Title

Hepatic cytochromes P450: structural degrons and barcodes, posttranslational modifications and cellular adapters in the ERAD-endgame.

Authors

Kim, Sung-Mi
Wang, YongQiang
Nabavi, Noushin
et al.

Peer reviewed

Hepatic cytochromes P450: structural degrons and barcodes, posttranslational modifications and cellular adapters in the ERAD-endgame

Sung-Mi Kim, YongQiang Wang, Noushin Nabavi, Yi Liu & Maria Almira Correia

Published online: 20 Jun 2016.

Submit your article to this journal

Article views: 57

View related articles

View Crossmark data

Taylor & Francis
Taylor & Francis Group

REVIEW ARTICLE

Hepatic cytochromes P450: structural degrons and barcodes, posttranslational modifications and cellular adapters in the ERAD-endgame

Sung-Mi Kim[a], YongQiang Wang[a], Noushin Nabavi[a*], Yi Liu[a] and Maria Almira Correia[a,b,c,d]

[a]Department of Cellular & Molecular Pharmacology, University of California San Francisco, San Francisco, CA, USA; [b]Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, CA, USA; [c]Department of Bioengineering and Therapeutic Sciences, University of California San Francisco, San Francisco, CA, USA; [d]The Liver Center, University of California San Francisco, San Francisco, CA, USA

ABSTRACT

The endoplasmic reticulum (ER)-anchored hepatic cytochromes P450 (P450s) are enzymes that metabolize endo- and xenobiotics i.e. drugs, carcinogens, toxins, natural and chemical products. These agents modulate liver P450 content through increased synthesis or reduction via inactivation and/or proteolytic degradation, resulting in clinically significant drug-drug interactions. P450 proteolytic degradation occurs via ER-associated degradation (ERAD) involving either of two distinct routes: Ubiquitin (Ub)-dependent 26S proteasomal degradation (ERAD/UPD) or autophagic lysosomal degradation (ERAD/ALD). CYP3A4, the major human liver/intestinal P450, and the fast-turnover CYP2E1 species are degraded via ERAD/UPD entailing multisite protein phosphorylation and subsequent ubiquitination by gp78 and CHIP E3 Ub-ligases. We are gaining insight into the nature of the structural determinants involved in CYP3A4 and CYP2E1 molecular recognition in ERAD/UPD [i.e. K_{48}-linked polyUb chains and linear and/or "conformational" phosphodegrons consisting either of consecutive sequences on surface loops and/or disordered regions, or structurally-assembled surface clusters of negatively charged acidic (Asp/Glu) and phosphorylated (Ser/Thr) residues, within or vicinal to which, Lys-residues are targeted for ubiquitination]. Structural inspection of select human liver P450s reveals that such linear or conformational phosphodegrons may indeed be a common P450-ERAD/UPD feature. By contrast, although many P450s such as the slow-turnover CYP2E1 species and rat liver CYP2B1 and CYP2C11 are degraded via ERAD/ALD, little is known about the mechanism of their ALD-targeting. On the basis of our current knowledge of ALD-substrate targeting, we propose a tripartite conjunction of K_{63}-linked Ub-chains, P450 structural "LIR" motifs and selective cellular "cargo receptors" as plausible P450-ALD determinants.

Abbreviations: AAA: ATPases associated with various cellular activities; ALD: autophagic lysosomal degradation; BFA: bafilomycin A; CHIP: C-terminus of Hsc70-interacting protein; CuOOH: cumene hydroperoxide; CPR: cytochrome P450 reductase; E1: Ub-activating enzyme; E2: Ub-conjugating enzyme; E3: Ub-ligase complex; ER: endoplasmic reticulum; ERAD: ER-associated degradation; gp78/AMFR: glycoprotein 78/autocrine motility factor receptor; HA: hemagglutinin; HMM: High molecular mass; 3-MA: 3-methyladenine; MAP-LC3: microtubule-associated protein 1 light chain 3; LIRs: LC3-interacting motifs; Lys-C: lysyl endopeptidase C; NBR1: neighbor of BRCA1 gene1; p62/SQSTM1: sequestosome-1; PKA: protein kinase A; PKC: protein kinase C; RING: Really interesting new gene; Rpn: (non-ATPase) lid 26S subunits; Rpt: AAA ATPase base 26S subunits; ROS: reactive O2 species; TAO: troleandomycin; Ub: ubiquitin; UPD: Ub-dependent 26S proteasomal degradation; VCP: Valosin-containing protein; VIM: VCP-interacting motif

Introduction

Cellular pathways for hepatic P450 degradation

Budding yeast and higher eukaryotic cells possess many different proteolytic processes for the basal turnover/disposal of senescent and/or structurally damaged organelles, and misfolded, aggregated and/or functionally inactivated proteins, thereby providing not only quality control but also enabling the salvage of their constituent building blocks (i.e. amino acids from proteins, fatty acids from lipoproteins, sugars from glycoproteins) for recycling and/or reutilization as energy sources. Of these, a relatively well-characterized process to date is the endoplasmic reticulum (**ER**)-associated degradation (**ERAD**), an ER-quality control system (Christianson & Ye, 2014; Hampton & Garza, 2009; Hirsch

et al., 2009; Nakatsukasa et al., 2008; Olzmann et al., 2013; Vembar & Brodsky, 2008), with three distinct mechanistic pathways in the yeast *S. cerevisiae* (Ahner & Brodsky, 2004; Carvalho et al., 2006; Taxis et al., 2003; Vashist & Ng, 2004): ERAD-**L** for the disposal of mis-folded/damaged ER-lumenal (**L**) proteins, ERAD-**M** for the misfolded/damaged polytopic ER-membrane (**M**)-anchored proteins such as HMG CoA reductase, and ERAD-**C** for that of monotopic ER-membrane anchored proteins with misfolded/damaged cytosolic (**C**) domains such as in principle, the hepatic cytochromes P450 (P450s). Each of these ERAD pathways, particularly in *S. cerevisiae,* is known to entail specific accessory adapters and/or participating ubiquitination modules that pre-dominantly target the ER-proteins to ubiquitin (**Ub**)-dependent 26S proteasomal degradation (**UPD**) (Christianson & Ye, 2014; Olzmann et al., 2013). However, in mammalian cells, the subsequent recogni-tion that while a given ER-protein in its native state incurred ERAD-L, ERAD-M or ERAD-C depending on its ER-topology, but opted for the autophagic-lysosomal degradation (ALD) when aggregated, gave rise to the ERAD-I and ERAD-II nomenclature for the former 3 ERAD pathways requiring UPD, and the latter pathway requir-ing ALD instead of UPD, respectively (Fujita et al., 2007).

Nine P450s (CYPs 3A4, 2C9, 1A2, 2E1, 2D6, 2C8, 2C19, 2A6 and 2B6, listed in decreasing order of their relative hepatic abundance) are primarily responsible for the oxidative metabolism of drug/xenobiotic substrates in the human liver (Guengerich, 2015). These hepatic P450s, along with their rabbit, rat and mouse orthologs, qualify as excellent prototypes of ERAD-C substrates by virtue both of their monotopic Type I ER-topology (i.e. a bulky cytosolic catalytic domain anchored to the ER-membrane via a single N-terminal 30–33-residue long amphipathic helix) and their cytosolic domain prone to oxidative/structural lesions stemming from their oxidative function that engenders highly reactive O_2 species (ROS) (Ekstrom & Ingelman-Sundberg, 1989; Goasduff & Cederbaum, 1999; Gorsky et al., 1984; Zhukov & Ingelman-Sundberg, 1999). However, in spite of these common features, not all P450 proteins pre-dominantly subscribe to the canonical ERAD-C/ERAD-I process, henceforth referred to as ERAD/UPD pathway. Thus, although all CYPs 3A as well as all structurally/functionally inactivated P450s are predominantly ERAD/UPD targets (Correia et al., 1987, 1992a, 1992b, 2005; Correia, 2003; Correia & Liao, 2007; Dai & Cederbaum, 1995; Faouzi et al., 2007; Korsmeyer et al., 1999; Liao et al., 2006; Lee et al., 2008; Murray & Correia, 2001; Morishima et al., 2005; Roberts, 1997; Schmiedlin-Ren, 1997; Sohn et al., 1991; Tierney et al., 1992; Wang et al., 1999), native P450s such as rat liver CYPs 2B1 and 2C11

are predominantly ERAD-II substrates requiring ALD instead of UPD, henceforth referred to as the ERAD/ALD pathway (Liao et al., 2005; Masaki et al., 1987; Murray et al., 2002; Ronis & Ingelman-Sundberg, 1989; Ronis et al., 1991). On the other hand, human or rat liver CYP2E1 utilizes both ERAD/UPD and ERAD/ALD path-ways, depending on whether it is suicidally inactivated or native/substrate-free, or native/substrate-bound, respectively (Bardag-Gorce et al., 2002; Morishima et al., 2005; Roberts et al., 1995; Song et al., 1989; Wang et al., 2011). Thus, although a preferential P450 routing into either pathway apparently exists, the pathway not chosen may simply represent a convenient alternative backup, in eventualities such as ER-stress when ERAD/UPD is overloaded and clogged with misfolded/aggre-gated proteins awaiting clearance, and competition for the ERAD/UPD cellular machinery is insurmountable. It is worth noting however, that P450 ERAD in cultured hep-atocytes may not always faithfully reflect the preferen-tial physiological sorting of any given P450 *in vivo*, as cultured cells are usually subjected to much higher O_2-concentrations than normally encountered in the liver of the intact animal, and thus more vulnerable to oxidative insults.

P450 ERAD/UPD

The elucidation of hepatic CYP3A and CYP2E1 ERAD has provided the essential blueprint for this P450 degrad-ation pathway. Such ERAD involves posttranslational phosphorylation of the P450 proteins by cytosolic kin-ases (Korsmeyer et al., 1999; Wang et al., 2001; Wang et al., 2009, 2011, 2012, 2015), their ubiquitination by the concerted action of the E1-Ub-activating enzyme, E2 Ub-conjugating enzyme/E3 Ub-ligase complexes (Kim et al., 2010; Morishima et al., 2005; Pabarcus et al., 2009; Wang et al., 2009, 2011, 2012, 2015), and subsequent degradation by the cytosolic 26S proteasome (Correia et al. 1992b, 2005; Correia, 2003; Correia et al., 2005; Correia & Liao, 2007; Faouzi et al., 2007; Liao et al., 2006; Murray & Correia, 2001; Morishima et al., 2005; Roberts, 1997; Tierney et al., 1992; Figure 1). The 26S proteasome consists of a 20S proteolytic barrel topped at each end by a 19S regulatory cap, with Ub-chain recognition/deu-biquitinating Rpn (non-ATPase) lid subunits (specifically Rpn10 and Rpn13 Ub-chain receptors/Rpn11 deubiquiti-nase) as well as Rpt AAA (ATPases associated with vari-ous cellular activities) ATPase base subunits for protein unfolding. As the Rpt ATPases grasp and unfold the ubiquitinated proteins, the Rpn10 and Rpn13 Ub-receptors situated immediately above the base engage the Ub-chains, enabling Rpn13 to deubiquitinate and recycle the Ub-chains for fresh ubiquitination cycles.
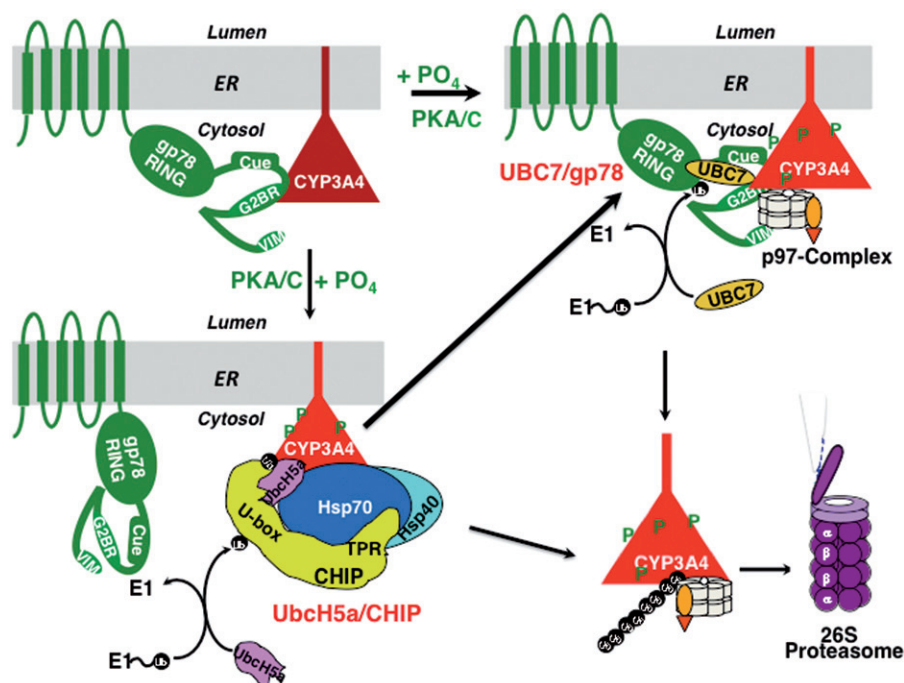
Figure 1. CYP3A4 ERAD/UPD: Known cellular participants. See discussion for details.

The bare, unfolded protein target is then threaded into the narrow 20S central proteolytic cavity with a pore diameter of merely 13–14 Å (Finley, 2009; Pickart & Cohen, 2004; Rechsteiner, 2005).

In common with the ERAD of polytopic ER-integral and ER-luminal proteins, ERAD/UPD of these P450 proteins also requires their extraction out of the ER into the cytosol by the p97/VCP (Valosin-containing protein) AAA-ATPase, an abundant cytosolic chaperone (Bar-Nun, 2005; Chou et al., 2011; Dai & Li, 2001; Elkabetz et al., 2004; Ikeda et al., 2009; Jentsch & Rumpf, 2007; Meyer et al., 2000; Richly et al., 2005; Xia et al., 2016; Ye et al., 2005), and/or the 19S proteasomal cap Rpt4 AAA-ATPase subunit (Lipson et al., 2008), before subsequent proteasomal degradation.

Several inhibitors [i.e. vinyl sulfone, MG-132, MG-262, lactacystin, epoxomycin, PS-341 (Bortezomib/Velcade; a boronated MG-132)] that specifically target the 20S proteasomal chymotrypsin-like (β5)-, caspase-like (β1)-, and/or trypsin-like (β2)-subunit N-terminal Thr-proteases either reversibly or irreversibly are commercially available, for use as diagnostic probes of this ERAD/UPD-pathway (Dick et al., 1997; Lee & Goldberg, 1998; Rechsteiner, 2005; Rock et al., 1994). However, not all of these agents inhibit these three 20S proteasomal proteolytic activities equivalently. Some are more selective and/or potent inhibitors of just one rather than all three protease activities. Additionally, it is important to recognize that these inhibitors block UPD at a step beyond P450 protein ubiquitination. Thus, an accurate assessment of their effectiveness as ERAD/UPD blockers with consequent stabilization of the relevant P450s requires monitoring the stabilization of both the parent ($\approx$ 50 kDa) and the ubiquitinated P450 species [>65 kDa high molecular mass (HMM)].

Cellular protein kinases and protein phosphorylation in P450 ERAD

The 1962 seminal finding that catecholamine administration could depress hepatic drug metabolism in rats (Fouts, 1962) implicated cAMP-signaling, thus instigating the subsequent documentation that the cyclic AMP analog N^6, O^2-dibutyryl cAMP could similarly lower hepatic microsomal P450 content *in vivo*, due to P450 protein phosphorylation that denatured the hemoprotein to its inactive, spectrally detectable P420 species (Hutterer et al., 1975). This finding spurred a flurry of subsequent investigations by various research groups over several decades on the precise site, role and/or purpose of such P450 phosphorylation (*literature extensively reviewed by* Aguiar et al., 2005; Correia, 2003; Correia et al., 2014; Correia & Liao, 2007; Korsmeyer et al., 1999).

More germane to the present P450 ERAD discussion, however, are the findings of Ingelman-Sundberg and colleagues (Eliasson et al., 1990, 1992, 1994; Johansson et al., 1991) who showed both in cultured hepatocytes and in *in vitro* reconstituted systems that such microsomal CYP2E1 and CYP3A1 phosphorylation actually predisposes these P450s, but not CYP2B1, to an

ERAD-like process, that although independent of either ubiquitination and/or the 26S proteasome (*and thus de facto UPD-independent*), required intrinsic ER-kinases and ER-integral Mg^{2+}-ATP-activated proteolytic systems. Accordingly, these investigators showed that glucagon or 8-bromo-cAMP-treatment of cultured rat hepatocytes resulted in the phosphorylation and rapid degradation of these P450s that was blocked by their select substrates/inhibitors (Eliasson et al., 1992, 1994). Using microsomal P450s *in vitro*, they further documented the involvement of an endogenous microsomal serine kinase, proceeding independently of any external kinase, but which in purified systems could be replaced by the catalytic subunit of a bovine cAMP-dependent protein kinase (PKA) (Eliasson et al., 1992, 1994). Such P450 protein phosphorylation was enhanced by incubation with NADPH and was associated with a spectroscopically detectable denaturation to P420 and consequent functional inactivation, which was blocked by select CYP2E1 and CYP3A substrates/inhibitors such as EtOH, imidazole or acetone and clotrimazole, troleandomycin (TAO) or erythromycin, respectively (Eliasson et al., 1992, 1994). Through HPLC-analyses of peptide digests, the authors further identified the P450 phosphorylation sites as CYP2E1 Ser129 (Eliasson et al., 1990, 1992), a finding consistent with the dibasic PKA-consensus motif (RR-X-S), and CYP3A4 Ser-393, a less common PKA-consensus site (Eliasson et al., 1994). This group proceeded to purify two forms of membrane bound serine proteinases (MW $\approx$ 32 000 kDa; pH 8.0 optimum) from rat liver microsomes that were documented to degrade not only CYP2E1 over a 19 h-period with a profile similar to that of liver microsomal CYP2E1 (Zhukov et al., 1993), but also CYP3A1 and CYP2B1 (Eliasson et al., 1994).

Equally pertinent to this discussion are the findings (Lohr & Kuhn-Welten, 1997) that an ER-bound PKA was responsible for the phosphorylation of testicular microsomal CYP17 and its subsequent targeting to an unidentified cytosolic ATP-dependent protease, most likely, the 26S proteasome. Our own studies, however, of cumene hydroperoxide (CuOOH)-inactivated CYP2B1 (Korsmeyer et al., 1999) and CYP3A4 (Wang et al., 2001) identified not only microsomal and cytosolic kinases such as PKA and PKC, but also invariably an UPD process. In retrospect, these findings foretold a role for both ER/cytosolic kinases as well as E2/E3-complexes and the 26S proteasome, as in our current understanding of P450 ERAD/UPD.

More recent LC-MS/MS analyses of human liver samples by Redlich et al. (2008) confirmed that Ser129 is indeed a CYP2E1 phosphorylation site. Our own LC-MS/MS analyses of *in vitro* phosphorylated human liver CYP2E1 peptide digests further revealed that Ser129 is a major (>98%) PKA-phosphorylation site both in the native and CuOOH-inactivated proteins (Wang et al., 2011). This residue is also correspondingly phosphorylated by PKC to >23% and 32%, respectively (Wang et al., 2011). However, the advent of much more sensitive LC-MS/MS methodology has led to the recognition that Ser129 is but one of 16 different CYP2E1 S/T-residues that are phosphorylated. Because CYP2E1 Ser129 mutation to Ala or Gly had little effect on its turnover upon transfection into COS 7 cells (Freeman & Wolf, 1994), we believe that some of these other 16 phosphorylated residues must be involved, as phosphorylation does indeed significantly enhance both CYP2E1 and CYP3A4 ERAD/UPD (Correia et al., 2014; Wang et al., 2009, 2011, 2012, 2015). Indeed, similar analyses of *in vitro* phosphorylated CYP3A4 revealed that PKA phosphorylates 5 Ser residues (S116, S119, S134, S259 and S478), whereas PKC phosphorylates 14 Ser/Thr residues (T92, S100, T103, S116, S119, S131, S134, T136, S139, S259, T264, T284, S398, S420). It is noteworthy that S393 identified earlier as a PKA-phosphorylation site (Eliasson et al., 1994) is absent in CYP3A4, but its vicinal S398 (present in most CYPs 3A) is detectably phosphorylated by PKC, but not PKA (Wang et al., 2012).

Of these 15 recently identified CYP3A4 phosphorylation sites, S134 was previously identified in human liver samples (Redlich et al., 2008), whereas we had identified T264 and S420 as PKC phosphorylated residues (Wang et al., 2001; Wang et al., 2009) and S478 as PKA-phosphorylation site (Wang et al., 2009). When the relevance of the phosphorylatable residues T264, S420 and S478 to CYP3A4 ubiquitination was probed through site-directed mutagenesis, their mutation to Ala was found to considerably reduce its extent (Wang et al., 2012). Furthermore, of these three, the PKA-phosphorylatable S478-residue had by far the most pronounced impact, even though it is phosphorylated just by PKA to a very low extent ($\approx$ 0.32% and 1.29% in the native and CuOOH-inactivated proteins, respectively; Wang et al., 2012). Thus, it appears that the precise site rather than the extent of S/T-phosphorylation is a critical determinant. Interestingly, CYP3A5 that has a phosphomimetic D478 instead of S478 is constitutively ubiquitinated to a much greater extent than CYP3A4, but not degraded quite as rapidly (Wang et al., 2012). Although, the relevance of the other 12 phosphorylated CYP3A residues has not been quite as extensively probed, yet we now know that S259 and T284, as constituents of two CYP3A4 phosphodegrons (*see below*) are also quite relevant to its ubiquitination by E2/E3-complexes (Wang et al., 2015). Collectively, these findings argue that phosphorylation is an important determinant of P450 ERAD/UPD, albeit not the only one.

E2/E3 Ub-ligase complexes participating in P450 ERAD/UPD

Just two well-defined E2/E3-complexes operate in budding yeast i.e. Ubc6p/Ubc7p/Cue1p/Hrd1p complex engaged in ubiquitinating substrates in the ERAD-M and ERAD-L pathways, whereas the Ubc6p/Ubc7p/Doa10p complex is responsible for ubiquitinating ERAD-C substrates as well as cytosolic proteins (Christianson & Ye, 2014; Hirsch et al., 2009; Kostova et al., 2007; Metzger et al., 2012; Ravid et al., 2006). By contrast, mammalian cells posess a greatly expanded repertoire of E2/E3-complexes devoted to ERAD, including the Hrd1p-orthologs Hrd1 and gp78/AMFR (autocrine motility factor receptor), the Doa10p-ortholog TEB4/MARCH VI, RNF5/RMA1, RNF170 and CHIP (C-terminus of Hsc70-interacting protein) (Ballinger et al., 1999; Chen et al., 2006, 2012; Edkins, 2013; Fang et al., 2001; Hirsch et al., 2009; Jiang et al., 2001; Kikkert et al., 2004; Kostova et al., 2007; McDonough & Patterson, 2003; Metzger et al., 2012; Murata et al., 2001; Olzmann et al., 2013; Peng et al., 2004; Rosser et al., 2007). With the exception of CHIP that is cytosolic, these other E3s are all integral membrane proteins. Initial studies in *S. cerevisiae* strains with genetic Hrd1p- and Doa10p-deletions excluded these particular E3s in CYP3A4 ERAD/UPD (Liao et al., 2006; Murray & Correia, 2001), a finding that was validated when the corresponding purified, recombinant mammalian orthologs (Hrd1 and TEB4) were tested in *in vitro* functionally reconstituted CYP3A4-E2/E3-ubiquitination systems (Pabarcus et al., 2009). These studies of CYP3A4-ubiquitination also included in parallel, two other plausible E3 Ub-ligases: gp78 and CHIP, which were both capable of ubiquitinating CuOOH-inactivated CYP3A4 (Pabarcus et al., 2009).

gp78/AMFR

This polytopic E3 is found integrated via its N-terminal 308 residues both in the ER- and plasma membranes, with its functional domain in the cytosol wherein it interacts with its cognate E2 Ubc7/Ube2g2 and the rest of the requisite ubiquitination machinery to target cytosolic domains of integral ER-proteins (i.e. P450s) (Chen et al., 2006, 2012; Das et al., 2009, 2013; Fang et al., 2001; Hirsch et al., 2009; Kostova et al., 2007; Kim et al., 2010; Metzger et al., 2012; Olzmann et al., 2013; Pabarcus et al., 2009) as well as cytosolic proteins [i.e. tryptophan 2,3-dioxygenase (TDO)] (Kim et al., 2016a). gp78 is critically involved in substrate recognition and subsequent catalytic transfer of the Ubc7-elaborated polyUb chain *en bloc* onto the substrate (Chen et al., 2006, 2012; Fang et al., 2001; Hirsch et al., 2009; Kim et al., 2010; Kostova

et al., 2007; Li et al., 2007, 2009; Metzger et al., 2012; Olzmann et al., 2013; Pabarcus et al., 2009). Its cytosolic, functionally active C-terminal domain (309–643 residues) is composed of an intrinsic RING-finger Ub-ligase (residues 341–378), Cue1-like domain (residues 456–497) for binding Ub and elongating polyUb-chains, Ubc7/Ube2g2-binding (G2BR; residues 579–600), substrate recognition and putative p97/VCP-interacting motif (VIM; residues 626–643) (Chen et al., 2006, 2012; Das et al., 2009, 2013; Fang et al., 2001; Kostova et al., 2007; Metzger et al., 2012). Except for VIM, all the other gp78-domains are essential for Ubc7-dependent CYP3A4-ubiquitination (Wang et al., 2015). Lentiviral shRNAi-mediated gp78-knockdown in cultured rat hepatocytes verified its physiological role in CYP3A ERAD (Kim et al., 2010). Such gp78-knockdown not only significantly stabilized both ER-integral parent and ubiquitinated CYP3A species, but also enhanced their functional content (Kim et al., 2010). This indicated that although other CYP3A-ERAD-associated E3s were present, they could not compensate for the cellular knockdown of gp78.

Chemical crosslinking coupled with LC-MS/MS analyses of Ubc7/gp78-ubiquitinated CYP3A4 peptide digests revealed that CYP3A4-K251, flanking its negatively charged (E258-pS259-E262-D263-pT264) DEST-cluster, interacts with gp78-K313 residing in a positively charged (R307-R308-R310-H312-K313) patch flanking the Ubc7-interacting gp78-RING finger domain, whereas CYP3A4-K257 is found crosslinked to gp78-K586 in another positively charged Q584-R585-K586 patch within the gp78-C-terminal Ubc7-interacting-G2BR domain (Wang et al., 2015). Simultaneous site-directed mutagenesis of both these positively charged gp78 patches were found to attenuate not only its CYP3A4 ubiquitination *in vitro* (Wang et al., 2015), but also that of human CYP2E1 (Figure 2), another P450 ubiquitinated by the Ubc7-gp78 complex (Wang et al., 2011). Notably, CYP3A4-K251, K257 and K266 along with several other CYP3A4-residues (K127, K141, K168, K209, K413, K421, K424 and K487) were also found cross-linked to its cognate E2 (Ubc7), consistent with both the critical ultimate Ub-donor role of Ubc7, and the dynamic nature of the inherent CYP3A4-Ubc7/gp78 electrostatic interactions within this ubiquitination system (Wang et al., 2015).

CHIP

This cytosolic U-box E3 Ub-ligase engages UbcH5a as its cognate E2-partner and functions cooperatively as a co-chaperone of Hsp70/Hsp40 in UPD substrate-recruitment (Ballinger et al., 1999; Clapp et al., 2012; Edkins, 2013; Jiang et al., 2001; McDonough & Patterson, 2003; Morishima et al., 2005; Murata et al., 2001; Peng et al.,
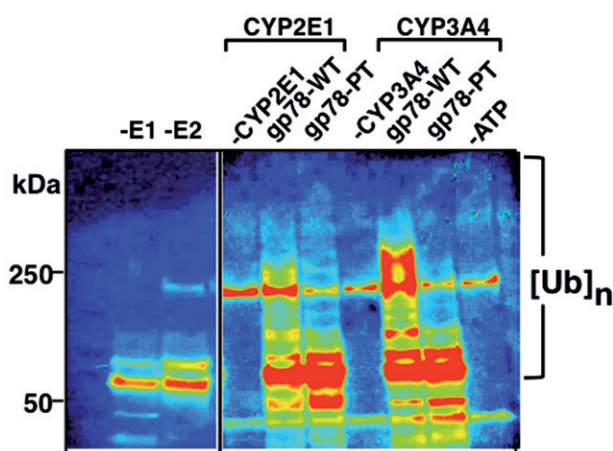
Figure 2. gp78-mediated ubiquitination of CYP2E1: Involvement of the same gp78 basic patches involved in its intermolecular electronic interactions with CYP3A4. This was documented in *in vitro* functionally reconstituted E1/E2/E3 systems in the presence or absence of either CYP2E1 or CYP3A4 or ATP, using either recombinant gp78 wild type (WT) or its patch mutant (PT) wherein its positively charged residues R307-R308-R310-H312-K313/Q584-R585-K586 (known to interact with certain CYP3A4 DEST clusters) were mutated to Ala via site-directed mutagenesis (Wang et al., 2015). Color code wheel: Red > orange > yellow > green > blue > indigo > violet.

2004; Rosser et al., 2007). It is a 303-residue long, 38.4 kDa protein containing three tandem tetratricopeptide repeats (TPR) in its N-terminal domain for Hsc/Hsp70- and Hsp90-binding, and a C-terminal RING-finger like U-box that is evolutionarily very highly conserved. Because yeasts lack CHIP (Vembar & Brodsky, 2008), its role in CYP3A4 ERAD could not be predicted *a priori* from our studies in *S. cerevisiae* (Liao et al., 2006; Murray & Correia, 2001). However, CHIP was documented to ubiquitinate various proteins including CFTR, its co-chaperone Hsp70, the androgen, estrogen and glucocorticoid receptors (Ballinger et al., 1999; Edkins, 2013; Jiang et al., 2001; McDonough & Patterson, 2003; Rosser et al., 2007; Younger et al., 2006), and most tellingly, the P450-like hemoprotein neuronal nitric-oxide synthase (Clapp et al., 2012; Peng et al., 2004), as well as the recombinant rabbit CYP2E1 and CYP2B4 *in vitro* (Morishima et al., 2005). This collective evidence coupled with our preliminary finding that CYP3A4 was indeed stabilized in a temperature sensitive Hsp70-defective yeast mutant (*ssa1–45*; Vembar & Brodsky, 2008), prompted the examination of its ability to ubiquitinate human CYP3A4 (Kim et al., 2010; Pabarcus et al., 2009; Wang et al., 2009, 2012, 2015) and CYP2E1 (Morishima et al., 2005; Wang et al., 2011) in *in vitro* functionally reconstituted ubiquitination systems. Such *in vitro* CYP3A4 and CYP2E1-ubiquitination suggested its involvement in P450 ERAD *in vivo* (Figure 1).

Accordingly, shRNAi-elicited hepatic CHIP knockdown in cultured rat hepatocytes of ≈ 80% led to a corresponding ≈ 2.5-fold stabilization of functionally active CYP3A (Kim et al., 2010) and CYP2E1 ER-content (Kim et al., 2016b), thereby verifying the physiological role of CHIP in P450 ERAD. Once again its function, as that of gp78, was also neither redundant nor compensated by any of the numerous cellular E3s (including hepatic gp78), functional in ERAD.

Chemical crosslinking coupled with LC-MS/MS analyses of CYP3A4 ubiquitinated by the fully reconstituted Hsp70/Hsp40/UbcH5a/CHIP-system revealed just two direct links of CYP3A4-K257 with CHIP-K125, and CYP3A4-K421 with CHIP-K22, whereas 9 different crosslinks were found between CYP3A4 residues (K127, K141, K168, K257, K266, K421) and one of 4 UbcH5a-residues (K4, K8, K133, K144) (Wang et al., 2015). Just one crosslink between CYP3A4-K266 and Hsp70-K512 was detected (Wang et al., 2015), not all surprisingly given that Hsp70 is known to interact with its targets largely via hydrophobic domains. Interestingly, CYP3A4-K257 and K266 residues flank the same surface acidic (E258-pS259-E262-D263-pT264) DEST-cluster that is relevant for its Ubc7-gp78 interactions.

Intriguingly, the CHIP complex is able to elaborate K_{11}-, K_{48}- and K_{63}-Ub-linkages in *in vitro* functionally reconstituted P450-ubiquitination systems (Wang et al., 2012). This suggests that it may also be capable of diverting K_{48}-Ub-linked ubiquitinated P450 proteins to ERAD/UPD, and K_{63}-linked ubiquitinated P450 proteins to ERAD/ALD (*see discussion below*). Indeed on the basis of studies with structural deletion CHIP-mutants, a role for CHIP as a molecular switch between these two cellular degradation pathways has been proposed in its targeting of α-synuclein to its UPD via its Hsp70-interacting TPR domain, and to ALD via its catalytic U-box (Shin et al., 2005).

Complementary rather than redundant roles of gp78 and CHIP E3 ligases in P450 ERAD

Several examples now exist in the literature indicating that in mammalian cells, more than one E2/E3 Ub-ligase complex can concomitantly target the same proteins as substrates (Imai et al., 2002; Jo et al., 2011; Lu et al., 2011; Morito et al., 2008; Shmueli et al., 2009; Wang et al., 2014, 2015; Ying et al., 2011; Younger et al., 2006; Zhang et al., 2015), thus revealing that they are not quite redundant and/or need not compete for the substrate target in question, but rather act synergistically and/or cooperatively. In doing so, it is unclear whether they select a given substrate molecule in parallel, simultaneously targeting different and/or multiple K-residues

in discrete surface domains, or whether they act in tandem according to some preordained "*hierarchical organization of prioritized substrate-E3 relationships*" (Christianson & Ye, 2014). Several lines of evidence strongly suggest that a similar hierarchical targeting may indeed occur in P450 ERAD: First, upon shRNAi-elicited gp78-knockdown, although CYP3A ER-export into the cytosol and thus its UPD were considerably reduced in cultured rat hepatocytes, significant CYP3A ubiquitination (detected as HMM ^{35}S-species) was found to still persist both in the ER and cytosol (Kim et al., 2010). Thus gp78 knockdown had reduced, but did not altogether abolish CYP3A ubiquitination and consequent ER to cytosol export (Kim et al., 2010). By contrast, similar shRNA-mediated CHIP knockdown completely abrogated both CYP3A ubiquitination and its ER to cytosol export, even though gp78 was functionally unaffected (Kim et al., 2010). These findings revealed that "*in vivo*" CHIP may function quite early as an E3 to prime CYP3A ubiquitination in the ER, and that after such initial CHIP-mediated CYP3A priming, gp78 may serve as an E4 to extend the already initiated Ub-chains (Chen et al., 2012; Kostova et al., 2007; Morito et al., 2008; Wang et al., 2014). Second, the findings that although each functionally reconstituted E2/E3-system could individually support CYP3A4-ubiquitination, but simultaneous inclusion of both these E2/E3 systems robustly potentiated their CYP3A4-ubiquitination with even greater elongation of HMM CYP3A4 Ub-conjugates, underscores their synergistic interactions (Wang et al., 2015). Furthermore, the exclusion of just CHIP in the first 30 min of this dual E2/E3-incubation attenuated the overall CYP3A4-ubiquitination and its extension to higher HMM-ubiquitinated species to a much greater extent than the omission of just gp78 during this initial 30 min-period. This similarly points to sequential interactions in a predetermined hierarchical order wherein CHIP serves as the E3 to prime P450 for ubiquitination, with gp78 then acting as an E4 to elongate these Ub-chains (Wang et al., 2015). Additionally, although each of these two E3-ligases can individually target specific K-residues in topologically different CYP3A4 surface clusters, their target sites (i.e. CYP3A4 K127, K168, K492) are not mutually exclusive (Wang et al., 2012). Further support for such complementary rather than redundant roles of these E2/E3 systems is also derived from their intermolecular interactions with some of the same CYP3A4 K-residues (K257, K266) flanking or within the acidic (E258-pS259-E262-D263-pT264) surface cluster (Wang et al., 2015). Thus, Ala-mutation of the residues within this cluster greatly attenuated CYP3A4 ubiquitination by the dual E2/E3-system. These findings suggest that although gp78 can *per se* effectively ubiquitinate

P450s (and its other substrates) in *in vitro* reconstituted systems, it can function both as an E3 and an E4 in ERAD/UPD (Kim et al., 2010; Pabarcus et al., 2009; Wang et al., 2009, 2011, 2012, 2015). A similar E4-role has been ascribed to CHIP in its promotion of Parkin-mediated ubiquitination of the unfolded Pael receptor (Imai et al., 2002).

P450 ERAD/ALD

Unlike P450 ERAD/UPD, relatively little is known about the sorting mechanisms and cellular participants involved in P450 ERAD/ALD. ALD is a highly conserved eukaryotic process, once thought to involve the bulk degradation of senescent, long-lived proteins/organelles as well as cytosolic components following starvation (Cuervo, 2011; Klionsky et al., 2010; Mizushima et al., 2008; Rabinowitz & White, 2010; Rubinsztein et al., 2012). It is now recognized to be both a rather tightly regulated and selective process entailing macroautophagy with subsequent lysosomal degradation (Bento et al., 2016; Cuervo, 2011; Klionsky et al., 2010; Mizushima et al., 2008, 2010; Madrigal-Matute & Cuervo, 2015; Ohsumi, 2014; Rabinowitz & White, 2010; Rubinsztein et al., 2012; Wen & Klionsky, 2016; Yang & Klionsky, 2009). Macroautophagy (or autophagy for short) involves the concerted function of over 35 **au**tophagy-related **g**enes (ATGs; Bento et al., 2016; Cuervo, 2011; Klionsky et al., 2010; Madrigal-Matute & Cuervo, 2015; Mizushima et al., 2008; Mizushima et al., 2010; Ohsumi, 2014; Rabinowitz & White, 2010; Rubinsztein et al., 2012; Wen & Klionsky, 2016; Yang & Klionsky, 2009) that generate multiprotein complexes that act cooperatively and sequentially to deliver the cargo for lysosomal degradation (Figure 3). This process, distinct from microautophagy (Madrigal-Matute & Cuervo, 2015) or chaperone-mediated autophagy (Madrigal-Matute & Cuervo, 2015), is initiated by the formation of an isolation membrane or "***phagophore***" of somewhat still uncertain and thus debatable cellular origin. This step requires both the Unc-51-like kinase (ULK1) complex that phosphorylates a Beclin l-complexed class III phosphoinositol-3 kinase [PI3K], a target of 3-methyladenine (3-MA), LY294002 or wortmannin (Mizushima et al., 2010). Upon choosing its cargo (i.e. a P450), the isolation membrane elongates and then fuses to form a double-membrane vesicle known as the "***autophagosome***". This step is critically dependent on the Atg5-Atg12-Atg16-WD-repeat (WIPI) protein-dependent conversion of microtubule-associated protein 1 light chain 3 (MAP-**LC3**, or LC3 for short, a mammalian homolog of yeast Atg8) from **LC3-I** (free form) to **LC3-II** (membrane-bound form) upon
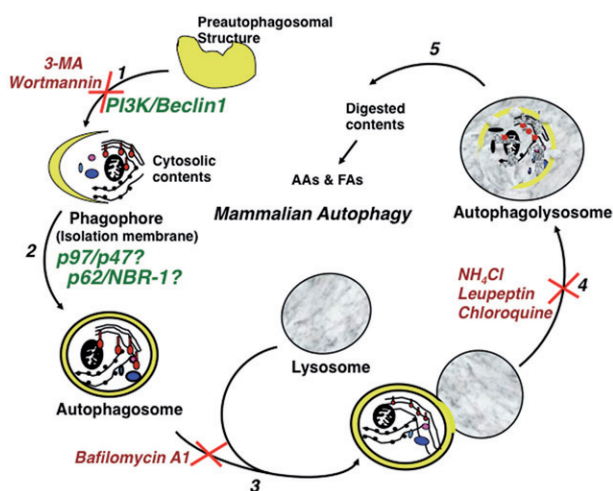
Figure 3. Autophagy in mammalian cells. The major steps in ALD of cellular proteins and organelles are illustrated. ALD-inhibitors that block the various steps and serve as diagnostic probes are also shown. See discussion for details.

conjugation with phosphatidylethanolamine (**PE**), a lipid enriched in the isolation membrane. Indeed, LC3-I to LC3-II conversion is a critical hallmark used as a biochemical and/or morphologic marker of cellular autophagic induction (Mizushima et al., 2010). Cargo selection and subsequent sequestration is dependent on LC3-interacting motifs "**LIRs**" of target substrates and/or accessory adapters such as p62 and/or NBR-1, the cargo receptors for ubiquitinated substrates and/or protein aggregates (Birgisdottir et al., 2013; Johansen & Lamark, 2011; Lamark et al., 2009; Stolz et al., 2014). Fusion of the cargo-sequestering autophagosome with a lysosome generates the ***autolysosome***, wherein lysosomal proteases degrade the cargo, and the end products are either recycled or consumed as an energy source. Although only the defined milestones common to this ALD process are underscored here (Figure 3), it is highly likely that P450s like other ALD substrates (Bento et al., 2016; Klionsky et al., 2010; Rubinsztein et al., 2012), incur a well-orchestrated, multi-step journey along cytoskeletal elements (microtubules and kinesin/dynein motors) from the ER to the lysosome, paved by various different ATG-protein complexes, kinases, cargo receptors, and/or membrane-tethering factors. Additionally, an alternate subfamily of ATG8-homologs GABARAPs/GATE-16, also involved in mammalian selective autophagy (Slobodkin & Elazar, 2013), could also participate in P450 ALD. The identity of each of these specific P450 ALD participants is currently unknown. In addition to PI3K-inhibitors listed above, other diagnostic ALD inhibitors include (i) bafilomycin A (BFA) that inhibits autophagosomal fusion and thus its maturation into the autolysosome; (ii) NH$_4$Cl or chloroquine that alkalinizes the acidic intralysosomal pH required for

proteolysis; and/or (iii) lysosomal protease inhibitors leupeptin, pepstatin, or E-64 (Mizushima et al., 2010). Collective experimental evidence including the use of 3-MA/NH$_4$Cl as diagnostic probes has revealed that certain P450s are largely degraded via ERAD/ALD, whereas only a few others are normally so degraded (Correia, 2003; Correia & Liao, 2007; Faouzi et al., 2007; Liao et al., 2005; Masaki et al., 1987; Murray et al., 2002; Ronis & Ingelman-Sundberg, 1989; Ronis et al., 1991; Roberts, 1997; Wang et al., 2011; Figure 3; *see below*).

Molecular/cellular determinants of P450 ERAD/UPD versus ERAD/ALD selectivity?

Studies with diagnostic UPD/ALD inhibitors in cultured human, mouse and rat hepatocytes (Figure 4) coupled with those in *S. cerevisiae* strains with specific defects/deletions of crucial UPD/ALD components, indicate that CYPs 3A, either native or inactivated, largely incur UPD (Correia et al. 1987, 1992a, 1992b, 2005; Correia, 2003; Correia & Liao, 2007; Faouzi et al., 2007; Korsmeyer et al., 1999; Liao et al., 2006; Murray & Correia, 2001; Roberts, 1997; Schmiedlin-Ren, 1997; Wang et al., 1999), whereas native CYPs 2B1 and 2C11 largely incur ALD (Correia, 2003; Correia & Liao, 2007; Liao et al., 2005; Masaki et al., 1987; Murray et al., 2002; Ronis & Ingelman-Sundberg, 1989; Ronis et al., 1991; Roberts, 1997; Wang et al., 2011), and CYP2E1 incurs both processes (Dai & Cederbaum, 1995; Morishima et al., 2005; Roberts, 1997; Ronis & Ingelman-Sundberg, 1989; Ronis et al., 1991; Sohn et al., 1991; Tierney et al., 1992; Wang et al., 2011). Indeed, rat liver CYP2E1 and its mammalian orthologs have been the first P450 proteins shown to incur biphasic turnover with a rapid ($t_{1/2}$, 7 h) and slow ($t_{1/2}$, 37 h) phase that reflect its degradation via ERAD/UPD and ERAD/ALD, respectively (Correia, 2003, *and references therein*; Roberts et al., 1995; Song et al., 1989). The rapid phase apparently stems from structural damage inflicted by ROS generated by its futile oxidative cycling in the absence or on withdrawal of an appropriate substrate (i.e. EtOH or acetone), or even with a poorly accepted substrate that permits ROS leakage from the active site. By contrast, substrate binding at its active site stabilizes the CYP2E1 protein conformation and prolongs its half-life (Correia, 2003; Roberts et al., 1995; Song et al., 1989, *and references therein*), diverting it into the slower ERAD/ALD pathway.

These collective observations along with the wide range of P450 protein half-lives (Correia, 2003; *and references therein*) imply that in spite of their equivalent ER-co-residency, some degree of selectivity normally exists in P450 sorting for degradation. What determines such differential P450 molecular sorting into the ERAD/UPD
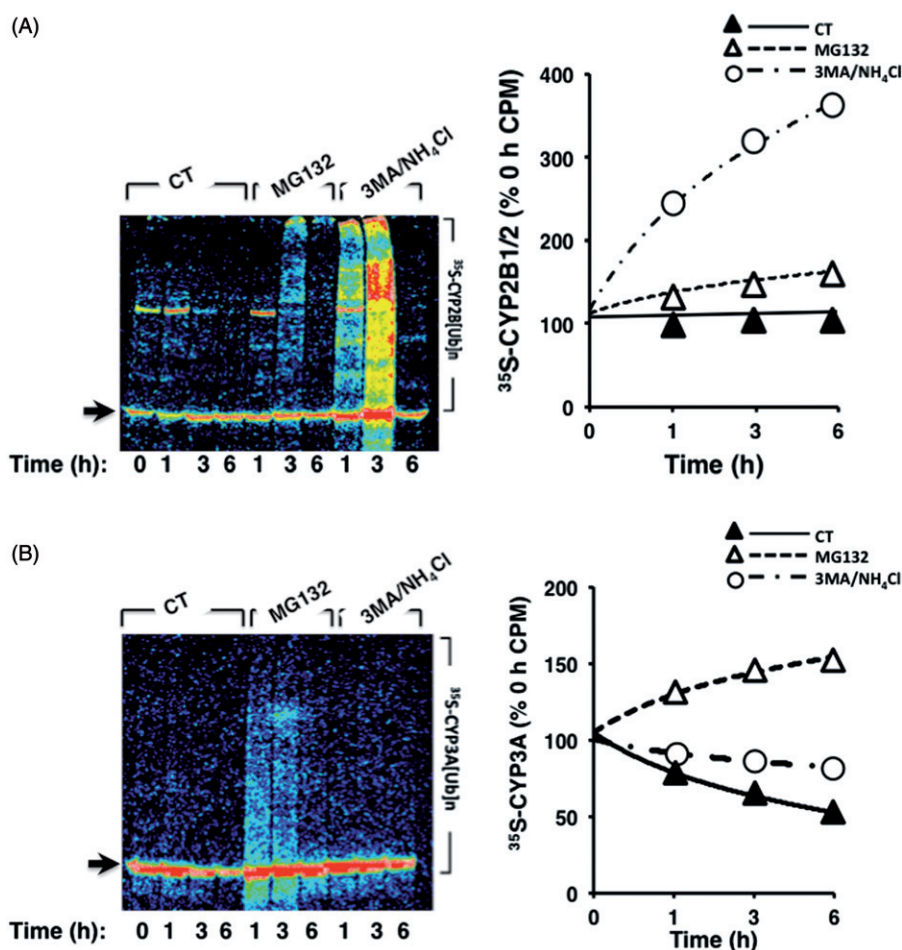
Figure 4. ^{35}S-pulse-chase analyses of CYP3A and CYP2B time-course in cultured rat hepatocytes. Cells were pulsed upon pretreatment for 4 days with either phenobarbital, 1 mM (A) or dexamethasone, 10 μM (B), following which they were pulse-chased with ^{35}S-methionine/cysteine for 1 h in a methionine-cysteine-free WEM culture medium as described previously (Kim et al., 2010). The culture medium was then exchanged with a medium containing cold methionine/cysteine, and harvested at 0, 1, 3 and 6 h thereafter. In parallel, similarly ^{35}S-pulse-chased cultures were treated at time 0 h with either MG132 (10 μM) or 3-MA (5 mM)/NH$_4$Cl (50 mM). Cell lysates were immunoprecipitated with either anti-CYP2B1 (A) or anti-CYP3A23 (B) antibodies. Aliquots of immunoprecipitates were subjected to SDS-PAGE and fluorography with Typhoon imaging (*left panels*). Smaller aliquots (10 μL) were subjected to liquid scintillation counting (*right panels*). CT: control. Arrows depict the parent ≈ 50 kDa ^{35}S-labeled P450 species.

and ERAD/ALD pathways? Plausible determinants include: (i) Proclivity to futile oxidative cycling, and thus a propensity for structural lesions in the catalytic domain by engendered ROS that target the P450 protein to UPD, with a consequently shortened half-life. Accordingly, genetic deletion of cytochrome P450 reductase (CPR), the *sine qua non* P450 redox partner, and consequent abolition of P450 oxidative function indeed results in the elevation of P450 hepatic content, most likely via protein stabilization (Gu et al., 2003; Henderson et al., 2003). This is also true when CPR function is chemically inhibited in cell culture (Goasduff & Cederbaum, 1999; Zhukov & Ingelman-Sundberg, 1999). Furthermore, just substrate (EtOH or acetone)-binding to the CYP2E1 active site and catalysis, aborts its futile cycling to ROS, thereby prolonging CYP2E1 half-life and stabilizing the protein (Bardag-Gorce et al., 2006;

Chien et al., 1997; Roberts et al., 1995; Song et al., 1989). Additionally, quasi-irreversible P450 inactivators such as TAO that coordinate tightly to the CYP3A-ferrous heme and abort its catalytic turnover, also dramatically prolong CYP3A half-life, resulting in the elevation of hepatic CYP3A content (Watkins et al., 1986), another valid example of P450 induction via protein stabilization. However, in spite of this compelling evidence favoring oxidative turnover as an ERAD/UPD determinant, arguments for additional requirements exist. For instance, heterologous expression of human CYP3A4 and CYP2E1 in *S. cerevisiae* whose basal CPR content is minimal, does not deter their UPD or switch it completely to ALD, as expected from their greatly diminished oxidative function (Liao et al., 2006; Murray & Correia, 2001; Wang et al., 2011). This argues for the existence of additional intrinsic structural features, barcodes or "*degrons*"

and/or posttranslational modifications to be involved in P450 recognition, which determine whether any given P450 is targeted to UPD or ALD. Accordingly, the observed switch of heterologously expressed rat liver CYP2B1 in *S. cerevisiae* from documented ERAD/ALD to ERAD/UPD upon appendage of a CYP3A4 C-terminal heptapeptide (Liao et al., 2005), reveals that a structurally-flexible C-terminal tail was all that was required for CYP2B1 to engage the 26S proteasome. Thus, it is conceivable that some structural P450 modifications acquired post-translationally (i.e. phosphorylation, ubiquitination, acetylation?) or intrinsic structural features unraveled by protein unfolding (i.e. concealed degrons or LIRs; *see below*) may dictate this P450 sorting process. Details of the nature of certain P450 structural determinants such as surface linear and/or conformational phosphodegrons and/or Lys$_{48}$-linked polyubiquitination that target proteins largely, if not exclusively to ERAD/UPD are now emerging (Wang et al., 2011, 2012 and 2015).

UPD-recognition via linear and/or conformational phosphodegrons

Degrons are usually specific short linear amino acid sequences (often containing phosphorylatable S/T-residues that engender phosphodegrons), structural motifs (unstructured/floppy C- and/or N-termini, disordered loops) and/or cytosol-exposed amino acids (K, R) that serve as hooks to engage different components of the cellular degradation machinery thereby leading to the molecular recognition and accelerated degradation of a given protein. Such phosphodegrons appear to play a key role in CYP3A4 E2/E3-recognition. Accordingly, CYP3A4-ubiquitination in an *in vitro* functionally reconstituted UBC7/gp78- or CHIP-system, followed by tryptic/lysyl endopeptidase C (Lys-C) digestion of the ubiquitinated protein, and subsequent LC-MS/MS analyses of the protein digest to monitor Ub-derived GG- or LRGG-remnant modified CYP3A4 peptides, led to the identification of various CYP3A4 ubiquitinated Lys (K)-residues (Wang et al., 2012, 2015). Scrutiny of the available CYP3A4 crystal structures (Ekroos & Sjogren, 2006; Sevriouka & Poulos, 2012; Williams et al., 2003; Yano et al., 2004) reveal that these ubiquitinated K-residues, along with previously identified PKA- or PKC-phosphorylated Ser and Thr (pS/pT)-residues reside within or are vicinal to surface clusters of acidic [Asp (D), Glu (E)] residues (Figure 5). Based on this observation and various other converging lines of literature evidence (*discussed in Wang et al., 2015*) on the nature of other observed P450 electrostatic protein–protein interactions (Bridges et al., 1998; Gao et al., 2006; Lin et al., 2012; Zhao et al.,

2012), distinguishing structural features of E2-enzyme-substrate interactions (Catic et al., 2004; Miao et al., 2010), as well as the high propensity of observed D/E/S/T-residues flanking ubiquitination sites within linear peptides identified through global Ub-remnant profiling analyses of the vast cellular "ubiquitinome" (Peng et al., 2003; Kim et al., 2011; Udeshi et al., 2012, 2013; Wagner et al., 2012; Xu et al., 2010), we proposed that such negatively charged surface DEST-clusters through electrostatic interactions with positively charged residues of these E2/E3 systems would be critical to CYP3A4 molecular recognition (Correia et al., 2014; Wang et al., 2012, 2015). Indeed, structural analyses of CYP3A4 K-ubiquitination verified that it not only occurs on contiguous linear motifs (i.e. K282-E283-pT284-E285) along disordered cytosol-exposed surface loops that conform to canonical phosphodegrons, but also involves interactions with D/E surface clusters spatially associated with phosphorylatable S/T residues (i.e. E258-pS259-E262-D263-pT264; K115-pS116-E122-D123-E124-E125) that are assembled together by the P450 tertiary structure and thus represent "*conformational*" phosphodegrons (Figure 5). Such P450 S/T phosphorylation by imparting additional negative charge would further boost the overall negative charge of a specific DEST cluster (Correia et al., 2014; Wang et al., 2012, 2015), thus serving as the molecular switch to enhance P450 recognition by an E2/E3-complex, thereby accelerating P450 proteasomal degradation, and controlling the timing of its ERAD. Such a role of CYP3A4 phosphodegrons in its recognition by gp78- and CHIP-ubiquitination complexes was validated though several complementary approaches: Thus, (i) as expected, high salt treatment was found to disrupt CYP3A4 electrostatic interactions with subcomponents of each E2/E3-complex thereby attenuating their CYP3A4 ubiquitination (Wang et al., 2015). (ii) Chemical-crosslinking of the surface K-residues coupled with LC-MS/MS analyses identified the specific intermolecular interaction-sites. Thus, CYP3A4-K-residues cross-linked to these E2/E3-components were found either flanking or vicinal to its negatively charged surface clusters (*identified above*), while the gp78-K-residues cross-linked to CYP3A4-K-residues resided within positively charged "basic patches" (Wang et al., 2015). (iii) Site-directed mutagenesis of the positively charged residues within these gp78 basic patches greatly attenuated gp78-mediated CYP3A4-ubiquitination, thereby verifying the involvement of the requisite electrostatic interactions (Wang et al., 2015). (iv) Site-directed mutagenesis to Ala of D/E/S/T-residues individually or in combination within relevant negatively charged CYP3A4 surface-clusters was also found to attenuate gp78- and/or CHIP-mediated
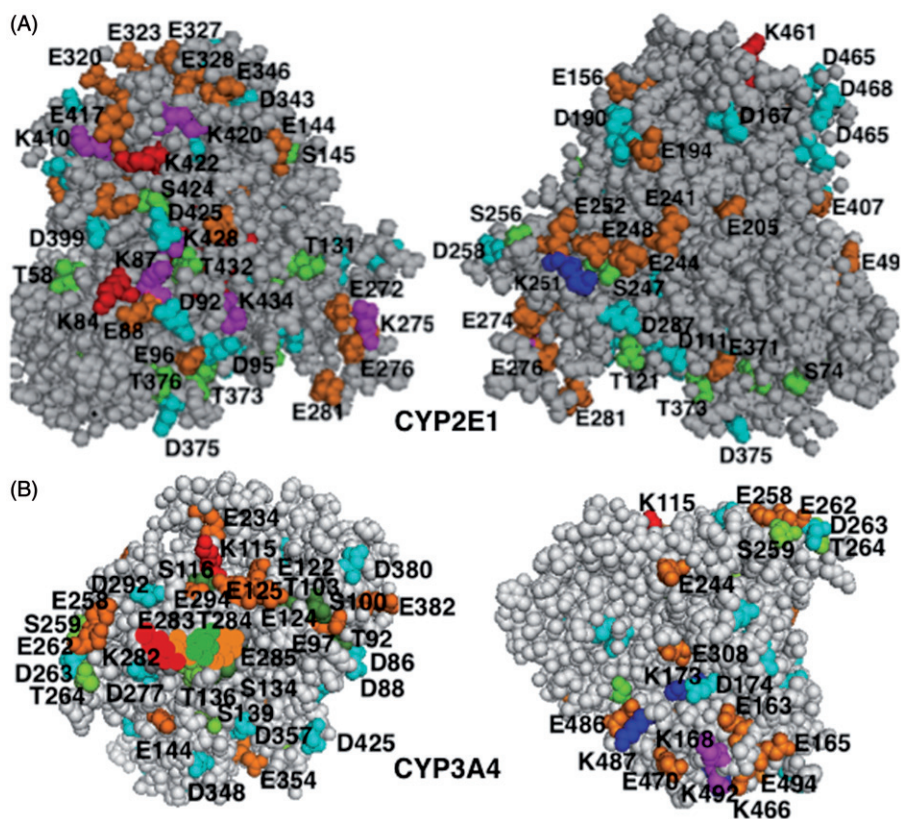
Figure 5. CYP2E1 and CYP3A4 structures with linear and ''conformational'' phosphodegrons: Previously identified phosphorylated S/T-residues (green) and ubiquitinated K-residues are shown. The K-residues ubiquitinated by gp78-complex are colored red, those ubiquitinated by CHIP-complex in blue, and those ubiquitinated by both E3-complexes in magenta. Note that these residues lie within or in the vicinity of linear loops or clusters of pS/pT (green), D and E residues colored cyan and orange, respectively.

CYP3A4-ubiquitination (Wang et al., 2015). Accordingly, these analyses identified the E283-pT284-E285 cluster as specifically relevant to CYP3A4 interaction with gp78, and its E258-pS259-E262-D263-pT264 cluster to that with CHIP (Wang et al., 2015).

Although, CYP2E1 interactions with these E2/E3 ligases have not been quite as extensively scrutinized, our initial inspection of the CYP2E1 structure (Porubsky et al., 2008) revealed that the previously identified CYP2E1 PKA/PKC-phosphorylation (pS/pT) sites and its UBC7/gp78 and CHIP-ubiquitination K-sites also reside within or vicinal to negatively charged DEST surface clusters (Figure 5; Correia et al., 2014; Wang et al., 2011). Furthermore, the positively charged gp78-patches required for CYP3A4-ubiquitination are similarly relevant to its CYP2E1-ubiquitination (Figure 2). Preliminary findings indicate that human liver CYPs 2C9, 2C19 and 2D6 are also targeted to gp78- and/or CHIP-mediated ubiquitination in *in vitro* reconstituted systems (*YQ Wang & M. A. Correia, preliminary findings*). Structural mapping of each of these P450s (Reynald et al., 2012; Rowland et al., 2006; Wang et al., 2012; Wester et al., 2004) reveals that many of the surface K-residues lie either within or vicinal

to negatively charged DEST-clusters, thereby suggesting this to be a common feature of these other gp78/CHIP-ubiquitinatable P450s (Figure 6).

Lys$_{48}$-linked polyUb chains

Ub contains seven internal K-residues (K6, K11, K27, K29, K33, K48 and K63), and proteomic analyses reveal that any of these residues can be utilized to form polymeric Ub-chains that apparently are physiologically relevant (Kulathu & Komander, 2012). The most common of these Ub-chains is Lys$_{48}$-linked polyubiquitination (wherein the isopeptide linkage occurs between Lys$_{48}$ of the first Ub within the chain and/or conjugated to the protein substrate and terminal Gly$_{76}$ of the next Ub-molecule), which is generally believed to target proteins to the 26S proteasome by tethering to the polyUb-receptors (Rpn10/Rpn13 subunits) of the 19S proteasomal cap (Finley, 2009; Pickart & Cohen, 2004; Rechsteiner, 2005). The ideal Ub-chain length for such proteasomal targeting is $\approx$4–20 (Finley, 2009; Pickart & Fushman, 2004; Pickart & Cohen, 2004; Rechsteiner, 2005), although even shorter chains may suffice (Lu et al., 2015).
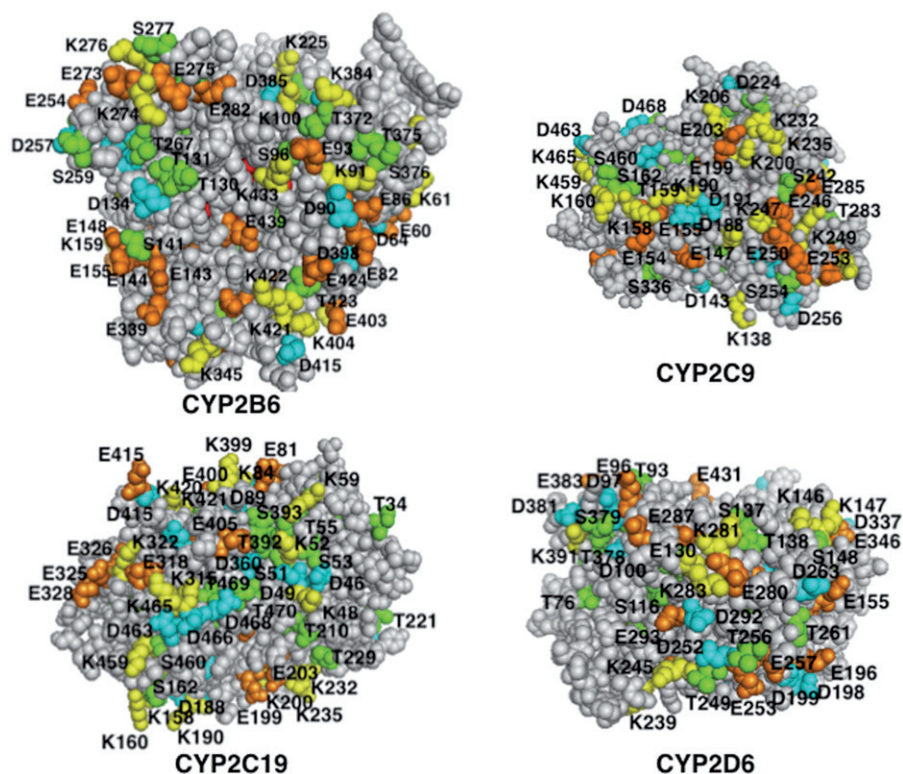
Figure 6. Plausible linear and "conformational" phosphodegrons of other major human liver P450s: Surface DEST clusters are shown with vicinal K-residues shown in yellow, S/T phosphorylation sites in green, D and E residues in cyan and orange, respectively. Note that although neither the ubiquitination nor the phosphorylation sites have yet to be conclusively identified, their situation within or vicinal to DEST clusters is highly intriguing.

These are directly elaborated on each available Lys-εNH_2 (or sometimes an N-terminal-αNH_2) group of the protein substrate, with larger/longer Ub-conjugates being preferred and degraded at a faster rate (Rechsteiner, 2005). Non-canonical Ub-linkages with substrate cysteine, serine, and threonine residues also occur, but these linkages are unstable and seldom survive the LC-MS/MS conditions required for their detection and interrogation of their physiological relevance (Cadwell & Coscoy, 2005; Ishikura et al., 2010; Vosper et al., 2009; Wang et al., 2007). In the case of CYP3A4 and CYP2E1, such Lys_{48}-linked Ub-chains are found on K-residues in surface loops and/or disordered regions, within or vicinal to DEST-clusters, elaborated exclusively by UBC7/gp78 E2/E3-complexes, but also to a respectable extent by the CHIP-system (Wang et al., 2011, 2012, 2015). Similarly, Lys_{29}-chains conjugated directly to a protein substrate are found to target to its proteasomal destruction (Rechsteiner, 2005). On the other hand, atypical Lys_{27}-linked chains target misfolded proteins to the 26S proteasome via Hsp70-Bag1 chaperone complex, wherein Bag1 rather than the substrate is the target of such decoration (Rechsteiner, 2005). By contrast, Lys_6-linked Ub-chains are involved in DNA repair, whereas Lys_{63}-linked chains are involved in DNA repair, endocytosis and ALD (*see below*).

P450 ERAD/ALD-recognition

By contrast to ERAD/UPD, the nature/identity of the P450 ALD-determinants remains relatively obscure and thus a promising area for future investigation. Autophagic targeting generally involves at the least 3 interdependent mechanisms that we propose are relevant to P450 ALD (Figure 7): (i) **LIRs** or **LRS**, LC3-interacting regions as adapters (Birgisdottir et al., 2013; Johansen & Lamark, 2011; Lamark et al., 2009; Stolz et al., 2014); (ii) **Monoubiquitination** or **Lys_{63}-linked polyubiquitination** (Kim et al., 2008; Kirkin et al., 2009; Linares et al., 2013; Mukhopadhyay & Riezman, 2007; Seibenhener et al., 2004); and (iii) cargo receptors/adapters such as **p62**/sequestosome-1 (SQSTM1) and/or **NBR1** (*neighbor of BRCA1 gene1*) that bear the essential **LIR** motif and serve to target mono- or Lys_{63}-ubiquitinated substrates to selective autophagy (Kim et al., 2008; Kirkin et al., 2009; Linares et al., 2013; Mukhopadhyay & Riezman, 2007; Olzmann et al., 2007; Pankiv et al., 2007; Seibenhener et al., 2004).
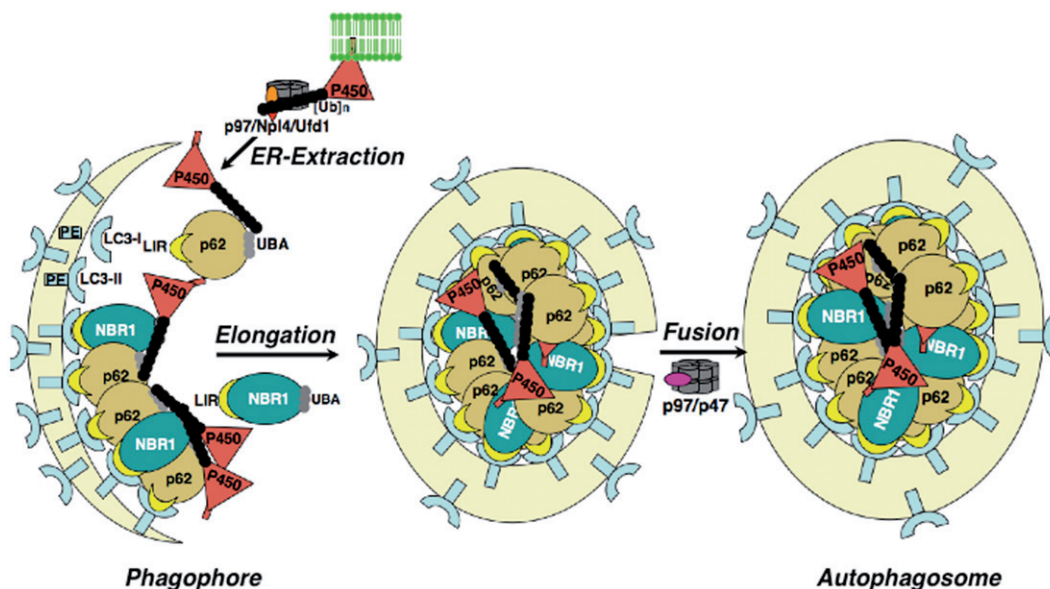
Figure 7. Plausible mechanisms of P450 autophagic targeting: Roles of hepatic p97/Npl4/Ufd1 complexes in ER-extraction, p62 and NBR1 heteromers in autophagic targeting of ubiquitinated P450s via their LIR- and UBA-domains to the phagophore, and of p97/p47 complexes in subsequent autophagosome biogenesis through fusion of the phagophore ends.

LIRs

The central feature of this structural protein barcode is a relatively conserved 8-amino acid linear sequence $(X_{-3}X_{-2}X_{-1}W_0X_1X_2LX_3)$, required to dock at the hydrophobic Ub-like domain of LC3 and thus to target the substrate to the nascent phagophore (Birgisdottir et al., 2013; Ichimura et al., 2008; Johansen & Lamark, 2011; Lamark et al., 2009; Noda et al., 2008; Stolz et al., 2014). In this consensus motif, the W_0-site absolutely has to be an aromatic residue (W, F or Y), while the L-site includes a large hydrophobic residue i.e. L, I, or V (Johansen & Lamark, 2011). $X_{-3}X_{-2}X_{-1}$ and X_1 or X_2 could be, but not strictly, acidic D/E and/or phosphorylatable S/T residues. Thus, the full consensus LIR motif may be written as **D/E-X-D/E-X-D/E-X-W/F/Y-D/E-X-D/E-X-L/I/V**, and is frequently abbreviated to the essential core **W/F/YXXL/I/V** sequence (Birgisdottir et al., 2013; Ichimura et al., 2008; Noda et al., 2008). It is also conceivable that some globular structures such as the P450s may contain "conformational" LIRs, wherein the W_0/XXL/I/V motif is assembled together by the protein's tertiary fold.

Inspection of various primary CYP2B amino acid sequences reveals several relatively conserved linear LIR motifs. However, the CYP2B4 crystal structure (i.e. apparently conserved in CYP2B6 and possibly other CYPs 2B; Halpert, 2011; Scott et al., 2003, 2004; Shah et al., 2011) reveals that some LIRs are surface exposed ($REKY_{62}GDV_{66}F$, $FQGY_{111}GVI_{114}FAN$ $GERW_{121}RAL$, $KDPVF_{195}LRLL_{199}$, $RQIY_{235}RNL_{238}QE$, $Y_{309}GFLLMLKY_{317}$ $PHV_{320}T$), while others are buried and would become available only upon protein unfolding (Figure 8). This is

also true of CYP2E1 (Porubsky et al., 2008) and CYP2D6 (Wang et al., 2012), two human P450s targeted bimodally to both ERAD/UPD and ERAD/ALD (Figure 9; see below). Inspection of the CYP3A4 sequence reveals that it too contains several LIR motifs. However, structural comparison reveals a key difference in that the critical aromatic W_0-residue of most CYP3A4 LIRs unlike those of CYP2B4- or CYP2E1-LIRs are concealed within the protein fold (Porubsky et al., 2008; Scott et al., 2003, 2004; Williams et al., 2003; Yano et al., 2004; Figure 9), and thus may not be readily accessible to the autophagic machinery, unless the protein is unfolded. It is noteworthy that LIR motifs are required but may not be sufficient for autophagic targeting.

Monoubiquitination/Lys$_{63}$-linked polyubiquitination

Increasing evidence indicates that ALD-substrates are tagged via mono- or polyubiquitination [wherein the isopeptide linkage is between Lys_{63} of the first Ub attached to the target (or within the chain) and Gly_{76} of the next Ub] (Kim et al., 2008; Kirkin et al., 2009; Linares et al., 2013; Mukhopadhyay & Riezman, 2007; Seibenhener et al., 2004). Such K_{63}-linked polyubiquitination is involved in their p62-/NBR-1-recruitment and subsequent targeting to ALD (Birgisdottir et al., 2013; Johansen & Lamark, 2011; Lamark et al., 2009; Lamark et al., 2009; Linares et al., 2013; Seibenhener et al., 2004; Stolz et al., 2014). Our findings (Wang et al., 2011, 2012) that CYPs 3A4 and 2E1 are not only ubiquitinated *in vitro* by the UbcH5a/CHIP/Hsp70 E2-E3 complex with almost equivalent K_{48}- and K_{63}-linkages, but also several
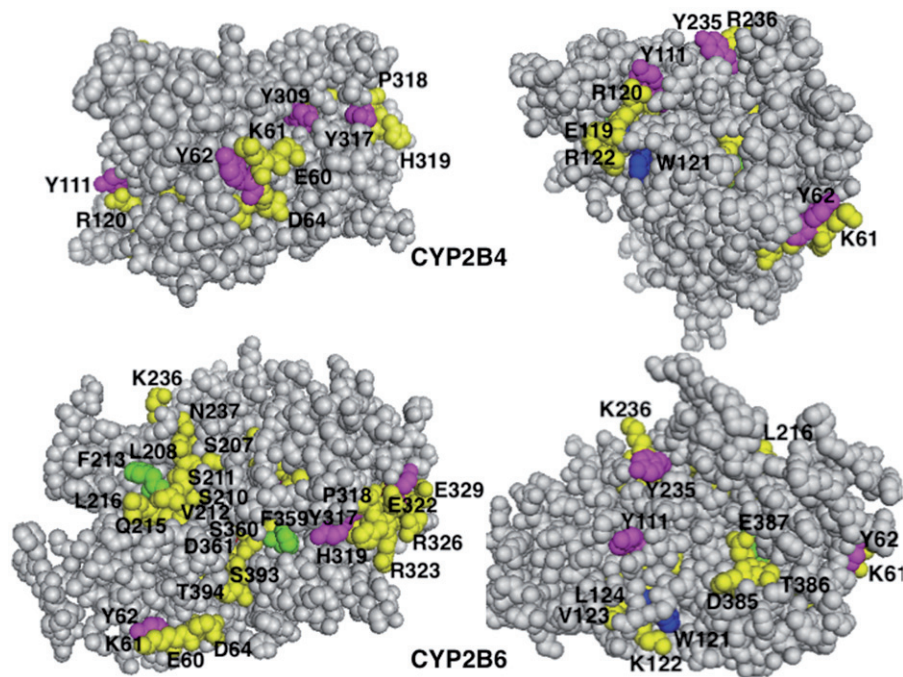
Figure 8. CYP2B4 versus CYP2B6 surface LIR-motifs. Two different orientations of CYP2B4, and CYP2B6 are shown based on their individual structures (*See discussion*). The critical W_0 aromatic residue of the essential LIR W/F/YXXL/I/V core motif is depicted as follows: Trp (blue), Phe (green), and Tyr (magenta), and the other LIR residues in yellow. The extended LIR motif (D/E/X-D/E/X-D/E/X-W/F/Y-D/E/X-D/E/X-L/I/V) is also depicted in yellow, when present in the P450 primary sequence.

human liver P450s incur both types of such linkages in HepG2 cells upon transfection (Figure 10), suggest that in the absence of functionally accessible LIRs, K_{63}-linked polyubiquitination may serve to target these proteins to ALD via p62 and/or NBR1 cargo receptors (Figure 7).

p62 and/or NBR1 autophagic cargo receptors

Several (>24) autophagic cargo receptors such as p62/sequestosome-1, NBR1, Bnip3 (Bcl-2/adenovirus E1B 19 kDa interacting protein 3) and NDP52 (nuclear dot protein 52 kDa) have been identified in mammals (Bento et al., 2016; Birgisdottir et al., 2013; Ichimura et al., 2008; Johansen & Lamark, 2011; Katsuragi et al., 2015; Lamark et al., 2009; Noda et al., 2008; Pankiv et al., 2007; Stolz et al., 2014; von Muhlinen et al., 2010). Their function is usually complementary, but redundant in some instances (Bento et al., 2016; Birgisdottir et al., 2013; Stolz et al., 2014). To qualify as autophagic receptors these proteins must recruit the phagophore to the cargo, leading to its selective autophagosomal engulfment. p62/SQSTM1 and NBR1 are two structurally related mammalian proteins that serve as cargo receptors for selective autophagy of ubiquitinated substrates and/or ubiquitinated aggregates (aggrephagy) (Bento et al., 2016; Birgisdottir et al., 2013; Johansen & Lamark, 2011; Lamark et al., 2009; Pankiv et al., 2007; Stolz et al., 2014).

Their relevant structural features include (i) a LIR motif for docking with LC3 of the phagophore; (ii) a C-terminal Ub-associated domain (UBA) for binding ubiquitinated substrates; and (iii) a N-terminal PB-1 domain for oligomerization (Bento et al., 2016; Birgisdottir et al., 2013; Johansen & Lamark, 2011; Lamark et al., 2009; Pankiv et al., 2007; Stolz et al., 2014). They function cooperatively as heteromeric complexes of p62 polymers and NBR1 oligomers that corral ubiquitinated substrates via their UBA domain and ferry them to the nascent phagophore, guided by their LC3-docking LIR motif. However, in some instances, the p62 LIR domain is dispensable and just oligomerization of its PB-1 domain apparently suffices (Itakura & Mizushima, 2011). Such oligomerization is thought to enable sequestration and clustering of the selected cargo. They are both selective autophagic substrates, and their levels are regulated by ALD rather than UPD (Bento et al., 2016; Birgisdottir et al., 2013; Johansen & Lamark, 2011; Komatsu et al., 2007; Lamark et al., 2009; Olzmann et al., 2007; Pankiv et al., 2007; Stolz et al., 2014). Both p62 and NBR1 mediate protein aggregation and are found in various pathologic inclusions i.e. Mallory Bodies of alcoholic/nonalcoholic steatohepatitis, and aggregates of neurodegenerative Alzheimer's, Huntington's and Parkinson's diseases (Bjorkoy et al., 2005; Denk et al., 2006; Johansen & Lamark, 2011; Komatsu et al., 2007; Strnad et al., 2008).
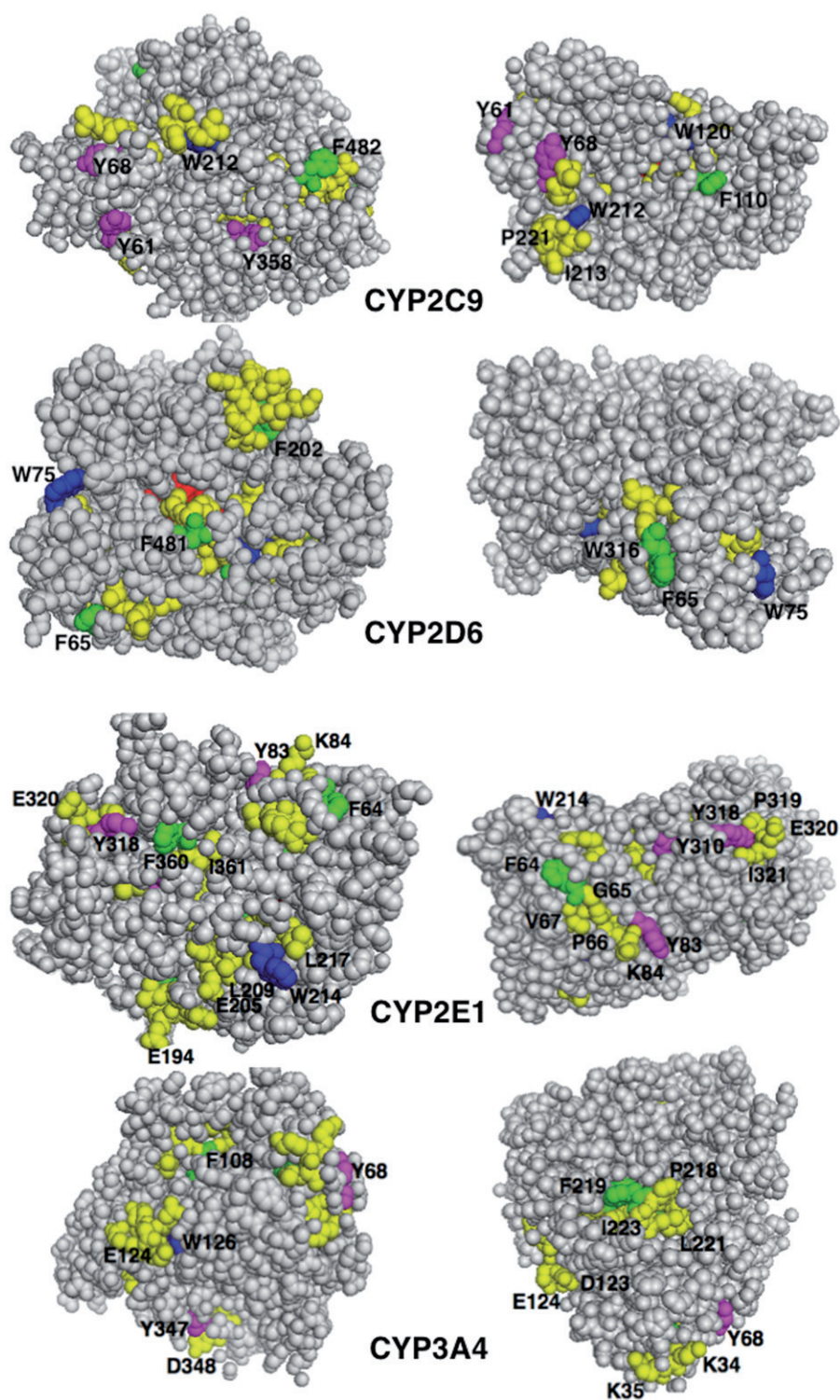
Figure 9. Surface LIR-motifs of other human P450s. Two different orientations of CYP2C9, CYP2D6, CYP2E1 and CYP3A4 are shown based on their individual structures (*See discussion*). The critical W_0 aromatic residue and the other LIR residues of the essential LIR W/F/YXXL/I/V core motif are depicted as follows: Trp (blue), Phe (green), and Tyr (magenta), and the other LIR residues in yellow. The extended LIR motif (D/E/X-D/E/X-D/E/X-W/F/Y-D/E/X-D/E/X-L/I/V) is also depicted in yellow, when present in the P450 primary sequence. Note that these critical W_0-residues are largely buried in CYP3A4.
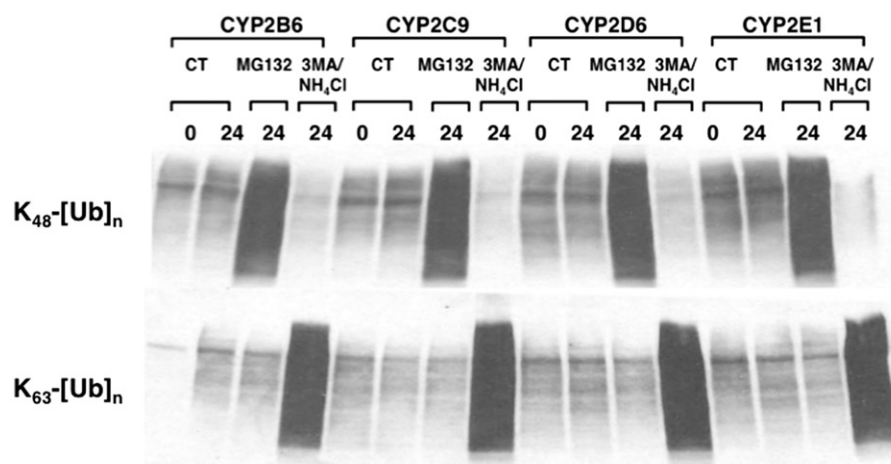
Figure 10. K_{48}- versus K_{63}-linked P450 ubiquitination in HepG2 cells: Cells were transfected for 48 h with a C-terminally tagged Myc/His$_6$ P450 plasmid. The vector-containing medium was removed, cells washed, and then fresh medium added with MG-132, or 3MA/NH$_4$Cl or no inhibitor (Control, CT). Six hours later, cells were harvested and lysates immunoprecipitated with antibodies to anti-Myc antibodies, and immunoblotted with specific anti-K_{48}-Ub or anti-K_{63}-Ub IgGs. Note that all these P450s undergo both types of ubiquitination. However, K_{48}-linked Ub-chains accumulate upon inhibition with the proteasomal inhibitor MG-132, whereas K_{63}-linked Ub-chains accumulate upon 3MA/NH$_4$Cl-mediated ALD inhibition. This may indicate that the type of ubiquitination *per se* is not a determinant of P450 UPD or ALD. Additional factors (structural degrons, barcodes or adapters) must be involved.

Their relatively high abundance in the liver (Komatsu et al., 2007), and their proclivity to target Lys$_{63}$-ubiquitinated substrates makes them potential candidates in the selective autophagy of P450 proteins, particularly those not so highly endowed with LIRs.

A role for p97 in P450 ALD?

p97, also known albeit incorrectly as VCP (Valosin-Containing Protein), or Cdc48p in yeast, is at the cross-roads of various cellular pathways (Anderson et al., 2015; Bug & Meyer, 2012; Deshaies, 2014; Meyer et al., 2012). It functions as discussed above in UPD, as a heterotrimeric complex with two additional heterodimeric adapters, Ufd1 and Npl4, that assist in the recruitment of polyubiquitinated target substrates to the p97-complex (Bar-Nun, 2005; Chou et al., 2011; Dai & Li, 2001; Elkabetz et al., 2004; Ikeda et al., 2009; Jentsch & Rumpf, 2007; Meyer et al., 2000; Richly et al., 2005; Xia et al., 2016; Ye et al., 2005). In *S. cerevisiae,* this heterotrimeric p97-complex is required for CYP3A ERAD/UPD (Liao et al., 2006), a finding verified through crosslinking analyses (Faouzi et al., 2007) and p97-targeted RNAi in cultured rat hepatocytes. Besides UPD, p97 functions in diverse cellular processes including homotypic membrane fusion, vesicular transport, and non-UPD-mediated degradation of cytosolic proteins (Chou et al., 2011; Dreveny et al., 2004; Wojcik et al., 2006; Woodman, 2003; Xia et al., 2016). Highly intriguing reports suggest a role for p97 in ALD (Chou et al., 2011). Accordingly, in yeast, Cdc48, (the p97 homolog) together with its other

adapter p47 has been shown to function in autophagosome biogenesis (Krick et al., 2010) and/or maturation (Tresse et al., 2010). The dual role of p97 in ERAD/UPD and ERAD/ALD reveals that it is strategically positioned to regulate the relative P450 flux through each of these degradation pathways (Chou et al., 2011; Ju et al., 2009; Tresse et al., 2010). Whether it plays each role through recruitment of different adapters (Ufd1/Npl4 in ERAD/UPD vs p47 in ERAD/ALD) remains to be determined. Equally plausible is that p97 could function concurrently as a complex with its UPD adapters Ufd1/Npl4 to extract ubiquitinated P450s from the ER and make them available to p62/NBR-1 or the phagophore, and/or as a complex with its p47 ALD adapter in autophagosome biogenesis/maturation (Figure 7), specific issues that remain to be elucidated.

Relative UPD versus ALD-targeting of select human liver P450s and their rat or mouse orthologs: an update

In common with most aberrant/misfolded/damaged cellular proteins, most mammalian P450s that are suicidally and/or structurally damaged incur ERAD/UPD, (Correia et al. 1987, 1992a, 1992b, 2005; Correia, 2003; Correia & Liao, 2007; Dai & Cederbaum, 1995; Faouzi et al., 2007; Korsmeyer et al., 1999; Liao et al., 2006; Lee et al., 2008; Murray & Correia, 2001; Morishima et al., 2005; Roberts, 1997; Schmiedlin-Ren, 1997; Sohn et al., 1991; Tierney et al., 1992; Wang et al., 1999). However, native P450s not only are tagged with K_{48}- and K_{63}-linked Ub-chains,
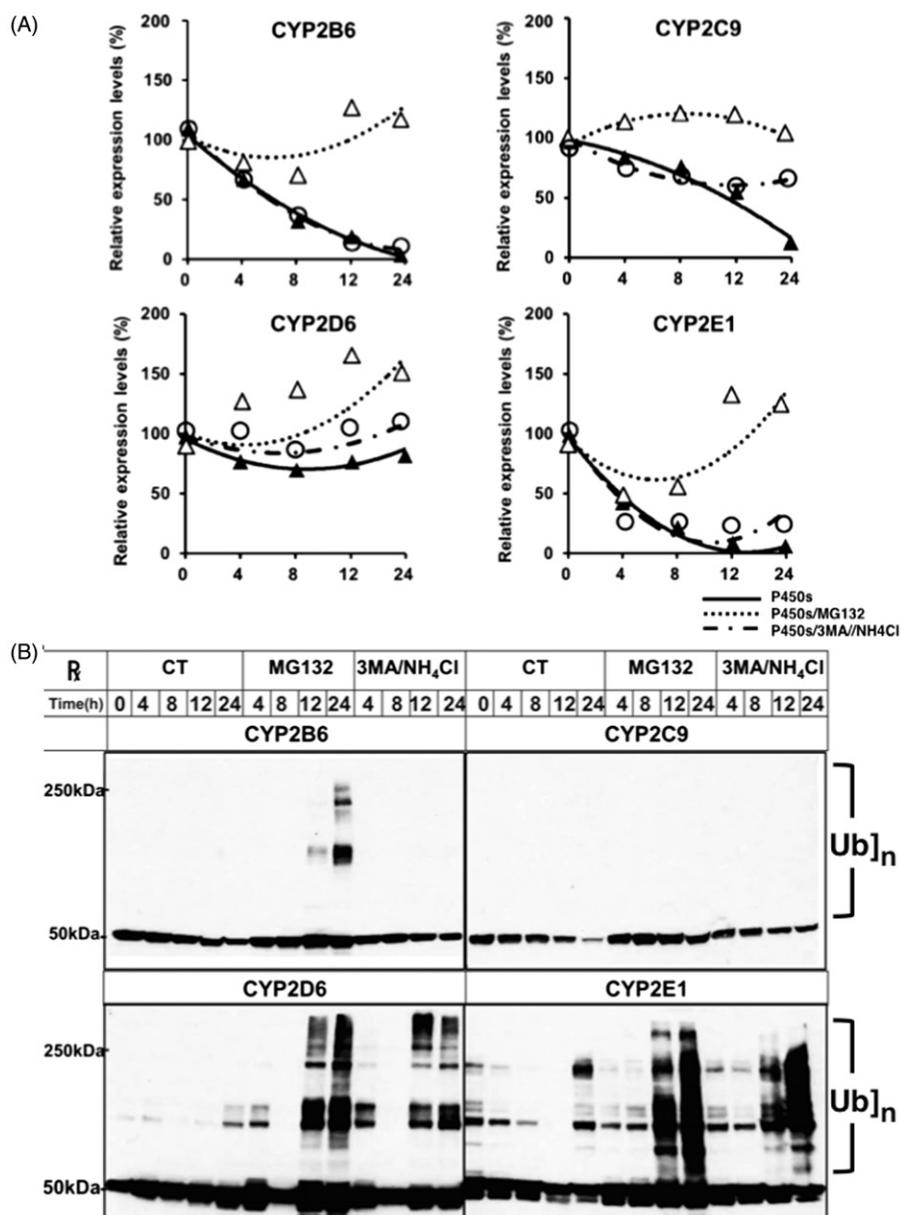
Figure 11. Cycloheximide-chase analyses of P450 turnover with UPD- or ALD-diagnostic probes. HepG2 cells were cultured and transfected with each P450 plasmid vector for 48 h as in Figure 10. After washing and medium exchange, cells were treated with cycloheximide (100 μM) with or without MG132 or 3MA/NH₄Cl as in *Figure 10*, followed by harvesting of cells at various times, and immunoblotting of lysates with an anti-Myc antibody. (A) Temporal plots of parent ≈ 50 kDa P450 species, following densitometric quantification. (B) Overexposure of the same immunoblots to reveal HMM ubiquitinated Myc-tagged-species. Note that all these P450s are stabilized by the UPD-inhibitor, MG132 to some extent. However, only CYP2D6 and CYP2E1 parent and HMM-species are significantly stabilized by the ALD probe 3MA/NH₄Cl. Even CYP2C9 (≈ 50 kDa species) shows some stabilization upon 3MA/NH₄Cl-treatment at 24 h. No stabilization of CYP2B6 is appreciably detected.

but also turnover bimodally via both ERAD/UPD and ERAD/ALD (Figure 11). Thus, although a clear preference for one pathway over the other exists, the specific physiological determinants of such preferences are less well understood. The discussion that follows attempts to begin to fill this void and gain some insight from our current knowledge of the proteolytic turnover of a few select native P450s that significantly impact clinical therapeutics and drug-drug interactions:

CYP2B6 and its mammalian orthologs

Cycloheximide-pulse-chase analyses coupled with the use of a diagnostic UPD (MG132) or ALD (3MA/NH₄Cl) probe in HepG2 cells transfected with a C-terminally Myc-tagged human liver CYP2B6 expression vector have revealed that CYP2B6 largely incurs UPD, as no appreciable stabilization of the parent (≈ 50 kDa) species is detected upon treatment with the ALD inhibitors (3MA/
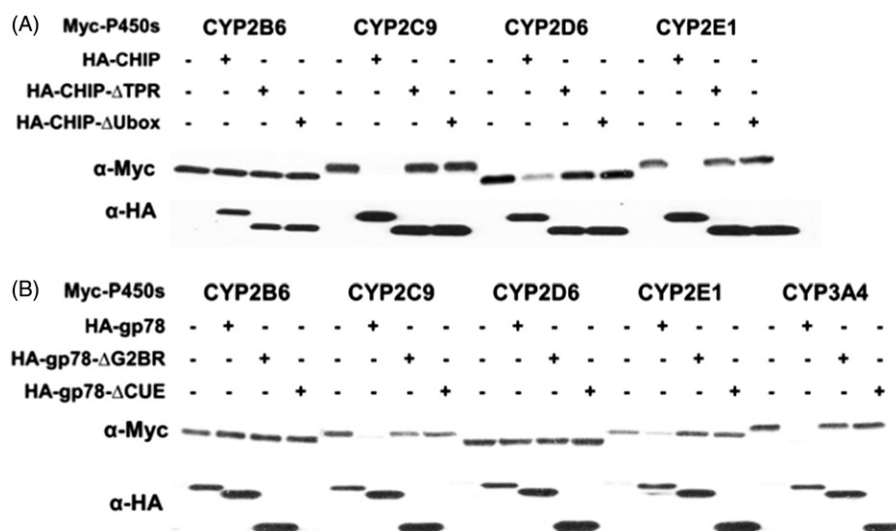
Figure 12. Relative role of CHIP- or gp78-E3 ligases in human P450 degradation: Myc/His6-tagged P450 plasmids were cotransfected with either HA-tagged CHIP or gp78 E3 ligases or their structural deletion constructs in HepG2 cells. As previously, CYP2E1 and CYP3A4 degradation is known to require both E3 ligase complexes for maximal degradation, and served as controls. Degradation of CYP2C9 similarly required both functionally relevant CHIP and gp78 E3-ligases, as the deletion of the CHIP catalytic U-box or Hsp70-interacting domain or of the gp78-E2-interacting or Cue-domains aborted their degradation whereas CYP2D6 degradation required CHIP, but not gp78-E3-ligase. CYP2B6 is ubiquitinated (*Figure 10*), but its degradation appears to be independent of either E3 ligase.

NH_4Cl) with time. Although CYP2B6 is ubiquitinated via both K_{48}- and K_{63}-Ub-chains (Figure 10), no accumulation of CYP2B6 ubiquitinated species was detected however upon treatment with 3MA/NH_4Cl, even at longer times (24 h) (Figure 11), when such an accumulation would have been observed, if CYP2B6 were indeed to be significantly degraded via ERAD/ALD in intact cells. Co-transfection of Myc-tagged CYP2B6 with either HA-tagged gp78 or HA-tagged CHIP in HepG2 cells for 48 h, and subsequent monitoring over a 6 h-period, excluded either of these two E3 Ub-ligases in CYP2B6 ERAD/UPD, thereby revealing that it's UPD was dependent on an as yet unidentified E3 ligase (Figure 12). Consistent with its preference for ERAD/UPD, inspection of the CYP2B6 crystal structure (Halpert, 2011; Shah et al., 2011) reveals that it contains several K-residues within or vicinal to surface DEST clusters (Figure 6). However, as expected from its low propensity for ALD, its surface LIR motifs are largely Phe- or Tyr-based (wherein the critical W_0 of the LIR motif is F or Y, the weaker W_0-residue), and the only Trp-bearing W_{121}KVL-LIR is buried within the protein-fold, unlike those of CYP2B4 (and possibly CYP2B1) (Figure 8). A notable caveat is that these CYP2B6 analyses relied on a C-terminally His_6/Myc-tagged CYP2B6, and it is plausible that such a C-terminal appendage may have enabled its ERAD/UPD rather than ERAD/ALD engagement (as observed previously with CYP2B1 appended with a CYP3A4 C-terminal heptapeptide; Liao et al., 2005), a possibility being currently examined.

By contrast to CYP2B6, CYP2B1, its closely related rat liver ortholog, has long been shown to be predominantly an ALD substrate (Liao et al., 2005; Masaki et al., 1987; Ronis & Ingelman-Sundberg, 1989; Ronis et al., 1991). This preferential ALD sorting appears to hold upon its heterologous expression in *S. cerevisiae* as well (Liao et al., 2005), but is lost upon extension of the CYP2B1 C-terminus (Liao et al., 2005). Furthermore, ^{35}S-pulse-chase analyses of PB-pretreated cultured rat hepatocytes reveal that CYP2B1 is stabilized largely by 3MA/NH_4Cl, but to an appreciable extent also by MG132, thereby indicating that CYP2B1 turnover is bimodal in cultured rat hepatocytes opting predominantly for ERAD/ALD, but ERAD/UPD as well to a significant extent (Figure 4). This ALD-preference however switches to UPD upon CYP2B1 inactivation (Lee et al., 2008; Korsmeyer et al., 1999). CYP2B4 is quite robustly ubiquitinated *in vitro* in a functionally reconstituted CHIP-system (Morishima et al., 2005), consistent with the possibility that this E3 is capable of elaborating K_{63}-linked Ub-chains that could target the protein to ALD (Birgisdottir et al., 2013; Johansen & Lamark, 2011; Lamark et al., 2009; Linares et al., 2013; Seibenhener et al., 2004; Stolz et al., 2014).

CYP2C9 and other CYP2C proteins

Cycloheximide-pulse-chase analyses coupled with the use of a diagnostic UPD (MG132) or ALD (3MA/NH_4Cl) probe in HepG2 cells transfected with a C-terminally Myc-tagged human liver CYP2C9 expression vector have revealed that this P450 also apparently turns over bimodally: A fraction of the parent ≈ 50 kDa incurs ERAD/UPD

early and thus is largely stabilized by MG132, and a fraction that undergoes ERAD/ALD and is stabilized around 12–24 h by 3MA/NH$_4$Cl (Figure 11A). Although Myc-pull-down studies indicate that CYP2C9 undergoes both K$_{48}$- or K$_{63}$-linked ubiquitination (Figure 10), no accumulation of ubiquitinated CYP2C9 species is detected upon UPD or ALD inhibition (Figure 11B), although stabilization of the parent species is clearly detected (Figure 11A). Furthermore, both CHIP and gp78 E3-complexes are involved in its ubiquitination, which requires their functionally active catalytic U-box and RING-domains, respectively. Accordingly, the CYP2C9 protein is significantly degraded upon co-transfection of either HA-tagged E3 ligase into HepG2 cells (Figure 12). Whether in common with CYP3A4 (Wang et al., 2015), this CYP2C9 CHIP/gp78-mediated ubiquitination is hierarchical and/or synergistic in character, remains to be determined. Consistent with this dual E3-involvement, inspection of a CYP2C9 crystal structure (Wester et al., 2004) reveals that it is studded with K-residues also situated within or vicinal to negatively charged surface DEST-clusters (Figure 6). Such structural scrutiny also reveals the presence of Trp-, Phe- and Tyr-based LIR motifs (Figure 9), thereby possibly accounting for its sorting to the ALD pathway as well. Preliminary findings reveal that native CYP2C19 is also ubiquitinated by both CHIP and gp78-E3-ligases. On the other hand, rat liver CYP2C11 incurs ERAD/ALD upon heterologous expression in *S. cerevisiae* (Murray et al., 2002), but upon suicide inactivation it is ubiquitinated and apparently proteasomally degraded, as revealed by the stabilization of the ubiquitinated CYP2C11 species by the proteasomal inhibitor hemin (Correia et al., 2005). A SILAC-based, Ub-remnant profiling/proteomic analyses of wild type and CHIP-deficient (CHIP$^{-/-}$) hepatocytes revealed that mouse liver CYPs 2C67 and 2C70 are appreciably ubiquitinated by CHIP, and CYPs 2C29, 2C50, 2C54, and 2C37 to a lesser extent (*S-M Kim & M. A. Correia, unpublished observations*). Their relative sorting to ERAD/UPD vs ERAD/ALD, if any, remains to be elucidated.

CYP2D6 and its orthologous CYP2D proteins

Parallel cycloheximide-pulse-chase analyses coupled with the use of a diagnostic UPD (MG132) or ALD (3MA/NH$_4$Cl) probe in HepG2 cells transfected with a C-terminally Myc-tagged human liver CYP2D6 expression vector have revealed that this P450 also turns over, albeit slowly, via both UPD and ALD with a fraction of the parent ≈ 50 kDa that is largely stabilized by MG132, and another fraction that is stabilized by 3MA/NH$_4$Cl (Figure 11A). CYP2D6 incurs both types of K$_{48}$- and K$_{63}$-linked ubiquitination (Figure 10), and this was confirmed

upon treatment with either MG132 or 3MA/NH$_4$Cl, as both probes stabilized HMM ubiquitinated CYP2D6 species to a significant extent (Figure 11B). Co-transfection studies in HepG2 cells with HA-tagged E3-ligases revealed that CYP2D6 is a CHIP-, but not a gp78-ubiquitination substrate (Figure 12). Structural inspection of CYP2D6 (Wang A et al., 2012) also reveals the presence of K-residues associated with surface DEST clusters (Figure 6), as well as surface LIR-motifs, hallmarks of a suitable autophagic target (Figure 9).

Preliminary examination of its mouse CYP2D10 ortholog using a SILAC-based, Ub-remnant profiling/proteomic approach in wild type and CHIP-deficient (CHIP$^{-/-}$) hepatocytes (*S-M Kim & M. A. Correia, unpublished observations*), revealed that genetic ablation of CHIP greatly reduced CYP2D10 ubiquitination, thereby validating this CYP2D ortholog as a CHIP target. Furthermore, preliminary studies in cultured hepatocytes from an autophagy-deficient (ATG5$^{-/-}$) mouse also revealed a significant stabilization of CYP2D10 (*DY Kwon, Y. Liu and M. A. Correia, preliminary findings*), thereby revealing that it is a plausible ALD-substrate.

CYP2E1 and orthologous proteins

The reportedly biphasic/bimodal turnover of human liver CYP2E1 was confirmed through our cycloheximide-pulse-chase analyses coupled with the use of a diagnostic UPD (MG132) or ALD (3MA/NH$_4$Cl) probe in HepG2 cells transfected with a C-terminally Myc-tagged human liver CYP2E1 expression vector (Figure 11A), as well as in in cultured human hepatocytes and *S. cerevisiae* following heterologous expression (Wang et al., 2011). The significant accumulation of CYP2E1 ubiquitinated species upon inhibition of either UPD or ALD is consistent with its biphasic/bimodal turnover (Figure 11B). In common with CYP3A4, it is a substrate of both CHIP- and gp78-E3-ligases, and this was confirmed in cell culture as well as upon cotransfection with either HA-tagged E3-ligase (Figure 12). Its recognition by these E3-ligases apparently also involves electrostatic interactions through negatively charged surface conformational phosphodegrons, consistent with the findings that its ubiquitination by gp78 was considerably attenuated upon site-directed mutation of positively charged gp78-patches (Figure 2). Inspection of CYP2E1 structure (Porubsky et al., 2008) reveals that its ubiquitinated K-residues are spatially associated with negatively charged surface DEST-clusters (Figure 5). However, it also has highly cytosol-accessible LIR-motifs that are Trp-, Phe- and Tyr-based (Figure 9).

Mouse CYP2E1 has been documented to turn over upon CCl$_4$-mediated inactivation via ERAD/UPD

(Tierney et al., 1992), and recombinant rabbit CYP2E1 to be a CHIP substrate (Morishima et al., 2005). Preliminary studies employing a SILAC-based, Ub-remnant profiling/proteomic approach in cultured wild type and CHIP-deficient mouse hepatocytes, revealed that genetic ablation of CHIP also greatly reduced CYP2E1 ubiquitination, thereby validating this CYP2E1 ortholog as a *bona fide* CHIP target.

CYP3A4 and orthologous CYP3A proteins

Previous ^{35}S-methionine/cysteine pulse-chase analyses of the CYP3A protein degradation in cultured rat hepatocytes (Faouzi et al., 2007) or incubation of rat hepatocytes in suspension (Wang et al., 1999) have revealed that rat liver CYPs 3A, either native or suicidally inactivated, are predominantly turned over via UPD as evident by the stabilization of the parent ($\approx$ 55 kDa) and ubiquitinated species ($\approx$ 65 to >250 kDa) species by the proteasomal inhibitors MG132 or MG262 (Figure 4; Faouzi et al., 2007; Wang et al., 1999). A small but detectable stabilization of the parent protein ($\approx$ 55 kDa) is also observed from 3–6 h upon treatment with the ALD inhibitors 3MA/NH$_4$Cl (Figure 4), thereby suggesting that ALD may also normally contribute to a small extent to their physiological turnover. This preferential ERAD/UPD sorting also holds upon heterologous expression of CYP3A4 in *S. cerevisiae* (Liao et al., 2006; Murray & Correia, 2001), and upon ^{35}S-pulse-chase analyses in HEK293T cells (Wang et al., 2009) or HepG2 cells (Wang et al., 2012), transfected with a CYP3A4 expression vector, yielding half-lives (t$_{1/2}$) of such transfected wild-type CYPs 3A4 and 3A5 proteins of 13.2 h and 15.9 h, respectively, in HepG2 cells (Wang et al., 2012). Consistent with a role for post-translational CYP3A4 phosphorylation, PKA or PKC inhibitors attenuated this CYP3A4 turnover (Wang et al., 2012), as did the combined mutation of phosphorylatable CYP3A4 S478, T264 and S420 residues (Wang et al., 2012). As discussed above both CHIP and gp78 E3 Ub-ligase complexes are involved in CYP3A4 targeting to ERAD/UPD, and their recognition of this substrate entails linear as well as conformational phosphodegrons along cytosol-exposed surface loops and/or disordered regions (Wang et al., 2015). Consistent with its predominant ERAD/UPD targeting, CYP3A4's LIR motifs, although present, are not all that cytosol-accessible (Figure 9).

Pathophysiological relevance of P450 degradation

Throughout their cellular lifespan, from their birth as nascent chains that fold into mature functional hemoproteins integrated into the ER, their subsequent oxidative insults stemming from their catalytic function and/or accidental encounters with suicide inactivators, to their age-defined senescence, cellular P450s are subject to proteolytic turnover via ERAD/UPD and/or ERAD/ALD, as discussed above. ERAD/UPD not only disposes structurally damaged/inactive P450s as cellular garbage (Correia, 2003; *and references therein*), but also plays a major role in the clinically relevant polymorphic expression of human liver P450s through clearance of various defective, misfolded and/or proteolytically susceptible P450 variants (Liao et al., 2010; *and references therein*). Thus, the R186G mutant encoded by the naturally occurring CYP2C8*8 allelic variant is unstable upon expression in COS-1 cells, but this instability can be greatly attenuated in the presence of MG-132 (Hichiya et al., 2005). Such clearance of aberrant P450 variants is vital both for quality control and to prevent proteotoxic stress due to the intracellular accumulation of abnormal/aberrant and/or structurally damaged proteins. Furthermore, such accelerated clearance of polymorphic P450 variants may also have a beneficial protective role. For example, CYP1B1.4 (bearing a N453S mutation) is poorly expressed relative to its CYP1B1 N453 variants genetically associated with increased risk of endometrial cancer. Upon expression in COS-1 cells and pulse-chase analysis, CYP1B1.4 N453S mutant is indeed degraded much faster than CYP1B1.1 N453 via ERAD/UPD (Bandiera et al., 2005). Consistently, individuals carrying this CYP1B1.4 N453S mutation apparently exhibit a lower incidence of such cancers than those carrying the CYP1B1.1 N453 variants (Bandiera et al., 2005).

More importantly, and quite not all that well appreciated is the fact that ERAD/UPD significantly contributes to the regulation of basal hepatic microsomal P450 content and thus its function, and is consequently a therapeutically relevant determinant of clinical hepatic drug metabolism and elimination and associated pharmacokinetic/pharmacodynamic drug-drug interactions (Chien et al., 1997; Kalgutkar et al., 2007; Liao et al., 2010; Xu et al., 2009; Yang et al., 2008). Accordingly, ^{35}S-pulse-chase analyses of cultured rat hepatocytes reveals that ERAD/UPD controls the amount of the newly synthesized/folded protein by limiting the fraction of nascent CYP3A4 chains that is actually incorporated into the ER-membrane at any given time, and thence folded into the mature functional P450 protein (Faouzi et al., 2007; Wang et al., 1999). Indeed, a significant fraction of these *de novo* synthesized ^{35}S-CYP3A chains are ubiquitinated immediately upon ^{35}S-pulse-chase and detected as HMM ^{35}S-CYP3A species, targeted for proteasomal degradation (Faouzi et al., 2007; Wang et al., 1999).

Another important feature of this P450 regulation by ERAD/UPD is the observed marked increase in hepatic ER-integral CYP3A content that is fully functional following lentiviral shRNAi directed against either gp78 or CHIP E3 Ub-ligase (Kim et al., 2010; see below). This reveals that each of these E3s in addition to targeting irreversibly damaged P450s, also targets either native P450s or an intermediate species, and thus regulates functional hepatic P450 content. These findings provide compelling support for ERAD/UPD as an important physiological regulator of hepatic P450 content and function, and not just a mechanism for disposal of fatally damaged and/or functionally dead P450s. Consequently, any elevation of functional P450 content through impairment or disruption of hepatic P450 ERAD would not only be therapeutically relevant, but also pathologically relevant given the propensity of some P450s such as CYP2E1 largely, and CYP3A4 to a smaller extent to generate pathogenic ROS.

Indeed, this high propensity for generating ROS has implicated CYP2E1 in the pathogenesis of toxic liver damage, alcoholic liver disease, NASH (nonalcoholic steatohepatitis), diabetes, and obesity (Bardag-Gorce et al., 2006; Cederbaum, 2006; Cederbaum et al., 2013; Schattenberg & Czaja, 2005; 2014). Although CYP2E1 normally comprises only $\approx$ 5–7% of total human hepatic P450 content, its abnormal elevation above this basal content ($>$7%) in these conditions either via transcriptional induction or protein stabilization is thought to promote liver injury (Bardag-Gorce et al., 2006; Cederbaum, 2006; Cederbaum et al., 2013; Guengerich, 2015; Schattenberg & Czaja, 2005, 2012). Thus, tight regulation of basal CYP2E1 content through carefully balanced control of its de novo synthesis as well as ERAD is clinically desirable. Indeed, we find that disruption of CYP2E1 ERAD/UPD through genetic ablation of CHIP in mice (CHIP$^{-/-}$) is associated with hepatic CYP2E1 elevation and consequent oxidative liver damage, up-regulated pathogenic JNK-signalling and microvesicular steatosis (Kim et al., 2016b). On the other hand, ERAD/ALD is equally important in CYP2E1 regulation as documented by the finding that its disruption via genetic ablation or siRNA-elicited knockdown of specific ALD-relevant genes (Atg7) in CYP2E1-expressing E47 HepG2 cells, stabilizes CYP2E1 thereby promoting acute chemically induced CYP2E1-dependent cytoxicity (Wu & Cederbaum, 2013). This acute cytoxicity is manifested through increased ROS production, oxidative stress, reduced E47 cell viability, and necrosis that were all further magnified upon ALD disruption by 3-MA-elicited autophagic inhibition (Wu & Cederbaum, 2013).

An additional pathophysiologic consideration is that ERAD/UPD may also be responsible for generating pathogenically relevant serum P450 autoantibodies in individuals with chronic active and drug-induced hepatitis and hypersensitivity reactions (Beaune et al., 1987; Bourdi et al., 1992, 1996; Boitier & Beaune, 2000; Dansette et al., 1998; Eliasson & Kenna, 1996; Leeder et al., 1996; Riley et al., 1993; Thervet et al., 2005; Uetrecht, 1999, 2005), associated with the therapeutic intake of CYP3A (carbamazepine and phenytoin), CYP2E1 (halothane) and CYP2C9 (tienilic acid) substrates. Thus, proinflammatory and/or oxidative stress conditions that are known to induce the immunoproteasome "i-proteasome" (through exchange of the constitutive catalytic β1-, β2- and β5–20S-proteasomal subunits with the inducible LMP2, MECL1 and LMP7-subunits of the i-proteasome; Ferrington & Gregerson, 2012; Kloetzel, 2004; Michalek et al., 1993; Monaco, 1995), may enable the generation of P450-peptides that are covalently drug-modified and/or with a hydrophobic C-terminus that can be recognized by the MHC class I molecules for antigenic presentation at the cell surface.

The degradation of cellular proteins including hepatic P450s and/or organelles via ERAD/UPD and/or ERAD/ALD is a basal, physiologic housekeeping function. Because these processes control a myriad of vital cellular functions, their dysfunction is intricately linked not only to P450-linked therapeutic misadventures, but also to many human diseases such as cancer, neurodegeneration (Alzheimer's, Parkinson's, and Huntington's diseases), myodegeneration, diabetes, Paget's disease of bone, cystic fibrosis, microbial infection, immunity and ageing (Bjorkoy et al., 2005; Cuervo, 2011; Komatsu, 2012; Komatsu et al., 2012; Manley et al., 2013; Madrigal-Matute & Cuervo, 2015; Mizushima et al., 2008; Nezis et al., 2012; Puissant et al., 2012; Rabinowitz & White, 2010; Zhang et al., 2015; reviewed by Guerriero & Brodsky, 2012). Disruption of these inherently protective and homeostatic mechanisms, can lead to apoptosis or cell death.

Indeed, this very likelihood that disruption of these physiologic ERAD processes can trigger ER stress and consequent proteotoxic crisis and apoptosis has prompted the exploitation of proteasomal inhibitors in cancer therapy (Deshaies, 2014; Johnson, 2015). Unlike their normal counterparts, cancer cells operate at highly accelerated protein turnover rates, and inhibition of their protein degradation could drive them quite easily into an unfolded protein response/ER-stress that triggers apoptosis. Both reversible proteasomal inhibitors such as bortezomib, its orally active analog MLN9708, and delanzomib, as well as irreversible inhibitors such as the epoxy-ketone inhibitor, carfilzomib and its orally active analog oprozomib, and the naturally occurring β-lactone marizomib

(salinosporamide A) are either in use or actively considered in cancer chemotherapy (Deshaies, 2014; Johnson, 2015). Although these compounds are effective against hematological/plasma cell malignancies such as multiple myeloma and mantle cell lymphoma, they are ineffective against solid tumors. One reason is that proteasomal blockade causes proteins to accumulate in the cytosol after their ER-extraction rather than within the ER, thereby minimizing the levels of ER-stress within the ER, and the required magnitude of "proteotoxic crisis" (Deshaies, 2014). The other even more important one is that because the proteasome is an essential homeostatic protein, its inhibition leads to its rebound synthesis (Deshaies, 2014), thus requiring higher, potentially toxic, pharmacological dosages of its inhibitors for an efficacious cancer therapeutic response.

This realization has fostered the consideration and pharmaceutical development of p97 inhibitors. As discussed earlier, p97 is not only at the crossroads of several physiologic pathways, but it is actively involved in extracting proteins from both the ER-lumen and ER-membrane. Thus, blockade of its function would heighten the magnitude of the ER-stress, and the consequent extent of the proteotoxic crisis and apoptosis. Indeed, several p97 inhibitors have been developed in recent years (*reviewed in Deshaies, 2014*). Of these, the most potent, selective and orally bioavailable p97 inhibitor is CB-5083, a very promising agent that not only disrupts UPD, but also activates ER-stress and autophagic pathways in non-small-cell lung cancer cells (Anderson et al., 2015). Given its pharmacologic/pharmacokinetic profile and the documentation of its effectiveness in both hematological as well as solid tumor animal models (Anderson et al., 2015), this compound is currently in Phase I trials both in patients with relapsed and refractory multiple myeloma and in patients with advanced solid tumors (Anderson et al., 2015). For the sake of completeness, it must be stated that compounds that target E3 Ub-ligases are also currently being considered as cancer chemotherapeutic agents, in an attempt to block the proteolytic turnover of their tumor suppressor substrates and prolong their life spans (Deshaies, 2014). Thus, although the disruption of the physiologic ERAD function can be detrimental to normal cells, it can be strategically exploited to mitigate various pathogenic/pathologic conditions. In conclusion, the therapeutic relevance of P450 turnover notwithstanding, given the physiological and pathophysiological relevance of these vital ERAD pathways, as their prototypic substrates, the ER-integral P450s serve at a more fundamental level, as excellent probes for their mechanistic dissection and characterization.

References

Aguiar M, Masse R, Gibbs BF. (2005). Regulation of cytochrome P450 by posttranslational modification. Drug Metab Rev 37:379–404.

Ahner A, Brodsky JL. (2004). Checkpoints in ER-associated degradation: excuse me, which way to the proteasome? Trends Cell Biol 14:474–478.

Anderson DJ, Le Moigne R, Djakovic S, et al. (2015). Targeting the AAA ATPase p97 as an Approach to Treat Cancer through Disruption of Protein Homeostasis. Cancer Cell 28:653–665.

Ballinger CA, Connell P, Wu Y, et al. (1999). Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. Mol Cell Biol 19:4535–4545.

Bandiera S, Weidlich S, Harth V, et al. (2005). Proteasomal degradation of human CYP1B1: effect of the Asn453Ser polymorphism on the post-translational regulation of CYP1B1 expression. Mol Pharmacol 67:435–443.

Bardag-Gorce F, Li J, French BA, French SW. (2002). Ethanol withdrawal induced CYP2E1 degradation in vivo, blocked by proteasomal inhibitor PS-341. Free Radic Biol Med 32:17–21.

Bardag-Gorce F, French BA, Nan L, et al. (2006). CYP2E1 induced by ethanol causes oxidative stress, proteasome inhibition and cytokeratin aggresome (Mallory body-like) formation. Exp Mol Pathol 81:191–201.

Bar-Nun S. (2005). The role of p97/Cdc48p in endoplasmic reticulum-associated degradation: from the immune system to yeast. Curr Top Microbiol Immunol 300:95–125.

Beaune P, Dansette PM, Mansuy D, et al. (1987). Human anti-endoplasmic reticulum autoantibodies appearing in a drug-

induced hepatitis are directed against a human liver cytochrome P-450 that hydroxylates the drug. Proc Natl Acad Sci USA 84:551–555.

Bento CF, Renna M, Ghislat G, et al. (2016). Mammalian autophagy: How does it work? Annu Rev Biochem. [Epub ahead of print]. doi: 10.1146/annurev-biochem-060815-014556.

Birgisdottir AB, Lamark T, Johansen T. (2013). The LIR motif - crucial for selective autophagy. J Cell Sci 126:3237–3247.

Bjorkoy G, Lamark T, Brech A, et al. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol 171:603–614.

Boitier E, Beaune P. (1999). Cytochromes P450 as targets to autoantibodies in immune mediated diseases. Mol Aspects Med 20:84–137.

Boitier E, Beaune P. (2000). Xenobiotic-metabolizing enzymes as autoantigens in human autoimmune disorders. An update. Clin Rev Allergy Immunol 18:215–239.

Bourdi M, Chen W, Peter RM, et al. (1996). Human cytochrome P450 2E1 is a major autoantigen associated with halothane hepatitis. Chem Res Toxicol 9:1159–1166.

Bourdi M, Gautier JC, Mircheva J, et al. (1992). Anti-liver microsomes autoantibodies and dihydralazine-induced hepatitis: specificity of autoantibodies and inductive capacity of the drug. Mol Pharmacol 42:280–285.

Bridges A, Gruenke L, Chang YT, et al. (1998). Identification of the binding site on cytochrome P450 2B4 for cytochrome b5 and cytochrome P450 reductase. J Biol Chem 273:17036–17049.

Bug M, Meyer H. (2012). Expanding into new markets-VCP/p97 in endocytosis and autophagy. J Struct Biol 179:78–82.

Cadwell K, Coscoy L. (2005). Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase. Science 309:127–130.

Carvalho P, Goder V, Rapoport TA. (2006). Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. Cell 126:361–373.

Catic A, Collins C, Church GM, Ploegh HL. (2004). Preferred in vivo ubiquitination sites. Bioinformatics 20:3302–3307.

Cederbaum AI. (2006). CYP2E1-biochemical and toxicological aspects and role in alcohol-induced liver injury . Mt Sinai J Med 73:657–672.

Cederbaum AI, Lu Y, Wang X, Wu D. (2013). Synergistic toxic interactions between CYP2E1, LPS/TNFα, and JNK/p38 MAP kinase and their implications in alcohol-induced liver injury. Adv Exp Med Biol 815:145–172.

Chen B, Mariano J, Tsai YC, et al. (2006). The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site. Proc Natl Acad Sci USA 103:341–346.

Chen Z, Du S, Fang S. (2012). gp78: a multifaceted ubiquitin ligase that integrates a unique protein degradation pathway from the endoplasmic reticulum. Curr Protein Pept Sci 13:414–424.

Chien JY, Thummel KE, Slattery JT. (1997). Pharmacokinetic consequences of induction of CYP2E1 by ligand stabilization. Drug Metab Dispos 25:1165–1175.

Chou TF, Brown SJ, Minond D, et al. (2011). Reversible inhibitor of p97, DBeQ, impairs both ubiquitin-dependent and autophagic protein clearance pathways. Proc Natl Acad Sci USA 108:4834–4839.

Christianson JC, Ye Y. (2014). Cleaning up in the endoplasmic reticulum: ubiquitin in charge. Nat Struct Mol Biol 21:325–335.

Clapp KM, Peng HM, Jenkins GJ, et al. (2012). Ubiquitination of neuronal nitric-oxide synthase in the calmodulin-binding site triggers proteasomal degradation of the protein. J Biol Chem 287:42601–42610.

Correia MA, Decker C, Sugiyama K, et al. (1987). Degradation of rat hepatic cytochrome P-450 heme by 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine to irreversibly bound protein adducts. Arch Biochem Biophys 258:436–451.

Correia MA, Yao K, Wrighton SA, et al. (1992a). Differential apoprotein loss of rat liver cytochromes P450 after their inactivation by 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine: a case for distinct proteolytic mechanisms? Arch Biochem Biophys 294:493–503.

Correia MA, Davoll SH, Wrighton SA, Thomas PE. (1992b). Degradation of rat liver cytochromes P450 3A after their inactivation by 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine: characterization of the proteolytic system. Arch Biochem Biophys 297:228–238.

Correia MA. (2003). Hepatic cytochrome P450 degradation: mechanistic diversity of the cellular sanitation brigade. Drug Metab Rev 35:107–143.

Correia MA, Sadeghi S, Mundo-Paredes E. (2005). Cytochrome P450 ubiquitination: branding for the proteolytic slaughter? Annu Rev Pharmacol Toxicol 45:439–464.

Correia MA, Liao M. (2007). Cellular proteolytic systems in P450 degradation: evolutionary conservation from Saccharomyces cerevisiae to mammalian liver. Expert Opin Drug Metab Toxicol 3:33–49.

Correia MA, Wang Y, Kim SM, Guan S. (2014). Hepatic cytochrome P450 ubiquitination: conformational phosphodegrons for E2/E3 recognition? IUBMB Life 66:78–88.

Cuervo AM. (2011). Cell biology. Autophagy's top chef. Science 332:1392–1393.

Dai Y, Cederbaum AI. (1995). Inactivation and degradation of human cytochrome P4502E1 by CCl4 in a transfected HepG2 cell line. J Pharmacol Exp Ther 275:1614–1622.

Dai RM, Li CC. (2001). Valosin-containing protein is a multiubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. Nat Cell Biol 3:740–744.

Dansette PM, Bonierbale E, Minoletti C, et al. (1998). Drug-induced immunotoxicity. Eur J Drug Metab Pharmacokinet 23:443–451.

Das R, Mariano J, Tsai YC, et al. (2009). Allosteric activation of E2-RING finger-mediated ubiquitylation by a structurally defined specific E2-binding region of gp78. Mol Cell 34:674–685.

Das R, Liang YH, Mariano J, et al. (2013). Allosteric regulation of E2:E3 interactions promote a processive ubiquitination machine. EMBO J 32:2504–2516.

Deshaies RJ. (2014). Proteotoxic crisis, the ubiquitin-proteasome system, and cancer therapy. BMC Biol 12:94

Dick LR, Cruikshank AA, Destree AT, et al. (1997). Mechanistic studies on the inactivation of the proteasome by lactacystin in cultured cells. J Biol Chem 272:182–188.

Dreveny I, Pye VE, Beuron F, et al. (2004). p97 and close encounters of every kind: a brief review. Biochem Soc Trans 32:715–720.

Edkins AL. (2013). CHIP: a co-chaperone for degradation by the proteasome. Subcell Biochem 78:219–242.

Ekroos M, Sjogren T. (2006). Structural basis for ligand promiscuity in cytochrome P450 3A4. Proc Natl Acad Sci USA 103:13682–13687.

Ekstrom G, Ingelman-Sundberg M. (1989). Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450IIE1). Biochem Pharmacol 38:1313–1319.

Eliasson E, Johansson I, Ingelman-Sundberg M. (1988). Ligand-dependent maintenance of ethanol-inducible cytochrome P-450 in primary rat hepatocyte cell cultures. Biochem Biophys Res Commun 150:436–443.

Eliasson E, Johansson I, Ingelman-Sundberg M. (1990). Substrate-, hormone-, and cAMP-regulated cytochrome P450 degradation. Proc Natl Acad Sci USA 87:3225–3229.

Eliasson E, Mkrtchian S, Ingelman-Sundberg M. (1992). Hormone- and substrate-regulated intracellular degradation of cytochrome P450 (2E1) involving MgATP-activated rapid proteolysis in the endoplasmic reticulum membranes. J Biol Chem 267:15765–15769.

Eliasson E, Mkrtchian S, Halpert JR, Ingelman-Sundberg M. (1994). Substrate-regulated, cAMP-dependent phosphorylation, denaturation, and degradation of glucocorticoid-inducible rat liver cytochrome P450 3A1. J Biol Chem 269:18378–18383.

Eliasson E, Kenna JG. (1996). Cytochrome P450 2E1 is a cell surface autoantigen in halothane hepatitis. Mol Pharmacol 50:573–582.

Elkabetz Y, Shapira I, Rabinovich E, Bar-Nun S. (2004). Distinct steps in dislocation of luminal endoplasmic reticulum-associated degradation substrates: roles of endoplamic reticulum-bound p97/Cdc48p and proteasome. J Biol Chem 279:3980–3989.

Fang S, Ferrone M, Yang C, et al. (2001). The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. Proc Natl Acad Sci USA 98:14422–14427.

Faouzi S, Medzihradszky KF, Hefner C, et al. (2007). Characterization of the physiological turnover of native and inactivated cytochromes P450 3A in cultured rat hepatocytes: A role for the cytosolic AAA ATPase p97? Biochemistry 46:7793–7803.

Ferrington DA, Gregerson DS. (2012). Immunoproteasomes: Structure, function, and antigen presentation. Prog Mol Biol Transl Sci 109:75–112.

Finley D. (2009). Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu Rev Biochem 78:477–513.

Fouts JR. (1962). Interaction of drugs and hepatic microsomes. Fed Proc 21:1107–1111.

Freeman JE, Wolf CR. (1994). Evidence against a role for serine 129 in determining murine cytochrome P450 Cyp2e-1 protein levels. Biochemistry 33:13963–13966.

Fujita E, Kouroku Y, Isoai A, et al. (2007). Two endoplasmic reticulum-associated degradation (ERAD) systems for the novel variant of the mutant dysferlin: ubiquitin/proteasome ERAD(I) and autophagy/lysosome ERAD(II). Hum Mol Genet 16:618–629.

Gao Q, Doneanu CE, Shaffer SA, et al. (2006). Identification of the interactions between cytochrome P450 2E1 and cytochrome b5 by mass spectrometry and site-directed mutagenesis. J Biol Chem 281:20404–20417.

Goasduff T, Cederbaum AI. (1999). NADPH-dependent microsomal electron transfer increases degradation of CYP2E1 by the proteasome complex: Role of reactive oxygen species. Arch Biochem Biophys 370:258–270.

Goldberg AL, Rock KL. (1992). Proteolysis, proteasomes and antigen presentation. Nature 357:375–379.

Gorsky LD, Koop DR, Coon MJ. (1984). On the stoichiometry of the oxidase and monooxygenase reactions catalyzed by liver microsomal cytochrome P-450. Products of oxygen reduction. J Biol Chem 259:6812–6817.

Gu J, Weng Y, Zhang QY, et al. (2003). Liver-specific deletion of the NADPH-cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase. J Biol Chem 278:25895–25901.

Guengerich FP. (2015) Human cytochrome P450 enzymes. In: Cytochrome P450: Structure, Mechanism and Biochemistry, Ortiz de Montellano P, ed. Switzerland: Springer International Publishing, 523–785.

Guerriero CJ, Brodsky JL. (2012). The delicate balance between secreted protein folding and endoplasmic reticulum-associated degradation in human physiology. Physiol Rev 92:537–576.

Halpert JR. (2011). Structure and function of cytochromes P450 2B: from mechanism-based inactivators to X-ray crystal structures and back. Drug Metab Dispos 39:1113–1121.

Hampton RY, Garza RM. (2009). Protein quality control as a strategy for cellular regulation: lessons from ubiquitin-mediated regulation of the sterol pathway. Chem Rev 109:1561–1574.

Henderson CJ, Otto DM, Carrie D, et al. (2003). Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. J Biol Chem 278:13480–13486.

Hichiya H, Tanaka-Kagawa T, Soyama A, et al. (2005). Functional characterization of five novel CYP2C8 variants, G171S, R186X, R186G, K247R, and K383N, found in a Japanese population. Drug Metab Dispos 33:630–636.

Hirsch C, Gauss R, Horn SC, et al. (2009). The ubiquitylation machinery of the endoplasmic reticulum. Nature 458:453–460.

Hutterer F, Dressler K, Greim H, et al. (1975) Effect of cyclic AMP on the phenobarbital-induced increase in cytochrome P450 and hypertrophy of the endoplasmic reticulum of the rat liver. In Cytochromes P450 and b5. Structure, Function and Interactions, Cooper DY, Rosenthal O, Snyder R, and Witmer C, eds. New York: Plenum Press, 117–126.

Ichimura Y, Kumanomidou T, Sou YS, et al. (2008). Structural basis for sorting mechanism of p62 in selective autophagy. J Biol Chem 283:22847–22857.

Ikeda Y, Demartino GN, Brown MS, et al. (2009). Regulated endoplasmic reticulum-associated degradation of a polytopic protein: p97 recruits proteasomes to Insig-1 before extraction from membranes. J Biol Chem 284:34889–34900.

Imai Y, Soda M, Hatakeyama S, et al. (2002). CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. Mol Cell 10:55–67.

Ishikura S, Weissman AM, Bonifacino JS. (2010). Serine residues in the cytosolic tail of the T-cell antigen receptor

alpha-chain mediate ubiquitination and endoplasmic reticulum-associated degradation of the unassembled protein. J Biol Chem 285:23916–23924.

Itakura E, Mizushima N. (2011). p62 Targeting to the autophagosome formation site requires self-oligomerization but not LC3 binding. J Cell Biol 192:17–27.

Jentsch S, Rumpf S. (2007). Cdc48 (p97): a "molecular gearbox" in the ubiquitin pathway? Trends Biochem Sci 32:6–11.

Jiang J, Ballinger CA, Wu Y, et al. (2001). CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. J Biol Chem 276:42938–42944.

Jo Y, Lee PC, Sguigna PV, DeBose-Boyd RA. (2011). Sterol-induced degradation of HMG CoA reductase depends on interplay of two Insigs and two ubiquitin ligases, gp78 and Trc8. Proc Natl Acad Sci USA 108:20503–20508.

Johansen T, Lamark T. (2011). Selective autophagy mediated by autophagic adapter proteins. Autophagy 7:279–296.

Johansson I, Eliasson E, Ingelman-Sundberg M. (1991). Hormone controlled phosphorylation and degradation of CYP2B1 and CYP2E1 in isolated rat hepatocytes. Biochem Biophys Res Commun 174:37–42.

Johnson DE. (2015). The ubiquitin-proteasome system: opportunities for therapeutic intervention in solid tumors. Endocr Relat Cancer 22:T1–T17.

Ju JS, Fuentealba RA, Miller SE, et al. (2009). Valosin-containing protein (VCP) is required for autophagy and is disrupted in VCP disease. J Cell Biol 187:875–888.

Kalgutkar AS, Obach RS, Maurer TS. (2007). Mechanism-based inactivation of cytochrome P450 enzymes: chemical mechanisms, structure-activity relationships and relationship to clinical drug-drug interactions and idiosyncratic adverse drug reactions. Curr Drug Metab 8:407–447.

Katsuragi Y, Ichimura Y, Komatsu M. (2015). p62/SQSTM1 functions as a signaling hub and an autophagy adaptor. Febs J 282:4672–4678.

Kikkert M, Doolman R, Dai M, et al. (2004). Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. J Biol Chem 279:3525–3534.

Kim PK, Hailey DW, Mullen RT, Lippincott-Schwartz J. (2008). Ubiquitin signals autophagic degradation of cytosolic proteins and peroxisomes. Proc Natl Acad Sci USA 105:20567–20574.

Kim SM, Acharya P, Engel JC, Correia MA. (2010). Liver cytochrome P450 3A ubiquitination in vivo by gp78/autocrine motility factor receptor and C terminus of Hsp70-interacting protein (CHIP) E3 ubiquitin ligases: physiological and pharmacological relevance. J Biol Chem 285:35866–35877.

Kim SM, Karkashon S, Yeh SR, Correia MA. (2016a). Structural determinants of human tryptophan 2,3-dioxygenase protein degradation. Exp Biol 260:816.4.

Kim SM, Grenert JP, Patterson C, Correia MA. (2016b) CHIP-/- mouse liver: Adiponectin-AMPK-FOXO-activation overrides CYP2E1-elicited JNK1-activation, delaying onset of NASH: Therapeutic implications (Submitted).

Kim W, Bennett EJ, Huttlin EL, et al. (2011). Systematic and quantitative assessment of the ubiquitin-modified proteome. Mol Cell 44:325–340.

Kirkin V, McEwan DG, Novak I, Dikic I. (2009). A role for ubiquitin in selective autophagy. Mol Cell 34:259–269.

Klionsky DJ, Codogno P, Cuervo AM, et al. (2010). A comprehensive glossary of autophagy-related molecules and processes. Autophagy 6:438–448.

Kloetzel PM. (2004). The proteasome and MHC class I antigen processing. Biochim Biophys Acta 1695:225–233.

Komatsu M, Waguri S, Koike M, et al. (2007). Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 131:1149–1163.

Komatsu M. (2012). Liver autophagy: physiology and pathology. J Biochem 152:5–15.

Komatsu M, Kageyama S, Ichimura Y. (2012). p62/SQSTM1/A170: physiology and pathology. Pharmacol Res 66:457–462.

Korsmeyer KK, Davoll S, Figueiredo-Pereira ME, Correia MA. (1999). Proteolytic degradation of heme-modified hepatic cytochromes P450: A role for phosphorylation, ubiquitination, and the 26S proteasome? Arch Biochem Biophys 365:31–44.

Kostova Z, Tsai YC, Weissman AM. (2007). Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation. Semin Cell Dev Biol 18:770–779.

Krick R, Bremer S, Welter E, et al. (2010). Cdc48/p97 and Shp1/p47 regulate autophagosome biogenesis in concert with ubiquitin-like Atg8. J Cell Biol 190:965–973.

Kulathu Y, Komander D. (2012). Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. Nat Rev Mol Cell Biol 13:508–523.

Lamark T, Kirkin V, Dikic I, Johansen T. (2009). NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets. Cell Cycle 8:1986–1990.

Lee DH, Goldberg AL. (1998). Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol 8:397–403.

Lee CM, Kim BY, Li L, Morgan ET. (2008). Nitric oxide-dependent proteasomal degradation of cytochrome P450 2B proteins. J Biol Chem 283:889–898.

Leeder JS, Gaedigk A, Lu X, Cook VA. (1996). Epitope mapping studies with human anti-cytochrome P450 3A antibodies. Mol Pharmacol 49:234–243.

Li W, Tu D, Brunger AT, Ye Y. (2007). A ubiquitin ligase transfers preformed polyubiquitin chains from a conjugating enzyme to a substrate. Nature 446:333–337.

Li W, Tu D, Li L, et al. (2009). Mechanistic insights into active site-associated polyubiquitination by the ubiquitin-conjugating enzyme Ube2g2. Proc Natl Acad Sci USA 106:3722–3727.

Liao M, Zgoda VG, Murray BP, Correia MA. (2005). Vacuolar degradation of rat liver CYP2B1 in Saccharomyces cerevisiae: further validation of the yeast model and structural implications for the degradation of mammalian endoplasmic reticulum P450 proteins. Mol Pharmacol 67:1460–1469.

Liao M, Faouzi S, Karyakin A, Correia MA. (2006). Endoplasmic reticulum-associated degradation of cytochrome P450 CYP3A4 in Saccharomyces cerevisiae: further characterization of cellular participants and structural determinants. Mol Pharmacol 69:1897–1904.

Liao M, Kang P, Murray BP, Correia MA. (2010) Cytochrome P450 degradation and its clinical relevance. In: Lu C, Li AP, eds. Enzyme Inhibition in Drug Discovery & Development. NJ: John Wiley & Sons, 363–406.

Lin HL, Kenaan C, Zhang H, Hollenberg PF. (2012). Reaction of human cytochrome P450 3A4 with peroxynitrite: nitrotyrosine formation on the proximal side impairs its interaction

with NADPH-cytochrome P450 reductase. Chem Res Toxicol 25:2642–2653.

Linares JF, Duran A, Yajima T, et al. (2013). K63 polyubiquitination and activation of mTOR by the p62-TRAF6 complex in nutrient-activated cells. Mol Cell 51:283–296.

Lipson C, Alalouf G, Bajorek M, et al. (2008). A proteasomal ATPase contributes to dislocation of endoplasmic reticulum-associated degradation (ERAD) substrates. J Biol Chem 283:7166–7175.

Lohr JB, Kuhn-Velten WN. (1997). Protein phosphorylation changes ligand-binding efficiency of cytochrome P450c17 (CYP17) and accelerates its proteolytic degradation: putative relevance for hormonal regulation of CYP17 activity. Biochem Biophys Res Commun 231:403–408.

Lu JP, Wang Y, Sliter DA, et al. (2011). RNF170 protein, an endoplasmic reticulum membrane ubiquitin ligase, mediates inositol 1,4,5-trisphosphate receptor ubiquitination and degradation. J Biol Chem 286:24426–24433.

Lu Y, Lee BH, King RW, et al. (2015). Substrate degradation by the proteasome: a single-molecule kinetic analysis. Science 348:1250834.

Madrigal-Matute J, Cuervo AM. (2015). Regulation of liver metabolism by autophagy. Gastroenterology 150:328–339.

Majeski AE, Dice JF. (2004). Mechanisms of chaperone-mediated autophagy. Int J Biochem Cell Biol 36:2435–2444.

Manley S, Williams JA, Ding WX. (2013). Role of p62/SQSTM1 in liver physiology and pathogenesis. Exp Biol Med (Maywood) 238:525–538.

Masaki R, Yamamoto A, Tashiro Y. (1987). Cytochrome P-450 and NADPH-cytochrome P-450 reductase are degraded in the autolysosomes in rat liver. J Cell Biol 104:1207–1215.

McDonough H, Patterson C. (2003). CHIP: a link between the chaperone and proteasome systems. Cell Stress Chaperones 8:303–308.

Metzger MB, Hristova VA, Weissman AM. (2012). HECT and RING finger families of E3 ubiquitin ligases at a glance. J Cell Sci 125:531–537.

Meyer HH, Shorter JG, Seemann J, et al. (2000). A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. EMBO J 19:2181–2192.

Meyer H, Bug M, Bremer S. (2012). Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. Nat Cell Biol 14:117–123.

Miao H, Jiang W, Ge L, et al. (2010). Tetra-glutamic acid residues adjacent to Lys248 in HMG-CoA reductase are critical for the ubiquitination mediated by gp78 and UBE2G2. Acta Biochim Biophys Sin (Shanghai) 42:303–310.

Michalek MT, Grant EP, Gramm C, et al. (1993). A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation. Nature 363:552–554.

Mizushima N, Levine B, Cuervo AM, Klionsky DJ. (2008). Autophagy fights disease through cellular self-digestion. Nature 451:1069–1075.

Mizushima N, Yoshimori T, Levine B. (2010). Methods in mammalian autophagy research. Cell 140:313–326.

Monaco JJ. (1995). Pathways for the processing and presentation of antigens to T cells. J Leukoc Biol 57:543–547.

Morishima Y, Peng HM, Lin HL, et al. (2005). Regulation of cytochrome P450 2E1 by heat shock protein 90-dependent stabilization and CHIP-dependent proteasomal degradation. Biochemistry 44:16333–16340.

Morito D, Hirao K, Oda Y, et al. (2008). Gp78 cooperates with RMA1 in endoplasmic reticulum-associated degradation of CFTRDeltaF508. Mol Biol Cell 19:1328–1336.

Mukhopadhyay D, Riezman H. (2007). Proteasome-independent functions of ubiquitin in endocytosis and signaling. Science 315:201–205.

Murata S, Minami Y, Minami M, et al. (2001). CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep 2:1133–1138.

Murray BP, Correia MA. (2001). Ubiquitin-dependent 26S proteasomal pathway: a role in the degradation of native human liver CYP3A4 expressed in Saccharomyces cerevisiae? Arch Biochem Biophys 393:106–116.

Murray BP, Zgoda VG, Correia MA. (2002). Native CYP2C11: heterologous expression in Saccharomyces cerevisiae reveals a role for vacuolar proteases rather than the proteasome system in the degradation of this endoplasmic reticulum protein. Mol Pharmacol 61:1146–1153.

Nakatsukasa K, Huyer G, Michaelis S, Brodsky JL. (2008). Dissecting the ER-associated degradation of a misfolded polytopic membrane protein. Cell 132:101–112.

Nezis IP, Stenmark H. (2012). p62 at the interface of autophagy, oxidative stress signaling, and cancer. Antioxid Redox Signal 17:786–793.

Noda NN, Kumeta H, Nakatogawa H, et al. (2008). Structural basis of target recognition by Atg8/LC3 during selective autophagy. Genes Cells 13:1211–1218.

Ohsumi Y. (2014). Historical landmarks of autophagy research. Cell Res 24:9–23.

Olzmann JA, Li L, Chudaev MV, et al. (2007). Parkin-mediated K63-linked polyubiquitination targets misfolded DJ-1 to aggresomes via binding to HDAC6. J Cell Biol 178:1025–1038.

Olzmann JA, Kopito RR, Christianson JC. (2013). The mammalian endoplasmic reticulum-associated degradation system. Cold Spring Harb Perspect Biol 5:013185.

Pabarcus MK, Hoe N, Sadeghi S, et al. (2009). CYP3A4 ubiquitination by gp78 (the tumor autocrine motility factor receptor, AMFR) and CHIP E3 ligases. Arch Biochem Biophys 483:66–74.

Pankiv S, Clausen TH, Lamark T, et al. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 282:24131–24145.

Peng J, Schwartz D, Elias JE, et al. (2003). A proteomics approach to understanding protein ubiquitination. Nat Biotechnol 21:921–926.

Peng HM, Morishima Y, Jenkins GJ, et al. (2004). Ubiquitylation of neuronal nitric-oxide synthase by CHIP, a chaperone-dependent E3 ligase. J Biol Chem 279: 52970–52977.

Pickart CM, Fushman D. (2004). Polyubiquitin chains: polymeric protein signals. Curr Opin Chem Biol 8:610–616.

Pickart CM, Cohen RE. (2004). Proteasomes and their kin: proteases in the machine age. Nat Rev Mol Cell Biol 5:177–187.

Porubsky PR, Meneely KM, Scott EE. (2008). Structures of human cytochrome P-450 2E1. Insights into the binding of inhibitors and both small molecular weight and fatty acid substrates. J Biol Chem 283:33698–33707.

Puissant A, Fenouille N, Auberger P. (2012). When autophagy meets cancer through p62/SQSTM1. Am J Cancer Res 2:397–413.

Rabinowitz JD, White E. (2010). Autophagy and metabolism. Science 330:1344–1348.

Ravid T, Kreft SG, Hochstrasser M. (2006). Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. EMBO J 25:533–543.

Rechsteiner M. (2005) The 26S proteasome. In: Protein Degradation Mayer J, Ciechanover A, Rechsteiner M, eds. Vol. 1. Weinheim: Wiley-VCH Veralag GmbH & Co. KGaA, 220–247

Redlich G, Zanger UM, Riedmaier S, et al. (2008). Distinction between human cytochrome P450 (CYP) isoforms and identification of new phosphorylation sites by mass spectrometry. J Proteome Res 7:4678–4688.

Reynald RL, Sansen S, Stout CD, Johnson EF. (2012). Structural characterization of human cytochrome P450 2C19: active site differences between P450s 2C8, 2C9, and 2C19. J Biol Chem 287:44581–44591.

Richly H, Rape M, Braun S, et al. (2005). A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. Cell 120:73–84.

Riley RJ, Smith G, Wolf CR, et al. (1993). Human anti-endoplasmic reticulum autoantibodies produced in aromatic anti-convulsant hypersensitivity reactions recognise rodent CYP3A proteins and a similarly regulated human P450 enzyme(s). Biochem Biophys Res Commun 191:32–40.

Roberts BJ, Song BJ, Soh Y, et al. (1995). Ethanol induces CYP2E1 by protein stabilization. Role of ubiquitin conjugation in the rapid degradation of CYP2E1. J Biol Chem 270:29632–29635.

Roberts BJ. (1997). Evidence of proteasome-mediated cytochrome P-450 degradation. J Biol Chem 272:9771–9778.

Rock KL, Gramm C, Rothstein L, et al. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 78:761–771.

Ronis MJ, Ingelman-Sundberg M. (1989). Acetone-dependent regulation of cytochrome P-450j (IIE1) and P-450b (IIB1) in rat liver. Xenobiotica 19:1161–1165.

Ronis MJ, Johansson I, Hultenby K, et al. (1991). Acetone-regulated synthesis and degradation of cytochrome P450E1 and cytochrome P4502B1 in rat liver [corrected]. Eur J Biochem 198:383–389.

Rosser MF, Washburn E, Muchowski PJ, et al. (2007). Chaperone functions of the E3 ubiquitin ligase CHIP. J Biol Chem 282:22267–22277.

Rowland P, Blaney FE, Smyth MG, et al. (2006). Crystal structure of human cytochrome P450 2D6. J Biol Chem 281:7614–7622.

Rubinsztein DC, Codogno P, Levine B. (2012). Autophagy modulation as a potential therapeutic target for diverse diseases. Nat Rev Drug Discov 11:709–730.

Schattenberg JM, Czaja MJ. (2012). Regulation of the effects of CYP2E1-induced oxidative stress by JNK signaling. Redox Biol 3:7–15.

Schattenberg JM, Wang Y, Singh R, et al. (2005). Hepatocyte CYP2E1 overexpression and steatohepatitis lead to impaired hepatic insulin signaling. J Biol Chem 280: 9887–9894.

Schmiedlin-Ren P, Edwards DJ, Fitzsimmons ME, et al. (1997). Mechanisms of enhanced oral availability of CYP3A4 substrates by grapefruit constituents. Decreased enterocyte CYP3A4 concentration and mechanism-based inactivation by furanocoumarins. Drug Metab Dispos 25:1228–1233.

Scott EE, He YA, Wester MR, et al. (2003). An open conformation of mammalian cytochrome P450 2B4 at 1.6-A resolution. Proc Natl Acad Sci USA 100:13196–13201.

Scott EE, White MA, He YA, et al. (2004). Structure of mammalian cytochrome P450 2B4 complexed with 4-(4-chlorophenyl)imidazole at 1.9-A resolution: insight into the range of P450 conformations and the coordination of redox partner binding. J Biol Chem 279:27294–27301.

Seibenhener ML, Babu JR, Geetha T, et al. (2004). Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol Cell Biol 24:8055–8068.

Sevrioukova IF, Poulos TL. (2012). Structural and mechanistic insights into the interaction of cytochrome P4503A4 with bromoergocryptine, a type I ligand. J Biol Chem 287:3510–3517.

Shah MB, Pascual J, Zhang Q, et al. (2011). Structures of Cytochrome P450 2B6 bound to 4-Benzylpyridine and 4-(4-Nitrobenzyl)pyridine: Insight into inhibitor binding and rearrangement of active site side chains. Mol Pharmacol 80:1047–1055.

Shin Y, Klucken J, Patterson C, et al. (2005). The co-chaperone carboxyl terminus of Hsp70-interacting protein (CHIP) mediates alpha-synuclein degradation decisions between proteasomal and lysosomal pathways. J Biol Chem 280:23727–23734.

Shmueli A, Tsai YC, Yang M, et al. (2009). Targeting of gp78 for ubiquitin-mediated proteasomal degradation by Hrd1: cross-talk between E3s in the endoplasmic reticulum. Biochem Biophys Res Commun 390:758–762.

Slobodkin MR, Elazar Z. (2013). The Atg8 family: multifunctional ubiquitin-like key regulators of autophagy. Essays Biochem 55:51–64.

Sohn DH, Yun YP, Park KS, et al. (1991). Post-translational reduction of cytochrome P450IIE by CCl4, its substrate. Biochem Biophys Res Commun 179:449–454.

Song BJ, Veech RL, Park SS, et al. (1989). Induction of rat hepatic N-nitrosodimethylamine demethylase by acetone is due to protein stabilization. J Biol Chem 264:3568–3572.

Stolz A, Ernst A, Dikic I. (2014). Cargo recognition and trafficking in selective autophagy. Nat Cell Biol 16:495–501.

Strnad P, Zatloukal K, Stumptner C, et al. (2008). Mallory-Denk-bodies: lessons from keratin-containing hepatic inclusion bodies. Biochim Biophys Acta 1782:764–774.

Taxis C, Hitt R, Park SH, et al. (2003). Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. J Biol Chem 278:35903–35913.

Thervet E, Legendre C, Beaune P, Anglicheau D. (2005). Cytochrome P450 3A polymorphisms and immunosuppressive drugs. Pharmacogenomics 6:37–47.

Tierney DJ, Haas AL, Koop DR. (1992). Degradation of cytochrome P450 2E1: selective loss after labilization of the enzyme. Arch Biochem Biophys 293:9–16.

Tresse E, Salomons FA, Vesa J, et al. (2010). VCP/p97 is essential for maturation of ubiquitin-containing autophagosomes

and this function is impaired by mutations that cause IBMPFD. Autophagy 6:217–227.

Udeshi ND, Mertins P, Svinkina T, Carr SA. (2012). Large-scale identification of ubiquitination sites by mass spectrometry. Nat Protoc 8:1950–1960.

Udeshi ND, Svinkina T, Mertins P, et al. (2013). Refined preparation and use of anti-diglycine remnant (K-ε-GG) antibody enables routine quantification of 10,000s of ubiquitination sites in single proteomics experiments. Mol Cell Proteomics 12:825–831.

Uetrecht JP. (1999). New concepts in immunology relevant to idiosyncratic drug reactions: the "danger hypothesis" and innate immune system. Chem Res Toxicol 12:387–395.

Uetrecht J. (2005). Current trends in drug-induced autoimmunity. Autoimmun Rev 4:309–314.

Vashist S, Ng DT. (2004). Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. J Cell Biol 165:41–52.

Vembar SS, Brodsky JL. (2008). One step at a time: endoplasmic reticulum-associated degradation. Nat Rev Mol Cell Biol 9:944–957.

von Muhlinen N, Thurston T, Ryzhakov G, et al. (2010). NDP52, a novel autophagy receptor for ubiquitin-decorated cytosolic bacteria. Autophagy 6:288–289.

Vosper JM, McDowell GS, Hindley CJ, et al. (2009). Ubiquitylation on canonical and non-canonical sites targets the transcription factor neurogenin for ubiquitin-mediated proteolysis. J Biol Chem 284:15458–15468.

Wagner SA, Beli P, Weinert BT, et al. (2012). Proteomic analyses reveal divergent ubiquitylation site patterns in murine tissues. Mol Cell Proteomics 11:1578–1585.

Wang HF, Figueiredo Pereira ME, Correia MA. (1999). Cytochrome P450 3A degradation in isolated rat hepatocytes: 26S proteasome inhibitors as probes. Arch Biochem Biophys 365:45–53.

Wang X, Herr RA, Chua WJ, et al. (2007). Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. J Cell Biol 177:613–624.

Wang Y, Guan S, Acharya P, et al. (2011). Ubiquitin-dependent proteasomal degradation of human liver cytochrome P450 2E1: identification of sites targeted for phosphorylation and ubiquitination. J Biol Chem 286:9443–9456.

Wang X, Medzihradszky KF, Maltby D, Correia MA. (2001). Phosphorylation of native and heme-modified CYP3A4 by protein kinase C: a mass spectrometric characterization of the phosphorylated peptides. Biochemistry 40:11318–11326.

Wang Y, Liao M, Hoe N, et al. (2009). A role for protein phosphorylation in cytochrome P450 3A4 ubiquitin-dependent proteasomal degradation. J Biol Chem 284:5671–5684.

Wang Y, Guan S, Acharya P, et al. (2012). Multisite phosphorylation of human liver cytochrome P450 3A4 enhances Its gp78- and CHIP-mediated ubiquitination: a pivotal role of its Ser-478 residue in the gp78-catalyzed reaction. Mol Cell Proteomics 11:M111. 010132.

Wang A, Savas U, Hsu MH, et al. (2012). Crystal structure of human cytochrome P450 2D6 with prinomastat bound. J Biol Chem 287:10834–10843.

Wang Y, Ha SW, Zhang T, et al. (2014). Polyubiquitylation of AMF requires cooperation between the gp78 and TRIM25 ubiquitin ligases. Oncotarget 5:2044–2051.

Wang Y, Kim SM, Trnka MJ, et al. (2015). Human liver cytochrome P450 3A4 ubiquitination: molecular recognition by UBC7-gp78 autocrine motility factor receptor and UbcH5a-CHIP-Hsc70-Hsp40 E2-E3 ubiquitin ligase complexes. J Biol Chem 290:3308–3332.

Watkins PB, Wrighton SA, Schuetz EG, et al. (1986). Macrolide antibiotics inhibit the degradation of the glucocorticoid-responsive cytochrome P-450p in rat hepatocytes in vivo and in primary monolayer culture. J Biol Chem 261:6264–6271.

Wen X, Klionsky DJ. (2016). An overview of macroautophagy in yeast. J Mol Biol 428:1681–1699.

Wester MR, Yano JK, Schoch GA, et al. (2004). The structure of human cytochrome P450 2C9 complexed with flurbiprofen at 2.0-A resolution. J Biol Chem 279:35630–35637.

Williams PA, Cosme J, Ward A, et al. (2003). Crystal structure of human cytochrome P450 2C9 with bound warfarin. Nature 424:464–468.

Wojcik C, Rowicka M, Kudlicki A, et al. (2006). Valosin-containing protein (p97) is a regulator of endoplasmic reticulum stress and of the degradation of N-end rule and ubiquitin-fusion degradation pathway substrates in mammalian cells. Mol Biol Cell 17:4606–4618.

Woodman PG. (2003). p97, a protein coping with multiple identities. J Cell Sci 116:4283–4290.

Wu D, Cederbaum AI. (2013). Inhibition of autophagy promotes CYP2E1-dependent toxicity in HepG2 cells via elevated oxidative stress, mitochondria dysfunction and activation of p38 and JNK MAPK. Redox Biol 1:552–565.

Xia D, Tang WK, Ye Y. (2016). Structure and function of the AAA + ATPASE p97/Cdc48p. Gene 583:67–77.

Xu L, Chen Y, Pan Y, et al. (2009). Prediction of human drug-drug interactions from time-dependent inactivation of CYP3A4 in primary hepatocytes using a population-based simulator. Drug Metab Dispos 37:2330–2339.

Xu G, Paige JS, Jaffrey SR. (2010). Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling. Nat Biotechnol 28:868–873.

Yang J, Liao M, Shou M, et al. (2008). Cytochrome p450 turnover: regulation of synthesis and degradation, methods for determining rates, and implications for the prediction of drug interactions. Curr Drug Metab 9:384–394.

Yang Z, Klionsky DJ. (2009). An overview of the molecular mechanism of autophagy. Curr Top Microbiol Immunol 335:1–32.

Yano JK, Wester MR, Schoch GA, et al. (2004). The structure of human microsomal cytochrome P450 3A4 determined by X-ray crystallography to 2.05-A resolution. J Biol Chem 279:38091–38094.

Ye Y, Shibata Y, Kikkert M, et al. (2005). Inaugural Article: Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. Proc Natl Acad Sci USA 102:14132–14138.

Ying Z, Wang H, Fan H, Wang G. (2011). The endoplasmic reticulum (ER)-associated degradation system regulates aggregation and degradation of mutant neuroserpin. J Biol Chem 286:20835–20844.

Younger JM, Chen L, Ren HY, et al. (2006). Sequential quality-control checkpoints triage misfolded cystic fibrosis transmembrane conductance regulator. Cell 126:571–582.

Zhang T, Kho DH, Wang Y, et al. (2015). Gp78, an E3 ubiquitin ligase acts as a gatekeeper suppressing nonalcoholic steatohepatitis (NASH) and liver cancer. PLoS One 10:e0118448.

Zhao C, Gao Q, Roberts AG, et al. (2012). Cross-linking mass spectrometry and mutagenesis confirm the functional importance of surface interactions between CYP3A4 and holo/apo cytochrome b(5). Biochemistry 51:9488–9500.

Zhukov A, Werlinder V, Ingelman-Sundberg M. (1993). Purification and characterization of two membrane bound serine proteinases from rat liver microsomes active in degradation of cytochrome P450. Biochem Biophys Res Commun 197:221–228.

Zhukov A, Ingelman-Sundberg M. (1999). Relationship between cytochrome P450 catalytic cycling and stability: fast degradation of ethanol-inducible cytochrome P450 2E1 (CYP2E1) in hepatoma cells is abolished by inactivation of its electron donor NADPH-cytochrome P450 reductase. Biochem J 340:453–458.