LETTERS

Dioxin receptor is a ligand-dependent E3 ubiquitin ligase

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Fat-soluble ligands, including sex steroid hormones and environmental toxins, activate ligand-dependent DNA-sequence-specific transcriptional factors that transduce signals through target-gene-selective transcriptional regulation[1]. However, the mechanisms of cellular perception of fat-soluble ligand signals through other target-selective systems remain unclear. The ubiquitin–proteasome system regulates selective protein degradation, in which the E3 ubiquitin ligases determine target specificity[2–4]. Here we characterize a fat-soluble ligand-dependent ubiquitin ligase complex in human cell lines, in which dioxin receptor (AhR)[5–9] is integrated as a component of a novel cullin 4B ubiquitin ligase complex, CUL4B^{AhR}. Complex assembly and ubiquitin ligase activity of CUL4B^{AhR} *in vitro* and *in vivo* are dependent on the AhR ligand. In the CUL4B^{AhR} complex, ligand-activated AhR acts as a substrate-specific adaptor component that targets sex steroid receptors for degradation. Thus, our findings uncover a function for AhR as an atypical component of the ubiquitin ligase complex and demonstrate a non-genomic signalling pathway in which fat-soluble ligands regulate target-protein-selective degradation through a ubiquitin ligase complex.

The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels. This target selectivity depends on the recognition of specific DNA elements by sequence-specific transcription factors[1] and the recognition of degradation substrates by E3 ubiquitin ligases[2–4]. These transcription factors and ligases serve primarily as specific adaptors that subsequently recruit transcriptional co-regulators and E2 ubiquitin-conjugating enzymes, respectively, to appropriate targets. The selective biological effects of fat-soluble ligands have been reported to be mediated by two classes of sequence-specific transcription factors, nuclear receptors[1] and arylhydrocarbon receptor (AhR) belonging to the basic helix–loop–helix (bHLH)/Per-Arnt-Sim (PAS) family[5–9].

AhR ligands modulate oestrogen and sex hormone, signalling both positively and negatively[8,10–13]. Functional impairments of male and female reproductive organs in AhR-deficient mice indicate the possible importance of AhR in sex hormone signalling[10,14]. Different AhR agonists[9], including 3-methylcholanthrene (3MC) and 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD), modulate oestrogen-dependent oestrogen receptor (ER)-α transactivation through the association of activated AhR/Arnt with ER-α[15]. Similarly, the transcriptional activity of nuclear androgen receptor (AR) was modulated by association with activated AhR (Supplementary Fig. S2a). However, ligand-bound AhR did not block oestrogen-induced co-activator recruitment on the oestrogen-responsive promoter (Supplementary Fig. S2b). This implies another mode of function for ligand-activated AhR beyond transcriptional regulation.

On activation of AhR by 3MC, we observed that protein levels of endogenous ER-α (in mammary tumour MCF-7 cells), ER-β (in ovarian tumour KGN cells) and AR (in prostate cancer LNCaP cells) were drastically decreased (Fig. 1a–c, and Supplementary Fig. 3a) without a change in messenger RNA levels (data not shown), irrespective of the presence of their cognate hormones. Other AhR agonists[9] (namely β-naphthoflavone (β-NF), environmental toxins such as TCDD and benzo[a]pyrene, and the endogenous metabolite indirubin) were similarly effective in protein degradation for ER-α (Fig. 1b) and ER-β/AR (data not shown), in agreement with a previous report on downregulated levels of uterine ER-α protein in rats treated with TCDD[16]. An AhR partial agonist/antagonist α-naphthoflavone (α-NF) was unable to accelerate the degradation of either AhR or ER-α (Fig. 1b, and Supplementary Fig. S3b).

AhR ligand-induced degradation (Fig. 1a–c) and functional repression (Supplementary Fig. S2c, d) of sex steroid receptors were abrogated in the presence of a proteasome inhibitor MG132. Consistently, poly-ubiquitination of ER-α was promoted by the activated AhR regardless of the presence of oestrogen (Fig. 1d, and Supplementary Fig. S3c). Pulse-chase kinetic analysis indicated that 3MC-induced degradation of ER-α was coupled to that of AhR[8,17,18] (Supplementary Fig. S3d). Moreover, the self-ubiquitination activity of the ligand-bound AhR immunocomplex was detected in an E1/E2-dependent manner (Supplementary Fig. S3e). Together with 3MC-dependent recognition of sex steroid receptors by AhR[8,12,13,15], these properties of AhR resemble those of classical adaptor components of the E3 ubiquitin ligase complexes, such as F-box proteins[3] or von Hippel–Lindau protein[19]. We therefore reasoned that activated AhR might act as an E3 ubiquitin ligase complex component.

To address this idea, AhR-containing complexes were purified from HeLa cells expressing Flag–AhR treated with 3MC or α-NF[15,20]. AhR formed large complexes in the presence of 3MC (Supplementary Fig. S4a–c). Further purification revealed five major 3MC-dependent complexes containing AhR (Fig. 1e). Complexes A and C contained well-known co-activators TRAP220/DRIP205/Med220 and p300 (ref. 1) (Supplementary Fig. S4d, e). Endogenous ER-α was detected in complexes B and C; however, ubiquitinated components were seen only in complex B (Fig. 1f, g).

Complex B was composed of the ubiquitin ligase core components cullin 4B (CUL4B)[3,21,22], damaged-DNA-binding protein 1 (DDB1)[23–27] and Rbx1 (Roc1)[3], together with subunits of the proteasomal 19S regulatory particle (19S RP), Arnt and transducin-β-like 3 (TBL3) (Fig. 1h). These components eluted with AhR in the presence of 3MC but not in the presence of α-NF (Fig. 1i, and Supplementary Fig. S4f). Neither CUL4A nor known substrate-specific adaptor components of CUL4A, such as DDB2, CSA and DET1[23,24], were present

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in the AhR–CUL4B complex. As the cullin amino terminus binds adaptor components and the carboxy terminus interacts with an E2 enzyme-binding subunit Rbx1 (ref. 3), we performed tandem purification of the AhR–CUL4B complex with glutathione *S*-transferase (GST)-tagged CUL4B-N (N-terminal domain of CUL4B) and Flag–AhR. This led to the identification of a core complex consisting of five components: DDB1, AhR, Arnt, TBL3 and CUL4B (Fig. 1j). Together with Rbx1, this complex is denoted by $CUL4B^{AhR}$.

Immunoprecipitation of AhR together with endogenous CUL4B from MCF-7 and LNCaP cells was observed only in the presence of 3MC (Fig. 2a, b). Consistently, ligand-dependent co-localization of AhR with CUL4 was seen in MCF-7 cells (Fig. 2c). Whereas CUL4B seemed to act as a scaffold mediating DDB1–TBL3 and AhR–DDB1 interactions in $CUL4B^{AhR}$ (Fig. 2d, lane 4), ligand-activated AhR induced the assembly of complex components (Fig. 2d, lanes 1–3). DDB1 did not bridge CUL4B association with TBL3 or AhR, apparently because of the absence of the signature WDXR/DWD box[22,25–27] of either TBL3 or AhR, which is essential for DDB1 binding (Fig. 2d, lane 5, and Supplementary Fig. S5a). Consistently, specific and 3MC-dependent interaction of the conserved C-terminal acidic domain of AhR with the N-terminal region of CUL4B, but not with DDB1, was observed in a GST pulldown assay (Supplementary Figs S5b and S6). Because a constitutively active AhR mutant (AhRΔPASB)[9] interacted with CUL4B in the absence of ligand (Supplementary Fig. S5b), ligand-dependent structural alteration presumably induces AhR–CUL4B interaction. An AhR mutant lacking the CUL4B-binding

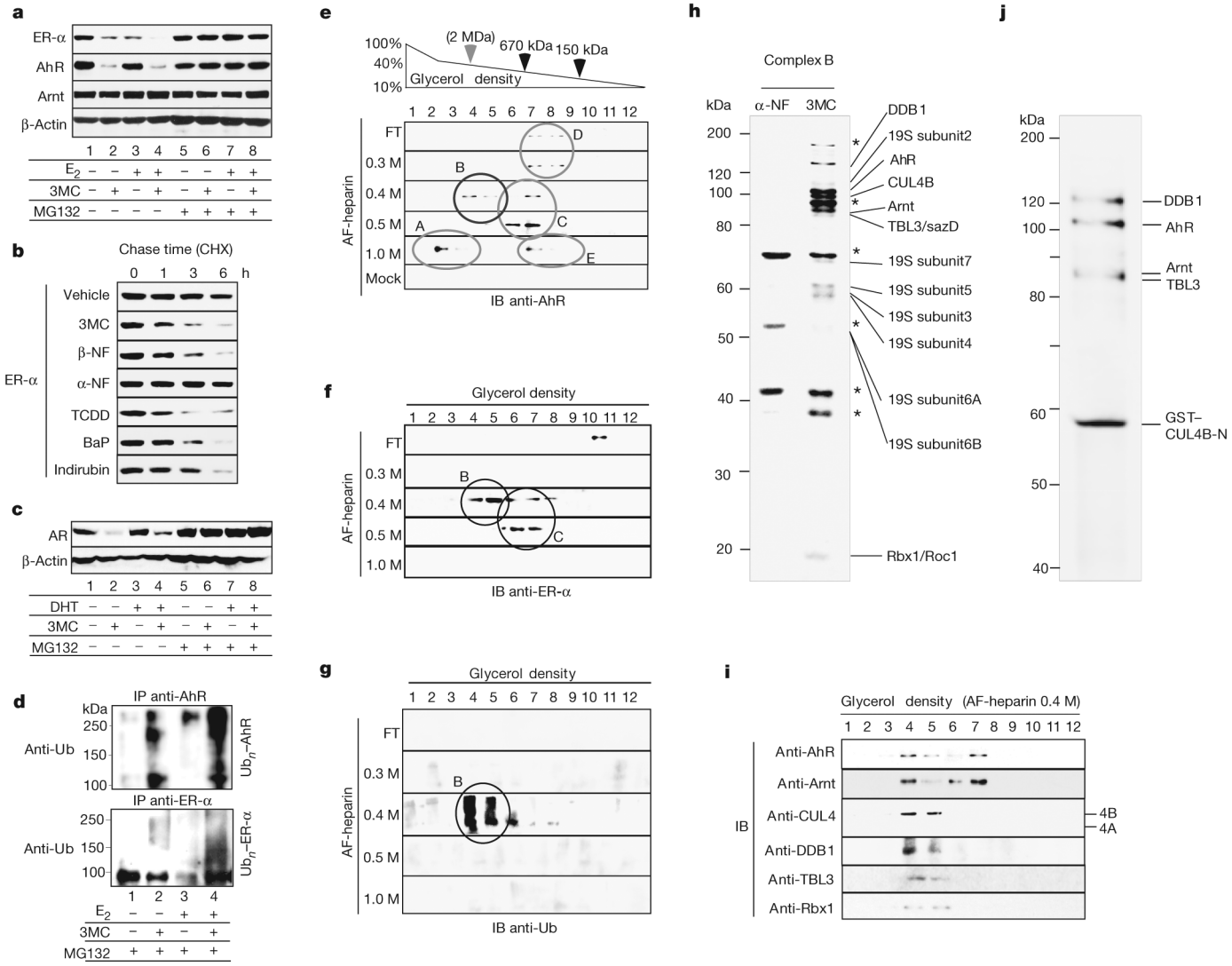

Figure 1 | Activated AhR acts as an E3 ubiquitin ligase. **a**–**c**, AhR-ligand-induced proteasomal degradation of ER-α (**a**, **b**) and AR (**c**). MCF-7 cells (**a**, **b**) and LNCaP cells (**c**) were incubated as indicated with E_2 (10 nM), DHT (10 nM) and/or 3MC (1 μM), β-NF (1 μM), benzo[a]pyrene (BaP; 100 nM), TCDD (10 nM), indirubin (10 nM) and α-NF (1 μM) in the presence or absence of MG132 (10 μM) and cycloheximide (CHX; 5 μM) for 3 h (**a**, **c**) or the indicated durations (**b**). Cell lysates were subjected to western blotting with specified antibodies. **d**, AhR-ligand-induced ubiquitination of ER-α. MCF-7 cells were incubated with the indicated ligands for 6 h. Western blots were subjected to dark exposure to detect poly-ubiquitinated forms of the receptors. IP, immunoprecipitation; Ub, ubiquitin. **e**, **f**, Biochemical separation and identification of AhR-associated complexes. Flag–AhR-associated proteins in the presence of 3MC or α-NF from HeLa cells stably expressing Flag–AhR were first fractionated by glycerol-density-gradient centrifugation (top, fractions 1–12), and then separated by Toyopearl AF-heparin column chromatography with the indicated KCl concentrations (FT, 1.0 M KCl). Samples from the 3MC-treated cells were resolved into five distinct complexes. IB, immunoblotting. **g**, Components of an AhR-associated complex are highly ubiquitinated. Western blots with anti-ubiquitin antibody. **h**, Identification of AhR-associated CUL4B ubiquitin ligase complex components. Components from complex B in **e** (fractions 4 and 5 from the glycerol-density-gradient centrifugation, eluted from an AF-heparin column at 0.4 M KCl) were resolved by SDS–PAGE, silver-stained and identified by matrix-assisted laser desorption ionization–time-of-flight MS analysis. **i**, Co-elution of the complex B components as a large complex. **j**, Association of activated AhR with the CUL4B complex. The $CUL4B^{AhR}$ complex from Flag–AhR-expressing HeLa cells treated with 3MC was affinity purified with GST-tagged N-terminal domain of CUL4B followed by anti-Flag antibody column fractionation.

acidic domain (AhRΔacid; Supplementary Fig. S6a) was indeed unable to promote ER-α ubiquitination *in vivo*, although the mutant retained 3MC-dependent transactivation function (Supplementary Fig. S5c). This indicates that the ubiquitin ligase function of AhR is independent of its transactivation function.

With two separately prepared components of recombinant AhR and CUL4B/DDB1/Rbx1 purified from *Spodoptera frugiperda* (Sf9) cells (Supplementary Fig. S7a), complex assembly *in vitro* was also dependent on 3MC (Fig. 2e). Furthermore, by *in vitro* ubiquitination assay (Supplementary Fig. S7b), the E3 ubiquitin ligase activity of CUL4B^{AhR} for ER-α was dependent on 3MC but not on 17β-oestradiol (E_2) (Fig. 2f). These data indicate that both the complex assembly and the ubiquitin ligase activity of CUL4B^{AhR} may be dependent on AhR agonists.

We then examined whether the recognition of sex steroid receptors for 3MC-dependent ubiquitination is indeed mediated by AhR. Co-immunoprecipitation analyses indicated that ligand-activated AhR was required for the recruitment of ER-α (Fig. 2a, d) or AR (Fig. 2b, and data not shown) to CUL4B^{AhR}. TBL3 and DDB1 did not seem essential for ER-α recruitment but stabilized the association of ER-α with CUL4B^{AhR} (Fig. 2d). Moreover, knockdown of CUL4B^{AhR} components (Supplementary Fig. S8) impaired the 3MC-induced ubiquitination and degradation of ER-α (Fig. 3a–d, and Supplementary Fig. S9a, b) and AR (Fig. 3e, Supplementary Fig. S9c and data not shown), and abolished the AhR-ligand-induced repression of ER-α transactivation (Supplementary Fig. S10a). Recognition of ER-α by activated AhR was retained, but ubiquitination of AhR-bound ER-α was abrogated, by knockdown of the other CUL4B^{AhR} components (Fig. 3d). An ER-α ΔA/B mutant[15] that lacks interaction with AhR, and an ER-α K7R mutant in which seven lysine residues had been replaced with arginine (Supplementary Fig. S6b), were resistant to AhR-dependent ubiquitination and transrepression (Fig. 3f, and Supplementary Fig. S10b). Taken together, these data suggest that ligand-activated AhR functions as a substrate-specific adaptor component of CUL4B^{AhR}. AhR is therefore a unique and atypical substrate-specific component of a cullin-based E3 complex, because AhR bears no known interaction motif with cullin complexes yet associates directly with CUL4B. Ubiquitination of ER-α-associated AhR was similarly abolished by the knockdown, and the overall ubiquitination and degradation of AhR[8,17,18] were partly affected (Supplementary Fig. S11a, b). This implies the existence of CUL4B^{AhR}-dependent (self-ubiquitination[3]) and CUL4B^{AhR}-independent pathways for AhR degradation.

Human ER-α (hER-α) degradation is reportedly accelerated by the binding of E_2 (ref. 1) or the phosphorylation of Ser 118 (ref. 28), whereas a partial antagonist, tamoxifen, has been shown to stabilize ER-α[1]. Nevertheless, 3MC-activated AhR efficiently induced the ubiquitination and subsequent degradation of tamoxifen-bound ER-α and ER-α-S118A mutant (Fig. 3f). Reciprocally, AhR was dispensable for E_2-dependent ER-α degradation (Supplementary Fig. S11c). These results indicate that the CUL4B^{AhR} system may act independently of innate protein degradation system(s) for ER-α. XAP2/ARA9/AIP[7,8,17], a chaperone that modulates the stability of unliganded AhR, seemed unlikely to mediate the accelerated degradation of ER-α by activated AhR (Supplementary Fig. S11d).

Last, we addressed the physiological significance of CUL4B^{AhR} for sex hormone signalling in intact animals. Injection with either 3MC (Fig. 4a) or β-NF (Fig. 4c) did not affect the expression of ER-α or AR mRNA (data not shown) but caused a decrease in protein levels of uterine ER-α in ovariectomized female wild-type mice and of prostate AR in castrated male wild-type mice (Fig. 4b) regardless of their treatment with cognate sex hormones. However, AhR deficiency (AhR$^{-/-}$ mice)[9,14] abolished such effects of AhR ligands but did not affect the modulation of stability of sex steroid receptors by their respective hormones (Fig. 4a, b). As a result of reduced sex steroid receptor levels after pretreatment with 3MC, E_2-dependent induction of c-*fos* in the uterus[15] and dihydrotestosterone (DHT)-dependent induction of *Probasin* in the prostate[10] were severely impaired (Fig. 4a, b). Cellular proliferation and gene induction in response to sex hormones in primary cultured epithelial cells from normal mouse uterus and prostate were consistently suppressed by 3MC (Supplementary Fig. S12a, b) and β-NF (Supplementary Fig. S12c), but no effect was detected in AhR$^{-/-}$ cells (Supplementary Fig. S12a, b). The significance of CUL4B^{AhR} complex components in the AhR-mediated suppression of sex hormone effects

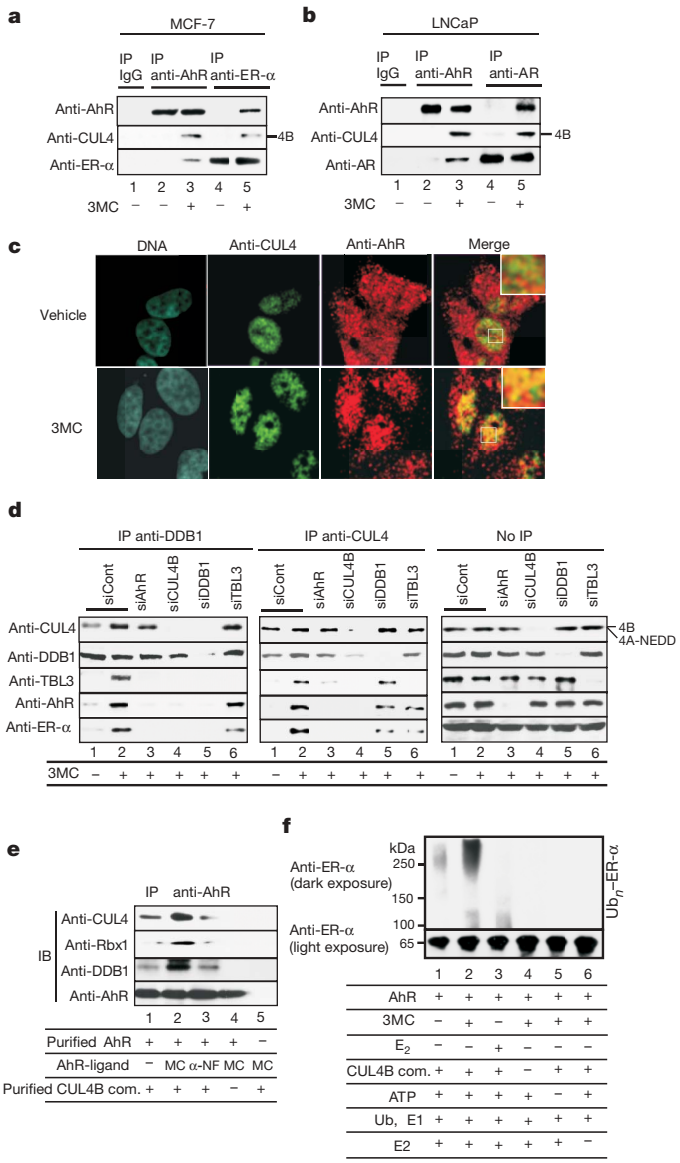

Figure 2 | AhR ligand-dependent assembly and ubiquitin ligase activity of CUL4B^{AhR}. **a**, **b**, 3MC-dependent association of endogenous CUL4B and AhR with ER-α and AR. Co-immunoprecipitation analyses from MCF-7 (**a**) and LNCaP (**b**) cells incubated with ligand and MG132 for 2 h. IP, immunoprecipitation. **c**, 3MC-dependent co-localization of AhR with CUL4. MCF-7 cells incubated with 3MC and MG132 for 2 h were immunostained with the indicated antibodies. **d**, Formation of the CUL4B^{AhR} complex. MCF-7 cells were transfected with specified short interfering RNAs (siRNAs) for 48 h, treated with 3MC and MG132 for 2 h, and immunoprecipitated with the indicated antibodies. **e**, Assembly of the CUL4B complex components with AhR is dependent on 3MC *in vitro*. Immunoprecipitation with anti-AhR antibodies of the indicated recombinant CUL4B complex components (CUL4B com.) was observed only in the presence of 3MC. IB, immunoblotting. **f**, CUL4B^{AhR} ubiquitinates ER-α *in vitro*. ER-α protein was incubated with and without recombinant CUL4B^{AhR} E3 complex components, ubiquitin (Ub), ATP, E1 and E2 enzymes as indicated, then subjected to western blotting.

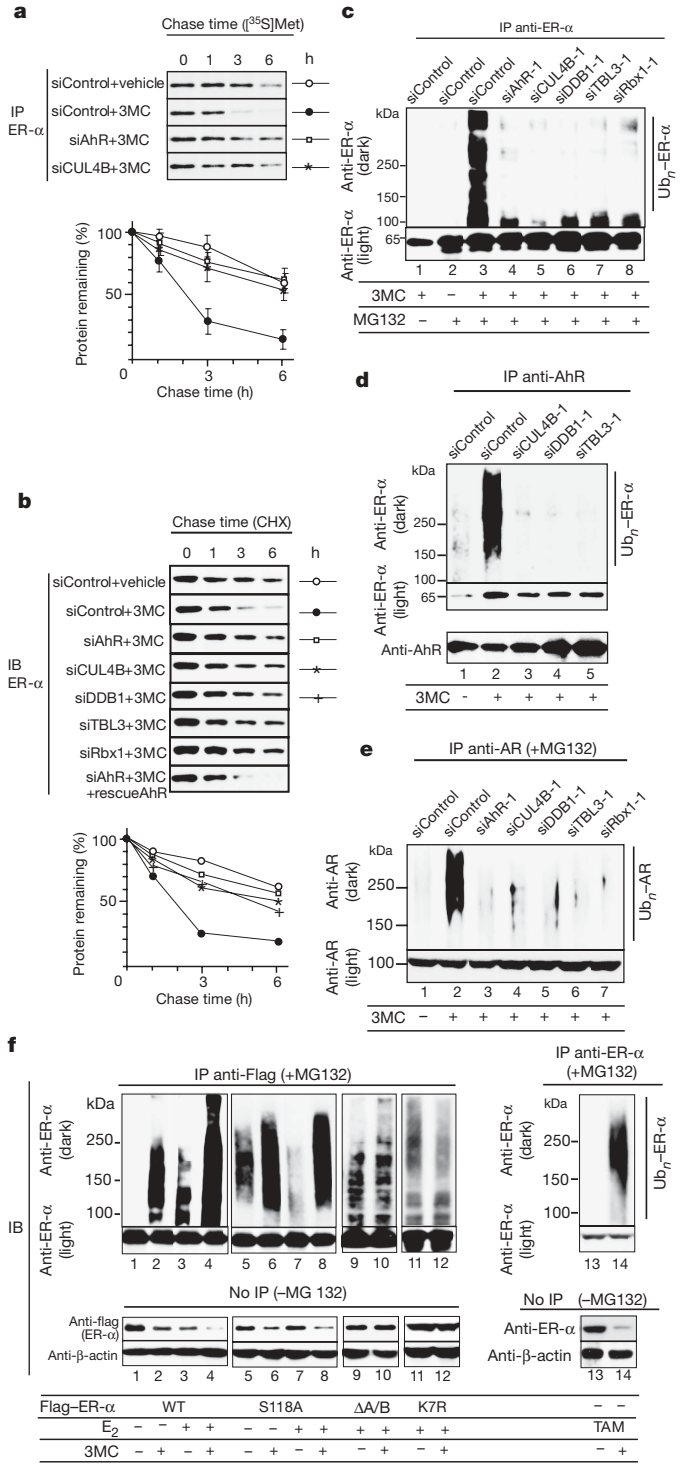

Figure 3 | Activated AhR is a substrate-specific adaptor component of the CUL4B^{AhR} complex. **a**–**c**, Components of CUL4B^{AhR} are required for 3MC-dependent ubiquitination and degradation of ER-α. MCF-7 cells were transfected with indicated siRNAs for 48 h, then used in pulse-chase analysis as in Supplementary Fig. S3d (**a**), in cycloheximide (CHX) chasing (**b**) and in the *in vivo* ubiquitination assay with ligand incubation for 6 h (**c**). All values are shown as means ± s.d. ($n = 3$) (**a**) or as means ($n = 3$) (**b**). The knockdown efficiency in the same lysates was confirmed in Supplementary Fig. S9a. IB, immunoblotting; IP, immunoprecipitation. **d**, AhR is the substrate-specific adaptor in the targeting of ER-α by CUL4B^{AhR}. MCF-7 cells transfected with the indicated siRNAs were lysed in TNE buffer and immunoprecipitated with anti-AhR antibody in the presence of MG132. Ubiquitination of the ER-α co-immunoprecipitated with AhR was detected by western blotting. **e**, LNCaP cells were subjected to the same analysis as in **a**–**c**. **f**, AhR-ligand-induced ER-α ubiquitination requires intact lysine residues and is independent of oestrogen binding or S118 phosphorylation of hER-α. Intact MCF-7 cells (right) or cells transfected with Flag–hER-α, AhR and their derivatives (left) were treated with the indicated ligands in the presence (top) or absence (bottom) of MG132 for 6 h, then subjected to western blotting. TAM, tamoxifen; WT, wild type.

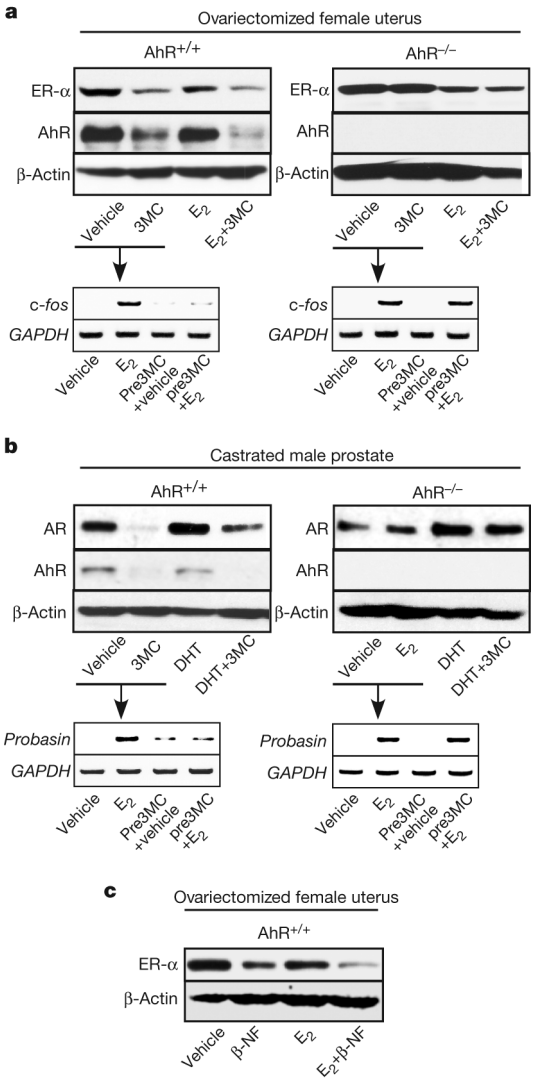

Figure 4 | Ligand-dependent ubiquitin ligase function of AhR *in vivo*. **a**, **b**, AhR activation enhances the degradation of ER-α and AR *in vivo*. Top: nine-week-old ovariectomized female mice (**a**) or castrated male mice (**b**) of the indicated genotypes were injected with vehicle or indicated ligands. After 4 h, uterus (**a**) or ventral prostate (**b**) was isolated and subjected to western blotting. Bottom: mice pretreated with vehicle or 3MC for 8 h were injected with either vehicle or E_2 (**a**), or DHT (**b**). After 4 h, the uterus or prostate was isolated for reverse transcriptase PCR. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **c**, Other AhR agonists produce a similar effect on oestrogen signalling to that of 3MC.

(Supplementary Fig. S12a, b) and the promotion of ER-α degradation in uterine cells (Supplementary Fig. S12d) was verified by knockdown of the components.

Here we have shown that a known sequence-specific transcription factor AhR acts as a ligand-dependent CUL4B-based E3 ubiquitin ligase for selectively targeting sex steroid receptors to bring about accelerated protein degradation. The transcription and ubiquitination functions of AhR seem to be responsible for a distinct set of biological events caused by endogenous and exogenous AhR ligands. In ubiquitin ligase complexes, substrate recognition by known

substrate-specific components is generally evoked by substrate modifications[2–4]. However, the recognition and subsequent ubiquitination of sex steroid receptors by AhR requires dioxin-type compounds as ligands but does not require the phosphorylation or ligand binding of sex steroid receptors. We have therefore shown that fat-soluble ligands directly control the function of a ubiquitin ligase complex for targeted protein destruction in animals (see Supplementary Fig. S1). In plants, auxin was recently found to control protein destruction through the auxin receptor SCF^{TIR1} (refs 29, 30). However, whereas SCF^{TIR1} is regulated by ligand-dependent substrate recognition by TIR1, $CUL4B^{AhR}$ is primarily regulated by the assembly of a ligand-dependent complex as well as substrate recognition. Considered together, ubiquitin-ligase-based perception mechanisms of fat-soluble ligands may be diverse in different species. It is possible that other nuclear receptors and binding proteins for fat-soluble ligands also serve as key components of ubiquitin ligases to mediate a non-genomic pathway of fat-soluble ligands to regulate target-protein-selective destruction.

METHODS

More detailed descriptions of all materials and methods are supplied in the Supplementary Information.

Biochemical purification and separation of AhR-associated complexes. The nuclear extracts preparation, anti-Flag affinity purification and mass spectrometry were performed as described previously[15,20]. For purification of the core $CUL4B^{AhR}$ complex, the nuclear extracts were first bound to the GST–CUL4B-N (amino acid residues 1–318) columns before being loaded on anti-Flag columns[20].

***In vitro* ubiquitination assay.** The *in vitro* ubiquitination assay was performed as described previously[23]. Purified Flag–AhR (0.2 μg) was incubated either with 3MC (10 μM) or vehicle (dimethylsulphoxide) for 30 min at 25 °C, then mixed with Flag–CUL4B/DDB1/Rbx1 complex (0.2 μg), and after further incubation for 30 min at 25 °C the substrate, ER-α (Calbiochem), was added.

Plasmids, antibodies, immunoprecipitation, *in vivo* ubiquitination, pulse-chasing, ligand responses in mice, and RNA-mediated interference experiments. Detailed methods used in this study can be found in the Supplementary Information.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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