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SPRINGER REVIEWS

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生体統御システムと内分泌撹乱

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6. 甲状腺ホルモン撹乱物質の生体作用の動物種差

加藤善久

6. 1. はじめに

ポリ塩化ビフェニル(PCB)は，食物連鎖を通して蓄積し，魚類，鳥類および多くの野生動物の組織のみならず，ヒトの血液，母乳，肝臓，脂肪組織などにも見出されている[1]．近年，PCBの曝露によって実験動物や野生生物だけでなく，ヒトにおいても甲状腺ホルモン(サイロキシン[T_4]，3,3',5-L-トリヨードサイロニン[T_3])の撹乱がひき起こされている[2]．ラットでは，いくつかのPCBの投与により血中T_4濃度の低下がひき起こされる[3,4]．一般に，PCBによるラットの血中T_4濃度の低下は，肝臓のUDP - グルクロノシルトランスフェラーゼ(UDP-GT)が誘導されることにより，T_4のグルクロン酸抱合体が増加し，その胆汁排泄が促進されることによると考えられている．しかし，PCBによる血中T_4濃度の低下と肝臓のT_4-UDP-GT活性の増加とは定量的に相関しない[3,4]など不明な点も多く，血中T_4濃度の低下メカニズムは十分に解明されていない．また，PCBの血中甲状腺ホルモンへの影響に関するほとんどの研究はラットを用いて行われたものであり，その低下作用における動物種差に関する研究は，ほとんど行われていない．筆者らはPCB投与による血中T_4濃度の低下メカニズムを明らかにするとともに，その低下のメカニズムに動物種差があるか否かを検討している．そこで，これまでの研究成果をここに紹介する．

6. 2. PCBの甲状腺ホルモンに対する作用：動物種差

筆者らは，種々の用量の2,2',4',5,5' - ペンタクロロビフェニル(ペンタCB)，2,2',3',4',5,6 - ヘキサクロロビフェニル(ヘキサCB)あるいはKanechlor-500(KC500，PCB製品であり，台湾油症の原因物質とされるPCB混合物)をマウスに投与し，投与量と血中総T_4濃度の関係から，マウスにおいて血中総T_4濃度を50%低下させる用量(ED_{50})を算出した．この用量をddY系マウス，Syrian系ハムスター，Wistar系ラットあるいはHartley系モルモットに投与した．血中総T_4濃度は，マウス，ハムスターおよびラットにペンタCB(11 mg/kg)を投与した後，マウスにヘキサCB(19 mg/kg)を投与した後，また4種の動物にKC500(37.5 mg/kg)を投与した後に，有意に低下した．血中総T_3濃度はマウスにペンタCBを投与したとき，あるいはモルモットにKC500を投与したときに有意に低下した．血中甲状腺刺激ホルモン濃度は，4種の動物にいずれのPCBを投与した場合にも変化しなかった．PCB投与による血中甲状腺ホルモン濃度の低下について，マウスで系統差のあることはわずかに報告されているが，このような動物種差に関する情報は乏しい．

6．3．血中T_4濃度の低下における肝臓UDP-GTの関与

一般に，PCBによるラット血中T_4濃度の低下の要因として，T_4のグルクロン酸抱合をはじめとするT_4の代謝系の促進が考えられる．そこで，3・2の動物のT_4-UDP-GT活性を測定した．T_4-UDP-GT活性は，マウスにヘキサCBを投与した後，あるいはモルモットにKC500を投与した後に有意に増加した．一方，ハムスターおよびラットに各PCBを投与したときには，この酵素活性は変化しなかった．さらに，KC500を投与した後，胆汁中T_4のグルクロン酸抱合体の排泄量は，ラットで有意に増加したが，マウス，ハムスター，モルモットでは変化しなかった．これらの結果から，マウスにヘキサCBを投与したときの血中T_4濃度の低下の要因には，肝臓のUDP-GTの誘導によるT_4の代謝促進を含む可能性がある．しかし，マウス，ハムスター，ラットおよびモルモットにペンタCBあるいはKC500を投与したときの血中T_4濃度の低下は，UDP-GTの誘導によるT_4の代謝促進では説明が困難である．

そこで，ラットにPCBを投与したときの血中T_4濃度の低下に，肝臓のUDP-GTによるT_4の代謝が関与しているか否かを明らかにするために，Wistar系ラットおよびGunnラット(遺伝的にUGT1サブファミリーを欠損したWistar系ラットの突然変異体)にペンタCB(112 mg/kg)あるいはKC500(100 mg/kg)を投与し，血中総T_4，遊離T_4濃度を測定した．その結果，それらは各PCB投与により両ラットで著しく低下した．このとき，Wistar系ラットにおいて，T_4の代謝を促進するUGT1 s，UGT1A1，UGT1A6の発現量およびT_4-UDP-GT活性は顕著に増加した．一方，Gunnラットではそれらに変化はまったく認められなかった(図6・1)．これらの結果から，Gunnラットにおいて，ペンタCBあるいはKC500を投与した後の血中T_4濃度の低下は，肝臓のT_4-UDP-GT活性の増加に依存しないことが示唆された．さらに，Wistar系ラットにPCBを投与したときの血中T_4濃度の低下も，少なくとも一部T_4-UDP-GTが非関与の機序による可能性が示唆された[5]．

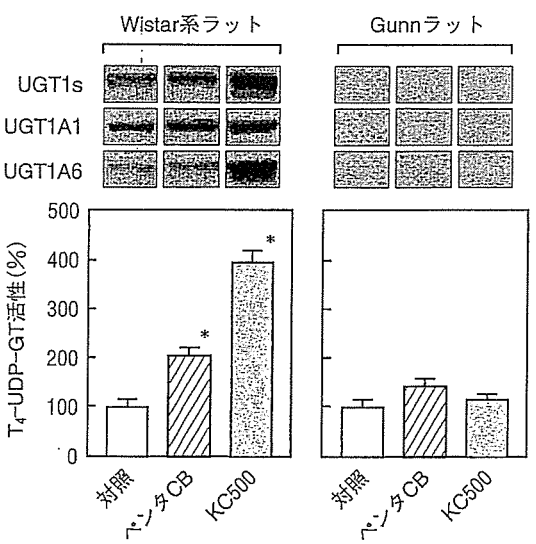

図6・1　Wistar系ラットおよびGunnラットにペンタCBおよびKC500を投与した後の肝ミクロソームのUGT分子種の発現量およびT_4-UDP-GT活性．平均±標準誤差．n=4～6．*p<0.05.

T_4の代謝を亢進させる生体内反応として，T_4のグルクロン酸抱合のほかに，脱ヨウ素化反応が知られている．そこで，4種の動物にペンタCB，ヘキサCB，KC500を投与し，肝臓のI型ヨードサイロニン脱ヨウ素化酵素活性を測定した．しかし，いずれの場合にも，この酵素活性の増加は認められなかった．Aroclor1254を用いて類似した結果も報告されている．したがって，PCBによる血中T_4濃度の低下にI型ヨードサイロニン脱ヨウ素化酵素は関与していないと考えられる．

6. 4. 血中T_4濃度の低下に対するT_4とトランスサイレチンとの結合阻害の関与

特定のPCBおよびそのヒドロキシル化体は血中T_4の輸送タンパクであるトランスサイレチン(TTR)と競合的に結合する．詳細なメカニズムは明らかにされていないが，それらとTTRとの結合が血中T_4の標的器官への輸送を撹乱し，血中T_4濃度を低下させる一因となることが報告されている[6,7]．そこで，4種の動物にKC500を投与し，投与後4日目に[^{125}I]T_4を静脈内投与し，血中[^{125}I]T_4と血中TTRあるいはアルブミンとの結合率を測定した．いずれの動物でも対照群では，[^{125}I]T_4はほとんどTTRと結合していた．マウスでは，KC500投与により，いずれのタンパクとの結合にも変化はまったく認められなかった．ハムスターでは，KC500(100 mg/kg)投与により，[^{125}I]T_4とTTRとの結合

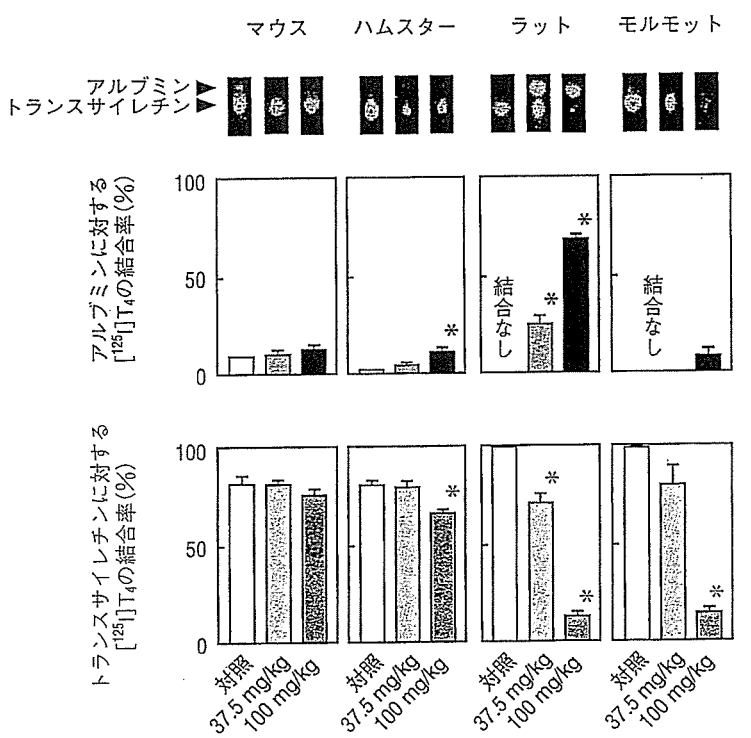

図6・2 Wistar系ラットにKC500を投与した後の[^{125}I]T_4と血中タンパク質との結合率．平均±標準誤差．n=3～4.*p<0.05(口絵11)．

率はわずかに減少した．一方，ラットおよびモルモットでは，KC500投与により，[^{125}I]T_4とTTRとの結合率は用量依存的に減少し，代わって[^{125}I]T_4とアルブミンとの結合率が増加した．この増加はラットにおいて顕著であった(図6・2，口絵11参照)．これらの結果から，ラットおよびモルモットでは，KC500によりT_4とTTRの結合阻害が起こっていることが示唆され，それが原因で，血中T_4濃度が低下した可能性が考えられる．一方，マウスでは，KC500により，T_4とTTRの結合阻害が起こっているとは考えにくく，血中T_4濃度の低下は，他のメカニズムによりひき起こされている可能性が示唆された．

6．5．甲状腺への直接作用，メチルスルフォン代謝物あるいはT_4と甲状腺ホルモン受容体との結合阻害の関与

PCBによる血中T_4濃度の低下の要因として，甲状腺濾胞上皮細胞におけるT_4の合成系の抑制，あるいは甲状腺からT_4の放出の抑制も考えられる．しかし，4種の動物にペンタCB，ヘキサCBあるいはKC500(それぞれED_{50})を投与したとき，甲状腺の腔胞変性，濾胞上皮細胞の肥大および過形成は，対照群と同程度であった．各PCBの今回用いた用量による血中T_4濃度の低下には，甲状腺への直接作用は考えにくい．

3-メチルスルフォニル(3-$MeSO_2$-)および4-$MeSO_2$-ペンタCB，および3-$MeSO_2$-ヘキサCBなどのPCBのメチルスルフォン代謝物は，UGT1A1/6を誘導することにより，血中T_4の代謝を亢進し，血中T_4濃度の低下をひき起こす[8]．そこで，各PCB投与による4種の動物の血中T_4濃度の低下に，メチルスルフォン代謝物が関与しているか否かを検討した．しかし，3種のPCBを投与したとき，各動物の血中T_4濃度の低下と血中あるいは肝臓中メチルスルフォン代謝物濃度との間には相関はみられなかった．

また，PCBは甲状腺ホルモン受容体(TR)を介する遺伝子発現を抑制することにより，甲状腺ホルモンの撹乱をひき起こすことが報告されている[9]．そこで，6種のPCB，16種の$MeSO_2$-PCBおよび20種のヒドロキシル化PCBとT_4とのTRへの競合的結合能を調べた．しかし，検討したPCBおよびPCB代謝物のなかにはTRへ結合するものを見出すことはできなかった[10]．

6．6．血中T_4の肝臓への移行とトランスポーターの関与

これまでの検討から，PCBによる血中T_4濃度の低下の作用機序について十分に説明することは困難であった．そこで，T_4の体内動態に注目してさらに検討を加えた．マウス，ハムスター，ラットあるいはモルモットにKC500を投与し，投与後4日目に[^{125}I]T_4を静脈内投与し，血中からの[^{125}I]T_4の消失速度を測定した．血中の[^{125}I]T_4の消失速度は，[^{125}I]T_4の投与後5分から増加し，T_4の全身クリアランスおよび分布容積は，用量依存的に増加した(表6・1)．また，C57Bl/6系マウスに異なる

表6・1　マウス，ハムスター，ラットおよびモルモットにKC500を投与した後の[^{125}I]T_4の薬物動態パラメーター

動物	処置	用量 (mg/kg)	全身クリアランス (ml/min)	分布容積 (ml)
マウス	対照		0.016 ± 0.003	4.8 ± 0.3
	KC500	37.5	0.015 ± 0.002	4.9 ± 0.3
	KC500	100	0.024 ± 0.003*	6.8 ± 0.5*
ハムスター	対照		0.034 ± 0.004	11.9 ± 1.0
	KC500	37.5	0.059 ± 0.009*	25.4 ± 1.7*
	KC500	100	0.086 ± 0.008*	43.7 ± 5.0*
ラット	対照		0.074 ± 0.007	14.4 ± 0.4
	KC500	37.5	0.143 ± 0.019	30.9 ± 4.0*
	KC500	100	0.241 ± 0.046*	61.2 ± 5.4*
モルモット	対照		0.085 ± 0.017	34.7 ± 3.8
	KC500	37.5	0.120 ± 0.027	56.6 ± 7.6*
	KC500	100	0.216 ± 0.019*	66.9 ± 7.3*

平均±標準誤差．n=4～6.*p<0.05.

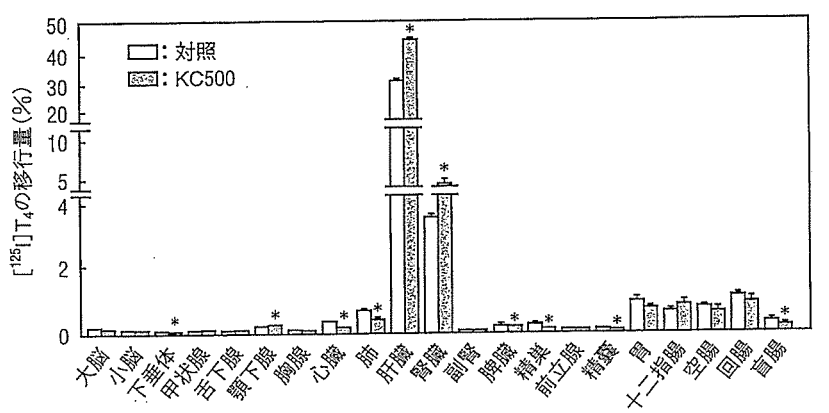

図6・3　KC500を処置したラットに，[^{125}I]T_4を静脈内投与した5分後の[^{125}I]T_4の組織分布．平均±標準誤差．n=4.*p<0.05.

タイプのPCB(3,3',4,4' - テトラクロロビフェニル，2,3',4,4',5 - ペンタクロロビフェニル，2,2',4,4',5,5'-ヘキサクロロビフェニル[非ダイオキシン様PCB])を投与し，同様の検討を試みたところ，いずれの場合にも血中T_4濃度は低下し，T_4の分布容積は増加した．これらの結果から，各動物にPCBを投与したとき，血中T_4濃度の低下は，血中から組織へのT_4の急速な移行によることが示唆された．そこで，血中から消失した[^{125}I]T_4がどこの組織に移行したかを明らかにするために，ラットにKC500を投与し，投与後4日目に[^{125}I]T_4を静脈内投与し，その5分後に各組織への[^{125}I]T_4の移行量を測定した．対照ラットの肝臓への[^{125}I]T_4の移行量は他の組織への移行量に比較して著しく多く，KC500投与により，その移行量は投与量の58%に増加した(図6・3)．また，KC500を処置したマウス，ハムスターおよびモルモットにおいても，肝臓への[^{125}I]T_4の移行量は，他の組織に比較して顕著に増加した．さらに，マウス，ハムスターおよびラットにフェノバルビタール(PB)を投与

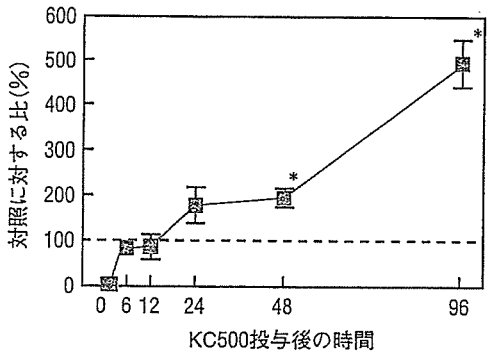

図6・4　ラットにKC500を投与した後の肝臓のLAT1のmRNAの発現量．平均±標準誤差．n=4～5.*p<0.01.

し，血中T_4濃度が低下するとき，T_4の全身クリアランスおよび分布容積は増加し，肝臓へのT_4の移行量は増加した．これらの結果から，用いた動物において，ダイオキシン様PCB(Ah受容体依存性の毒性発現をするPCB)および非ダイオキシン様PCB(PB response unit依存性の毒性発現をするPCB)ともに，PCB投与による血中T_4濃度の低下は，T_4の分布容積が増加すること，すなわちT_4が血中からすみやかに肝臓に移行することに起因することが示唆された．

最近，甲状腺ホルモンを基質とするトランスポーターが次々と明らかにされている．その例としては，有機アニオン輸送ポリペプチド(Oatp1～4)，Na^+/タウロコール酸共輸送ペプチド(Ntcp)，L型アミノ酸トランスポーター(LAT1～2)，モノカルボン酸トランスポーター(MCT8)があげられる．これらのうち，Oatp1～4，NtcpおよびMCT8はラットの肝臓に発現していること，肝臓内へのT_4の輸送に関与していることが報告されている．そこで，ラットにKC500を投与し，経時的に肝臓を摘出し，各トランスポーターのmRNAの発現量を測定した．KC500を処置したラット肝臓のOatp2 mRNAの発現量は，処置後4日目に1.4倍に増加した．また，LAT1 mRNAの発現量は，処置後2日目から有意に増加し，4日目には5.0倍に増加した(図6・4)．これらの結果は，肝臓のLAT1およびOatp2の発現増加が，血中から肝臓へのT_4の移行量の増加をもたらす要因になっている可能性を示唆している．

6．7．おわりに

これまでの多くの研究から，PCBによるラット血中T_4濃度の低下は，甲状腺における甲状腺ホルモンの合成・放出を抑制させること，甲状腺ホルモンの代謝クリアランスを促進させる(特にT_4-UDP-GTの誘導)こと，甲状腺ホルモンの末梢組織への移行(TTRを介するT_4の輸送)に異常をきたすことによってひき起こされることが報告されてきた．ところが，マウス，ハムスター，ラットおよびモルモットにペンタCB，ヘキサCBあるいはKC500を投与したとき，血中T_4濃度の低下は，甲状腺への直接作用，肝臓のT_4-UDP-GTおよび脱ヨウ素化酵素の誘導あるいはメチルスルフォン代謝物によるT_4の代謝促進，あるいはT_4とTRとの結合阻害では説明することが困難であった．そこで，PCBを投与した後，T_4の体内動態について検討したところ，PCBによる血中T_4濃度の低下は，血中

から肝臓へのT_4の移行量の増加に起因している可能性が示唆された．各PCBによる血中から肝臓へのT_4の移行量の違いは，各PCBのヒドロキシル化代謝物の生成量と，生成したヒドロキシル化代謝物とTTRとの親和性の強さ，また肝臓の甲状腺ホルモントランスポーターの発現量の増加割合などに依存する可能性が考えられる．また，血中から肝臓へのT_4の輸送のメカニズムは，PCBのタイプあるいは動物種により異なり，いくつかのT_4の輸送メカニズムが組み合わさっているものと考えられる．また，PCBによる胆汁中へのT_4のグルクロン酸抱合体の排泄量の変動は，肝臓へのT_4の移行量の違いと，T_4-UGP-GTの誘導の程度により左右され，血中T_4濃度の低下とは直接的な関連性は低いかもしれない．このように，PCBによる血中T_4濃度の低下機序やその動物種差を，単一の要因で説明することは難しく，これまでに検討してきた要因，あるいは未知の要因が複雑に絡み合って，各動物の血中T_4濃度の低下が惹起されるものと考えられる．今後，ヒトを含む多くの動物種のPCBによる血中甲状腺ホルモン濃度の減少に対する感受性を理解するために，末梢組織(特に肝臓)および脳における甲状腺ホルモンの取り込みメカニズム，甲状腺ホルモントランスポーターおよび甲状腺ホルモン応答遺伝子の機能とそれらの変動メカニズムについてさらに研究する必要がある．

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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

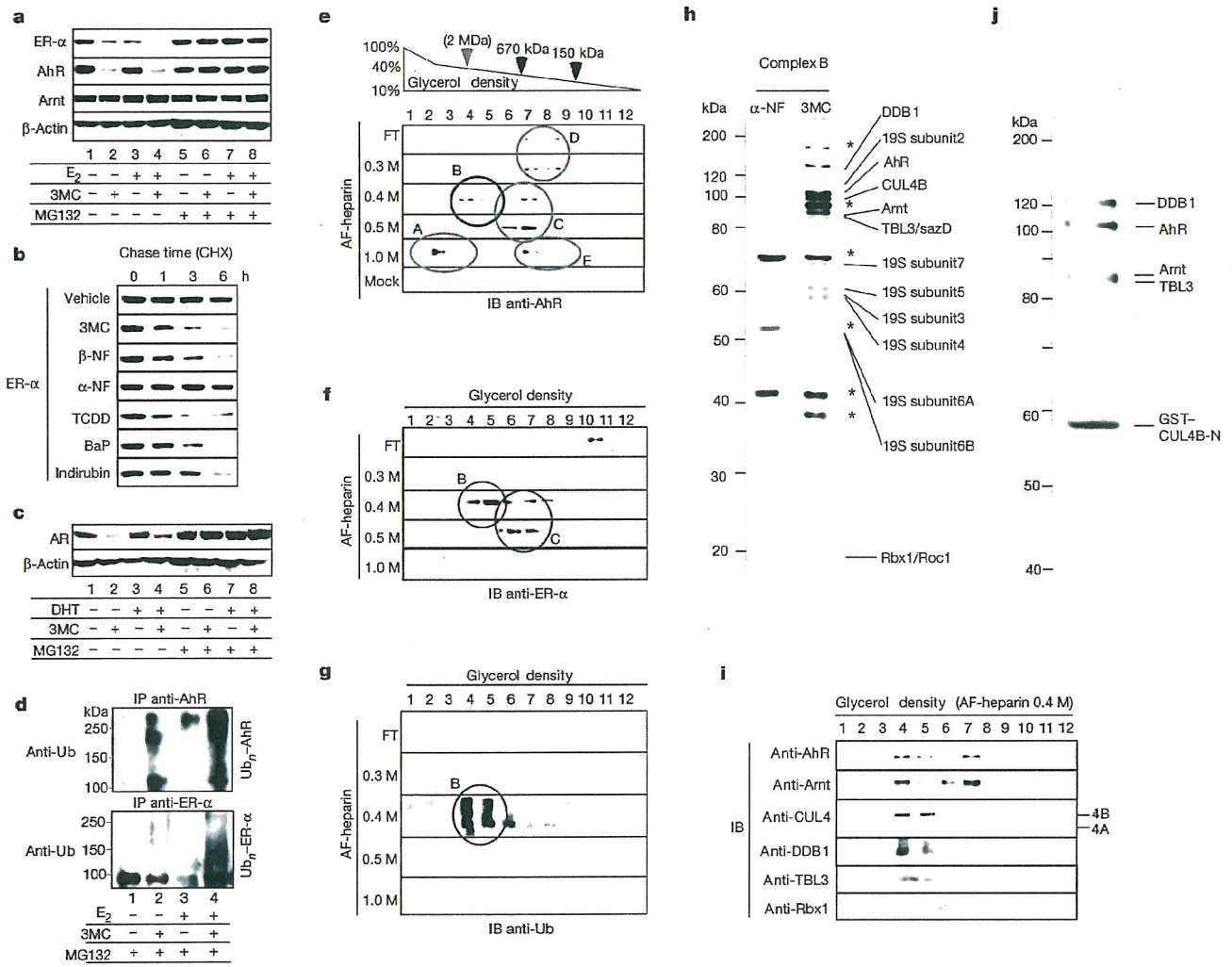

Figure 1 | Activated AhR acts as an E3 ubiquitin ligase. a–c, AhR-ligand-induced proteasomal degradation of ER-α (**a**, **b**) and AR (**c**). MCF-7 cells (**a**, **b**) and LNCaP cells (**c**) were incubated as indicated with E_2 (10 nM), DHT (10 nM) and/or 3MC (1 μM), β-NF (1 μM), benzo[a]pyrene (BaP; 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.

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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.

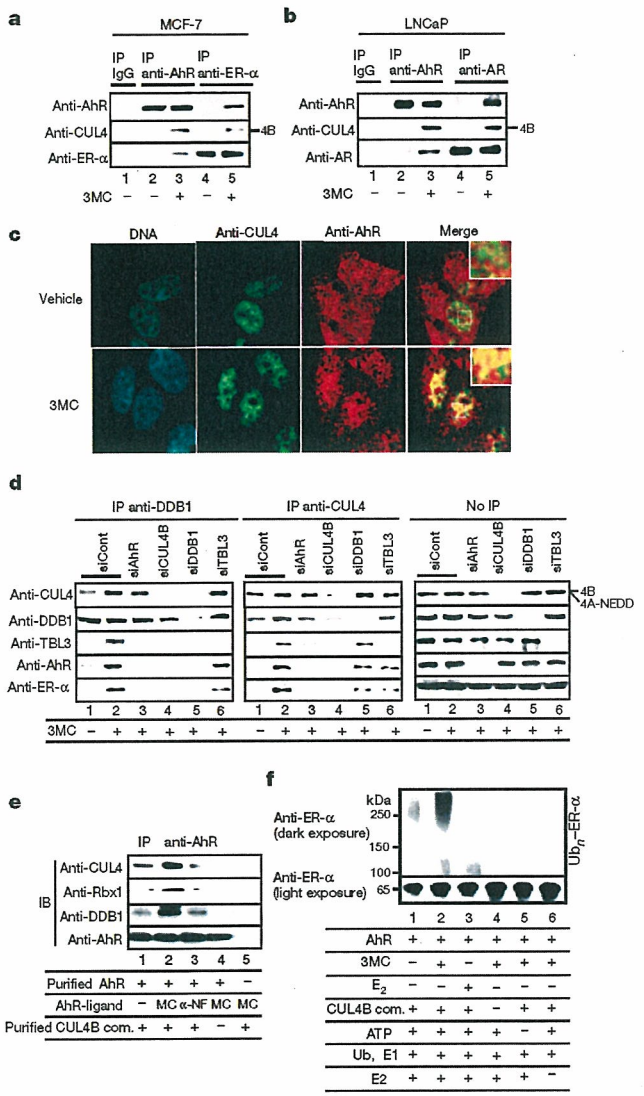

Figure 2 | AhR ligand-dependent assembly and ubiquitin ligase activity of $CUL4B^{AhR}$. **a**, **b**, 3MC-dependent association of endogenous CUL4B and AhR with ER-α and AR. Co-immunoprecipitation analyses from MCF-7 (**a**) and LNCaP (**b**) cells incubated with ligand and MG132 for 2 h. IP, immunoprecipitation. **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.

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

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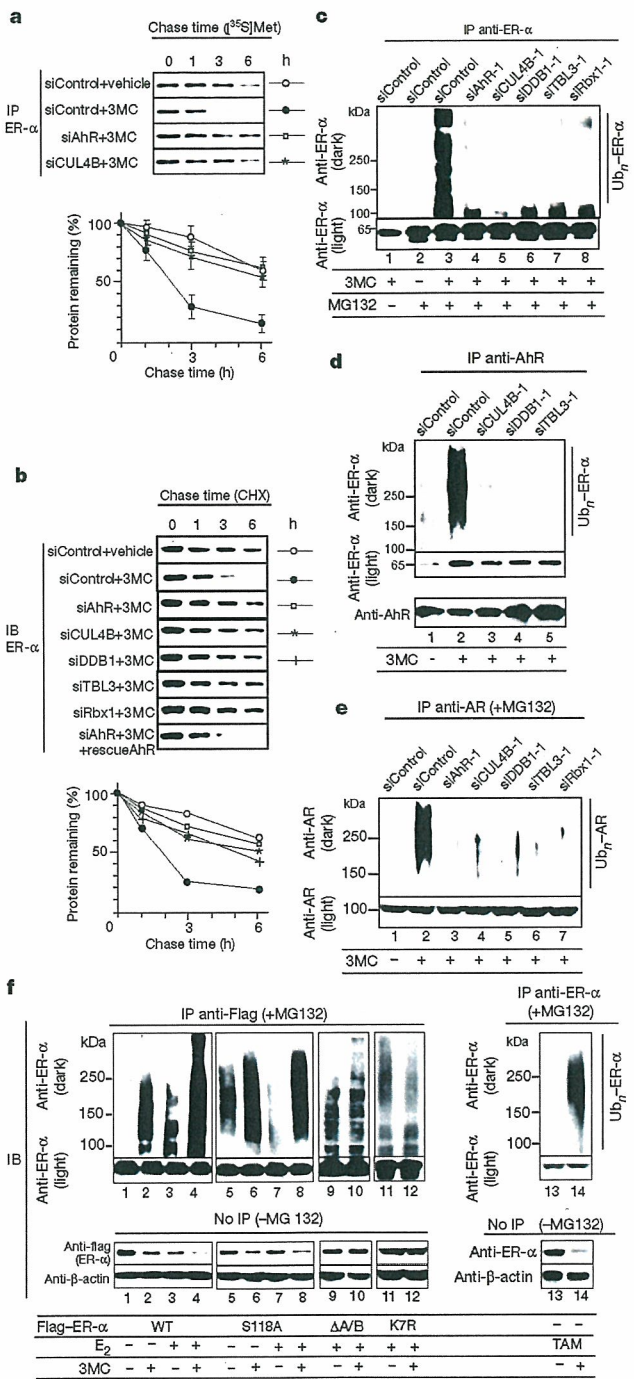

Figure 3 | Activated AhR is a substrate-specific adaptor component of the CUL4B^{AhR} complex. **a–c**, Components of CUL4B^{AhR} are required for 3MC-dependent ubiquitination and degradation of ER-α. MCF-7 cells were transfected with indicated siRNAs for 48 h, then used in pulse-chase analysis as in 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

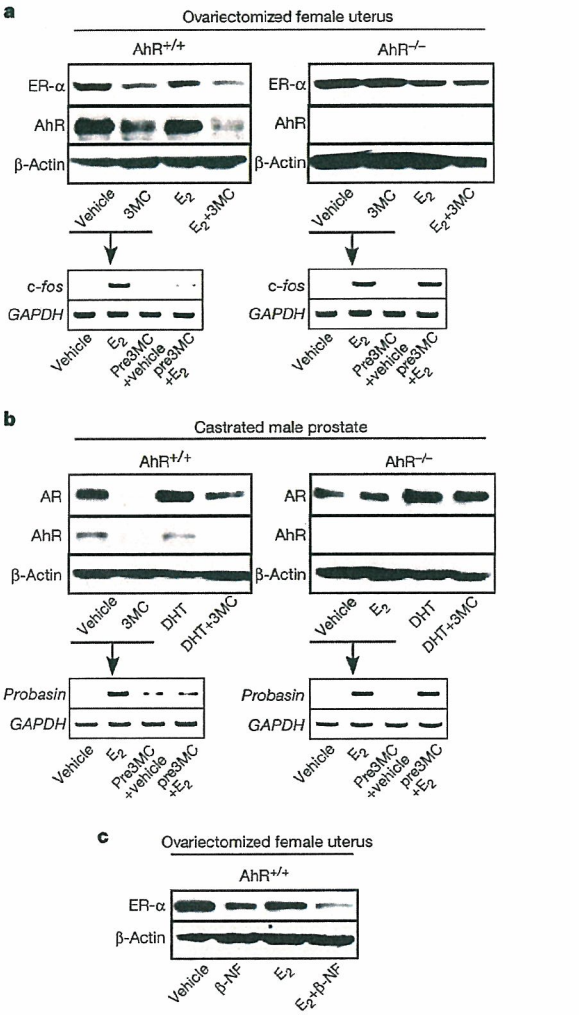

Figure 4 | Ligand-dependent ubiquitin ligase function of AhR *in vivo*. **a, b**, AhR activation enhances the degradation of ER-α and AR *in vivo*. Top: nine-week-old ovariectomized female mice (**a**) or castrated male mice (**b**) of the indicated genotypes were injected with vehicle or indicated ligands. 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

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.

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