation assay was performed to confirm whether AHR would physically interact with LC3B and p62 in HeLa cells in situ. We observed that AHR interacted with LC3B (Figure 2.5A) and p62 (Figure 2.5B) in both wild type and p23 knockdown HeLa cells, whereas negative control groups without the addition of antibodies against the interaction partners did not show signals. There were more AHR–LC3B and AHR–p62 interactions in p23 knockdown than in wild type HeLa cells (Figure 2.5A,B). We expected that the signal of the LC3B-AHR interaction should be enhanced in the presence of CQ since LC3B-II can be accumulated when acidic proteases in the lysosomes are inhibited by CQ. Indeed, treatment with CQ enhanced the AHR–LC3B interaction significantly in p23 knockdown HeLa cells but not in WT HeLa cells (Figure 2.5A). Macroautophagy (or autophagy) can selectively degrade K63-ubiquitinated target proteins (Mukhopadhyay & Riezman, 2007). Given that we observed an interaction between AHR and p62, which is a known ubiquitin-binding cargo receptor, we examined the ubiquitination status of AHR in wild type and p23 knockdown HeLa cells. We observed that AHR was K63-ubiquitinated in both cell lines without exogenous ligand treatment and significantly more K63-ubiquitination was detected in p23 knockdown HeLa cells (Figure 2.6A). Both cell lines showed minimal levels of K48-ubiquitinated AHR protein levels (Figure 2.6B, “KD NT” and “NT” lanes of image). Co-treatment with an AHR ligand 3MC in the presence of a proteasome inhibitor MG132 showed a more intense K48-ubiquitinated AHR protein (Figure 2.6B), which exhibited a pattern that was different from the K63-ubiquitination of AHR as shown in Figure 2.6A. Collectively, these data supported that the basal AHR protein undergoes K63-ubiquitination (but not K48-ubiquitination) in HeLa cells and this K63-ubiquitination is more apparent when p23 is down-regulated. AHR undergoes p62/LC3-mediated selective autophagy via K63-
ubiquitination in the absence of ligand treatment. Down-regulation of p23 promotes this degradation process, leading to a decrease in the basal AHR protein levels.

**Figure 2.5.** Proximity ligation assay results showing that AHR interacts with LC3B and p62 in wild type (WT) and p23 stable knockdown (p23KD) HeLa cells. (A) More AHR–LC3B interaction was detected in p23KD than in WT HeLa cells (NT images) and treatment with 40 μM CQ for 6 h increased the interaction (CQ versus NT images). (B) p23KD cells showed more AHR–p62 interaction than in WT HeLa cells (NT images). The graphs represent replicate data of means ± SD of one experiment, n=3. This experiment was repeated twice with similar results. Image J software was used to measure signal dots (red channel) and cell numbers (nucleus numbers in the blue channel) in each of the whole images captured (signals and nucleus on the edges were excluded). Statistical significance was determined by
two-way ANOVA with Tukey’s multiple comparisons test (A) and unpaired t-test (B). The images are representative of the replicate data with a scale bar at the right bottom of each image. NC, negative control (cells incubated without antibodies against interaction partners); NT, no addition as no treatment; CQ, chloroquine in water.

*Figure 2.6.* AHR undergoes K63 ubiquitination in p23 stable knockdown (p23KD) and wild type (WT) HeLa cells. (A) Immunoprecipitation of AHR by AHR polyclonal antibody SA210, followed by K63-TUBE Far-western analysis showing more K63-ubiquitinated AHR in p23KD than in WT HeLa cells. The graph represents replicate data (means ± SD, n = 6). The mean of WT HeLa was arbitrarily set as one for comparison. Data were analyzed by unpaired t-test to determine statistical significance. The images above are representative of the replicate data. (B) K48-TUBE was used for Far-western analysis showing minimal K48-ubiquitination of AHR in WT and p23KD HeLa cells. Treatment with 3MC (1 μM) and MG132 (10 μM) for 2 h showed accumulation of K48-ubiquitinated AHR. The pattern of K48-ubiquitinated AHR was different from K63-ubiquitinated AHR. The graph represents replicate data (means ± SD, n = 3). One WT HeLa NT value was arbitrarily set as one for comparison. Data were analyzed by one-way ANOVA with Tukey’s multiple comparisons test to determine statistical significance. The images above are representative of the replicate data. “WT NT” stands for wild-type HeLa cells with no treatment whereas “KD NT” stands for p23 knockdown HeLa cells with no treatment.
AHR Is Degraded Via Autophagy in Human Lung, Liver, and Breast Cancer Cell Lines

Next, we examined whether the p62/LC3-mediated autophagy of AHR in HeLa cells can be observed in other cell types. We observed that the AHR protein levels were significantly increased to about 1.5- to 1.7-fold in the presence of 40 µM CQ for six hours in a variety of cancer cell types—namely liver cancer cell line Hep3B, lung cancer cell line A549, and two breast cancer cell lines T-47D and MDA-MB-468. These results showed that similar to HeLa cells, AHR in these cells is likely degraded via autophagy in the absence of ligand treatment (Figure 2.7A). Results from proximity ligation assay confirmed that AHR interacted with LC3B in the presence or absence of CQ (Figure 2.7B). In addition, the interaction between AHR and p62 was detected in all four cell types (Figure 2.7C).
AHR is degraded via autophagy in human lung, liver, and breast cancer cell lines. (A) Treatment with 40 μM CQ dissolved in water for 6 h increased AHR protein levels in A549, Hep3B, T-47D, and MDA-MB-468 cells (means ± SD; n = 3 of one experiment, except for n = 5 for Hep3B). This experiment was repeated once with similar results. Each Western lane contained 30 μg of whole-cell lysate. Data are normalized by the total protein stain. NT, no addition as no treatment. An unpaired t-test was used to determine statistical significance. The images below are representative of the replicate data. Proximity ligation assay results showing that AHR interacted with (B) LC3B and (C) p62 in all four cell lines. All images are representative of the replicate data of three independent experiments. Each image contains a scale bar at the right bottom. NC, negative control (cells incubated without primary antibodies against interaction partners); NT, no addition as no treatment; CQ, chloroquine in water.
Chaperone-Mediated Autophagy Is Unlikely Involved in the Degradation of AHR in HeLa Cells

Next, we explored whether another selective autophagic pathway, namely chaperone-mediated autophagy, could degrade AHR in HeLa cells. This type of autophagy requires interaction with the lysosomal membrane-bound protein LAMP2A for the internalization of cargo protein into lysosomes for degradation. When HeLa cells were treated with 100 µM 6-AN, a chaperone-mediated autophagy activator, for 24 h, the AHR levels were not significantly altered (Figure 2.8). However, the LAMP2 immunoblot signal, which was detected using an antibody that recognizes both LAMP2A and LAMP2B, was significantly reduced after the 6-AN treatment. Collectively, we were not able to show any involvement of chaperone-mediated autophagy in the degradation of AHR in HeLa cells.

![WT HeLa](WT HeLa)

*Figure 2.8. Activation of chaperone-mediated autophagy does not degrade AHR in HeLa cells. Wild type (WT) HeLa cells were treated with a chaperone-mediated autophagy activator 6-aminonicotinamide (6-AN, 100 µM) for 24 h. Treatment with 6-AN did not change AHR protein levels (bottom left graph) but reduced LAMP2 levels (bottom right graph). Images above are representative of triplicate samples in one experiment and this experiment was repeated once with similar results. Each*
Western lane contained 30 μg of whole-cell lysate. Data are normalized by the total protein stain. The plots represent means ± SD, n = 3. An unpaired t-test was used to determine statistical significance.

**Discussion**

We used HeLa cells for this study since HeLa cells were one of the human cell lines, we first tried that exhibit apparent p23-dependent degradation of AHR (P. M. Nguyen et al., 2012a). HeLa cells increase the AHR content after the treatment with an autophagy inhibitor such as CQ, Baf A1, or 3MA. CQ and Baf A1 suppress the acidification of lysosomes and the fusion of autophagosomes with lysosomes (Steinman, Mellman, Muller, & Cohn, 1983) whereas 3MA inhibits autophagosome formation via inhibition of class III PI3K at the early stages of autophagy (Wu et al., 2010). The basal AHR protein can be ubiquitinated via K63-linkage and then degraded in LC3B-dependent autophagy. p62 likely acts as the cargo receptor of AHR to undergo autophagy since we can detect the AHR–p62 interaction by co-immunoprecipitation study and proximity ligation assay. Similarly, it has been reported that Dvl is degraded via the p62/LC3-mediated autophagy upon Wnt ligand activation (Gao et al., 2010). In addition, Tbx9 cell transcription factor is selectively degraded via the p62/LC3 pathway in an Atg5-dependent manner (Rivera Vargas et al., 2017). Selectivity is largely conferred by the ubiquitination architectures of the client proteins and the preference of the cargo receptor to certain ubiquitin chains and cargoes. The chain formation including chain length, mixed, or branched chains may attach more information for the ubiquitin code. Post-translational modification of both the adaptors and the ubiquitin chains also adds more layers of regulation (Kwon & Ciechanover, 2017; Ohtake & Tsuchiya, 2017; Zaffagnini & Martens, 2016).
Characterization of the ubiquitination machinery and the nature of the AHR–p62 interactions would provide a better picture of how AHR undergoes the LC3B-mediated autophagy.

As a co-chaperone of HSP90, p23 has been reported to stabilize HSP90 confirmation and assist AHR activation (A Kazlauskas et al., 1999). The expression of p23 is ubiquitous; however, there is limited, if any, information regarding the regulation of p23 gene expression. Additionally, there is no evidence showing that the p23 levels can be altered in the presence of an AHR ligand. Although p23 stabilizes AHR from degradation in cell lines (Jinyun Chen et al., 2019; P. M. Nguyen et al., 2012a), there is limited information on how p23 maintains the AHR protein levels in the absence of ligand treatment. In this study, the increase of the AHR protein levels in HeLa cells by CQ is higher when p23 is down-regulated, consistent with the notion that CQ inhibits autophagy, which is responsible for the p23-dependent AHR degradation. Proteins with a glutamine-rich region (such as AHR) are known to be more prone to aggregation, and these proteins can be degraded by autophagy (Ravikumar, Duden, & Rubinsztein, 2002). Since p62/LC3 is involved in autophagic degradation of misfolded proteins (Pankiv et al., 2007), reduction of the p23 content in HeLa cells may allow AHR to be more prone to aggregation and become accessible for ubiquitination. This hypothesis is supported by our findings that the response of AHR to autophagy inhibition, activation, and LC3B knockdown are more pronounced in HeLa cells when p23 is down-regulated. Moreover, the interaction between AHR and p62 (or LC3B) is more apparent in p23 knockdown HeLa cells. The autophagy-related gene expression, however, did not show any significant difference between wild type and p23 knockdown HeLa cells. Although we are not aware of any report showing the correlation between p23 and autophagy
activity, the basal LC3B-II levels and the autophagic flux are unambiguously higher when p23 is down-regulated in HeLa cells. Collectively, we concluded that the down-regulation of p23 promotes autophagy to selectively degrade AHR without altering the expression of any autophagy-related gene. Importantly, the down-regulation of p23 in HeLa cells causes more K63-ubiquitination of AHR in the absence of ligand treatment, revealing that reduction of the cellular p23 content to about 50% in HeLa cells is sufficient to promote the K63-ubiquitination of AHR, leading to the p62/LC3B-mediated selective autophagy.

Interestingly, it has been reported that HIF-1α—another member of the AHR PAS protein family—can be K63-ubiquitinated, and this ubiquitination leads to chaperone-mediated autophagy of HIF-1α during serum deprivation (Ferreira, Soares, Ramalho, Pereira, & Girao, 2015). HIF-1α is constantly degraded by 26S proteasome under normoxic conditions. In the absence of serum, the KFERQ-like motif of HIF-1α interacts with HSC70; K63-ubiquitination of HIF-1α apparently causes the interaction between HIF-1α and the lysosomal membrane-bound LAMP-2A—a necessity for internalization of HIF-1α into lysosomes for degradation. We examined the possible role of chaperone-mediated autophagy in AHR protein degradation in HeLa cells. However, activation of chaperone-mediated autophagy by 6-AN did not alter the AHR content in HeLa cells. In addition, starvation of HeLa cells for four hours caused an increase of the AHR content whereas prolonged starvation in rodents is known to cause chaperone-mediated autophagy (Cuervo, Knecht, Terlecky, & Dice, 1995). Collectively, chaperone-mediated autophagy is unlikely to be involved in the degradation of AHR in HeLa cells. Reduction of the LAMP2 levels in HeLa cells upon 6-AN treatment is surprising since activation of chaperone-mediated autophagy often increases the LAMP2A levels at the lysosomal
membrane (Kiffin, Christian, Knecht, & Cuervo, 2004). Realizing that the LAMP2 antibody we used to detect LAMP2A cannot differentiate LAMP2A from LAMP2B, we cannot rule out the possibility that LAMP2B may be suppressed by 6-AN treatment in HeLa cells.

This p62/LC3B-mediated AHR degradation is analogous to the cargo-induced model of selective autophagy, which involves the formation of the K63-ubiquitinated client proteins that are recognized by the cargo receptors (Rogov et al., 2014). Although both cargo-induced and starvation-induced autophagy involve the formation of autophagosome and fusion of the autophagosome with the lysosome, the signaling cascade and the involvement of autophagy-related (Atg) proteins are not exactly identical (Zaffagnini & Martens, 2016). It is evidence that AHR can be degraded when autophagy is induced by nutrient deprivation or treatment with metformin. However, autophagy induction via nutrient deprivation quickly increases the amount of AHR. During that time, HSP90 protects the AHR protein from degradation, consistently with the literature that inhibition of HSP90 leads to client protein degradation through autophagy (Jinwal et al., 2012; Qing, Yan, Qu, Liu, & Xiao, 2007; Qing, Yan, & Xiao, 2006) and inhibition of HSP90 by geldanamycin results in the proteasomal degradation of AHR (H. S. Chen, Singh, & Perdew, 1997). Down-regulation of HSP90 in HeLa cells exhibits more AHR degradation upon early nutrient deprivation; subsequently, AHR levels are steadily increased due to the up-regulation of the AHR gene transcription. We previously discovered that the protective role of p23 on the AHR levels does not require HSP90, since stable knockdown of HSP90 to 55% of its wild type content in HeLa cells does not alter the AHR levels and p23 mutants with modest HSP90 binding affinity can still effectively restore the AHR levels (Pappas et al., 2018). Although we observed that
degradation of AHR occurs after 15-min treatment with HBSS in HSP90 knockdown HeLa cells, this reduction is unique to nutrient deprivation and is temporary, probably involving the general HSP90 protection of protein folding under stress; it is rather different from the previous observation that the long-term, steady-state AHR levels are not affected by the reduction of HSP90. Studying the underlying mechanisms of how HeLa cells trigger the synthesis of AHR in response to autophagy induction by nutrient deprivation might reveal a potential role of AHR in the autophagy-related diseases, such as neurodegenerative diseases and tumorigenesis.

Conclusions

In summary, we uncovered a mechanism for AHR protein degradation in HeLa cells. We observed that AHR is K63-ubiquitinated and binding of AHR to p62 and LC3B-II occurs. Inhibition of autophagy increases AHR protein levels. These observations are more pronounced when p23 is down-regulated in HeLa cells, suggesting that reduction of the p23 content stimulates autophagy to degrade AHR. Other than HeLa cells, four other human cell lines also show the autophagy-dependent regulation of the AHR protein levels and exhibit AHR–LC3B-II and AHR–p62 interactions. Collectively, the AHR content is controlled by selective autophagy – a novel understanding of how human cells maintain the AHR protein levels. This mechanism can potentially be manipulated in an effort to control the AHR levels for clinical applications.

Materials and Methods

Reagents

3MA, bafilomycin A1, and metformin were purchased from Cayman Chemical (Ann Arbor, MI, USA). Chloroquine, rabbit IgG, Duolink proximity ligation assay kit, and anti-LC3B rabbit IgG were purchased from Sigma (St. Louis, MO, USA).
Cycloheximide, anti-AHR monoclonal mouse IgG (A-3x), used for proximity ligation assay and co-immunoprecipitation for p62 due to less interference in the region of p62 on Western analysis), anti-LAMP2 (H4B4), and anti-HSP90 goat IgG (N-17) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). HBSS was purchased from Gibco (Grand Island, NY, USA). Puromycin was purchased from Goldbio (St Louis, MO, USA). FBS was purchased from Gemini Bio (West Sacramento, CA, USA). HyClone DMEM, protein G Dynabeads, anti-p23 mouse IgG (JJ3), and all cell culture reagents (if not specified) were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Direct-zol RNA kit was purchased from Zymo Research (Irvine, CA, USA). MMLV high-performance reverse transcriptase was purchased from Epicentre (Madison, WI, USA). qPCR SYBR Green supermix was purchased from Bio-Rad (Hercules, CA, USA). Zymopure maxiprep kit was purchased from Zymo Research (Irvine, CA, USA). RT² Profiler PCR array plates were purchased from Qiagen (Germantown, MD, USA). K48- and K63-TUBE were purchased from LifeSensors (Malvern, PA, USA). p23 shRNA, HSP90 shRNA, and LC3B shRNA were purchased from Dharmaco (Lafayette, CO, USA). Anti-AHR rabbit IgG (SA210) and anti-p62 rabbit IgG were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Anti-β-actin mouse IgG was purchased from Ambion (Austin, TX, USA). All secondary IgG and streptavidin conjugated with IRDye 800CW or 680 were purchased from LI-COR Bioscience (Lincoln, NE, USA). The pCMV-VSV-G (8454) and pCMV-dR8.2 dvpr packaging plasmid (8455) were purchased from Addgene (Watertown, MA, USA).

Cell Culture

All cell lines were authenticated by ATCC and were maintained at 37 °C and 5% CO₂. HeLa, A549, and T-47D cells were cultured in DMEM supplemented with
10% fetal bovine serum, 2 mM GlutaMAX-I, 10 U/mL of penicillin, and 10 mg/mL of streptomycin. Hep3B and MDA-MB-468 cells were grown in Advanced MEM with 5% fetal bovine serum, 2 mM GlutaMAX-I, 10 U/mL of penicillin, and 10 mg/mL of streptomycin. For nutrient deprivation study, cells were rinsed with HBSS twice and then grown in HBSS for the designated period.

**Generation of HSP90, p23, and LC3B Stable Knockdown Cells**

Protocols for the generation of the shRNA-containing lentiviruses and lentiviral infection were previously described (P. M. Nguyen et al., 2012a). p23 knockdown HeLa cells were generated using p23-specific shRNA (#1475); HSP90 knockdown HeLa cells were generated using the HSP90α-specific shRNA (#6, 8563), and LC3B knockdown HeLa cells were generated using the MAP1LC3B-specific shRNA #153286 and #151769. In essence, AD-293 cells (about $7 \times 10^5$ cells) were plated in 5 mL of medium without antibiotics in a 25 cm$^2$ flask. After incubation at 37 °C and 5% CO$_2$ overnight, cells reached 50%–80% confluence. Fresh medium without antibiotics was exchanged. Cells were transfected using EndoFectin reagent (2:1 DNA ratio) with the plasmids cocktail as follows: 2.5 μg of specific shRNA plasmid, 1.875 μg of the pCMV-dR8.2 dvpr packaging plasmid, and 0.625 μg of the VSV-G envelope plasmid. Fresh complete medium was exchanged 15 h later. Medium which contained the virus was collected after 24 h and stored at 4 °C. Another 5 mL of fresh complete medium was added to cells and was collected 24 h afterwards. The combined medium was centrifuged at 400 g for 5 min to remove any AD-293 cells that were inadvertently collected. The resulting supernatant was used for infection. The infection of HeLa cells was performed by first seeding cells in a 75 cm$^2$ flask to 50%–70% confluence. Fresh complete medium containing 8 μg/mL of polybrene was exchanged. Supernatant containing lentiviral particles (0.5–1 mL)
was then added. The fresh complete medium was exchanged 24 h after infection. The selection was started by adding 1.5 μg/mL of puromycin 48–54 h after infection. Western analysis was performed to determine the target protein levels after 2–3 passages.

**Transient Transfection**

Cells were grown in 6-well plates and transfection was initiated when cells were about 90% confluence. Cells were transfected with 2.5 μg of DNA and 5 μL of EndoFectin reagent. Fresh complete medium was exchanged 24 h after transfection. Treatment with CQ was started at 48 h post-transfection and cells were harvested at 54 h post-transfection.

**Whole-Cell Lysate Preparation and Western Analysis**

Cell pellets were resuspended using lysis buffer (25 mM HEPES, pH 7.4, 0.4 M KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% NP-40, 1 mM PMSF, and 2 μg/mL of leupeptin) of 2.5× volume of cell pellets. After three cycles of freeze/thaw, lysates were kept on ice for 30 min and were then centrifuged at 16,000 g for 10 min at 4 °C. The supernatants were defined as whole-cell lysates and were subjected to BCA assay and LI-COR Western analysis. The protocol for Western analysis was described previously (Xie, Huang, Park, Pham, & Chan, 2014) with minor modification. Fifteen percent acrylamide gels were transferred for 3 h at 4 °C. After the wet transfer, total protein staining was performed using LI-COR Total Protein Stain. Membranes for the examination of LC3B levels for autophagic flux were dried overnight and wet with PBS before blocking. The transferred nitrocellulose membranes were blocked in PBS with 5% BSA for 1 h. Dilutions for antibodies were as follows: 1:1,000 for p23 (JJ3), p62, LC3B and AHR (A-3x); 1:2,000 for AHR (SA210); 1:5,000 for β-actin; 1:200 for HSP90 (N-17). If not specified, Western
bands were normalized using total protein stain. Results were obtained and analyzed using an LI-COR Odyssey CLx imaging system.

**RT-qPCR**

RT-qPCR was performed as described previously (Pappas et al., 2018). In brief, RNA was extracted using the Direct-zol kit. Reverse transcription was performed from 0.5 to 1 μg of RNA using Epicentre MMLV reverse transcriptase. Quantitative PCR was performed with: 1 μL of cDNA from reverse transcription solution, 10 μL of Bio-Rad iTaq SYBR green supermix, and 0.8 pmol sequence-specific primers (AHR primers are OL615, 5’-ACATCACCTACGCCAGTCGC-3’ and OL616, 5’-TCTATGCGCTTGGAAGGAT-3’ whereas β-actin primers are OL101, 5’-CCACACTGTGCCCATCTAGG-3’ and OL102, 5’-AGGATCTTTCATGAGTACGTCATAGC-3’) using a Bio-Rad CFX Connect real-time PCR machine with the following protocol: 40 cycles of 90 °C for 10 s/60 °C for 1 min with fluorescence readings taken at 60 °C. The $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001) was used to present the normalized values. For RT² Profiler PCR Array, reverse transcription was performed with 0.5 μg of RNA using Epicentre MMLV reverse transcriptase. PCR master mix (1,350 μL of Bio-Rad iTaq SYBR green supermix, 102 μL of cDNA synthesis solution, and 1,248 μL of RNase-free water) was prepared and an aliquot of 25 μL was added to each well of the RT2 Profiler PCR Array plate. A Bio-Rad CFX Connect real-time PCR machine was used with the following protocol: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec/60 °C for 1 min (ramp rate between 95 °C to 60 °C step was set as 1 °C/sec). Data analysis was performed at Qiagen GeneGlobe Data Analysis Center (www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page).
Immunoprecipitation and Co-Immunoprecipitation Experiments

Immunoprecipitation was performed as described previously (Pappas et al., 2018). In brief, cells were lysed with the method described above. Three deubiquitylase inhibitors, namely 1,10-phenanthroline (5 mM), NEM (10 mM), and PR-619 (50 μM) were added in the lysis buffer for immunoprecipitation experiment with K48- and K63-TUBE Far-western analysis. One to two milligrams of whole-cell lysates were used for immunoprecipitation (IP) and co-immunoprecipitation (co-IP) of AHR using anti-AHR antibody SA210 (1:200 by volume) or anti-AHR antibody A-3x (1:100 by volume for AHR–p62 co-IP) for 30 min at room temperature. The pre-equilibrated Protein G Dynabeads (1:200 by volume) were then added to each sample with the assay buffer: 25 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1% Tween-20 for IP (0.05% Tween-20 for co-IP), and 1 mg/mL of BSA. The samples were incubated with a rotation of 60 rpm overnight at 4 °C. The beads were then washed three times for 5 min each with the assay buffer and then eluted with electrophoresis sample buffer for SDS-PAGE, followed by LI-COR Western analysis.

K48- and K63-TUBE and Far-Western Analysis

HeLa cells were seeded and cultured overnight in a 75 cm² flask. Treat the cells with DMSO (0.1%), 3MC (1 μM), and/or MG132 (10 μM) for 2 h and then harvested with cold PBS. Cell lysates were obtained using a lysis buffer (see above) with a 4× volume of the cell pellet. Immunoprecipitation was performed as described above, followed by Far-western analysis. K48- or K63-TUBE (1:1,000) were incubated with the nitrocellulose membrane for 1 h at room temperature and then with IRDye-800 conjugated streptavidin (1:10,000) for 2 h at room temperature. The
wash step between incubation was the same as in Western analysis. Results were obtained and analyzed using an LI-COR Odyssey CLx imaging system.

**Proximity Ligation Assay**

Cells were seeded on round microscope glass coverslips placed in wells of a 12-well plate and grown to about 80% confluence. Cells were then rinsed with PBS twice and incubated with ice-cold 100% methanol for 5 min. After one rinse with PBS, cells were ready for proximity ligation assay using Duolink in situ kit. In brief, cells were incubated with Duolink blocking solution (40 μL) at 37 °C for 60 min, followed by incubation with the two antibodies (in Duolink antibody diluent) used for interaction study (40 μL) at 37 °C for 60 min using the following dilution: mouse anti-AHR A-3x (1:100), rabbit anti-p62 (1:100), and rabbit anti-LC3B (1:100). After that, we performed steps involving Duolink probe incubation, ligation, and amplification according to the manufacturer’s recommendation. Nucleus staining was performed by adding 1 μg/mL of DAPI in water to cells for a 1 min incubation. Coverslips were mounted using 6 μL of PBS and were sealed with nail polish. Samples were viewed using a KEYENCE BZ-X700 fluorescence microscope and results were analyzed by ImageJ software.

**Statistical Analysis**

GraphPad Prism 8 software (La Jolla, CA) was utilized for statistical analysis. Two-tailed unpaired t-test, multiple t-tests corrected with the Holm-Sidak method for multiple comparisons, and one-way and two-way (or mixed-model) ANOVA with Sidak, Tukey or Dunnett tests for multiple comparisons were used to determine statistical significance with \(^{*}p < 0.05, {^{**}}p < 0.01, {^{***}}p < 0.001, {^{****}}p < 0.0001\), and ns, not significant \((p > 0.05)\).
CHAPTER 3: GLYCOGEN SYNTHASE KINASE 3 BETA REGULATES THE HUMAN ARYL HYDROCARBON RECEPTOR CELLULAR CONTENT AND ACTIVITY

Abstract

The aryl hydrocarbon receptor (AHR) is a cytosolic receptor which is involved in diverse cellular events in humans. The most well-characterized function of AHR is its ability to upregulate gene transcription after exposure to its ligands, such as environmental toxicants, dietary antioxidants, drugs, and endogenous ligands. The cellular content of AHR is partly controlled by its degradation via the ubiquitin–proteasome system and the lysosome-dependent autophagy. We used human cervical cancer (HeLa) cells to investigate how AHR undergoes protein degradation and how its activity is modulated. Since the glycogen synthase kinase 3 beta (GSK3β)-mediated phosphorylation can trigger protein degradation and substrates of GSK3β contain stretches of serine/threonine residues which can be found in AHR, we examined whether degradation and activity of AHR can be controlled by GSK3β. We observed that AHR undergoes the GSK3β-dependent, LC3-mediated lysosomal degradation without ligand treatment. The AHR can be phosphorylated in a GSK3β-dependent manner at three putative sites (S436/S440/S444, S689/S693/T697, and S723/S727/T731), which leads to lysosomal degradation of the AHR protein. Inhibition of the GSK3β activity suppresses the ligand-activated transcription of an AHR target gene in HeLa, human liver cancer (Hep3B), and human breast cancer (MCF-7) cells. Collectively, our findings support that phosphorylation of AHR by GSK3β is essential for the optimal activation of its target gene transcription and this
phosphorylation may partake as an “off” switch by subjecting the receptor to lysosomal degradation.

Introduction

The aryl hydrocarbon receptor (AHR) is a cellular sensor of environmental pollutants/carcinogens, endogenous ligands, and numerous dietary flavonoids and their derivatives (Murray & Perdew, 2020). Upon ligand binding, the AHR cytosolic complex changes conformation to reveal its nuclear localization motif, resulting in nuclear entry. The nuclear AHR dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT) to form an active transcription factor: it binds to the dioxin response element, recruits co-activators locally to alter chromatin structure, and eventually allows optimal assembly of the preinitiation complex for gene transcription. Although AHR is best known for its action as a ligand-activated transcription factor which regulates transcription of xenobiotic metabolizing enzyme genes, the biological role of this receptor is rather complex, and yet very interesting. For example, recent reports show that AHR plays an intricate role in seemingly diverse biological processes and diseases, namely autoimmune diseases (Iyer et al., 2018; Kahalehili et al., 2020; Rosser et al., 2020), cancers and cell proliferation (García-Villatoro et al., 2020; Huerta-Yepez et al., 2020; Nothdurft et al., 2020; Sári et al., 2020; X. Zhang et al., 2021), cancer stem cell differentiation (Ly et al., 2019), respiratory disorders (Castañeda et al., 2018; Lu et al., 2017), atopic dermatitis (Smith et al., 2017; Takei et al., 2015; van den Bogaard et al., 2013), bone disorders (Y. Jia et al., 2019), antiviral response (Giovannoni et al., 2020), and adipocyte differentiation (Dou et al., 2019). This receptor is even implicated in the SARS-CoV-2 pathophysiology (Anderson, Carbone, & Mazzoccoli, 2021). Obviously AHR has become an attractive target for drug development; thus, any means to modulate its
function in selective tissues is highly desirable. AHR normally translocates into the nucleus after ligand binding, followed by degradation via the ubiquitin-proteasome system (Davarinos & Pollenz, 1999; Q. Ma & Baldwin, 2000; Roberts & Whitelaw, 1999a). Interestingly, even without addition of an exogenous ligand, we showed that AHR undergoes lysosomal degradation in various untransformed and immortalized cell lines (J. Chen, Yang, Russu, & Chan, 2021; Yang & Chan, 2020). An in-depth understanding of the regulation of the cellular AHR content should unveil ways to modulate the AHR function by controlling its protein levels.

Glycogen synthase kinase 3 beta (GSK3β) is a serine/threonine kinase which was first discovered to regulate gluconeogenesis by phosphorylation of glycogen synthase (EMBI, Rylatt, & Cohen, 1980). Since then, more than five hundred GSK3β substrates have been suggested based on the motif-based predictions (Linding et al., 2007), which make this kinase one of the most complicated kinases for cellular processes. One of its most notable substrates is β-catenin. Upon phosphorylation by GSK3β, β-catenin is degraded via the ubiquitin-proteasome system; GSK3β is therefore recognized as a key player in the regulation of β-catenin function, particularly in the promotion of cancer growth (Takahashi-Yanaga, 2013).

Our laboratory is interested in studying the underpinnings of AHR degradation in controlling its basal levels within a cell. Realizing that GSK3β is responsible for β-catenin degradation, we hypothesized that events such as GSK3β phosphorylation may play a role in AHR degradation since the carboxyl region of AHR is serine and threonine rich. Here, we provide evidence supporting that GSK3β is responsible for AHR phosphorylation, which directs AHR to lysosomal degradation. This phosphorylation is essential for the optimal transcription of AHR target gene upon ligand activation.
Results

GSK3β Regulates the AHR Protein Levels in HeLa Cells

When we examined the GSK3β phosphorylation sites of β-catenin and the general requirement of a GSK3β substrate (Beurel, Grieco, & Jope, 2015a), we identified three similar stretches of serine/threonine regions of the human AHR which would hint AHR as a potential substrate of GSK3β (Figure 3.1). With the understanding that the phosphorylated β-catenin by GSK3β undergoes protein degradation, we first examined whether the AHR protein levels can be reduced by GSK3β. After treating HeLa cells with 10–40 μM tidegusib (TDG), which is a specific GSK3β inhibitor (Beurel et al., 2015a), AHR protein levels were significantly increased to 1.7-fold (Figure 3.2A). Another GSK3β inhibitor lithium chloride (LiCl) also significantly increased the AHR protein levels to 1.5-fold (Figure 3.2B). Downregulation of GSK3β by a sequence-specific shRNA significantly increased the AHR protein levels to two-fold when compared to the scramble shRNA negative control (Figure 3.2C). The AHR protein levels were significantly suppressed to 60% of the wild-type content when the HA-tagged GSK3β was transiently expressed (Figure 3.2D). Collectively, these results strongly supported that GSK3β downregulates the AHR protein content in HeLa cells.

Figure 3.1. GSK3β phosphorylation sites. (A) GSK3β phosphorylation consensus sequence. (B) GSK3β phosphorylation sites of β-catenin. (C) Three putative GSK3β phosphorylation sites of the human AHR (amino acid 1–848) which show
best match to the consensus sequence. The numbers indicate the amino acid locations of the primary sequence. Orange font represents the serine and threonine sites for GSK3β phosphorylation. S45 (bold) of β-catenin represents the priming site of casein kinase 1. Phosphorylation of the priming site is necessary for GSK phosphorylation.

Figure 3.2. AHR protein levels are regulated by GSK3β in HeLa cells. (A) HeLa cells were treated with 10 μM, 20 μM or 40 μM TDG for 8 h. Western blot analysis
showed that AHR protein levels increased after TDG treatment. The images are representative of the replicate data (means ± SD, n = 3). DMSO group (treated for 8h) was arbitrarily set as one (with no error bar) for data normalization. One-way ANOVA with Dunnett multiple comparisons test was performed to determine statistical significance. (B) AHR protein levels increased after 6-h, 5 mM LiCl treatment. The images are representative of the replicate data (means ± SD, n = 3). No LiCl (no treatment) group was arbitrarily set as one (with no error bar) for data normalization. Un-paired t-test with Welch’s correction was used to determine the statistical significance. (C) Transient knockdown of GSK3β (after 72 h) increased AHR protein levels. The images are representative of the replicate data (means ± SD, n = 3). Cells transfected with scramble shRNA were used as the negative control and was arbitrarily set as one (with no error bar) for data normalization. Unpaired t-test with Welch’s correction was used to determine the statistical significance. (D) Transient expression of HA-GSK3β (after 72 h) decreased AHR protein levels. The images are representative of the replicate data (means ± SD, n = 3) in one experiment and was repeated two more times with similar results. No plasmid group (NP) represents cells undergoing the same transient transfection without a plasmid. Unpaired t-test was used to determine the statistical significance. For A to D, each Western lane contained 30 μg of whole-cell lysate. Data in A for 10–20 μM treatment groups were normalized by β-actin (as shown) whereas the rest of the data were normalized by total protein stain.

**GSK3β Causes the Autophagy-Mediated Lysosomal Degradation of AHR in HeLa Cells**

Next, we examined whether GSK3β would suppress the AHR levels by promoting AHR degradation. We performed experiments using a proteasome inhibitor MG132 and an autophagy inhibitor chloroquine (CQ). We observed that MG132 surprisingly decreased the amount of AHR protein in HeLa cells that were transiently transfected with either a plasmid carrying the HA fusion of GSK3β cDNA or with no plasmid (Figure 3.3A). CQ, however, increased the AHR protein levels in HeLa cells with or without HA-GSK3β expression (Figure 3.3A), suggesting that AHR undergoes the GSK3β-dependent lysosomal degradation, but not proteasomal degradation, in HeLa cells without addition of an AHR ligand. Treatment with CQ increased the AHR protein levels in the HeLa cells which had undergone transfection
but with no plasmid, suggesting that AHR is actively undergoing lysosomal degradation. To ensure that on our hand, MG132 could inhibit proteasomal degradation in Figure 3.3A, we examined the change of the β-catenin protein levels in HeLa cells and confirmed that MG132 reversed the GSK3β-mediated reduction of the β-catenin levels (Figure 3.3B).

Figure 3.3. AHR undergoes the LC-3-mediated autophagy after GSK3β phosphorylation. (A) AHR protein levels ± HA-GSK3β transient expression (72 h) were increased by CQ (40 μM for 6 h, last 6 h of the 72-h period of transient transfection) but not by MG132 (10 μM for 6 h, last 6 h of the 72-h period of transient transfection). The time and dose of CQ and MG132 were optimized for inhibition of degradation. The images are representative of the replicate data (means ± SD, n =
3) in one experiment and was repeated once with similar results. DMSO treatment
(group (treated for 6 h) was arbitrarily set as one for data normalization. No plasmid
(NP) group represents cells undergoing the same transient transfection as HA-
GSK3β but without plasmid. Two-way ANOVA with Dunnett multiple comparisons
test was performed to determine statistical significance. (B) β-catenin protein levels
decreased after HA-GSK3β transient expression (72 h) and were increased ± HA-
GSK3β by MG132 (10 μM for 6 h, last 6 h of the 72-h period of transient
transfection). The images are representative of the replicate data (means ± SD, n = 5).
DMSO treatment group was arbitrarily set as one for data normalization. No
plasmid (NP) group represents cells undergoing the same transient transfection as
HA-GSK3β but without plasmid. Two-way ANOVA with Sidak multiple comparisons
test was performed to determine statistical significance. (C) LC3B-II level with HA-
GSK3β transient expression (72 h) was higher than that in the no plasmid group
(NP). No plasmid (NP) group represents cells undergoing the same transient
transfection as HA-GSK3β but without plasmid. The fold change of LC3B-II increase
after 6-h, 40 μM CQ treatment was also higher in the HA-GSK3β group. The images
are representative of the replicate data (means ± SD, n = 3) in one experiment and
was repeated once with similar results. No treatment (NT) group (treated for 6 h)
was arbitrarily set as one for data normalization. Two-way ANOVA with Tukey
multiple comparisons test was performed to determine statistical significance. (D)
Immunoprecipitation of AHR from HeLa whole cell lysates ± GSK3β knockdown
using anti-AHR SA210 polyclonal antibody, followed by K63-TUBE Far-western
analysis showing less K63-ubiquitinated AHR in GSK3β knockdown group while
more AHR (104 kDa) being precipitated when compared to the scramble shRNA-
transfected group. The images are representative of the replicate data (means ±
SD, n = 3) with scramble shRNA group set as one for normalization of three
separate experiment (thus no error bar). For (A–C), each Western lane contained 30
μg of whole-cell lysate. Data were normalized by total protein stain.

Next, we examined whether GSK3β could trigger autophagy in HeLa cells by
measuring the LCB-II levels, which correspond to the autophagic flux. We observed
that the LC3B-II protein levels in HA-GSK3β expressing cells were increased (1.7-
fold) when compared to cells that had undergone transfection with no plasmid
(Figure 3.3C, NT). After 6 h of CQ treatment, the increase of LC3B-II was much
higher in the HA-GSK3β group, suggesting that GSK3β causes an increase of the
autophagic flux in HeLa cells. Next, we examined whether the K63-ubiquitination of
AHR would occur, which might lead to its lysosomal degradation. We performed
Far-western analysis using lysine (K) 63 specific tandem ubiquitin binding entity
(TUBE) conjugated to biotin. This K63 TUBE reagent has a 1000–10,000-fold binding affinity favoring the K63-linked ubiquitin than the other linkages. After immunoprecipitation using the SA-210 AHR antibody, GSK3β knockdown lysate showed higher amount of AHR while the K63-ubiquitinated AHR was less when compared to the scramble shRNA transfected group (Figure 3.3D). The ratio of K63-ubiquitinated AHR/total AHR decreased after GSK3β knockdown, supporting that GSK3β promotes the K63-ubiquitination of AHR. Collectively, our results support that GSK3β causes the AHR degradation in lysosome via the K63-dependent, LC3-mediated autophagy.

**GSK3β Phosphorylates AHR in HeLa Cells**

Next, we examined whether AHR could be phosphorylated by GSK3β. Phos-tag reagent, which has been successfully used by other researchers to detect phosphorylated proteins (Jinwal et al., 2011; Li et al., 2016), was used to detect the phosphorylated AHR. In principle, phos-tag reagent binds selectively to the phosphate group(s) of protein and retards the bound protein during electrophoresis when the reagent was embedded evenly in the acrylamide gel. First, we confirmed that the phosphorylated β-catenin could be detected using this approach with a 7.5% acrylamide gel containing 20 μM phos-tag reagent (Figure 3.4A). Then we used the same strategy to detect the phosphorylated AHR. There were three bands detected using the anti-AHR SA-210 antibody at the top region of the immunoblot, indicated by red dots (Figure 3.4B). These higher mobility bands were not observed in a conventional acrylamide gel without phos-tag reagent. Intensity of the putative phosphorylated AHR region showed a time- and dose-dependent decrease after phosphatase (λ-PP) treatment, supporting that these bands represent the phosphorylated AHR (Figure 3.4B). After λ-PP treatment, the top region intensities
were weaker while the unphosphorylated AHR band intensity increased. Thus, we quantified our data by the ratio of the intensity of the phosphorylated region to the intensity of the total AHR, which was the combined intensity of phosphorylated and unphosphorylated AHR. Inhibition of GSK3β by TDG yielded more unphosphorylated AHR and showed that the phosphorylated AHR/total AHR value decreased to about 50% (Figure 3.4C). Transient expression of the HA fusion of GSK3β increased the phosphorylated AHR region intensity and decreased the unphosphorylated AHR intensity. The phosphorylated AHR/total AHR value increased to 2-fold when HA-GSK3β was transiently expressed (Figure 3.4D). Taken together, AHR can be phosphorylated in a GSK3β-dependent manner.
Figure 3.4. Phosphorylation of AHR is affected by GSK3β activity. 7.5% acrylamide bis-Tris gel embedded with 20 μM phos-tag reagent was used to confirm the phosphorylation of AHR. (A) Phosphorylated (p-β-catenin) and unphosphorylated β-catenin can be separated, and the intensities of three phosphorylated bands (red dots) were significantly decreased by 1 μL (80 units) of λ-PP treatment for 2, 30 or 60 min at 30 °C. NT represents no treatment. The top region of AHR blot in B-D shows three prominent phosphorylated AHR bands (p-AHR, red dots). The whole top region indicated by the red bracket is defined as the phosphorylated region and unphosphorylated AHR is indicated with the black dot. The y-axis (p-AHR/total AHR) represents intensity of the phosphorylated bracket region divided by the sum of the intensities of the bracket region plus unphosphorylated AHR. (B) Time- and dose-dependent decrease of the p-AHR/total AHR value after 1 μL (80 units) or 5 μL (400 units) of λ-PP treatment for 2, 30 or 60 min for 30 °C. The images are representative of the replicate data (means ± SD, n = 3). Two-minute of no λ-PP treatment group was arbitrarily set as one (with no error bar) for data normalization. Two-way ANOVA with Dunnett multiple comparisons test was performed to determine statistical significance. (C) After 40 μM TDG treatment for 8 h, the p-AHR/total AHR values decreased. The images are representative of the replicate data (means ± SD, n = 4). DMSO group (treated for 8 h) was arbitrarily set as one (with no error bar) for data normalization. Unpaired t-test with Welch’s correction was used to determine the statistical significance. (D) Transient expression of HA-GSK3β increased the p-AHR/total AHR value. The images are representative of the replicate data (means ± SD, n = 5). No plasmid group (NP), which represents cells undergone same transient transfection protocol without the plasmid carrying HA-GSK3β cDNA, was arbitrarily set as one for data normalization. Unpaired t-test with Welch’s correction was used to determine the statistical significance. For (A–D), each Western lane contained 40 μg of whole-cell lysate. Data were normalized by total protein stain.
Mutational Analysis Reveals Three Putative GSK3β Phosphorylation Regions of AHR

To further investigate whether AHR would be a substrate of GSK3β, we used two truncated human AHR, namely CΔ553 (amino acid 1–295) and NΔ515 (amino acid 516–848), that were fused to GFP to approximate the phosphorylation location(s) (Figure 3.5A). Neither of these two GFP fusions responded to TDG inhibition (Figure 3.5B,C) whereas both the endogenous AHR and GFP-AHR controls did (Figure 3.5B–D). There are three stretches of serine and threonine residues of AHR that fulfill the requirement of an GSK3β substrate: they are S436/S440/S444, S689/S693/T697, and S723/S727/T731 (Figure 3.1). These locations match the consensus GSK3β recognition motif and the GSK3β phosphorylation sites of β-catenin. We mutated these serine and threonine residues to alanine and generated three GFP fusion mutants, namely M1 (S436A/S440A/S444A), M2 (S689A/S693A/T697A), and M3 (S723A/S727A/T731A) (Figure 3.5A). After treatment with 40 μM TDG, only HeLa cells transfected with the M2-GFP fusion showed no statistically significant increase whereas M1- and M3-GFP fusions showed modest increase after GSK3β inhibition by TDG (1.26- to 1.34-fold), when compared to the endogenous AHR and AHR-GFP fusion with a somewhat higher increase upon TDG treatment (1.39- to 1.54-fold) (Figure 3.5E–G).
Figure 3.5. Site-directed mutagenesis reveals three putative GSK3β phosphorylation sites of AHR. (A) A map showing GFP fusion expressing plasmids of full length human AHR and two truncated derivatives (pGFP2-N2-AHR, pGFP2-C2-CA553, and pGFP2-N2-NA515) and three AHR mutant GFP plasmids (S436A/S440A/S444A, M1; S689A/S693A/T697A, M2; S723A/S727A/T731A, M3). The names of the plasmid expressing the corresponding protein are listed on the left. Both GFP fusions of CA553 (B) and NA515 (C), 56 h after transient transfection, did not respond to TDG while the full-length AHR-GFP after transiently transfecting pGFP2-N2-AHR plasmid for 56 h (D) showed similar increase as the endogenous
AHR proteins after TDG (40 μM, 8 h) treatment. The images are representative of the replicate data (means ± SD, n = 3) in one experiment, which was repeated once with similar results. DMSO treatment group was arbitrarily set as one for data normalization. NT represents no transfection. One-way ANOVA with Sidak multiple comparisons test was performed to determine statistical significance. (E) M1-GFP mutant showed lowered increase (by 20%) when compared to endogenous AHR but similar increase as AHR-GFP (data from 3.5D) after TDG (40 μM, 8 h) treatment. (F) For M2-GFP mutant, the TDG (40 μM, 8 h) increase was minimal. The increase level was significantly lower comparing to AHR-GFP (data from 3.5D). (G) M3-GFP mutant showed 27% less increase comparing to the endogenous AHR and 13% less increase as AHR-GFP (data from 3.5D) after TDG (40 μM, 8 h) treatment. The images in (E–G) are representative of the replicate data (means ± SD, n = 6). DMSO treatment groups were arbitrarily set as one for data normalization. Two experiments of n = 3 each were performed so that error bars are present on the DMSO groups. The dotted lines separate the AHR-GFP transfection data that are presented in 3.5D for easy reference. One-way ANOVA with Sidak multiple comparisons test was performed to determine statistical significance. For B to G, each Western lane contained 30 μg of whole-cell lysate. Data were normalized by total protein stain. NΔ515-GFP (5C) was detected by the GFP antibody since this construct cannot be detected by SA210 AHR antibody while all other GFP fusions of AHR and derivatives were detected by SA210 AHR antibody.

Next, we examined whether resistance to GSK3β control would translate into resistance to lysosomal degradation. Our results showed that levels of GFP fusions of CΔ553 and NΔ515 after transient transfection into HeLa cells were not altered after CQ, which is an autophagy inhibitor, treatment whereas the endogenous AHR and AHR-GFP fusion showed higher levels after CQ treatment (Figure 3.6A–C). In addition, unlike the endogenous AHR in HeLa cells which showed about 2–2.5-fold increase upon CQ treatment, levels of the GFP fusions of all three mutants (M1-M3) after transient transfection were only modestly increased (1.23- to 1.27-fold) (Figure 3.6D–F) while the AHR-GFP levels were increased by 1.8-fold in the presence of CQ. Collectively, these results revealed three putative GSK3β phosphorylation regions of AHR which are essential for the lysosomal degradation of AHR.
Figure 3.6. GFP fusions of two truncated AHR and three mutants were less sensitive to lysosomal degradation. HeLa cells that were transiently transfected (for 54 h) with the plasmid expressing GFP-CΔ553 (A), NΔ515-GFP (B) or full length AHR-GFP (C) were treated with either no treatment (NT) or 40 μM CQ for 6 h. Only AHR-GFP showed similar increase as the endogenous AHR upon TDG treatment. The images are representative of the replicate data (means ± SD, n = 3) in one experiment, and was repeated once with similar results. No treatment group (NT) was arbitrarily set as one for data normalization. One-way ANOVA with Sidak multiple comparisons test was performed to determine statistical significance. HeLa cells that were transiently transfected (for 54 h) with the plasmid expressing M1-GFP (D), M2-GFP (E) or M3-GFP (F) showed a significant lower increase of 1.2- to 1.3-fold when compared to 2.0- to 2.5-fold increase of endogenous AHR upon treatment with 40 μM CQ for 6 h. These increases were significantly less when compared to AHR-GFP (data from 3.6C). The images in (D–F) are representative of the replicate data (means ± SD, n = 6). No treatment groups (NT) were arbitrarily set as one for data normalization. Two experiments of n = 3 each were performed so that error bars are present on the NT groups. The dotted lines separate the AHR-GFP transfection data that are presented in 3.6C for easy reference. One-way ANOVA with Sidak multiple comparisons test was performed to determine statistical significance. For (A–F),

- For (A–F),
each Western lane contained 30 μg of whole-cell lysate. Data were normalized by total protein stain. NΔ515-GFP (3.6B) was detected by the GFP antibody since this construct could not be detected by SA210 AHR antibody while all other GFP fusions of AHR and derivatives were detected by SA210 AHR antibody.

**p23 Is Essential for the GSK3β-Mediated Degradation of AHR in HeLa Cells**

We previously reported that the basal AHR protein levels are regulated by selective autophagy and this degradation is more pronounced in the stable p23 knockdown (p23KD) HeLa cells, resulting in about 50% of the AHR content when compared to the wild type content (Yang & Chan, 2020). When we treated the p23KD HeLa cells with TDG (10–40 μM), only 40 μM TDG increased AHR protein level significantly to 1.3-fold (Figure 3.7A), which was lower than what we observed in HeLa cells (1.7-fold, Figure 2A). LiCl (5 mM), on the other hand, could not alter the AHR protein levels in p23KD HeLa cells (Figure 3.7B). Transient expression of the HA fusion of GSK3β in p23KD HeLa cells decreased the β-catenin protein levels as expected. However, the AHR protein levels were not altered (Figure 3.7C).

Consistently, LiCl caused an increase of the β-catenin protein levels in p23 KD HeLa cells (Figure 3.7D) but elicited no effect on the AHR protein levels. To further confirm that p23 is essential for the GSK3β effect on AHR, we examined whether this GSK3β effect could be observed in p23 knockdown cells if the p23 levels were restored. We transiently transfected the plasmid expressing the GFP fusion of p23 into p23 stable knockdown HeLa cells and confirmed the expression of p23-GFP (Figure 3.7E). After transient expression of p23-GFP, the AHR protein levels became sensitive to 20 μM TDG treatment: the AHR protein levels were increased to 1.8-fold, which was similar to the response we observed in wild type HeLa cells (Figure 3.2), supporting that this AHR regulation by GSK3β is p23-dependent.
**Figure 3.7.** Down-regulation of p23 in HeLa cells causes AHR not responsive to GSK3β regulation. (A) AHR protein levels were not altered by 8-h treatment with 10 μM or 20μM TDG in p23 stable knockdown (p23KD) HeLa cells. Treatment with 40 μM TDG increased AHR protein levels to 1.3-fold. The images are representative of the replicate data (means ± SD, n = 3). DMSO group (treated for 8 h) was arbitrarily set as one (with no error bar) for data normalization. One-way ANOVA with Dunnett...
multiple comparisons test was performed to determine statistical significance. (B) Treatment with 5 mM LiCl for 6 h did not alter AHR protein levels in p23KD HeLa cells. The images are representative of the replicate data (means ± SD, n = 3). No LiCl (no treatment) group was arbitrarily set as one (with no error bar) for data normalization. Unpaired t-test with Welch’s correction was used to determine the statistical significance. (C) β-catenin protein levels decreased while AHR protein levels showed no significant difference after transient expression of HA-GSK3β (72 h after transient transfection) in p23KD HeLa cells. The images are representative of the replicate data (means ± SD, n = 3). No plasmid group (NP) represents cells undergoing the same transient transfection protocol without the HA-GSK3β expressing plasmid and was arbitrarily set as one (with no error bar) for data normalization. Unpaired t-test with Welch’s correction was used to determine the statistical significance. (D) β-catenin protein levels increased in p23KD HeLa cells after treatment with 5 mM LiCl for 6 h. The images are representative of the replicate data (means ± SD, n = 3 in one experiment, which was repeated once with similar results). No LiCl (no treatment) group was arbitrarily set as one for data normalization. Unpaired t-test was used to determine the statistical significance. (E) p23KD HeLa cells were transiently transfected with the plasmid expressing GFP or GFP-p23. Transient expression of GFP-p23 caused AHR protein levels to increase when cells were treated 20 μM TDG for 8 h. The images are representative of the replicate data (means ± SD, n = 3 in one experiment, which was repeated once with similar results). DMSO treatment group of GFP-transfected cells was arbitrarily set as one for data normalization. Two-way ANOVA with Sidak multiple comparisons test was performed to determine statistical significance. For (A–E), each Western lane contained 30 μg of whole-cell lysate. Data in A were normalized by β-actin (as shown) whereas the rest of the data were normalized by total protein stain.

GSK3β-Mediated AHR Phosphorylation Is Necessary for Optimal Activation of the AHR Target Gene Transcription in Human Cervical, Liver, and Breast Cancer Cells

Next, we examined whether the AHR function could be regulated by GSK3β. To do this, we measured the prototypical induction of the cytochrome P450 1A1 gene transcription by an AHR ligand in HeLa cells in the presence or absence of TDG. We observed that the cytochrome P450 1A1 (cyp1a1) transcript was induced in HeLa cells, as expected, by two well-known AHR ligands beta-naphthoflavone (βNF) and 3-methylcholanthrene (3MC) to 5- and 19-fold, respectively, and this induction was suppressed effectively by a classical AHR antagonist CH223191.
However, TDG alone did not alter the cyp1a1 transcript levels but significantly suppressed the βNF (5-fold to 3-fold) and 3MC (19-fold to 7-fold) dependent cyp1a1 gene transcription. The effect of TDG and CH223191 was synergistic, which was particularly apparent in the case of the 3MC-induced cyp1a1 gene transcription. This observation suggested that TDG and CH223191 likely inhibit the AHR function via different mechanisms. Downregulation of GSK3β expression via a GSK3β specific shRNA in HeLa cells significantly suppressed the ability of an AHR ligand (βNF, benzo[a]pyrene (BaP) or 3MC) to induce the cyp1a1 gene transcription (Figure 3.8C-E). Furthermore, knockdown of GSK3β abolished the TDG suppression of the 3MC-mediated AHR activity (Figure 3.8F). In addition to what we observed in HeLa cells, this suppression of the 3MC activation of cyp1a1 gene transcription by TDG was also observed in Hep3B (180-fold to 92-fold) and MCF-7 (1864-fold to 961-fold) cells (Figure 3.8G,H). Collectively, our results showed that phosphorylation of AHR by GSK3β is essential for optimal AHR activity in terms of the ligand-activated gene transcription.
Figure 3.8. Reduction of GSK3β activity suppresses the ligand-dependent activation of AHR target gene transcription. For A and B, HeLa cells were treated with 40 μM TDG for 9 h. At 5-h post-TDG treatment, cells were treated with either (A) 10 μM βNF, 10 μM CH223191 or both or (B) 1 μM 3MC, 10 μM CH223191 or both. TDG were able to partially block the increase of the cyp1a1 message levels triggered by βNF and 3MC. Co-treatment with TDG with CH223191 further
decreased the *cyp1a1* message levels. For A and B, the graphs represent replicate data (means ± SD, n = 3; n = 4 for DMSO, TDG + 3MC and TDG + 3MC + CH223191 groups in 3.8B) and DMSO group was arbitrarily set as one (with no error bar) for data normalization. HeLa cells were transiently transfected for 70 h with the plasmid carrying scramble shRNA or GSK3β specific shRNA. Cells were then treated with (C) DMSO or 10 μM βNF, (D) DMSO or 5 μM BaP, (E) DMSO or 1 μM 3MC for 4 h. Cells were harvested at 74-h post transfection/4-h post ligand treatment. The increase level of the *cyp1a1* message after ligands treatment were significantly lower in GSK3β knockdown groups. For C to E, graphs represent replicate data (means ± SD, n = 3) with DMSO group arbitrarily set as one (with no error bar) for data normalization. (F) 7.5-h treatment with TDG (40 μM, treated at 70–77.5 h post-transfection) could not suppress the 3MC (1 μM, 3.5 h, treated at 74–77.5 h post-transfection) induced *cyp1a1* messages in GSK3β shRNA transfected HeLa cells as observed in scramble shRNA transfected HeLa cells. The graph represents the replicate data (means ± SD, n = 3) with 3MC groups minus TDG were arbitrarily set as one (with no error bar) for data normalization. Hep3B (G) and MCF-7 (H) cells were treated 40 μM TDG for 9 h. At 5-h post-TDG treatment, cells were treated with 1 μM 3MC for 4 h. The induced *cyp1a1* message levels were suppressed by TDG in both cell lines. The graphs in G and H represent replicate data (means ± SD, n = 3) with DMSO group arbitrarily set as one (with no error bar) for data normalization. For (A–H), one-way ANOVA with Sidak multiple comparisons test was performed to determine statistical significance.

**Discussion**

GSK3β generally regulates its substrate stability (via phosphorylation) by promoting the binding of E3 ligases, such as β-transducin repeat containing proteins (β-TrCP), and eventually targeting its substrates for proteasomal degradation (Beurel et al., 2015a). We previously discovered that without ligand treatment, AHR protein levels are regulated by selective autophagy while inhibition of proteasomal degradation by MG132 further decreases the AHR protein content (Yang & Chan, 2020). This MG132 effect on AHR has also been observed with the GSK3β-mediated AHR degradation which involved the formation of the K63-linked ubiquitination of AHR, revealing the flexibility of degradation mechanisms that can be triggered by GSK3β phosphorylation.
GSK3β is involved in the regulation of mammalian circadian rhythm (Iitaka, Miyazaki, Akaike, & Ishida, 2005). In particular, there are at least two PAS proteins, namely PER2 and CLOCK, that are GSK3β substrates (Iitaka et al., 2005; Spengler, Kuropatwinski, Schumer, & Antoch, 2009). These proteins are in the same family as AHR, making it intriguing that AHR is also regulated by GSK3β. Another notable GSK3β substrate is β-catenin. There are many aspects of functional dependence between AHR and β-catenin. AHR has been shown as a β-catenin target gene in prostate cancer cells so that increased β-catenin activity leads to increased AHR expression (Chesire, Dunn, Ewing, Luo, & Isaacs, 2004). However, increased AHR function promotes proteasomal degradation of β-catenin since AHR is a E3 ligase which recognizes β-catenin as one of its substrate proteins for degradation (Kawajiri et al., 2009). Functionally speaking, AHR and β-catenin operate synergistically to activate the cyp1a1 gene transcription (Braeuning, Köhle, Buchmann, & Schwarz, 2011; Procházková et al., 2011). Interestingly, both AHR and β-catenin activities are controlled by protein degradation mechanisms: AHR undergoes proteasomal degradation after ligand activation whereas β-catenin undergoes proteasomal degradation, limiting its transactivation of the Wnt target genes. Since AHR contains several stretches of serine and threonine residues in its transactivation region which are similar to the GSK3β phosphorylation sites of β-catenin, we explored whether GSK3β would play any role in AHR degradation. Surprisingly, but interestingly, GSK3β causes AHR degradation via autophagy rather than the ubiquitin-proteasome system. In an effort to validate the degradation mechanism, we captured proteasomal degradation using MG132 by proving that the levels of β-catenin were restored upon MG132 treatment. In contrast, MG132 caused reduction of the AHR levels when GSK3β was inhibited by TDG, clearly supporting a mechanism that is
different from β-catenin degradation. This reduction is explained by the fact that MG132 can trigger autophagy (Bao, Gu, Ta, Wang, & Xu, 2016), resulting in further degradation of AHR. One may argue that accumulation of AHR could still be proteasome-mediated since increased p62 levels, which is caused by an autophagy inhibitor such as CQ, has been shown to increase the half-life of proteasome substrates (Korolchuk, Mansilla, Menzies, & Rubinsztein, 2009b). However, while such substrates accumulate after MG132 treatment (Korolchuk et al., 2009b), AHR did not, supporting that AHR undergoes lysosomal degradation via autophagy. We observed a higher autophagic flux in HA-GSK3β expressing HeLa cells. GSK3β phosphorylates HIV Tat-interactive protein 60 kDa (TIP60), which in turn activates Unc-51 like kinase-1 (ULK1), a key autophagy-related protein that plays an important role in autophagy initiation. Through this GSK3β-TIP60-ULK1 pathway, autophagy is induced under ER-stress or growth factor deprivation (Lin et al., 2012; Nie et al., 2016). However, if the GSK3β-mediated degradation of AHR were merely caused by induction of autophagy, p23 knockdown HeLa cells would have been more pronounced in the suppression of the AHR levels since AHR is more prone to degradation via autophagy when p23 is down-regulated (Yang & Chan, 2020). On the contrary, the GSK3β-mediated AHR degradation is less apparent when the p23 levels are downregulated. The relationship between GSK3β and p23 in affecting AHR degradation is reminiscent of the role of Axin, which is part of the β-catenin destruction complex, in facilitating the GSK3β-mediated degradation of β-catenin (Kim & Jho, 2010). Perhaps p23 plays a role in conforming the structure of AHR to be recognizable by GSK3β. Collectively, our results are consistent with the conclusion that GSK3β phosphorylates AHR in a p23-dependent manner and in turn causes its lysosomai degradation, which is heightened by active GSK3β.
The primary sequence of AHR has multiple locations containing two or more serine and threonine residues that are 3–4 amino acids apart—the minimal requirement for GSK3β recognition. Among them, there are three AHR regions that match the consensus GSK3β targeting sequence the best. We performed mutational analysis to convert every serine or threonine residue into alanine in all three (M1-M3) regions. Results from our deletion studies using CΔ553 (amino acid 1–295) and NΔ515 (amino acid 516–848) revealed that amino acids between 296 and 515 are likely essential for the GSK3β-mediated degradation of AHR since both deletion construct levels were unaltered upon TDG treatment. We, however, cannot rule out the possibility that protein folding could be distorted in these deletion constructs, preventing them to be recognized for lysosomal degradation. Nevertheless, since M1 is located within this 296–515 region, we initially predicted that M1, but not M2 and M3, would contain the GSK3β phosphorylation sites. We also realized that more than one region could be phosphorylated by GSK3β since Shaggy, a Drosophila orthologue of GSK3β, phosphorylates Cubitus interruptus (Ci) at two distinct regions that are more than 30 amino acids apart (J. Jia et al., 2002). Interestingly, all three mutants (M1-M3) showed some resistance to GSK3β inhibition by TDG with the order of M2 > M3 ~ M1, suggesting that M2 (rather than M1) likely contains the GSK3β phosphorylation sites. Moreover, all three mutants showed significant, but not complete, resistance to lysosomal degradation by CQ, suggesting that all three regions could mediate AHR lysosomal degradation. GSK3β phosphorylation often works in conjunction with another kinase, since GSK3β substrates are usually “primed” by a kinase, followed by GSK3β phosphorylation (Beurel, Grieco, & Jope, 2015b). However, the interplay among kinases targeting M1-M3 is much more complicated since the reported “primed” kinase site is only few
amino acids away from the GSK3β site, which cannot explain how M1–3 sites could mutually affect one another. We cannot rule out the possibility that M1 and M3 regions could be targeted by a kinase which might somehow direct AHR toward lysosomal degradation, or abrogation of phosphorylation at the M1/M3 mutated regions by a kinase might cause a conformational change that would interfere with the GSK3β phosphorylation at the M2 region. On the contrary, phosphorylation of AHR by GSK3β could cause a conformational change that would allow another kinase to further phosphorylate AHR.

Inhibition of extracellular signal-regulated (Erk) kinase by inhibitors, such as U0126 and PD98059, increases the AHR protein levels in mouse liver cancer Hepa1c1c7 cells (S. Chen, Operaña, Bonzo, Nguyen, & Tukey, 2005). Expression of a constitutively active form of MEK1 in Hepa1c1c7 cells reduces the AHR protein levels by promoting the Erk kinase-dependent phosphorylation of AHR. Interestingly, these researchers showed that the ligand-dependent function of AHR is suppressed by these Erk kinase inhibitors, similar to what we observed when GSK3β is inhibited or downregulated. However, it is apparent that these Erk inhibitors can physically interfere with the action of a more potent AHR ligand since U0126 is an AHR activator whereas PD98059 is an AHR antagonist (S. Chen et al., 2005). In our case, suppression of the AHR transcriptional activation is likely mediated directly through the action of GSK3β in phosphorylating AHR, since inhibition of GSK3β (by TDG) and downregulation of GSK3β showed similar degrees of suppression. The treatment with TDG in the GSK3β knockdown HeLa cells did not further suppress the AHR transactivation function. Moreover, the putative phosphorylation sites (M1-M3) of AHR overlap with its region required for physical recruitment of coactivators, making it plausible that phosphorylation positively affects the transcriptional
activation of AHR (S. Chen et al., 2005). Our results reveal interesting insights on how AHR signals: AHR is more active when it is phosphorylated; in other words, phosphorylation is a means to modulate AHR function. In addition, phosphorylation of AHR is also a trigger for its own lysosomal degradation, i.e., it acts as an “off” switch since the half-life of the phosphorylated AHR is governed by lysosomal degradation (Figure 3.9). AHR phosphorylation may work in conjunction with the proposed “off” switches of AHR—proteasomal degradation of AHR after ligand activation (Davarinos & Pollenz, 1999) and upregulation of the AHR repressor (ahrr) gene transcription by AHR (Evans et al., 2008).

**Figure 3.9.** A proposed model of GSK3β role on AHR function and degradation. AHR is phosphorylated by GSK3β in a p23-dependent manner in HeLa cells. This phosphorylation is required for optimal activation of the ligand-dependent AHR target gene transcription. After phosphorylation, AHR is K63-ubiquitinated and is targeted for the LC3-mediated selective autophagy. When the p23 content is compromised in HeLa cells, AHR is more prone to degradation via autophagy, bypassing the GSK3β phosphorylation of AHR.

**Conclusions**

Phosphorylation of AHR by GSK3β occurs in HeLa cells even without exogenous ligand treatment. After the GSK3β-mediated phosphorylation, AHR
undergoes lysosomal degradation and is more active in the ligand-activated gene transcription. Inhibition of GSK3β activity by TDG suppresses the transcriptional activation function of AHR in at least HeLa, Hep3B, and MCF-7 cells, suggesting that the role of GSK3β in AHR modulation is likely a general mechanism across many human cell types.

**Materials and Methods**

**Reagents**

TDG, CQ, CH223191, 3MC, βNF, polybrene, N-ethylmaleimide (NEM), and BaP were purchased from Sigma (St. Louis, MO, USA). LiCl was purchased from Fisher scientific (Rockford, IL, USA). K63-TUBE-biotin, PR-619, and 1,10-phenanthroline were purchased from LifeSensors (Malvern, PA, USA). MG132 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Puromycin was purchased from Gold Bio-technology (St. Louis, MO, USA). GSK3β shRNA, p23 shRNA, and scramble shRNA were purchased from Dharmaco (Lafayette, CO, USA). HA-GSK3β expressing plasmid (1015) was a gift from Scott Friedman (Addgene plasmid # 49491; http://n2t.net/addgene:49491; RRID: Addgene_49491) (Lang et al., 2013). pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid # 8454; http://n2t.net/addgene:8454; RRID: Addgene_8454). pCMV-dR8.2 dvpr was a gift from Bob Weinberg (Addgene plasmid # 8455; http://n2t.net/addgene:8455; RRID: Addgene_8455). The codon humanized pGFP2-N2 plasmid was purchased from BioSignal Packard (Montreal, QC, Canada). EndoFectin transfection reagent was purchased from GeneCopoeia (Rockville, MD, USA). ZymoPURE II Plasmid Maxiprep kit and Direct-zol RNA Miniprep kit were purchased from Zymo Research (Irvine, CA, USA). MMLV high-performance reverse transcriptase was purchased from Epicentre (Madison, WI, USA). iTaq Universal SYBR Green Supermix was
purchased from Bio-Rad (Hercules, CA, USA). QuikChange Lightning site-directed mutagenesis kit (210518) was purchased from Agilent (Santa Clara, CA, USA). Phos-tag acrylamide (AAL-107) was purchased from FUJIFILM Wako Chemicals (Richmond, VA, USA). λ-PP, anti-GSK3α/β mouse IgG (0011-A), anti-β-catenin mouse IgG (15B8), and anti-GFP mouse IgG (B-2) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Protein G Dynabeads and anti-p23 antibody (JJ3) were purchased from ThermoFisher Scientific (Rockford, IL, USA). Anti-AHR SA-210 rabbit polyclonal antibody was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Anti-β-actin mouse monoclonal antibody (AM4302) was purchased from Ambion (Austin, TX, USA). Anti-LC3B rabbit IgG (L7543) and IgG from rabbit serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitrocellulose membrane, REVERT 700 Total Protein Stain for Western blot normalization, IRDye 800CW, IRDye 680 anti-rabbit, anti-mouse secondary antibody, and IRDye 800CW streptavidin were purchased from LI-COR Bioscience (Lincoln, NE, USA). GlutaMAX-I, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). HyClone FBS and HyClone DMEM were purchased from Fisher scientific (Rockford, IL, USA).

Cell Culture

HeLa and Hep3B cell lines were authenticated by ATCC. MCF-7 cell line was obtained from ATCC. AD-293 cells were obtained from Agilent Technologies (Santa Clara, CA, USA). All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM GlutaMAX-I, 10 U/mL of penicillin, and 10 mg/mL of streptomycin at 37 °C and 5% CO₂.
**Generation of p23 Stable Knockdown Cells**

We used our previously published protocol (Yang & Chan, 2020) to generate new p23 stable knockdown HeLa cells for this study. In brief, on day 1, AD-293 cells (about $7 \times 10^5$ cells) were seeded in 5 mL of DMEM (10% FBS) without antibiotics in a 25 cm² flask and incubated at 37 °C and 5% CO₂ overnight. On day 2, cells should reach 50–80% confluence and in fresh medium without antibiotics. Transfection was performed in the late afternoon using EndoFectin transfection reagent (2:1 DNA ratio) with the plasmid cocktail as follows: 2.5 μg of p23 shRNA plasmid (#1475), 1.875 μg of the pCMV-dR8.2 dvpr packaging plasmid, and 0.625 μg of the VSV-G envelope plasmid. Fresh complete medium was exchanged 15 h after transfection (day 3). After 24 h, medium which contained the virus was collected (day 4) and stored at 4 °C. Another 5 mL of fresh complete medium was added to cells and was collected 24 h afterwards (day 5). The combined medium was centrifuged at 400× g for 5 min to remove any AD-293 cells that were inadvertently collected. The resulting supernatant was used for infection. Lentiviral infection of HeLa cells was performed by first seeding cells in a 75 cm² flask to 50–70% confluence. Fresh complete medium containing 8 μg/mL of polybrene was exchanged. Supernatant containing lentiviral particles (0.5 mL) was then added. The fresh complete medium was exchanged 24 h after infection. The selection was started by adding 1.5 μg/mL of puromycin 48–54 h after infection. Western analysis was performed to determine the p23 protein levels after 2–3 passages.

**Transient Transfection**

Cells were grown in 6-well plates overnight (16–18 h) and transfection was initiated when cells were about 90–95% confluence. Cells were transfected with 3 μg of plasmid (4 μg for HA-GSK3β) and 6 μL of EndoFectin reagent (8 μL for HA-
GSK3β). Fresh complete medium was exchanged 24 h after transfection. HA-GSK3β and GSK3β shRNA plasmids were transfected for 72 h. Usually, we initiated cell treatment at 70 h post transfection. pGFP plasmids were transfected for 48 h with cell treatment initiated at 48 h post transfection.

**Whole-Cell Lysate Preparation**

Cells were harvested using cold 1xPBS by mechanical scraping. Cell pellets were resuspended using cold lysis buffer (25 mM HEPES, pH 7.4, 0.4 M KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% NP-40, 1 mM PMSF, and 2 μg/mL of leupeptin) of 3 times the volume of cell pellets. After three cycles of freeze/thaw, lysates were kept on ice for 30 min and were then centrifuged at 16,000× g for 10 to 20 min at 4 °C. The supernatants were defined as whole-cell lysates and were subjected to BCA assay to determine the protein concentration.

**Western Blot Analysis**

The protocol for Western blot analysis was described previously (Yang & Chan, 2020) with minor modification. In brief, if 15% instead of 10% acrylamide gels were used, they were transferred via full submersion for an additional one hour (3 h total) at 4 °C. After the wet transfer, total protein staining was performed using LI-COR REVERT Total Protein Stain. Membranes for the examination of LC3B levels were dried for at least 40 min and wet with PBS before blocking. The transferred nitrocellulose membranes were blocked in PBS with 5% BSA for 1 h. Dilutions for antibodies were as follows: 1:1000 for anti-p23 JJ3 and anti-LC3B L7543; 1:2000 for anti-AHR SA210; 1:5000 for anti-β-actin AM4302; 1:200 for anti-GSK3β 0011-A and anti-β-catenin 15B8. If not specified, Western bands were normalized using total protein stain. Results were obtained and analyzed using a LI-COR Odyssey CLx imaging system.
RT-qPCR

RT-qPCR was performed as described previously (Pappas et al., 2018). In brief, when HeLa cells in a 75 cm² flask reached 90–100% confluence, 5% of cells (0.3 mL of 6 mL total cell suspension) were seeded onto each well of a 6-well plate. After incubation for 16–18 h, cells were treated with different reagents or transfected with the GSK3β shRNA at 90–95% confluence. For Hep3B and MCF-7 cells, when cells in 75 cm² flask reached 95% confluence, 8.3% of cells (0.5 mL of 6 mL total cell suspension) were seeded onto each well of a 6-well plate. After incubation for about 24 h, cells reached 80–90% confluence and were treated with different reagents. After treatment, media were aspirated, and RNA was extracted using the Direct-zol kit with TRI reagent. Reverse transcription was performed for 1 μg of RNA using Epicentre MMLV reverse transcriptase. Quantitative PCR was performed with: 1 μL of cDNA from reverse transcription solution, 10 μL of Bio-Rad iTaq SYBR green supermix, and 0.8 pmol sequence-specific primers (cyp1a1 primers are OL109, 5’-GGC CAC ATC CGG GAC ATC ACA GA-3’ and OL110, 5’-TGG GGA TGG TGA AGG GGA CGA A-3’; β-actin primers are OL101, 5’-CCA CAC TGT GCC CAT CTA GG-3’ and OL102, 5’-AGG ATC TTC ATG AGG TAG TCA GTC AG-3’ ) using a Bio-Rad CFX Connect real-time PCR machine with the following protocol: 40 cycles of 90 °C for 10 s/60 °C for 1 min with fluorescence readings taken at 60 °C. The 2^−ΔΔCq method (Livak & Schmittgen, 2001) was used to present the normalized values.

Immunoprecipitation and K63-TUBE Far-Western Analysis

HeLa cells were seeded and cultured overnight in 6-well plates. Transfection of the GSK3β shRNA was initiated when cells reached ~90% confluence and was continued for 72 h. Immunoprecipitation was performed as described previously.
(Yang & Chan, 2020). In brief, cells were lysed as described under 4.5 with 4 times the volume of pellet size. Three deubiquitylase inhibitors, namely 1,10-phenanthroline (5 mM), NEM (10 mM), and PR-619 (50 μM) were added in the lysis buffer for immunoprecipitation experiment with K63-TUBE Far-western analysis.

About 1.5–2.0 milligrams of whole-cell lysates were used for immunoprecipitation of AHR using the anti-AHR SA210 antibody (1:200 by volume) for 30 min at room temperature. The pre-equilibrated Protein G Dynabeads (1:200 by volume) were then added to each sample with the assay buffer: 25 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1% Tween-20, and 1 mg/mL of BSA. The samples were incubated with a rotation of 60 rpm overnight at 4 ºC. The beads were then washed three times for 5 min each with the assay buffer and then eluted with electrophoresis sample buffer for SDS-PAGE, followed by Far-western analysis. K63-TUBE biotin (1:1000) were incubated with the nitrocellulose membrane for 1 h at room temperature and then with IRDye-800 conjugated streptavidin (1:10,000) for 2 h at room temperature. The wash step between incubation was the same as in Western analysis. Results were obtained and analyzed using a LI-COR Odyssey CLx imaging system.

**Phos-Tag Gel SDS-PAGE**

Whole-cell lysates were treated with 0, 80 units (1 μL), or 400 units (5 μL) of λ-PP for 2, 30, or 60 min at 30 °C. To detect the phosphorylated AHR and β-catenin, a gel mix containing 7.5% acrylamide, 20 μM phos-tag reagent, and 80 μM ZnCl₂ was prepared to make the phos-tag acrylamide gel, according to the manufacturer’s protocol. Bis-Tris SDS-PAGE system was used with MOPS as the running buffer. SDS-PAGE were performed at 4 °C; 55 volts for the stacking gel and 110 volts for
the resolving gel. Wet transfer was performed at 4 °C for 4 h. Western blot analysis was performed as described above.

Table 1
*M1, M2, and M3 Using the pGFP2-N2-AHR Plasmid as the Template for QuikChange Mutagenesis*

<table>
<thead>
<tr>
<th>Target sequence</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>S436A-S440A-S444A</td>
<td>Forward (OL904): 5'-a aat ggc act gct gga aaa gac gct gct acc aca gcc act cta agc aag g-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse (OL905): 5'-c ttg gct tag agt ggc tqt ggt agc agc gtc ttt tcc agc agt gcc att t-3'</td>
</tr>
<tr>
<td></td>
<td>Forward (OL906): 5'-gag ttc ccc tac aaa gct gaa atg gat gct atg cct tat gca cag aac tt att tcc-3'</td>
</tr>
<tr>
<td>S689A-S693A-T697A</td>
<td>Reverse (OL907): 5'-gga aat aaa gtt ctg tgc ata agg cat agc atc cat ttc agc ttt gta ggg gaa ctc-3'</td>
</tr>
<tr>
<td></td>
<td>Forward (OL908): 5'-c tac cct atg ggg gct ttt gaa cca gcc cca tac ccc gct act tct agt t-3'</td>
</tr>
<tr>
<td>S723A-S727A-T731A</td>
<td>Reverse (OL909): 5'-a act aga agt agc ggg gta tgg ggc tgg ttc aaa agc ccc cat agg gta g-3'</td>
</tr>
<tr>
<td>AHR Sequencing part 1</td>
<td>OL859: 5'-TGGCACCAAAAATCAACGG GACTT-3'</td>
</tr>
<tr>
<td>AHR Sequencing part 2</td>
<td>OL374: 5'-CGCCAACATCACCCTACGCAGTC-3'</td>
</tr>
<tr>
<td>AHR Sequencing part 3</td>
<td>OL375: 5'-CTCCACCTTCAGCCACCACCCCATCATAC-3'</td>
</tr>
<tr>
<td>AHR Sequencing part 4a (For M1, M2 and M3)</td>
<td>OL376: 5'-AGGTCAACCCAGGGCTTTCAGG-3'</td>
</tr>
<tr>
<td>AHR Sequencing part 4b (For M2 confirmation)</td>
<td>OL910: 5'-GGTCACCCAGGGCTTTTTC-3'</td>
</tr>
<tr>
<td>AHR Sequencing part 5a (For M1, M2 and M3)</td>
<td>OL858: 5'-TCGCCGGACACGCTGAAC-3'</td>
</tr>
<tr>
<td>AHR Sequencing part 5b (For M2 confirmation)</td>
<td>OL911: 5'-GCCTTTCAATTGTCCACACG-3'</td>
</tr>
</tbody>
</table>
Site-Directed Mutagenesis

QuikChange II lightning kit was used to perform site-directed mutagenesis. All the procedures were performed strictly according to the manufacturer’s protocol. For the mutant strand synthesis reaction, 10 ng of the pGFP2-N2-AHR plasmid (J. Chen et al., 2021) were used as the template. Primers are listed in Table 1. The full-length cDNA sequences of all mutant plasmids were confirmed by sequencing (Functional Biosciences, Madison, WI).

Statistical Analysis

GraphPad Prism 9 software (La Jolla, CA, USA) was utilized for statistical analysis. Two-tailed unpaired t-test and one-way and two-way (or mixed-model) ANOVA with Sidak, Tukey or Dunnett tests for multiple comparisons were used to determine statistical significance with * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, and ns, not significant (p > 0.05). Some specific detail is also mentioned in the figure legends.
My projects focus on the following two questions: What are the mechanisms that control AHR protein levels and what is the role of p23 in AHR regulation. Previously, our lab discovered that knocking down of AHR co-chaperone p23 decreased AHR protein levels in mouse and human cell lines. I confirmed this decreased AHR protein levels caused by p23 knockdown in HeLa cells and used HeLa cells to study the degradation mechanism of basal AHR proteins. Cycloheximide (CHX) experiments showed that the degradation rate of AHR in p23KD HeLa cells is significantly higher than WT HeLa cells (Pappas et al., 2018). Treatment with proteasome inhibitor MG132 did not increase but further decreased AHR protein levels, suggesting another major protein degradation method: autophagy-lysosomal degradation. In chapter 2, I characterized and confirmed the key steps of selective autophagy in AHR protein degradation, including K63-ubiquitination of AHR, binding of AHR protein to autophagy adaptor protein p62 and autophagosome membrane-bound protein LC3B-II in vitro and in situ. Stable knockdown of LC3B also confirmed its involvement in AHR protein degradation. Compared to WT HeLa cells, p23KD HeLa cells showed a significantly higher autophagic flux, confirming the protective role of p23 in AHR degradation through selective autophagy. Another four human cancer cells lines also showed increase of AHR protein levels upon CQ treatment and the interaction between AHR and LC3B, as well as AHR and p62. Comparing to the other four cells lines, the interaction between AHR and LC3B in A549 cells is not as sensitive to CQ treatment as other cell lines.
I also sought for AHR regulation mechanism from post-translational modification perspective. With the reports on the correlation between AHR and classical GSK3β substrate β-catenin, and the multiple putative GSK3β recognition motif in AHR cDNA sequence, I investigated the role of GSK3β phosphorylation in AHR protein regulation. Alteration of GSK3β activity and expression levels can affect AHR protein levels. This regulation is mediated through autophagy-lysosomal degradation pathway. Surprisingly, GSK3β regulation is not the reason that cause increased AHR protein degradation in p23KD HeLa cells. Instead, p23KD HeLa cells are not sensitive to GSK3β modulation, which is AHR-specific and p23-dependent. Phosphorylation of AHR was confirmed using Phos-tag gel, showing that the intensities of putative phosphorylated AHR bands were subject to the control of λ-phosphatase dephosphorylation and GSK3β activity. Moreover, phosphorylation of AHR enhanced the transactivation function of AHR. Phosphorylation is not only the signal for ubiquitination and subsequent degradation, but it also optimizes AHR function as a transcription factor.

Based on the current data, there are several questions that need future attention: (1) More data are needed to confirm the phosphorylation sites of AHR. An in vitro GSK3β phosphorylation assay followed by LC-MS detection would be helpful to confirm the GSK3β-dependent phosphorylation of AHR and verify the proposed phosphorylation sites, which may provide more insights to explain the responses of two truncated AHR and three AHR mutations to GSK3β inhibition and autophagy degradation inhibition. (2) Is priming required for GSK3β phosphorylation of AHR? If so, what kinase(s) are responsible for the priming? (3) Other than the transactivation function of AHR, does phosphorylation affect other AHR roles, such as E3 ligase for ER and β-catenin? (4) What is the E3 ligase responsible for recognition of
phosphorylated AHR and the transfer of ubiquitin molecules to AHR? By targeting this ubiquitination step, can AHR protein level be specifically affected and thus help with AHR-related disease situations? (5) What is the underlying mechanism that p23 can protect AHR from selective autophagy and why lower p23 content leads to insensitivity of AHR to GSK3β regulation? p23 is a highly conserved acidic phosphoprotein (Garcia-Ranea, Mirey, Camonis, & Valencia, 2002). It was first discovered as the co-chaperone of HSP90 in the progesterone receptor complex (Johnson, Beito, Krco, & Toft, 1994), and has been found in a variety of HSP90 client protein complexes. As a co-chaperone protein, it is reported to help prevent protein aggregation in *in vitro* studies (Bose, Weikl, Bügl, & Buchner, 1996; Freeman, Toft, & Morimoto, 1996). One hypothesis is that p23 may protect AHR from aggregation given the fact that the poly-Q region, as in AHR C-terminal sequence, renders the protein more prone to aggregation and hence cleared by autophagy. More data on aggregation of AHR proteins would be useful to test this hypothesis. It is also possible that p23 can act as a scaffold protein like Axin in β-catenin construction complex. The interaction among p23, AHR and GSK3β needs to be further confirmed. (6) Data in chapter 2 together with results showed by my colleague Jinyun Chen and I (J. Chen et al., 2021) supported that AHR in triple negative breast cancer cells can interact with both LC3B and LAMP2A. However, data showed that there is robust CMA going on in HeLa cells using CMA substrate GAPDH as positive control (figure 4.1A left), but AHR in HeLa cells is not affected by CMA inducer 6AN (figure 2.8) and the interaction with CMA key protein LAMP2A (figure 4.1A right) is also much weaker comparing to that of GAPDH. AHR in HeLa cells is not sensitive to LAMP2A anti-sense RNA transfection, which cannot decrease LAMP2 protein levels either (could be technical; may consider using LAMP2A-specific shRNA for
knockdown). The LAMP2 bands of WT HeLa cells showed lower position compared to that of MDA-MB-468 cells (figure 4.1B). The signals that trigger different mechanisms of autophagy for AHR degradation remains to be further investigated.

Figure 4.1. AHR in HeLa cells is not controlled by chaperone-mediated autophagy (CMA). Proximity ligation assay results showed that (A left panel) GAPDH interacted with LAMP2 and this interaction is significantly enhanced by treatment with CMA activator 6-aminonicotinamide (6-AN, 100 μM) for 24 h while (A right panel) AHR showed minimal interaction with LAMP2. (B) LAMP2 anti-sense RNA transfection (55-hour) cannot alter LAMP2 nor AHR protein levels in HeLa cells. NT, no transfection; Anti/Endo, LAMP2A anti-sense RNA/EndoFectin transfection; Empty/Endo, empty plasmid/EndoFectin transfection; Anti/Eco, LAMP2A anti-sense RNA/EcoTransfect transfection; Empty/Eco, empty plasmid/EcoTransfect transfection.


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The materials and methods used in each chapter are described individually. Some details regarding some basic techniques are listed below.

**Generation of HSP90, p23, and LC3B Stable Knockdown Cells**

p23 knockdown HeLa cells were generated using p23-specific shRNA (#1475); HSP90 knockdown HeLa cells were generated using the HSP90α-specific shRNA (#6, 8563), and LC3B knockdown HeLa cells were generated using the MAP1LC3B-specific shRNA #153286 and #151769. In essence, AD-293 cells (about $7 \times 10^5$ cells) were plated in 5 mL of medium without antibiotics in a 25 cm$^2$ flask. After incubation at 37 °C and 5% CO$_2$ overnight, cells reached 50%–80% confluence. Fresh medium without antibiotics was exchanged. Cells were transfected using EndoFectin reagent (2:1 DNA ratio) with the plasmids cocktail as follows: 2.5 µg of specific shRNA plasmid, 1.875 µg of the pCMV-dR8.2 dvpr packaging plasmid, and 0.625 µg of the VSV-G envelope plasmid. Fresh complete medium was exchanged 15 h later. Medium which contained the virus was collected after 24 hours and stored at 4 °C. Another 5 mL of fresh complete medium was added to cells and was collected 24 h afterwards. The combined medium was centrifuged at 400 g for 5 min to remove any AD-293 cells that were inadvertently collected. The resulting supernatant was used for infection. Store the virus-containing medium at 4 °C for short term use. Aliquot the media into 1.5 mL tubes and freeze down in -80 °C for long term storage. After taken out from -80 °C, thaw on ice (can only be frozen and thawed once).

The infection of HeLa cells was performed by first seeding cells in a 25 cm$^2$ flask to 50%–70% confluence. Fresh complete medium containing 8 µg/mL of
polybrene (dissolved in water; sealed and stored in 4 \(^\circ\)C) was exchanged. Supernatant containing lentiviral particles (0.5–1 mL) was then added. The fresh complete medium was exchanged 24 h after infection. The selection was started by adding 1.5 µg/mL of puromycin 48–54 h after infection. Include one flask of wild type cells for selection as negative control. For other cell lines, determine the optimal puromycin concentration in advance: Seed cells in 12-well plates and culture overnight to reach about 80–90% confluence. Do serial dilution of puromycin at the range of 1-10 µg/mL. Check the cells every day and change fresh media containing puromycin if needed. The optimal concentration should be the one that kill all the cells within 3-5 days. Western analysis was performed to determine the target protein levels after 2–3 passages.

**Transient Transfection**

Cells were grown in 6-well plates and transfection was initiated when cells were about 90% confluence (after 16-18 hours culture for the best). The DNA, EndoFectin transfection reagent and opti-MEM should be taken out in advance and warmed to room temperature. Cells were transfected with 2.5 µg of DNA and 5 µL of EndoFectin reagent (2:1 DNA ratio). For cells that are difficult to transfect, optimize the DNA amount (usually within the range of 2.5-4 µg) and DNA amount: EndoFectin volume ratio (try 1:2 to 1:4). Dilute the DNA in opti-MEM and incubate for 5 minutes at room temperature. During this incubation, dilute EndoFectin with opti-MEM and mix well. Combine the EndoFectin dilution with DNA dilution and incubate for 15-20 min at room temperature to allow for the formation of DNA-EndoFectin complexes. Add the complexes into each well drop by drop avoiding direct adding to the cells. Gently rock the plate back and forth for homogenous distribution. Incubate the cells at 37 \(^\circ\)C. Exchange fresh complete media after 24h transfection (especially when
transfection shows toxicity to cell growth). Treatment or harvest of cells can be done at 48-72h post transfection. Optimize the time for different plasmid DNA and cell lines.

Whole-Cell Lysate Preparation

Cell pellets were resuspended using lysis buffer (25 mM HEPES, pH 7.4, 0.4 M KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% NP-40, 1 mM PMSF, and 2 µg/mL of leupeptin) of 2.5-3× volume of cell pellets. After three cycles of freeze/thaw, lysates were kept on ice for 30 min and were then centrifuged at 16,000 g for 10-20 min (use longer time when pellet size is big) at 4 °C. The supernatants were defined as whole-cell lysates and were subjected to BCA assay to determine protein concentration.

Table 2
BCA Assay 96-well Plate Standards Layout

<table>
<thead>
<tr>
<th>Concentration (mg/mL)</th>
<th>Column</th>
<th>Row 1 well content</th>
<th>BSA dilution (µL)</th>
<th>HEDG buffer (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A</td>
<td>0</td>
<td>10</td>
<td>1:10 BSA dilution</td>
</tr>
<tr>
<td>0.05</td>
<td>B</td>
<td>5</td>
<td>5</td>
<td>1:10 BSA dilution</td>
</tr>
<tr>
<td>0.1</td>
<td>C</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>D</td>
<td>2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>E</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>F</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>0.7</td>
<td>G</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>H</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Note. 1:10 BSA dilution=1:10 dilution of 10mg/mL BSA stock solution. 1:100 BSA dilution=1:10 dilution of 1:10 BSA dilution.

Bicinchoninic Acid Protein Assay (BCA)

Use 96-well plates for BSA standards and samples. Do duplicates for each standard or sample. The first two column are for standards with concentration ranging from 0 to 1.0 mg/mL BSA as shown in Table 2. The recipe for each
concentration is shown in Row1 well content of the table. The volume for each standard or sample is 10 μL. For samples wells, try to dilute the samples to make a final concentration in the range of 0.4-0.7 mg/mL. I usually do 1:10 dilution. After adding all the standards and samples, add 190 μL BCA reagent mix to each well using multichannel pipette and mix well, making a total volume of 200 μL. BCA reagent mix is composed of 98% BCA reagent A and 2% BCA reagent B (by volume). Incubate the plate at 37 °C for 30min and determine the absorbance at 562 nm using Epoch plate reader. Calculate protein concentration based on standard curve and absorbance.

**SDS-PAGE**

We use Bio-Rad Mini-PROTEAN tank for SDS-PAGE with homemade 1.5 mm acrylamide gels. See Table 3 below for 2-gel recipe. Seal the bottom of the gel using 0.7% agarose (14 mg agarose dissolved in 0.5 mL lower Tris and 1.5 mL dH₂O; microwave to dissolve and solidify for about 10 min). Pouring the running gel on top of the agarose layer until the top surface reach ~1.5 cm to the top edge of the short glass; seal by carefully and slowly adding water on top to give a horizontal gel surface. Let the gel stand for 30-60 min at room temperature until a clear line emerge between the water layer and the running gel. Pour the stacking gel and insert a 15-well of 10-well comb according to experiment design. Let the gel solidify for another 4-5 hours at room temperature. Wrap the gel with water and store at 4 °C for future use. Running condition: ~110 V for the stacking gel; ~180 V for the running gel (the lower voltage, the lower temperature, which may help improve the resolution and avoid distortion. Use 200 V maximal). Use 350 mL 1x TGS running buffer for the inner and outer compartment of tank. Stop running util the dye front reach ~2-5mm to the bottom of the gel.
<table>
<thead>
<tr>
<th></th>
<th>Running gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>8.2 mL</td>
<td>6.9 mL</td>
</tr>
<tr>
<td>Acrylamide/Bis (30/0.8)</td>
<td>6.7 mL</td>
<td>8 mL</td>
</tr>
<tr>
<td>Buffer (Tris)</td>
<td>5 mL (lower Tris)</td>
<td>1.25 mL (upper Tris)</td>
</tr>
<tr>
<td>10% AP</td>
<td>100 μL</td>
<td>50 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μL</td>
<td>5 μL</td>
</tr>
</tbody>
</table>

Note. Percentage of the gel is based on the target protein size.

**Li-COR Western Analysis**

Based on BCA assay result, prepare samples for SDS-PAGE with 20-40 μg total protein loaded (determine the optimal loading amount by doing serial dilution). Add HEDG buffer to get similar volume of samples. Mix the lysate sample 1:1 (v/v) with 1x treatment buffer. Vortex completely and heat at 95 °C for 1.5-3 min (depending on the volume of the samples). Briefly centrifuge to collect the samples at the bottom, which is ready for SDS-PAGE. Minimize loading time to reduce the diffusion of samples to avoid distortion of bands. Choose the percentage of the gel depending on the molecular weight of target proteins. Pre-wet the filter paper and nitrocellulose membrane before wet transfer. Set up the polyacrylamide gel-nitrocellulose membrane pair “sandwich” as illustrated in Figure A1. Transfer for ~120 min using 300 mA setting at 4 °C. Fifteen percent acrylamide gels were transferred for 3h at 4 °C. After the wet transfer, total protein staining was performed using LI-COR Revert™ 700 Total Protein Stain. Rinse the membrane in dH2O and incubate in ~ 5 mL stain solution with gentle shaking. Decant the stain solution and rinse the membrane twice with Wash solution, 30 sec each time. Rinse with water and proceed to imaging using the 700 nm channel. After imaging, incubate with
Revert solution 5-10 min. Rinse with water again and the membrane is ready to proceed for blocking. Membranes for the examination of LC3B levels for autophagic flux were dried overnight (membranes for small molecular weight proteins should be dried for at least 1 hour to improve the quantification accuracy; keep the drying time consistent; total protein stain can be done before or after dry process) and wet with PBS before total protein staining and blocking. The transferred nitrocellulose membranes were blocked in PBS with 5% BSA for 1 h. Incubate with primary antibody overnight in 4 °C. Wash 5 min × 5 with 1x PBST before proceeding to secondary antibody incubation (2 h at room temperature). Wash the membrane as previous. The last time wash should be PBS to lower the background signal brought by Tween-20. Dry the membrane and proceed to imaging. If not specified, Western bands were normalized using total protein stain. Results were obtained and analyzed using an LI-COR Odyssey CLx imaging system.

*Figure A1.* Filter paper-polyacrylamide gel-nitrocellulose membrane sandwich setup illustration.
**Zn\(^{2+}\)-Phos-tag™ SDS-PAGE**

Compared to conventional Laemmli SDS-PAGE described above, the Zn\(^{2+}\)-Phos-tag™ SDS-PAGE used the neutral Bis-Tris gel system to separate phosphorylated and unphosphorylated proteins for better resolution. After optimization based on sample type (e.g., whole cell lysate that contains various phosphorylated and unphosphorylated proteins needs lower concentration of phos-tag acrylamide) and target protein size (according to the manual, 6% acrylamide gel is recommended for proteins bigger than 60 kDa. I increased the percentage to 7.5% for easier handle of the soft gel). The method to pour the gel is the same as conventional acrylamide gel; just the recipe is different (Table 4). I prepared gel freshly before use (let the gel solidify for 5.5 h in total and wrap in water and put in 4 °C for 30 min). The manual recommended the molar ratio of metal ion (ZnCl\(_2\)) to phos-tag acrylamide in the gel starting at 1:2 and I increased to 1:4 for better capture of phosphorylated proteins. Used 15 μM phos-tag for freshly prepared phos-tag acrylamide solution and increased to 20 μM after the stock solution being stored at 4 °C for several weeks.

Sample preparation were similar as conventional SDS-PAGE. Add 2 mM ZnCl\(_2\) to the sample before loading (to reduce the effects caused by EDTA in the lysis buffer). MOPS were used as the running buffer. SDS-PAGE were performed at 4 °C; 55 volts for the stacking gel and 110 volts for the resolving gel. Wet transfer was performed at 4 °C for 4 h (add 0.175% SDS in the wet transfer buffer; change ice box in the tank every 1-1.5 hours to make sure the buffer stays cool). Dry the membrane for 30 min before proceeding to blocking. Western blot analysis was performed as described under the LI-COR western analysis section.
Table 4
Zn²⁺-Phos-tag™ SDS-PAGE Gel Recipe (1 gel)

<table>
<thead>
<tr>
<th>7.5% resolving gel</th>
<th>Phos-tag gel</th>
<th>Conventional gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 μM phos-tag</td>
<td>dH₂O</td>
<td>4.825 mL</td>
<td>4.945 mL</td>
</tr>
<tr>
<td></td>
<td>Acrylamide/Bis (30/0.8)</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>pH 6.8 Bis-Tris buffer</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
<td>0.625 mL</td>
</tr>
<tr>
<td>5 mM phos-tag solution</td>
<td>40 μL</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>10 mM ZnCl₂ solution</td>
<td>80 μL</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>10% AP</td>
<td>50 μL</td>
<td>50 μL</td>
<td>25 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 μL</td>
<td>5 μL</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>Total</td>
<td>10 mL</td>
<td>10 mL</td>
<td>2.5 mL</td>
</tr>
</tbody>
</table>

**Lambda Phosphatase Experiment**

The reaction of λ-phosphatase dephosphorylation was set up as follow: 5 μL 10x Lambda phosphatase buffer + 5 μL 10x MnCl₂ + 133.33 μg whole cell lysate + water to reach a total volume of 50 μL. Mix well and aliquot 16.6 μL into three RNase-free 0.2 mL PCR tubes and incubate at 30 °C for 2, 30 and 60 min using the PCR thermocycler. Take 15 μL (equals to 40 μg of protein) to load.

**RT-qPCR**

RNA extraction. Before experiments, briefly centrifuge all kit components stored in -20 °C. Centrifuge to collect contents at the bottom of the tube. For all the steps performed, use DNase/RNase-free filter tips. Lyse cells with TRI reagent. I always lyse cells fresh in 12-well or 6-well plate. Can also collect the cells and freeze in -80 °C before lysing. See Direct-zol kit manual for the corresponding volume of TRI reagent used for different size of samples. All the centrifuge steps during RNA extraction are 10,000-16,000 g (I usually do 16,000 g) at room temperature. For 12-well plate sample, add 400 μL TRI reagent per well and mix well by pipetting. Proceed to RNA Purification. Add an equal volume of ethanol (95-
100%) directly to one volume sample in TRI. Mix well by inverting and vortex. Load the mixture into a column in a collection tube. Centrifuge 30 sec. Transfer the column into a new collection tube and perform in-column DNase I treatment (recommended). Wash the column with 400 µL RNA Wash Buffer. Centrifuge 30 sec and discard the flow through. Prepare DNase I reaction mix in a clean tube (amount is per column): 5 µL DNase I (6 U/µL), 75 µL DNA Digestion Buffer. Mix by gentle inversion. Add 80 µL of the mix per column directly to the column matrix. Incubate at room temperature for 15 min and then centrifuge for 30 sec. Add 400 µL Direct-zol RNA PreWash to the column and centrifuge for 30 sec. Discard the flow through and repeat this step. Add 700 µL RNA Wash Buffer to the column and centrifuge of 2 min to ensure complete removal of the wash buffer. Discard the flow through and transfer the column to a RNase-free tube. Add 50 µL DNase/RNase-free water directly to the column matrix and centrifuge for 1 min. Alternatively, for more concentrated RNA, use ≥ 25 µL elution. Quantify with nanodrop. If not proceed to reverse transcription immediately, add RNase inhibitor 0.5 µL per tube and store at -80°C.

Epicentre MMLV reverse transcription. Denature the RNA samples and anneal the primers. For each reaction, combine the following components on ice in a RNase-free 0.2 mL PCR tube: 10 µL total reaction volume= 0.4 µL random primer + 100 pg-1 µg (use 0.5-1 µg for lower Cq number) + RNase-free water. Incubate at 65°C for 2 min and chill on ice (set as 4°C) for at least 1 min in the thermocycler. Briefly centrifuge and combine with the following components on ice for one reaction (total volume would be 20 µL): 3 µL RNase-free water, 2 µL 10x MMLV buffer, 2 µL 100 mM DTT, 0.5 µL ScriptGuard RNase inhibitor, 2 µL dNTP mix (2.5 mM), 0.5 µL MMLV high performance reverse transcriptase. Use the PCR thermocycler to
perform the protocol: 10 min room temperature followed by 37 °C 60 min; terminate the reaction by heating at 85 °C for 5 min and then 4 °C for at least 1 min.

Centrifuge briefly. The cDNA can be used immediately for real-time PCR or stored at -20 °C for future use.

Quantitative PCR (qPCR). qPCR was performed with 1 μL of cDNA from reverse transcription solution, 10 μL of Bio-Rad iTaq SYBR green supermix, and 0.8 pmol sequence-specific primers using a Bio-Rad CFX Connect real-time PCR machine with the following protocol: 40 cycles of 90 °C for 10 s/60 °C for 1 min with fluorescence readings taken at 60 °C. The $2^{-\Delta\Delta Cq}$ method was used to present the normalized values. For RT² Profiler PCR Array, reverse transcription was performed with 0.5 μg of RNA using Epicentre MMLV reverse transcriptase. PCR master mix (1,350 μL of Bio-Rad iTaq SYBR green supermix, 102 μL of cDNA synthesis solution, and 1,248 μL of RNase-free water) was prepared and an aliquot of 25 μL was added to each well of the RT² Profiler PCR Array plate. A Bio-Rad CFX Connect real-time PCR machine was used with the following protocol: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec/60 °C for 1 min (ramp rate between 95 °C to 60 °C step was set as 1 °C /sec). Data analysis was performed at Qiagen GeneGlobe Data Analysis Center (www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page).

**Immunoprecipitation and Co-Immunoprecipitation Experiments**

Cells were lysed with the method described under whole cell lysate preparation. Three deubiquitylase inhibitors, namely 1,10-phenanthroline (5 mM), NEM (10 mM), and PR-619 (50 μM) were added in the lysis buffer for immunoprecipitation experiment with K48- and K63-TUBE Far-western analysis. One to two milligrams of whole-cell lysates (about one to two 75 cm² flasks or one to
two 6-well plates of cells) were used for immunoprecipitation (IP) and co-
immunoprecipitation (co-IP) of AHR using anti-AHR antibody SA210 (1:200 by
volume) or anti-AHR antibody A-3x (1:100 by volume for AHR–p62 co-IP) for 30 min
at room temperature. When incubating, pre-equilibrate protein G magnetic beads at
room temperature as follows: (1) Before taking out beads, shake well to homogenize
the solution with beads. Aliquot needed volume of beads slurry into GeneMate
microfuge tubes (the tube walls need to be thin enough for magnet). Add 0.5 mL of
water. Magnet 1 min and discard supernatant. (2) Repeat this for three more times
(gently tap to mix the beads in water). (3) Wash with IP buffer, following steps in (1).
IP buffer recipe: 25 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT, 10% glycerol,
0.15 M NaCl, 0.1% Tween-20 for IP (0.05% Tween-20 for co-IP). For co-IP, add
tween-20 only for washing steps to preserve more interaction, depending on the
signal/background noise results. Add the samples and 1 mg/mL of BSA to the pre-
equilibrated Protein G Dynabeads (1:200 by volume) and q.s. with IP buffer to 1-1.2
mL. The samples were incubated with a rotation of 60 rpm overnight (16-18h) at 4
°C. The beads were then washed three times for 5 min each with 1 mL IP buffer and
then eluted with 35-40 μL electrophoresis sample buffer for SDS-PAGE, followed by
LI-COR Western analysis.

**K48- and K63-TUBE and Far-Western Analysis**

HeLa cells were seeded and cultured overnight in a 75 cm² flask and
harvested after treatment, if needed, with cold PBS by mechanical scraping. Cell
lysates were obtained using a lysis buffer (see whole cell lysate preparation) with a
4× volume of the cell pellet. Immunoprecipitation was performed as described
above, followed by Far-western analysis. K48- or K63-TUBE (1:1,000) were
incubated with the nitrocellulose membrane for 1 h at room temperature. Wash with
1x PBST 5 min for 5 times and then incubate with IRDye-800cw conjugated streptavidin (1:10,000) for 2 h at room temperature. The blocking step and wash steps between and after incubation were the same as in LI-COR Western analysis. Results were obtained and analyzed using an LI-COR Odyssey CLx imaging system. Membrane was scanned wet. After placing the membrane on the working area of LI-COR, scroll the membrane to make to membrane tightly placed flat on the glass top.

**Proximity Ligation Assay**

Cells were seeded on round microscope glass coverslips placed in wells of a 12-well plate and grown to about 80% confluence. Cells were then rinsed with PBS twice and incubated with ice-cold 100% methanol for 5 min. After one rinse with PBS, cells were ready for proximity ligation assay using Duolink in situ kit. Cells were first incubated with Duolink blocking solution (40 μL) at 37 °C for 60 min, followed by incubation with the two antibodies (in Duolink antibody diluent) used for interaction study (40 μL) at 37 °C for 60 min using the following dilution: mouse anti-AHR A-3x (1:100), rabbit anti-p62 (1:100), and rabbit anti-LC3B (1:100). Then, 5 min×3 washes using wash buffer A were performed with gentle shaking. Samples were next incubated with Duolink probes. Briefly mixed the PLUS and MINUS probes by vortex and did 1:5 dilution using Duolink antibody diluent. Aspirate all the residual wash buffer and apply 40 μL Duolink probes. Incubated the samples at 37 °C for 60 min. Wash the coverslip 4×5 min at room temperature with 1×wash buffer A. Added the ligase to the 1×ligation buffer at 1:40 dilution and mix well immediately before adding to samples. Incubated at 37 °C for 30 min. Wash the coverslip 3×5 min at room temperature with 1×wash buffer A. Added the polymerase to the 1×amplification buffer at 1:80 dilution and mix well immediately before adding to samples. Both the ligase and polymerase should be temporarily stored in the
freezing block. The amplification buffer is light sensitive. All solutions and samples containing the buffer should be protected from light. Incubated at 37 °C for 100 min.

Final wash steps included 2×10 min wash with 1× wash buffer B and then 0.01× wash buffer B for 2 min at room temperature (keep from light). Nucleus staining was performed by adding 1 μg/mL of DAPI (dilute freshly from 1000x stock solution; keep from light) in water to cells for a 1 min incubation. Coverslips were rinsed with 1× PBS and then mounted using ~6 μL of PBS and were sealed with nail polish.

Samples were viewed using a KEYENCE BZ-X700 fluorescence microscope. Wait for ~15 minutes before analyzing in the fluorescence microscope, using at least a 20x objective. Results were analyzed by ImageJ software.
APPENDIX B: HOMEMADE REAGENT RECIPE

The recipes of homemade reagent mentioned in APPENDIX B were listed below.

**Lower Tris Buffer (1.5 M Tris)**

181.7 g Tris in 800 mL H$_2$O. Adjust pH to 8.8 and qs 1000 mL.

**Upper Tris Buffer (0.5 M Tris)**

30.3 g Tris base in 400 mL H$_2$O. Adjust pH to 6.8 and qs 500 mL. pH 6.8 Bis-Tris buffer (1.4 mol/L Bis-Tris): Bis-Tris base 29.9 g in 50 mL water. Then add 5 mL concentrated HCl (12M) and q.s. to 100 mL (no need to adjust pH). Keep at 4 ºC in the dark.

**10 mM ZnCl$_2$ Solution**

14 mg in 10 mL water. Filter to remove impurities. Prepare just before use.

**10% AP**

1 g ammonium persulfate in 10 mL dH$_2$O. Aliquot to 0.5 mL fractions and stored at -20 ºC. After taken out, do not put back (avoid freeze-thaw). Use within two weeks.

**Acrylamide/Bisacrylamide 30/0.8 (30% acrylamide/ 0.8% Bisacrylamide)**

120 g acrylamide + 3.2 g Bisacrylamide, qs 400 mL (dissolve in ~200 mL H$_2$O then qs 400 mL)  
Caution: only use special spatula (only for acrylamide and Bis); weight in hood; wear gloves, mask and lab coat.

**HEDG Buffer**

25 mM HEPES (5.96 g) + 1 mM EDTA (2 mL of 0.5 M EDTA) + 1 mM DTT (154.25 mg) + 10% glycerol (100 mL), qs with water to 1 L. Adjust pH to 7.4.

**100 mM PMSF Stock**

Dissolve PMSF 17.4 mg into 1 mL isopropanol. Aliquot to 1 mL and store at -20 ºC.

**Treatment Buffer (1x) Minus βME**

6.3 g or 5 mL glycerol + 6.25 mL upper Tris buffer + 20 mL 10% SDS + 4 mL 0.25% Bromophenol Blue, qs with water to 50 mL. Take 950 µL and add 50 µL βME to make ready-to-use 1x treatment buffer.
MOPS Running Buffer

Tris base 4.242 g + MOPS 7.322 g + 0.1855 g NaHSO₃ (sodium bisulfite) + 3.5 mL 10% (w/v) SDS solution and q.s. to 350 mL. Prepare just before use.

Wet Transfer Buffer

3.03 g Tris + 14.4 g glycine + 200 mL methanol, qs with water to 1L
### Table 5

*A List of the 84 Autophagy-Related Genes Plus 5 Housekeeping Reference Genes Analyzed in Figure. 2.3B*

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_005163</td>
<td>AKT1</td>
<td>V-akt murine thymoma viral oncogene homolog 1</td>
</tr>
<tr>
<td>NM_017749</td>
<td>AMBRA1</td>
<td>Autophagy/beclin-1 regulator 1</td>
</tr>
<tr>
<td>NM_000484</td>
<td>APP</td>
<td>Amyloid beta (A4) precursor protein</td>
</tr>
<tr>
<td>NM_031482</td>
<td>ATG10</td>
<td>ATG10 autophagy related 10 homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>NM_004707</td>
<td>ATG12</td>
<td>ATG12 autophagy related 12 homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>NM_017974</td>
<td>ATG16L1</td>
<td>ATG16 autophagy related 16-like 1 (S. cerevisiae)</td>
</tr>
<tr>
<td>NM_033388</td>
<td>ATG16L2</td>
<td>ATG16 autophagy related 16-like 2 (S. cerevisiae)</td>
</tr>
<tr>
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*Note.* Asterisk (*) indicates a housekeeping gene.