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

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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

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tripartite conjunction of K_{63}-linked Ub-chains, P450 structural “LIR” motifs, and selective cellular “cargo receptors” as plausible P450-ALD determinants.

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 (Vembar & Brodsky, 2008; Nakatsukasa et al., 2008; Hampton & Garza, 2009; Hirsch et al, 2009; Olzmann et al., 2013; Christianson &Ye, 2014), with three distinct mechanistic pathways in the yeast *S. cerevisiae* (Taxis et al., 2003; Vashist & Ng, 2004; Ahner & Brodsky, 2004; Carvalho et al., 2006): ERAD-**L** for the disposal of misfolded/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 predominantly target the ER-proteins to ubiquitin (**Ub**)-dependent 26S proteasomal degradation (**UPD**) (Olzmann et al., 2013; Christianson & Ye, 2014). However, in mammalian cells, the subsequent recognition 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 requiring 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) (Gorsky et al., 1984; Ekstrom & Ingelman-Sundberg, 1989; Goasduff & Cederbaum, 1999; Zhukov & Ingelman-Sundberg, 1999). However, in spite of these common features, not all P450 proteins predominantly 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; Tierney et al., 1992; Sohn et

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al., 1991; Dai & Cederbaum, 1995; Roberts, 1997; Schmiedlin-Ren, 1997; Korsmeyer et al., 1997; Wang et al., 1999; Murray & Correia, 2001; Morishima et al., 2005; Liao et al., 2006; Correia & Liao, 2007; Faouzi et al., 2007; Lee et al., 2008), 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 (Masaki et al., 1987; Ronis & Ingelman-Sundberg, 1989; Ronis et al., 1991; Murray et al., 2002; Liao et al., 2005). On the other hand, human or rat liver CYP2E1 utilizes both ERAD/UPD and ERAD/ALD pathways, depending on whether it is suicidally inactivated or native/substrate-free, or native/substrate-bound, respectively (Song et al., 1989; Roberts et al., 1995; Bardag-Gorce, 2002; Morishima et al., 2005; 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/aggregated proteins awaiting clearance, and competition for the ERAD/UPD cellular machinery is insurmountable. It is worth noting however, that P450 ERAD in cultured hepatocytes may not always faithfully reflect the preferential 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 degradation pathway. Such ERAD involves posttranslational phosphorylation of the P450 proteins by cytosolic kinases (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 (Morishima et al., 2005; Pabarcus et al., 2009; Wang et al., 2009, 2011, 2012, 2015; Kim et al., 2010), and subsequent degradation by the cytosolic 26S proteasome (Correia et al. 1992b, 2005; Tierney et al., 1992; Roberts, 1997; Murray & Correia, 2001; Correia, 2003; Correia et al., 2005; Morishima et al., 2005; Liao et al., 2006; Correia & Liao, 2007; Faouzi et al., 2007; Fig. 1). The 26S proteasome consists of a 20S proteolytic barrel topped at each end by a 19S regulatory cap, with Ub-chain recognition/deubiquitinating Rpn (non-ATPase) lid subunits (specifically Rpn 10 and Rpn13 Ub-chain receptors/Rpn11 deubiquitinase) as well as Rpt AAA (ATPases associated with various 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. The bare, unfolded protein target is then threaded into the narrow 20S central proteolytic cavity with a pore diameter of merely 13–14 Å (Pickart & Cohen, 2004; Rechsteiner, 2005; Finley, 2009).

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 (Dai & Li, 2001; Meyer et al., 2000; Elkabetz et al., 2004; Richly et al., 2005; Ye et al., 2005; Bar-Nun, 2005; Ikeda et al., 2009; Jentsch & Rumpf, 2007; Chou et al., 2011; Xia et al.,

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2016), 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 (Rock et al., 1994; Dick et al., 1997; Lee & Goldberg, 1998; Rechsteiner, 2005). 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 (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; Korsmeyer et al., 1999; Correia et al., 2003, 2014, 2007 and Correia & Liao, 2007).

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,

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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 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 (Wang et al., 2009, 2011, 2012, 2015; Correia et al., 2014). 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 (that is 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

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(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 (Ravid et al., 2006; Kostova et al., 2007; Hirsch et al., 2009; Metzger et al., 2012; Christianson & Ye, 2014). 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; Murata et al., 2001; Fang et al., 2001; Jiang et al., 2001; McDonough & Patterson, 2003; Peng et al., 2004; Kikkert et al., 2005; Chen et al., 2006; Kostova et al., 2007; Rosser et al., 2007; Hirsch et al., 2009; Metzger et al., 2012; Chen et al., 2012; Olzmann et al., 2013; Edkins, 2013). 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 (Murray & Correia, 2001; Liao et al., 2006), 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 (Parbacus 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 (Parbacus 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) (Fang et al., 2001; Chen et al., 2006; Kostova et al., 2007; Das et al., 2009, 2013; Hirsch et al., 2009; Pabarcus et al. 2009; Kim et al., 2010; Metzger et al., 2012; Chen et al., 2012; Olzmann et al., 2013) 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 (Fang et al., 2001; Chen et al., 2006; Kostova et al., 2007; Li et al., 2007, 2009; Hirsch et al., 2009; Pabarcus et al. 2009; Kim et al., 2010; Metzger et al., 2012; Chen et al., 2012; Olzmann et al., 2013). Its cytosolic, functionally

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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) (Fang et al., 2001; Chen et al., 2006; Kostova et al., 2007; Das et al., 2009, 2013; Metzger et al., 2012; Chen 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 (Fig. 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; Murata et al., 2001; Jiang et al., 2001; McDonough & Patterson, 2003; Peng et al., 2004; Morishima et al., 2005; Rosser et al., 2007; Clapp et al., 2012; Edkins, 2013). 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* (Murray & Correia, 2001; Liao et al., 2006). However, CHIP was documented to ubiquitinate various proteins including CFTR, its co-chaperone Hsp70, the androgen, estrogen, and glucocorticoid receptors (Ballinger et al., 1999; Jiang et al., 2001; McDonough & Patterson, 2003; Younger et al., 2006; Rosser et al., 2007; Edkins, 2013), and most tellingly, the P450-like hemoprotein neuronal nitric-oxide synthase (Peng et al., 2004; Clapp et al., 2012), as well as the recombinant rabbit CYP2E1 and CYP2B4 *in vitro* (Morishima et al., 2005). This collective evidence coupled with our preliminary finding that

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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 (Pabarcus et al., 2009; Wang et al., 2009, 2012, 2015; Kim et al., 2010) 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* (Fig. 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 HPLC-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 that 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; Younger et al., 2006; Morito et al., 2008; Shmueli et al., 2009; Ying et al., 2011; Lu et al., 2011; Jo et al., 2011; Wang et al., 2014; Zhang et al., 2015; Wang 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

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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 (Kostova et al., 2007; Morito et al., 2008; Chen et al., 2012; 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 (Pabarcus et al., 2009; Kim et al., 2010; 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 (Mizushima et al., 2008; Klionsky et al., 2010; Rabinowitz & White, 2010; Cuervo, 2011; Rubinsztein et al, 2012). It is now recognized to be both a rather tightly regulated and selective process entailing macroautophagy with subsequent lysosomal degradation (Mizushima et al., 2008; Yang & Klionsky, 2009; Klionsky et al., 2010; Mizushima et al., 2010; Rabinowitz & White, 2010; Cuervo, 2011; Rubinsztein et al, 2012; Ohsumi, 2014; Madrigal-Matute & Cuervo, 2015; Bento et al., 2016; Wen & Klionsky, 2016). Macroautophagy (or autophagy for short) involves the concerted function of over 35 **a**u**t**ophagy-related **g**enes (ATGs; Mizushima et

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al., 2008; Yang & Klionsky, 2009; Klionsky et al., 2010; Mizushima et al., 2010; Rabinowitz & White, 2010; Cuervo, 2011; Rubinsztein et al, 2012; Ohsumi, 2014; Madrigal-Matute & Cuervo, 2015; Bento et al., 2016; Wen & Klionsky, 2016) that generate multiprotein complexes that act cooperatively and sequentially to deliver the cargo for lysosomal degradation (Fig. 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 I-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 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 (Lamark et al., 2009; Johansen & Lamark, 2011; Birgisdottir et al., 2013; 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 (Fig. 3), it is highly likely that P450s like other ALD substrates (Klionsky et al., 2010; Rubinsztein et al, 2012; Bento et al., 2016), 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_4Cl 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_4Cl as diagnostic probes has revealed that certain P450s are largely degraded via ERAD/ALD, whereas only a few others are normally so degraded (Masaki et al., 1987; Ronis & Ingelman-Sundberg, 1989; Ronis et al., 1991; Roberts, 1997; Murray et al., 2002; Correia, 2003; Liao et al., 2005; Correia & Liao, 2007; Faouzi et al., 2007; Wang et al., 2011; Fig. 3; *see below*).

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

Studies with diagnostic UPD/ALD inhibitors in cultured human, mouse and rat hepatocytes (Fig. 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; Roberts, 1997; Schmiedlin-Ren, 1997; Korsmeyer et al., 1997; Wang et al., 1999; Murray & Correia, 2001; Liao et al., 2006; Correia & Liao, 2007; Faouzi et al., 2007), whereas native CYPs 2B1 and 2C11 largely incur ALD (Masaki et al., 1987; Ronis & Ingelman-Sundberg, 1989; Ronis et al., 1991; Roberts, 1997; Murray et al., 2002; Correia, 2003; Liao et al., 2005; Correia & Liao, 2007; Wang et al., 2011), and CYP2E1 incurs both processes (Ronis & Ingelman-Sundberg, 1989; Ronis et al., 1991; Sohn et al., 1991; Tierney et al., 1992; Dai & Cederbaum, 1995; Roberts, 1997; Morishima et al., 2005; 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*; Song et al., 1989; Roberts et al., 1994). 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 (Song et al., 1989; Roberts et al., 1994; Correia, 2003, *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 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 (Song et al., 1989; Roberts et al., 1995; Chien et al., 1997; Bardag-Gorce et al., 2006). 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

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expected from their greatly diminished oxidative function (Murray & Correia, 2001; Liao et al., 2006; 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 (Yano et al., 2004; Williams et al., 2004; Ekroos & Sjogren, 2006; Sevriouka & Poulos, 2012) 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 (Fig. 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; Zhao et al., 2012; Lin 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; Xu et al, 2010; Kim et al., 2011, Wagner et al., 2012; Udeshi et al., 2012, 2013), 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 (Wang et al., 2012, 2015; Correia et al., 2014). 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

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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 (Fig. 5). Such P450 S/T phosphorylation by imparting additional negative charge would further boost the overall negative charge of a specific DEST cluster (Wang et al., 2012, 2015; Correia et al., 2014), 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 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 (Fig. 5; Wang et al., 2011; Correia et al., 2014). Furthermore, the positively charged gp78-patches required for CYP3A4-ubiquitination are similarly relevant to its CYP2E1-ubiquitination (Fig. 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 (Wester et al., 2004; Rowland et al., 2006; Reynald et al., 2012; Wang et al., 2012) reveals that many of the surface K-residues to 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 (Fig. 6).

Lys_{48}-linked polyUb chains

Ub contains 7 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

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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 (Pickart & Cohen, 2004; Rechsteiner, 2005; Finley, 2009). The ideal Ub-chain length for such proteasomal targeting is 4–20 (Pickart & Fushman, 2004; Pickart & Cohen, 2004; Rechsteiner, 2005; Finley, 2009), although even shorter chains may suffice (Lu et al., 2015). 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; Wang et al., 2007; Vosper et al., 2009; Ishikura et al., 2010). 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 (Fig. 7): (i) **LIRs** or **LRS**, LC3-interacting regions as adapters (Lamark et al., 2009; Johansen & Lamark, 2011; Birgisdottir et al., 2013; Stolz et al., 2014); (ii) **Monoubiquitination** or **Lys_{63}-linked polyubiquitination** (Seibenhener et al., 2004; Mukhopadhyay & Riezman, 2007; Kim et al., 2008; Kirkin et al., 2009; Linares et al., 2013); 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 (Seibenhener et al., 2004; Pankiv et al., 2007; Olzmann et al., 2007; Mukhopadhyay & Riezman, 2007; Kim et al., 2008; Kirkin et al., 2009; Linares et al., 2013).

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 (Ichimura et al., 2008; Noda et al., 2008; Lamark et al., 2009; Johansen & Lamark, 2011; Birgisdottir et al., 2013; 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

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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 (Ichimura et al., 2008; Noda et al., 2008; Birgisdottir et al., 2013). 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 (that is apparently conserved in CYP2B6 and possibly other CYPs 2B; Scott et al., 2003, 2004; Shah et al., 2011; Halpert, 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 (Fig. 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 (Fig. 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 (Yano et al., 2004; Williams et al., 2004; Scott et al., 2003, 2004; Porubsky et al., 2008; Fig. 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] (Seibenhener et al., 2004; Mukhopadhyay & Riezman, 2007; Kim et al., 2008; Kirkin et al., 2009; Linares et al., 2013). Such K_{63}-linked polyubiquitination is involved in their p62-/NBR-1-recruitment and subsequent targeting to ALD (Seibenhener et al., 2004; Lamark et al., 2009; Lamark et al., 2009; Johansen & Lamark, 2011; Birgisdottir et al., 2013; Linares et al., 2013; 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 human liver P450s incur both types of such linkages in HepG2 cells upon transfection (Fig. 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 (Fig. 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 (Pankiv et al., 2007; Ichimura et al, 2008; Noda et al., 2008; Lamark et al., 2009; von Muhlinen et al., 2010; Johansen & Lamark, 2011; Birgisdottir et al., 2013; Stolz et al., 2014; Katsuragi et al., 2015; Bento et al., 2016). Their function is usually complementary, but redundant in some instances (Birgisdottir et al., 2013; Stolz et

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al., 2014; Bento et al., 2016). 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) (Pankiv et al., 2007; Lamark et al., 2009; Johansen & Lamark, 2011; Birgisdottir et al., 2013; Stolz et al., 2014; Bento et al., 2016). 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 (Pankiv et al., 2007; Lamark et al., 2009; Johansen & Lamark, 2011; Birgisdottir et al., 2013; Stolz et al., 2014; Bento et al., 2016). 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 (Pankiv et al., 2007; Olzmann et al., 2007; Komatsu et al., 2007; Lamark et al., 2009; Johansen & Lamark, 2011; Birgisdottir et al., 2013; Stolz et al., 2014; Bento et al., 2016). 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; Komatsu et al., 2007; Strnad et al., 2008 Johansen & Lamark, 2011). 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 crossroads of various cellular pathways (Bug & Meyer, 2012; Meyer et al., 2012; Deshaies, 2014; Anderson et al., 2015). 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 (Meyer et al., 2000; Dai & Li, 2001; Elkabetz et al., 2004; Richly et al., 2005; Ye et al., 2005; Bar-Nun, 2005; Ikeda et al., 2009; Jentsch & Rumpf, 2007; Chou et al., 2011; Xia et al., 2016). 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 (Woodman, 2003; Dreveney et al., 2004; Wojcik et al., 2006; Chou et al., 2011; 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

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through each of these degradation pathways (Ju et al., 2009; Tresse et al., 2009, 2010; Chou et al., 2011). 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 (Fig. 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; Tierney et al., 1992; Sohn et al., 1991; Dai & Cederbaum, 1995; Roberts, 1997; Schmiedlin-Ren, 1997; Korsmeyer et al., 1997; Wang et al., 1999; Murray & Correia, 2001; Morishima et al., 2005; Liao et al., 2006; Correia & Liao, 2007; Faouzi et al., 2007; Lee et al, 2008). However, native P450s not only are tagged with K_{48}- and K_{63}-linked Ub-chains, but also turnover bimodally via both ERAD/UPD and ERAD/ALD (Fig. 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/NH_4Cl) 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/NH_4Cl) with time. Although CYP2B6 is ubiquitinated via both K_{48}- and K_{63}-Ub-chains (Fig. 10), no accumulation of CYP2B6 ubiquitinated species was detected however upon treatment with 3MA/NH_4Cl, even at longer times (24 h) (Fig. 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 its UPD was dependent on an as yet unidentified E3 ligase (Fig. 12). Consistent with its preference for ERAD/UPD, inspection of the CYP2B6 crystal structure (Shah et al., 2011; Halpert, 2011) reveals that it contains several K-residues within or vicinal to surface DEST clusters (Fig. 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) (Fig. 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

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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 (Masaki et al., 1987; Ronis & Ingelman-Sundberg, 1989; Ronis et al., 1991; Liao et al., 2005). 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 (Fig. 4). This ALD-preference however switches to UPD upon CYP2B1 inactivation (Korsmeyer et al., 1999; Lee et al., 2008). 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 (Seibenhener et al., 2004; Lamark et al., 2009; Lamark et al., 2009; Johansen & Lamark, 2011; Birgisdottir et al., 2013; Linares et al., 2013; 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_4Cl (Fig. 11A). Although Myc-pull-down studies indicate that CYP2C9 undergoes both K_{48}- or K_{63}-linked ubiquitination (Fig. 10), no accumulation of ubiquitinated CYP2C9 species is detected upon UPD or ALD inhibition (Fig. 11B), although stabilization of the parent species is clearly detected (Fig. 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 (Fig. 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 (Fig. 6). Such structural scrutiny also reveals the presence of Trp-, Phe- and Tyr-based LIR motifs (Fig. 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 ($CHIP^{-/-}$)

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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_4Cl) 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_4Cl (Fig. 11A). CYP2D6 incurs both types of K_{48}- and K_{63}-linked ubiquitination (Fig. 10), and this was confirmed upon treatment with either MG132 or 3MA/NH_4Cl, as both probes stabilized HMM ubiquitinated CYP2D6 species to a significant extent (Fig. 11B). Co-transfection studies in HepG2 cells with HA-tagged E3-ligases revealed that CYP2D6 is a CHIP-, but not a gp78-ubiquitination substrate (Fig. 12). Structural inspection of CYP2D6 (Wang A et al., 2012) also reveals the presence of K-residues associated with surface DEST clusters (Fig. 6), as well as surface LIR-motifs, hallmarks of a suitable autophagic target (Fig. 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_4Cl) probe in HepG2 cells transfected with a C-terminally Myc-tagged human liver CYP2E1 expression vector (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 2). Inspection of CYP2E1 structure (Porubsky et al., 2008) reveals that its ubiquitinated K-residues are spatially associated with negatively charged surface DEST-clusters (Fig. 5). However, it also has highly cytosol-accessible LIR-motifs that are Trp-, Phe- and Tyr-based (Fig. 9).

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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 (55 kDa) and ubiquitinated species (65 to >250 kDa) species by the proteasomal inhibitors MG132 or MG262 (Fig. 4; Wang et al., 1999; Faouzi et al., 2007). A small but detectable stabilization of the parent protein (55 kDa) is also observed from 3–6 h upon treatment with the ALD inhibitors 3MA/NH_4Cl (Fig. 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* (Murray & Correia, 2001; Liao et al., 2006), 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 (Fig. 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

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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/pharmacodynamics drug-drug interactions (Chien et al., 1997; Kalgutkar et al., 2007; Yang et al., 2008; Xu et al., 2009; Liao et al., 2010). 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 (Wang et al., 1999; Faouzi et al., 2007). 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 (Wang et al., 1999; Faouzi et al., 2007).

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 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; Schattenberg & Czaja, 2005; 2014; Guengerich, 2015). 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

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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; Riley et al, 1993; Leeder et al., 1996; Eliasson & Kenna, 1996; Dansette et al., 1998; Boitier & Beaune, 2000; Thervet et al., 2004; 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; Michalek et al., 1993; Monaco, 1995; Kloetzl, 2004; Ferrington & Grgerson, 2012), 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; Mizushima et al., 2008; Rabinowitz & White, 2010; Komatsu, 2012; Komatsu et al., 2012; Nezis et al., 2012; Puissant et al., 2012; Manley et al., 2013; Madrigal-Matute & Cuervo, 2015; 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 DE, 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

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oprozomib, and the naturally occurring β-lactone marizomib (salinosporamide A) are either in use or actively considered in cancer chemotherapy (Deshaies, 2014; Johnson DE, 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, DE, 2015). Given its pharmacologic/pharmacokinetic profile and the documentation of its effectiveness in both hematological as well as solid tumor animal models (Anderson, DE, 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, DE, 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.

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List of commonly used 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
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 O_2 species
TAO	troleandomycin
Ub	ubiquitin
UPD	Ub-dependent 26S proteasomal degradation

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VCP	Valosin-containing protein
VIM	VCP-interacting motif

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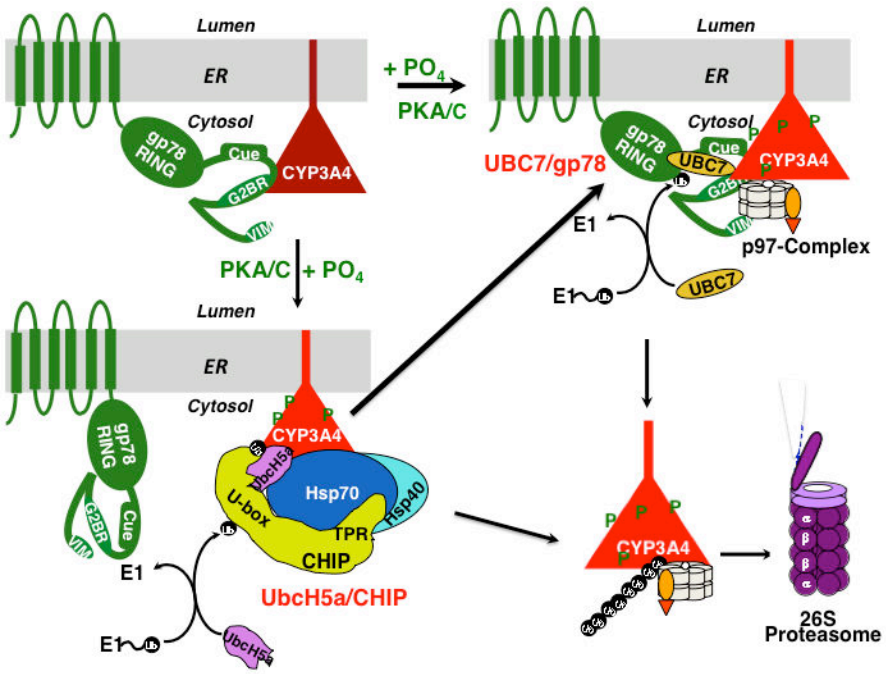

Fig. 1. CYP3A4 ERAD/UPD: Known cellular participants

See discussion for details.

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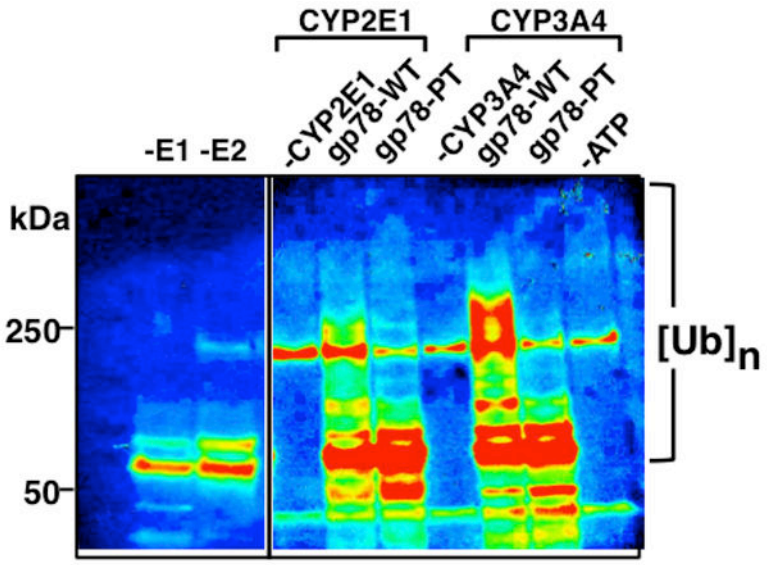

Fig. 2. gp78-mediated ubiquitination of CYP2E1: Involvement of the same gp78 basic patches involved in its intermolecular electronic interactions with CYP3A4

This was documented in *in vitro* functionally reconstituted E1/E2/E3 systems in the presence or absence of 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.

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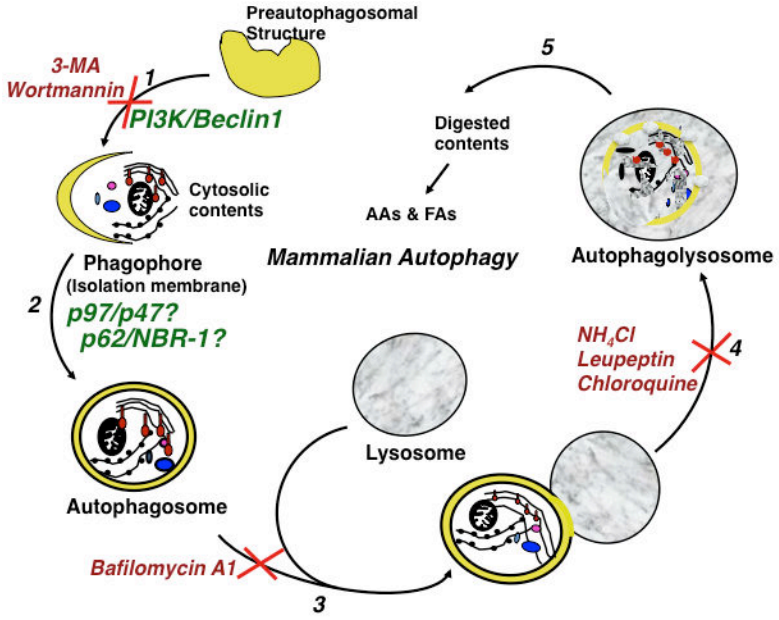

Fig. 3. Autophagy in mammalian cells

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.

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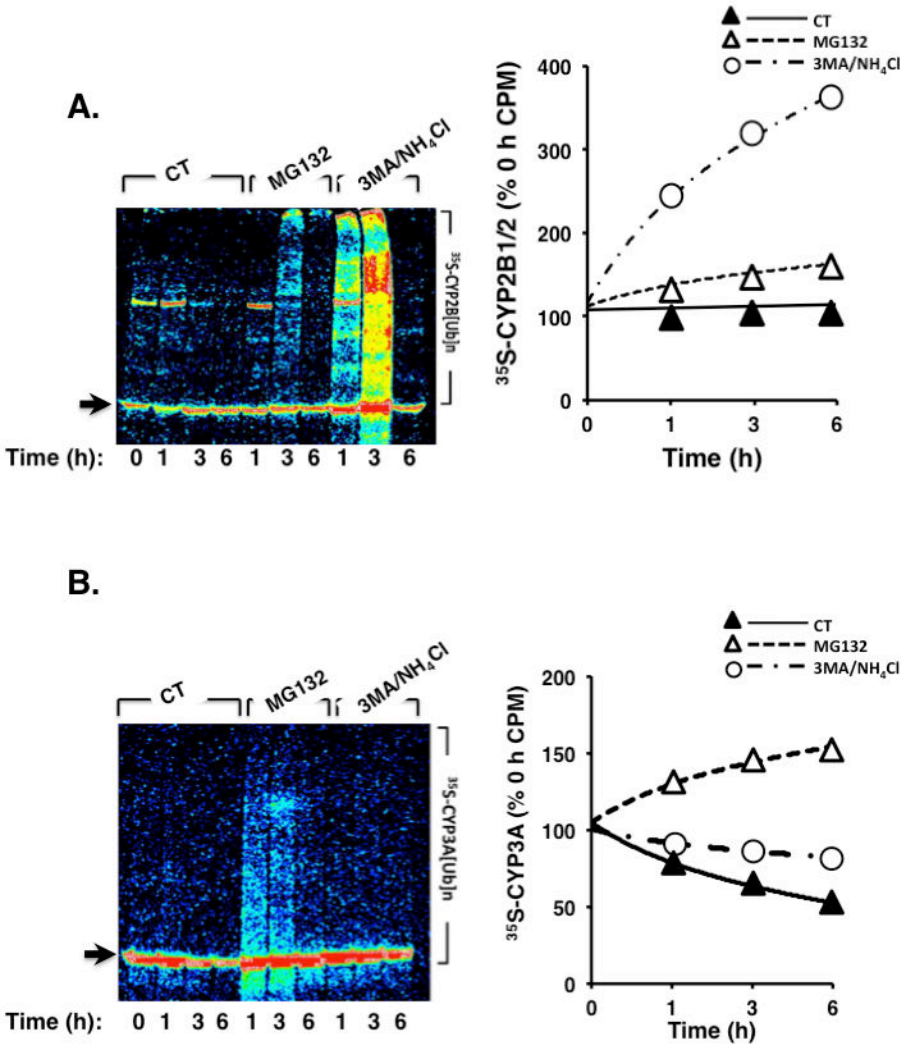

Fig. 4. ^{35}S-pulse-chase analyses of CYP3A and CYP2B time-course in cultured rat hepatocytes

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_4Cl (50 mM). Cell lysates were immunopreciptated 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.

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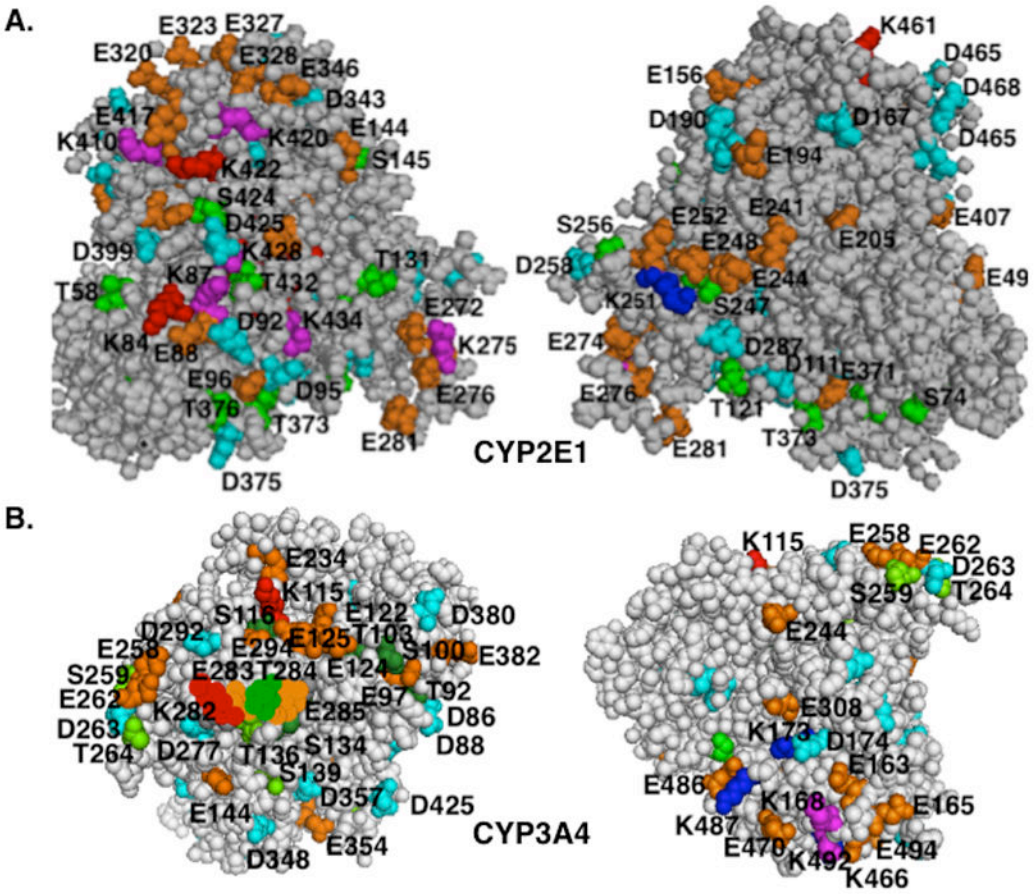

Fig. 5. CYP2E1 and CYP3A4 structures with linear and "conformational" phosphodegrons

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.

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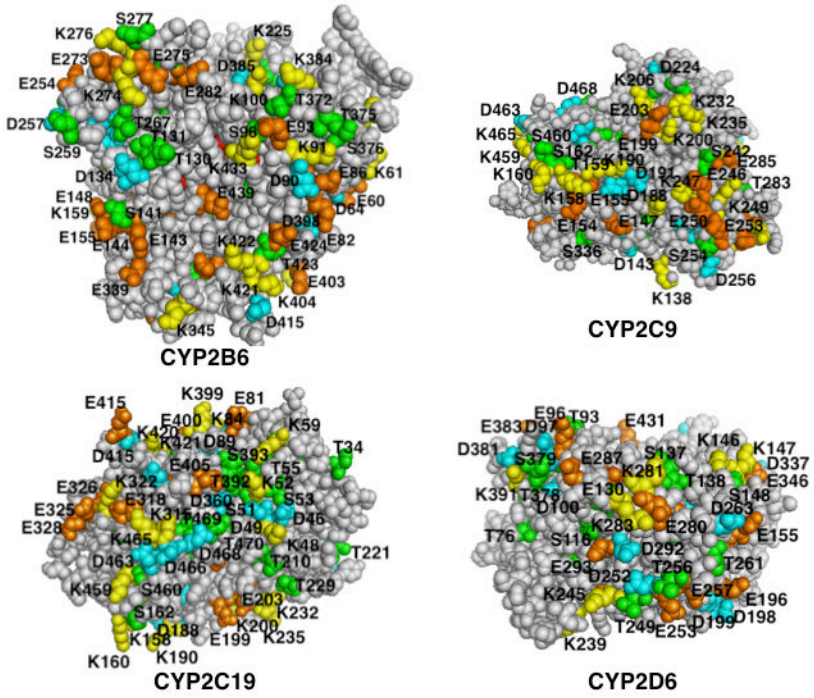

Fig. 6. Plausible linear and "conformational" phosphodegrons of other major human liver P450s

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.

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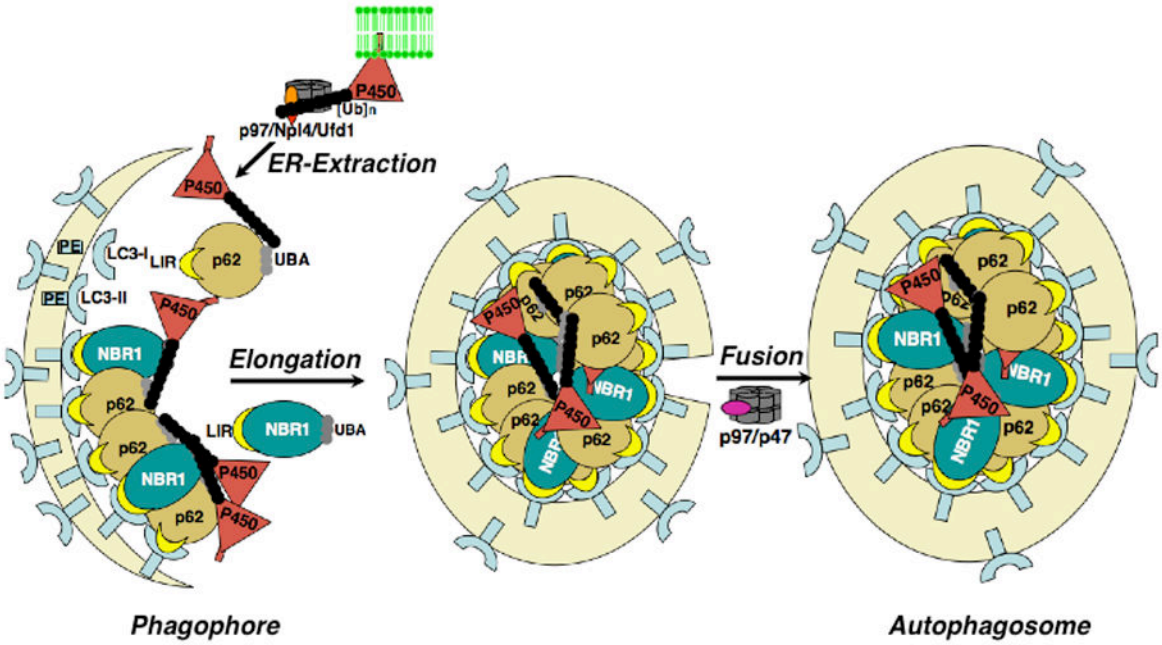

Fig. 7. Plausible mechanisms of P450 autophagic targeting

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.

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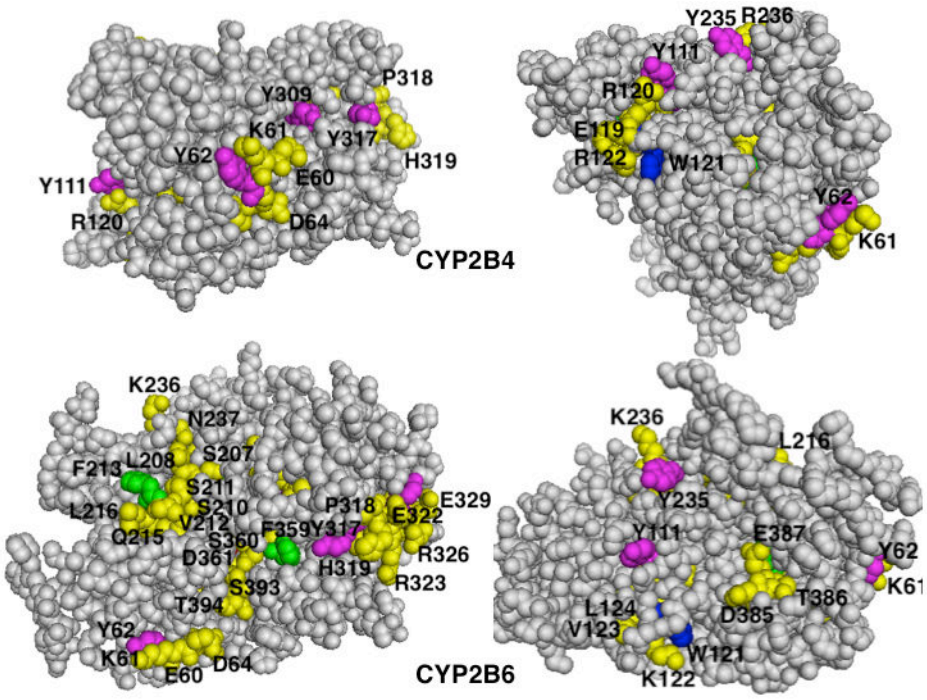

Fig. 8. CYP2B4 versus CYP2B6 surface LIR-motifs

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. Note that these critical W_0-residues are largely buried in CYP2B6, but not CYP2B4.

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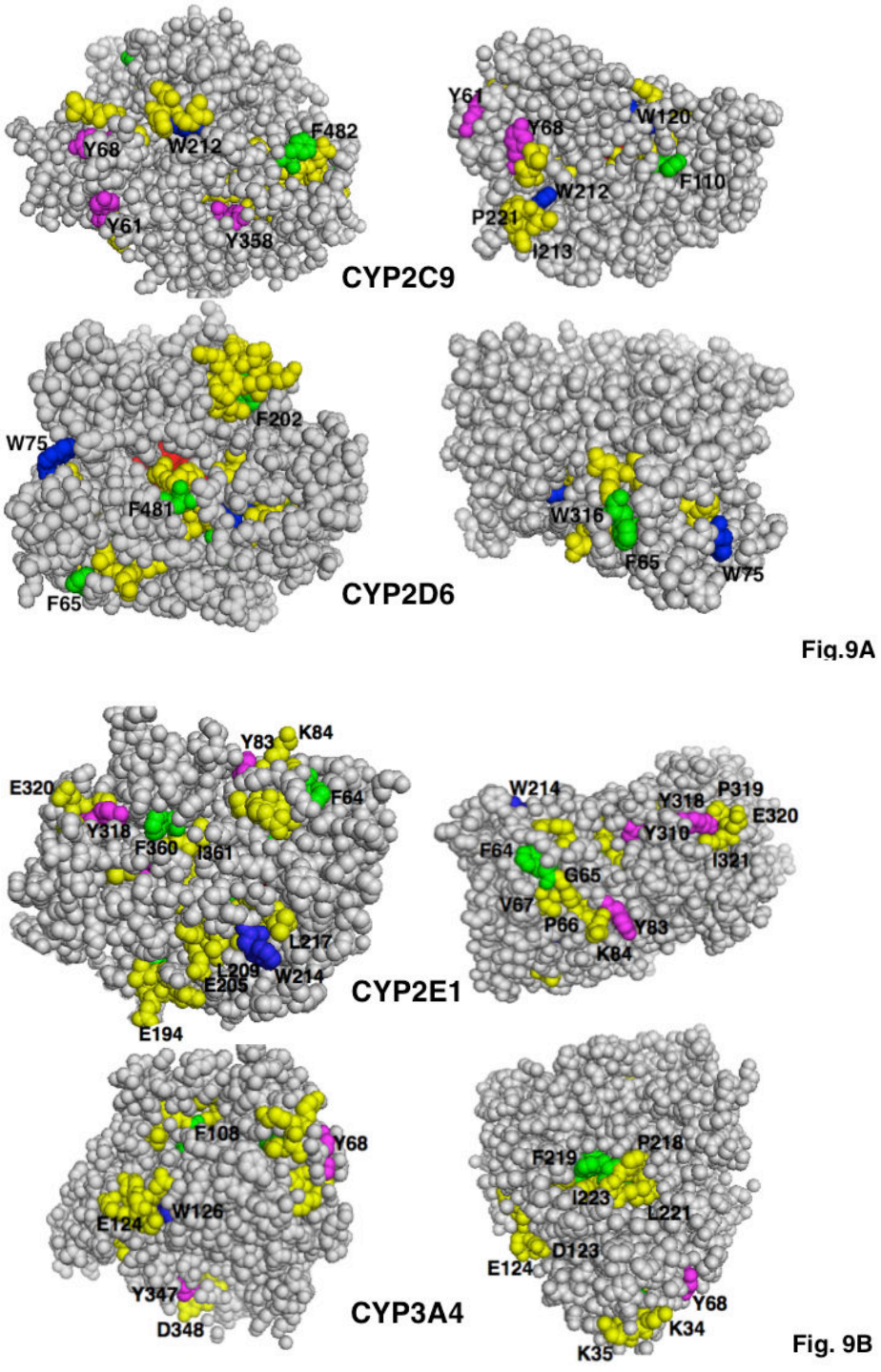

Fig. 9. Surface LIR-motifs of other human P450s

Two different orientations of CYP2C9 and CYP2D6 (**A**) and CYP2E1 and CYP3A4 (**B**) 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

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also depicted in yellow, when present in the P450 primary sequence. Note that these critical W_0-residues are largely buried in CYP3A4.

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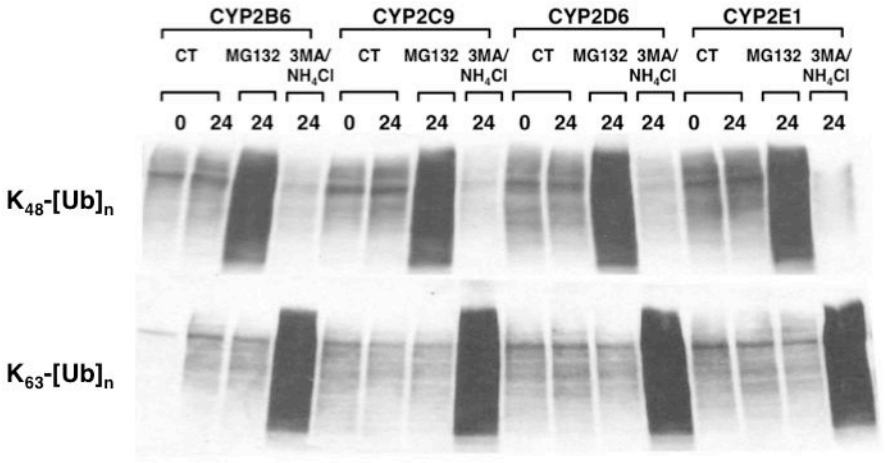

Fig. 10. K_{48}- versus K_{63}-linked P450 ubiquitination in HepG2 cells

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_4Cl or no inhibitor (Control, CT). Six h 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_4Cl-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.

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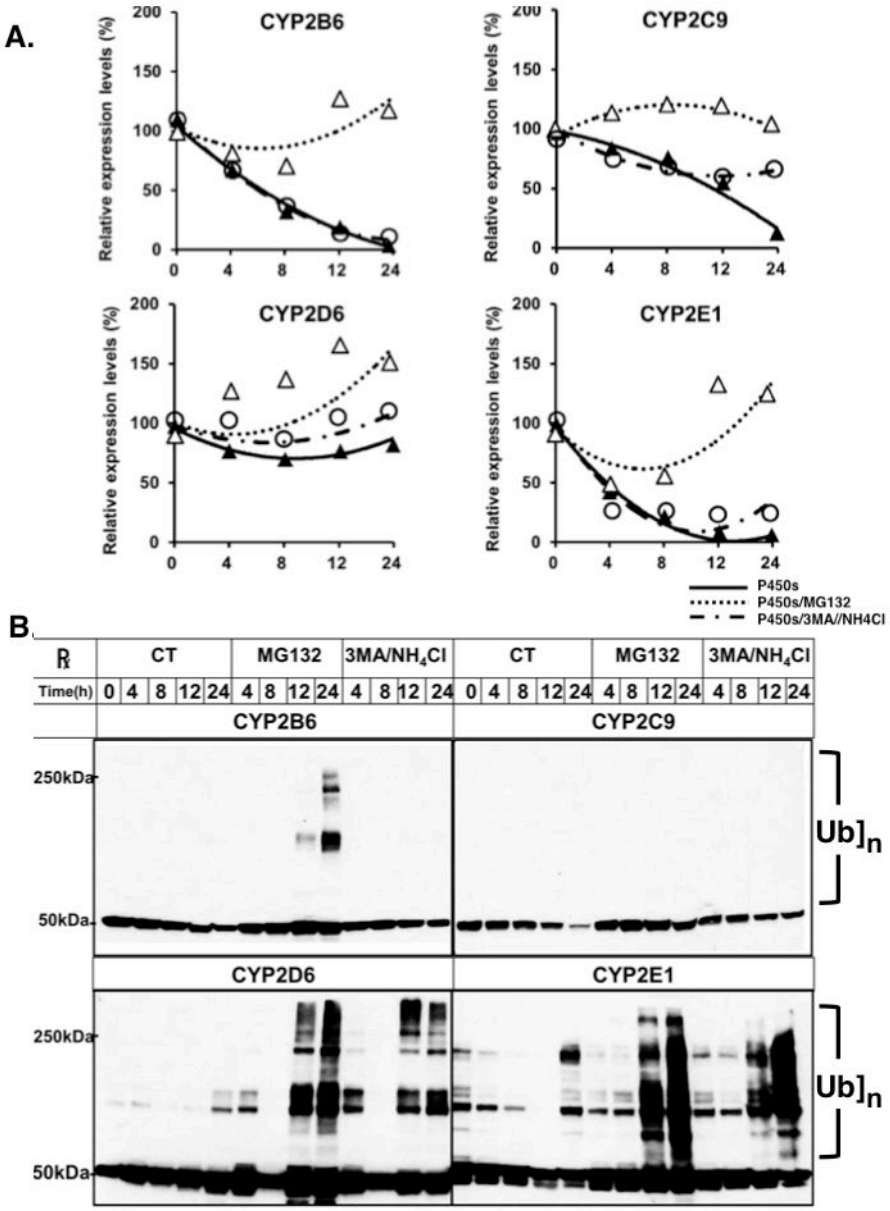

Fig. 11. Cycloheximide-chase analyses of P450 turnover with UPD- or ALD-diagnostic probes

HepG2 cells were cultured and transfected with each P450 plasmid vector for 48 h as in Fig. 10. After washing and medium exchange, cells were treated with cycloheximide (100 μM) with or without MG132 or 3MA/NH_4Cl as in Fig. 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/NH_4Cl. Even CYP2C9 (50 kDa species) shows some stabilization upon 3MA/NH_4Cl-treatment at 24 h. No stabilization of CYP2B6 is appreciably detected.

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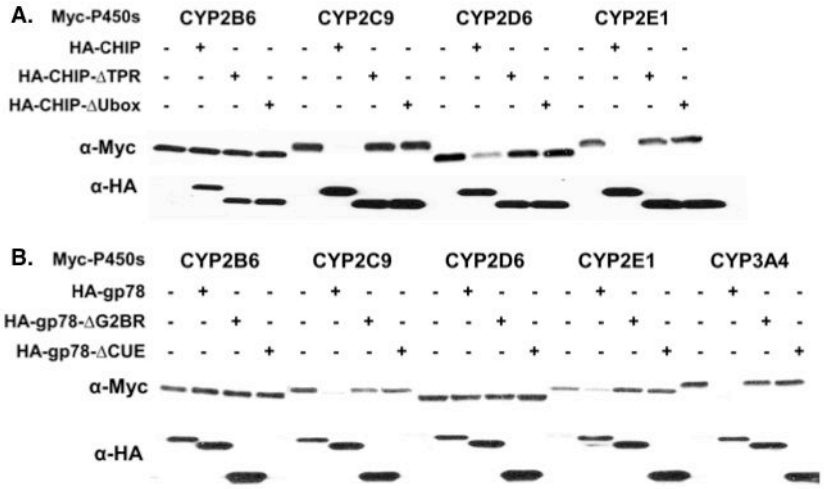

Fig. 12. Relative role of CHIP- or gp78-E3 ligases in human P450 degradation

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 and CYP2D6 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. CYP2B6 is ubiquitinated (Fig. 10), but its degradation appears to be independent of either E3 ligase.