

Tryptophanemia is controlled by a tryptophan-sensing mechanism ubiquitinating tryptophan 2,3-dioxygenase

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Maintaining stable tryptophan levels is required to control neuronal and immune activity. We report that tryptophan homeostasis is largely controlled by the stability of tryptophan 2,3-dioxygenase (TDO), the hepatic enzyme responsible for tryptophan catabolism. High tryptophan levels stabilize the active tetrameric conformation of TDO through binding noncatalytic exosites, resulting in rapid catabolism of tryptophan. In low tryptophan, the lack of tryptophan binding in the exosites destabilizes the tetramer into inactive monomers and dimers and unmasks a four-amino acid degron that triggers TDO polyubiquitination by SKP1-CUL1-F-box complexes, resulting in proteasome-mediated degradation of TDO and rapid interruption of tryptophan catabolism. The nonmetabolizable analog alpha-methyl-tryptophan stabilizes tetrameric TDO and thereby stably reduces tryptophanemia. Our results uncover a mechanism allowing a rapid adaptation of tryptophan catabolism to ensure quick degradation of excess tryptophan while preventing further catabolism below physiological levels. This ensures a tight control of tryptophanemia as required for both neurological and immune homeostasis.

tryptophan | tryptophan 2,3-dioxygenase | ubiquitination

Blood levels of essential amino acids are remarkably constant despite large variations in diet supply, but the mechanisms ensuring amino acid homeostasis remain poorly understood (1). Systemic homeostasis is particularly important for tryptophan given its key roles as a neurotransmitter precursor and a regulator of immune responses (2–5). In humans, tryptophanemia is stably maintained around $60 \pm 15 \mu\text{M}$ (mean $\pm$ SD) (6). Tryptophan catabolism involves dioxygenation leading to the production of kynurenine and derivatives (7, 8). This first and rate-limiting step can be catalyzed by two enzymes: TDO and indoleamine 2,3-dioxygenase (IDO1). Despite functional homology, these two enzymes differ in sequence, structure, expression, and physiological role. TDO (gene name *TDO2*) is a tetrameric enzyme expressed in the liver and responsible for degradation of excess dietary tryptophan (7, 9, 10). IDO1 is monomeric, only expressed in immune and inflammatory sites and mostly involved in immunoregulation (7, 11–13). Tryptophan catabolism by IDO1 can locally suppress T lymphocyte responses by depleting tryptophan and producing kynurenine. This immunosuppressive effect is exploited by tumors to resist immune rejection, and IDO1 inhibitors have been developed for cancer immunotherapy (3, 14). While IDO1 activity produces detectable levels of kynurenine in the blood, TDO does not as the kynurenine produced by TDO undergoes further degradation in the liver along the kynurenine pathway, leading to NAD and/or quinolinic acid (8). However, TDO activity is needed to control tryptophanemia. TDO-knockout (TDO-KO) mice and TDO-deficient humans have plasmatic tryptophan concentrations eight- to ninefold higher than wild-type mice or healthy humans (9, 15). As a result, TDO-KO mice better reject tumors and have higher levels of serotonin and other tryptophan metabolites in the brain, resulting in anxiolytic modulation and increased neurogenesis (9, 16). TDO is also expressed in some

human tumors and may contribute to tumoral immune resistance (10, 16–18).

Results

Stabilization of TDO by Its Substrate Tryptophan. While culturing TDO-positive human tumor cell lines, we first observed that the level of TDO protein decreased with culture time, in parallel with the reduction of tryptophan levels in the medium. We therefore tested the hypothesis that TDO protein levels were controlled by the amount of tryptophan. We incubated human glioblastoma cell line A172, which constitutively expresses *TDO2*, in medium with or without tryptophan and evaluated TDO protein levels by western blot using TDO-specific monoclonal antibody III, which recognizes a unique band that was observed only in TDO-expressing cells but not in derivatives in which TDO had been genetically inactivated (10). We observed that in the absence of tryptophan, TDO disappeared within the first 2 h (Fig. 1A), while the *TDO2* messenger RNA (mRNA) level did not change (Fig. 1B). In the presence of tryptophan, TDO disappeared after prolonged incubation time, and this was slowed down by the addition of TDO inhibitor 680C91 (19), which effectively maintained high levels of tryptophan in the culture medium (Fig. 1C and D). By culturing cells in a range of tryptophan concentrations between 0 and 100 μM , we observed that the level of TDO protein correlated

Significance

Besides being a protein building block, the essential amino acid tryptophan is a neurotransmitter precursor and a potent immunomodulator. Therefore, its systemic concentration needs to be constant in spite of irregular dietary supply. How this is achieved is unclear. Dietary tryptophan is degraded in the liver by tryptophan 2,3-dioxygenase (TDO). We report that tryptophan itself regulates TDO stability: Abundant tryptophan binds to noncatalytic exosites and stabilizes active tetrameric TDO. Hence, tryptophan is rapidly degraded and tryptophanemia contained. When tryptophan is scarce, it detaches from exosites, inducing tetramer dissociation and unmasking a degron triggering TDO polyubiquitination and proteasome-mediated degradation. Tryptophan catabolism is interrupted and blood level maintained. Matching catabolism to dietary supply, this mechanism ensures rapid and tight control of tryptophanemia.

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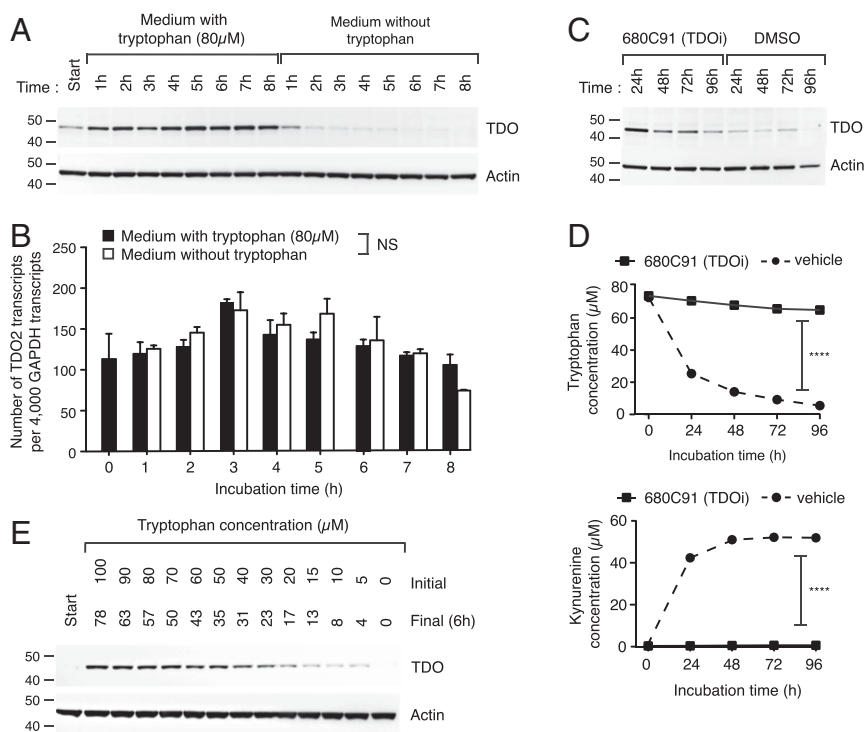


Fig. 1. Control of TDO protein levels by tryptophan. (A) A172 human glioblastoma cells were initially cultured in medium with tryptophan (start), and then incubated in medium with or without 80 μ M tryptophan for the indicated time. TDO protein level was evaluated by western blot using mAb clone III (10). One representative out of four independent experiments is presented. (B) TDO2 mRNA levels were measured in the same samples by quantitative RT-PCR and normalized to GAPDH (mean + SD of duplicates). NS: $P > 0.05$ for medium with tryptophan versus without tryptophan (two-way ANOVA). (C) A172 cells were incubated in medium containing tryptophan (80 μ M) and treated or not with TDO inhibitor (TDOi) 680C91 (33 μ M) for the indicated time. TDO protein level was evaluated by western blot as above. One representative out of four independent experiments is shown. (D) Kynurenine or tryptophan concentrations were measured by HPLC in the supernatant of the cells tested in C (mean + SD of duplicates). **** $p < 0.0001$ for 680C91 and time parameters (two-way ANOVA). (E) A172 cells were incubated in medium with tryptophan for 72 h until the exhaustion of tryptophan (start). Cells were then incubated for 6 h in media containing the indicated tryptophan concentrations. TDO protein level was evaluated by western blot and related to the final tryptophan concentration in the supernatant measured by HPLC. One representative out of four independent experiments is shown.

strictly and positively with the tryptophan concentration (Fig. 1E). Incubation of cells in hypoxia did not decrease the TDO levels, indicating that oxygen, the other substrate involved in TDO activity, did not regulate TDO protein levels (SI Appendix, Fig. S1).

We then investigated whether this control by tryptophan also occurred in vivo on hepatic TDO. We fed starved mice with tryptophan by oral gavage and killed them at different time points. TDO levels in the liver increased within 30 min after gavage and returned to normal levels after a few hours (Fig. 2A and B). During this time course, the levels of hepatic TDO followed the systemic concentration of tryptophan (Fig. 2C), while the levels of *Tdo2* transcript in the liver remained constant (Fig. 2D). These results suggest that high tryptophanemia increases hepatic TDO levels, resulting in rapid normalization of systemic tryptophan.

Ubiquitination and Proteasome-Mediated Degradation of TDO in the Absence of Tryptophan. To determine whether this control of the TDO protein occurred at the level of translation or post-translationally, we analyzed TDO stability in A172 cells treated with cycloheximide to block protein synthesis. The TDO half-life, which was over 8 h in the presence of tryptophan, decreased to 1 h 22 min in the absence of tryptophan (Fig. 3A and SI Appendix, Table S1). Interestingly, addition of proteasome inhibitor bortezomib increased TDO half-life in the absence of tryptophan (Fig. 3B and SI Appendix, Table S1). As bortezomib only partially inhibits proteasome activity, it was not able to fully prevent TDO degradation. Altogether, these results indicated that the absence of tryptophan induced a rapid degradation of TDO by the proteasome.

We then assessed whether the lack of tryptophan triggered TDO polyubiquitination. We used the tandem ubiquitin binding entities assay (TUBE) (20, 21) to purify ubiquitinated proteins from A172 cells, and we observed TDO polyubiquitination in the absence but not in the presence of tryptophan (Fig. 3C). To confirm the involvement of polyubiquitination, we analyzed TDO half-life in cells treated with MLN7243, an inhibitor of the E1 ubiquitin-activating enzyme (22). This treatment, which efficiently blocked protein ubiquitination, almost completely prevented TDO degradation in the absence of tryptophan (Fig. 3D and SI Appendix, Table S1). Similar results were obtained with another inhibitor of protein polyubiquitination (SI Appendix, Fig. S2).

Ubiquitination of TDO by SKP1-CUL1-F-Box Complexes. We then sought to identify the E3 ubiquitin ligase(s) responsible for TDO polyubiquitination in the absence of tryptophan. We used pevonedistat (also called MLN4924) (23), a NEDD8-activating enzyme inhibitor, to distinguish between the HECT and the RING family of E3 ubiquitin ligases. Indeed, only cullin RING ubiquitin ligases (CRLs), which represent the largest group of RING E3 ligases, need to be activated by neddylation and therefore remain inactive in the presence of pevonedistat (24). We observed that pevonedistat increased the half-life of TDO in the absence of tryptophan, suggesting the involvement of CRLs in TDO polyubiquitination (Fig. 4A and SI Appendix, Table S1).

CRLs are multiprotein complexes built around a cullin scaffold, whose C terminus recruits a catalytic small RING protein (RBX1 or 2) that interacts with E2 enzymes, while the N terminus interacts with a receptor responsible for substrate recognition,

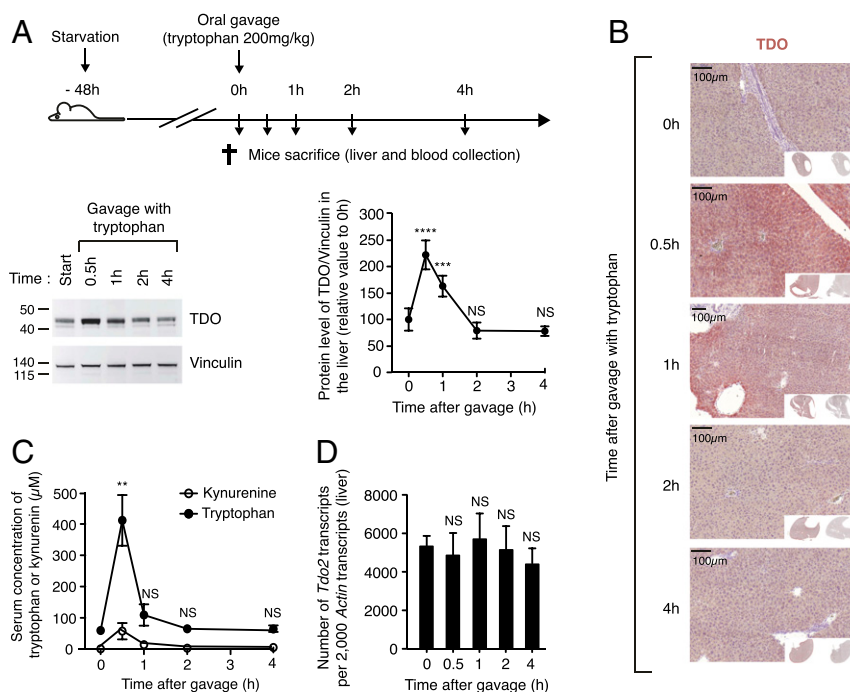


Fig. 2. Tryptophanemia controls liver TDO protein levels. (A) C57BL/6 mice were starved for 48 h to stabilize the systemic concentration of tryptophan and the level of TDO protein in the liver before receiving tryptophan 200 mg/kg body weight by oral gavage ($n = 20$). Four mice were killed at each indicated time point, and TDO protein levels in the liver were evaluated by western blot and normalized to vinculin. One representative mouse out of four is illustrated for each time point (Left), and the graph (Right) represents the results (mean $\pm$ SD) of four mice for each time point. $^{NS}P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ for different time points versus initial situation (Unpaired t test with Welch correction). This experiment was performed independently three times. (B) TDO protein level was also evaluated by immunohistochemistry on formalin-fixed paraffin-embedded tissue sections of mouse livers using a mouse-rabbit chimeric anti-TDO mAb V whose specificity was validated using TDO-KO mice (36). (Insets) Whole section stained with (Left) or without (Right) primary antibody. (C) Systemic concentrations of tryptophan and kynurenine were evaluated by HPLC in the sera of mice from A. Statistical analyses were performed as in A. (D) *Tdo2* mRNA levels were measured by quantitative RT-PCR in the liver and normalized to actin. Statistical analyses were performed as in A.

such as an F-box protein (25). There are several cullin variants, including CUL1, CUL3, CUL4A, CUL4B, and CUL5. To identify which CRL was involved in TDO polyubiquitination, we used C-terminally truncated forms of cullins, which are inactive as they are unable to interact with RBX proteins and E2 enzymes but can still bind the substrate receptor and therefore act as dominant-negative cullins (26). We transiently expressed the dominant-negative cullins in human embryonic kidney cells HEK293-EBNA stably transfected with TDO. We observed that transfection of the dominant-negative CUL1 increased the half-life of TDO in the absence of tryptophan compared to cells transfected with the empty vector (Fig. 4B and *SI Appendix, Table S2*). In contrast, expression of truncated CUL3, CUL4A, CUL4B, and CUL5 had no effect on TDO half-life (Fig. 4B–D and *SI Appendix, Table S2*). We failed to derive cells permanently inactivated for CUL1 either with a dominant-negative CUL1 or with CRISPR-Cas9, suggesting that such inactivation was lethal for HEK293 cells. Altogether, our results suggest that CRL1, usually called SKP1-CUL1-F-box complex, is responsible for TDO polyubiquitination in the absence of tryptophan.

Tryptophan and Alpha-Methyl-Tryptophan Stabilize the Tetrameric Conformation of TDO. Alpha-methyl-tryptophan is a tryptophan analog that cannot be degraded by TDO. It was shown previously that both tryptophan and alpha-methyl-tryptophan can enhance TDO activity in rat liver homogenates (27). We therefore tested whether this nonmetabolizable analog could stabilize TDO. We observed that alpha-methyl-tryptophan considerably increased and sustained TDO protein levels in long-term cultures of A172 cells (Fig. 5A). Because alpha-methyl-tryptophan does not inhibit TDO enzymatic activity, the stabilization of TDO resulted

in higher tryptophan degradation by the cells, particularly at the late time points (Fig. 5A, Right). These results suggested the presence of two types of tryptophan-binding sites in the TDO protein: catalytic sites, which are responsible for tryptophan degradation, and noncatalytic sites, which would also accommodate alpha-methyl-tryptophan and would regulate TDO stability. This is consistent with the crystal structure of TDO, which was published in the course of our work, and showed that in addition to the four heme-containing catalytic sites, tetrameric TDO has four exosites that also bind tryptophan (28). Alpha-methyl-tryptophan cannot bind to the catalytic site because of steric hindrance of the methyl with the heme, but it does bind to the exosite (28). These results suggested that the binding of tryptophan or alpha-methyl-tryptophan to these exosites could stabilize TDO by reducing its degradation by the proteasome.

We then investigated whether alpha-methyl-tryptophan also stabilized hepatic TDO *in vivo*. We fed starved mice by oral gavage with alpha-methyl-tryptophan and killed them after different time points to evaluate TDO protein levels in the liver by western blot. We observed increased and sustained TDO levels that accumulated over time (Fig. 5B). Interestingly, this increased stability of TDO translated into a considerably reduced systemic concentration of tryptophan, from 60 μ M to 20 μ M (Fig. 5C). This indicated that the TDO that was stabilized by alpha-methyl-tryptophan was catalytically active. Together, these results show that the systemic concentration of tryptophan is limited by the post-translational stability of TDO. When tryptophan is abundant, the binding of tryptophan in the exosites stabilizes TDO, thereby increasing tryptophan catabolism.

Next, we explored how the absence of tryptophan induces TDO polyubiquitination and degradation. TDO has a tetrameric

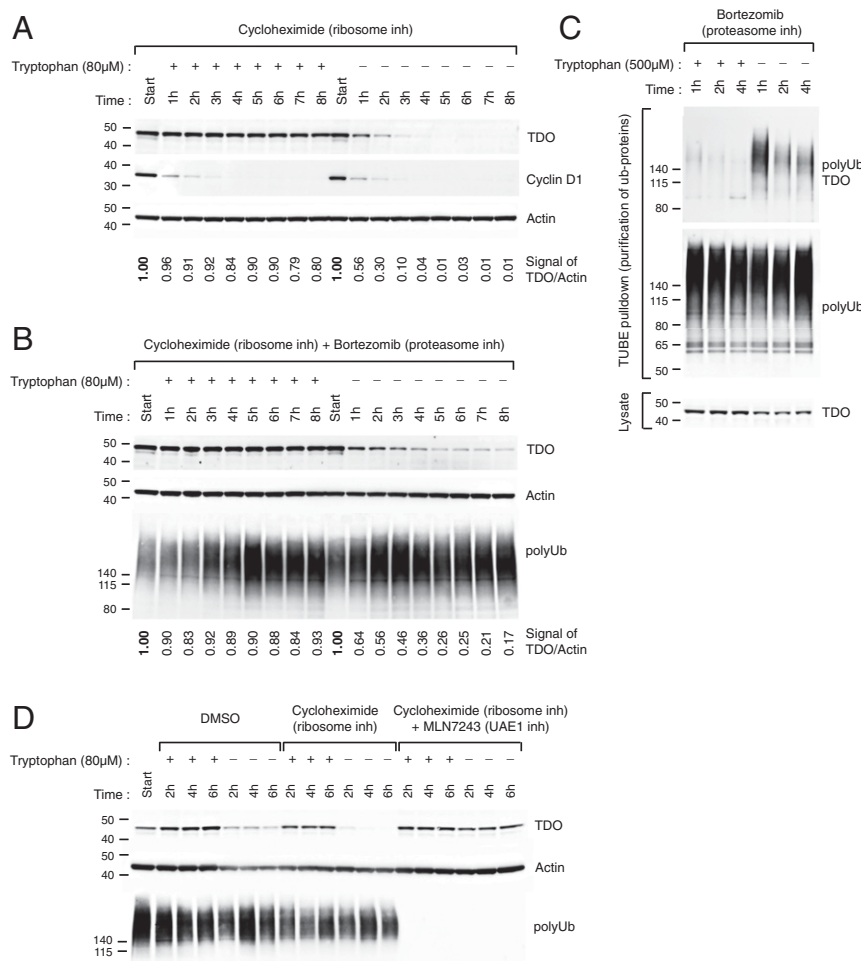


Fig. 3. Ubiquitination and proteasome-mediated degradation of TDO in the absence of tryptophan. (A) A172 cells initially grown in medium containing tryptophan (start) were incubated in medium containing cycloheximide (50 μ g/mL) with or without 80 μ M tryptophan for the indicated times. TDO protein levels were evaluated by western blot analysis using mAb clone III. The cyclin D1 level was used as a control for inhibition of protein synthesis by ribosome inhibitor cycloheximide. Signal of TDO/Actin was quantified and related to the value of 0 h. (B) TDO stability was evaluated in the presence of cycloheximide (50 μ g/mL) and proteasome inhibitor, bortezomib (1 μ M), in the same conditions as in A. Polyubiquitinated protein (polyUb) level was used as a control for proteasome inhibition. (C) A172 cells were incubated in medium with or without 500 μ M tryptophan and with bortezomib (1 μ M) to prevent TDO protein degradation for 1 to 4 h. Ubiquitinated proteins were purified by the TUBE assay. Purified proteins and lysates were then analyzed by immunoblotting with a TDO rabbit polyclonal antibody. (D) A172 cells were initially cultured with tryptophan (start) and then incubated in medium with or without 80 μ M tryptophan for the indicated times in the presence of cycloheximide (50 μ g/mL) and MLN7243 (10 μ M), an inhibitor of the E1 ubiquitin-activating enzyme (UAE1). TDO protein level was evaluated by western blot. The level of polyubiquitinated proteins was measured as a control for UAE1 inhibition. All these experiments were performed independently three times.

conformation allowing the degradation of four substrates bound at the same time in the catalytic sites. We hypothesized that the binding of tryptophan to the four exosites stabilizes the tetrameric conformation and thereby masks a degron, which is a degradation motif recognized by ubiquitin ligases. We used gel filtration chromatography in native conditions to analyze the conformation of TDO in A172 cells cultured with or without tryptophan in the presence of bortezomib. In the presence of tryptophan, TDO exhibited a molecular mass close to 192 kDa, which corresponded to the theoretical mass of the tetramer (Fig. 5D). In contrast, in the absence of tryptophan, TDO had a lower mass ranging from 96 to 48 kDa, which corresponded to the dimer and the monomer, respectively (Fig. 5D).

To determine whether tryptophan stabilizes the tetrameric conformation of TDO by binding to the exosites, we tested whether alpha-methyl-tryptophan also stabilized its tetrameric structure by performing a western blot in native conditions. Alpha-methyl-tryptophan considerably stabilized the tetrameric conformation of TDO, which otherwise reduced to smaller forms consistent with a

dimeric or monomeric conformation (Fig. 5E). Altogether, these results suggest that tryptophan stabilizes the tetrameric conformation of TDO by binding to the exosites, thereby favoring efficient tryptophan catabolism. When tryptophan becomes scarce, the structure of TDO evolves into monomeric and dimeric conformations, predicted to be inactive (29). This conformational change might unmask degrons recognized by SKP1-CUL1-F-box complexes, which address TDO to the proteasome.

A Four-Amino Acid Degron in the Noncatalytic Tryptophan-Binding Site Is Unmasked upon Dissociation of the TDO Tetramer.

The putative degron motif had to be shared between mouse and human TDO and masked in the tetrameric conformation with tryptophan bound to the exosites. We therefore deleted a series of residues located either at the interface between the monomers or in the exosite. Most mutations introduced did not affect TDO stability or destabilized TDO even in the presence of tryptophan, probably because their folding was compromised. Strikingly, the deletion of residues 200 to 213 stabilized TDO when tryptophan

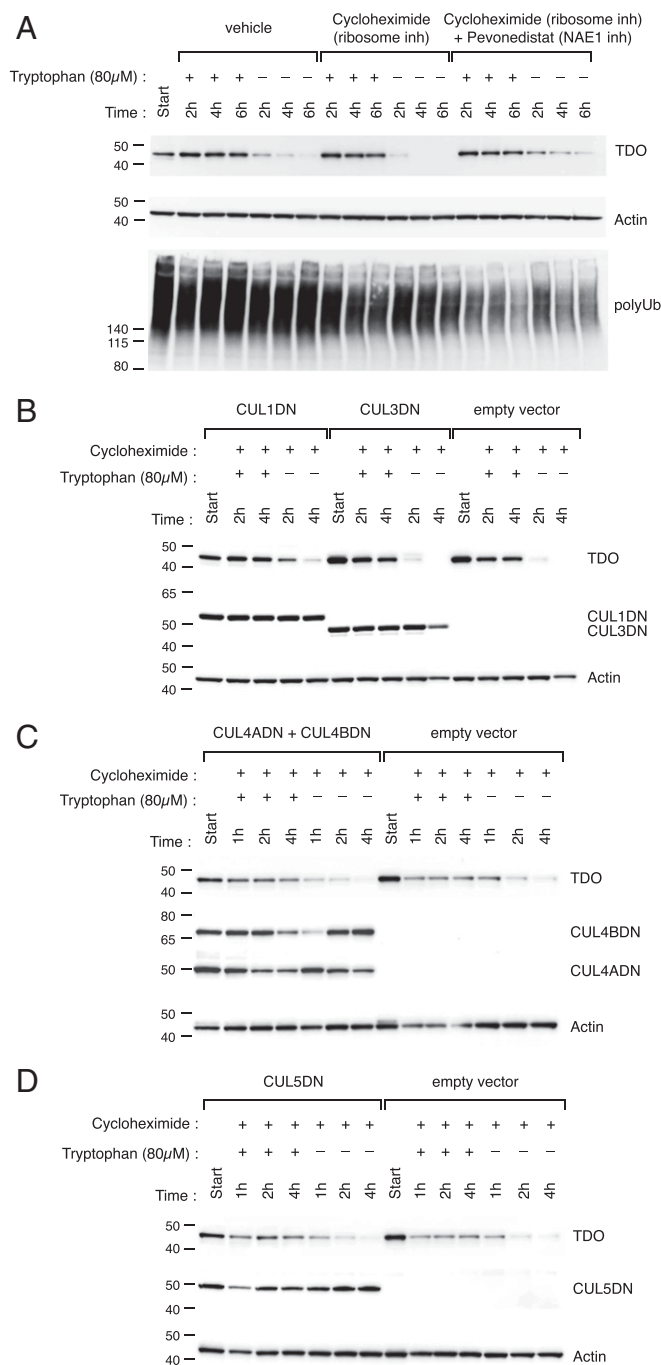


Fig. 4. Role of CRL1 in TDO degradation. (A) A172 cells were first cultured in medium containing tryptophan (start) and then incubated in medium with or without 80 μ M tryptophan for the indicated times. Cells were treated with cycloheximide (50 μ g/mL) and pevonedistat/MLN4924 (5 μ g/mL), an inhibitor of the NEDD8-activating enzyme (NAE1). The levels of TDO protein and of polyubiquitinated proteins were evaluated by western blot. A partial decrease of the polyubiquitinated proteins is characteristic of cullin RING ubiquitin ligase inhibition. One representative out of three independent experiments is shown. (B–D) Stably transfected HEK293 cells expressing TDO2 (HEK293 hTDO c119) were transiently transfected with the dominant-negative forms of cullins (CULDN) as indicated or with empty vector (pcDNA3). Cells were initially cultured in medium containing tryptophan (start) then incubated for the indicated times in medium containing cycloheximide (50 μ g/mL) in the presence or the absence of tryptophan (80 μ M). Protein levels of TDO and CULDN were evaluated by western blot with anti-TDO mAb clone III and with an anti-FLAG antibody, respectively. Experiments were performed independently three times for CUL1 and CUL3 and two times for the other cullins.

was absent without affecting TDO levels in the presence of tryptophan (Fig. 6A). Partial stabilization of TDO was also obtained with the deletion of the structurally close residues 98 to 105 (Fig. 6A). According to the crystal structure, these residues are included in the TDO exosite and appear to be masked by bound tryptophan (Fig. 6B) (28). These sequences, which are similar in mouse and human TDO, do not comprise any lysine and therefore are unlikely to contain the TDO ubiquitination site(s). Because the TDO mutants were not functional, the intracellular concentration of tryptophan might remain high in the cells expressing this inactive TDO even in medium without tryptophan (Fig. 6A). Although this could potentially lead to TDO stabilization independent from the deletion, this was unlikely given the fact that another inactive TDO protein, which was deleted in catalytic residues 169 to 187, was unstable in cells cultured without tryptophan (Fig. 6C). It was also possible that the 200 to 213 deletion did not remove a degron but only stabilized the tetrameric conformation of TDO irrespective of tryptophan binding and thereby prevented TDO degradation by masking a degron located elsewhere. However, the 200 to 213 deletion did not stabilize the tetrameric conformation (SI Appendix, Fig. S3). Therefore, amino acids 200 to 213 were likely to contain the degron. To define more precisely which amino acid(s) are involved in TDO stability, we then performed an alanine scanning for residues 200 to 213. Alanine substitution of residues 208 to 211, which constitute the core of the exosite, stabilized TDO in the absence of tryptophan, while mutation of other residues, such as 204 to 206, did not (Fig. 6D). Together, these results suggest that the exosite of each TDO subunit contains a degron, with sequence WLER_{208 to 211}, that is masked by the binding of tryptophan or alpha-methyl-tryptophan (Fig. 6B). In the absence of tryptophan, this degron is unmasked and allows the interaction of TDO with ubiquitin ligase SKP1-CUL1-F-box.

Discussion

Our results indicate a mechanism allowing stable tryptophanemia despite varying levels of tryptophan supply in the diet. High tryptophan availability stabilizes TDO in the liver, allowing efficient tryptophan catabolism. In contrast, low tryptophan levels trigger proteasome-mediated degradation of TDO, thereby stopping tryptophan catabolism and preventing hypotryptophanemia. We describe the molecular mechanism of this posttranslational control of TDO stability. Our work extends the findings of Lewis-Ballester et al., whose crystallographic study of human TDO described the presence of four noncatalytic exosites that bind tryptophan or alpha-methyl-tryptophan with high affinity and stabilize the TDO protein (28). These authors also suggested that TDO was degraded by the proteasome and showed that recombinant TDO can be ubiquitinated *in vitro* by recombinant E3 ubiquitin ligases Ubc7/gp78, Ubc7/Hrd1, and UbcH5a/CHIP/Hsc70/Hsp40. Although they proposed that tryptophan binding to the exosites might retard proteasome-mediated degradation, the authors did not describe this mechanism in molecular terms.

Our results provide molecular insights into this process by showing that tryptophan binding to exosites stabilizes the homotetrameric structure of TDO. In the absence of tryptophan, TDO dissociates into inactive monomers that are polyubiquitinated and degraded by the proteasome. We also show that the exosites contain a four-amino acid degron that targets TDO for degradation by the proteasome. This degron, with sequence WLER_{208 to 211}, is masked by the binding of tryptophan in the exosites, resulting in protection of TDO from degradation. In the absence of tryptophan, unmasking of this degron results in TDO ubiquitination by the E3 ligase SKP1-CUL1-F-box. The degron itself does not contain a lysine residue, so ubiquitination takes place at different sites. In their *in vitro* reconstituted hTDO ubiquitination reactions, Lewis-Ballester et al. identified 15 lysines that could be ubiquitinated *in vitro*—regardless of tryptophan presence or

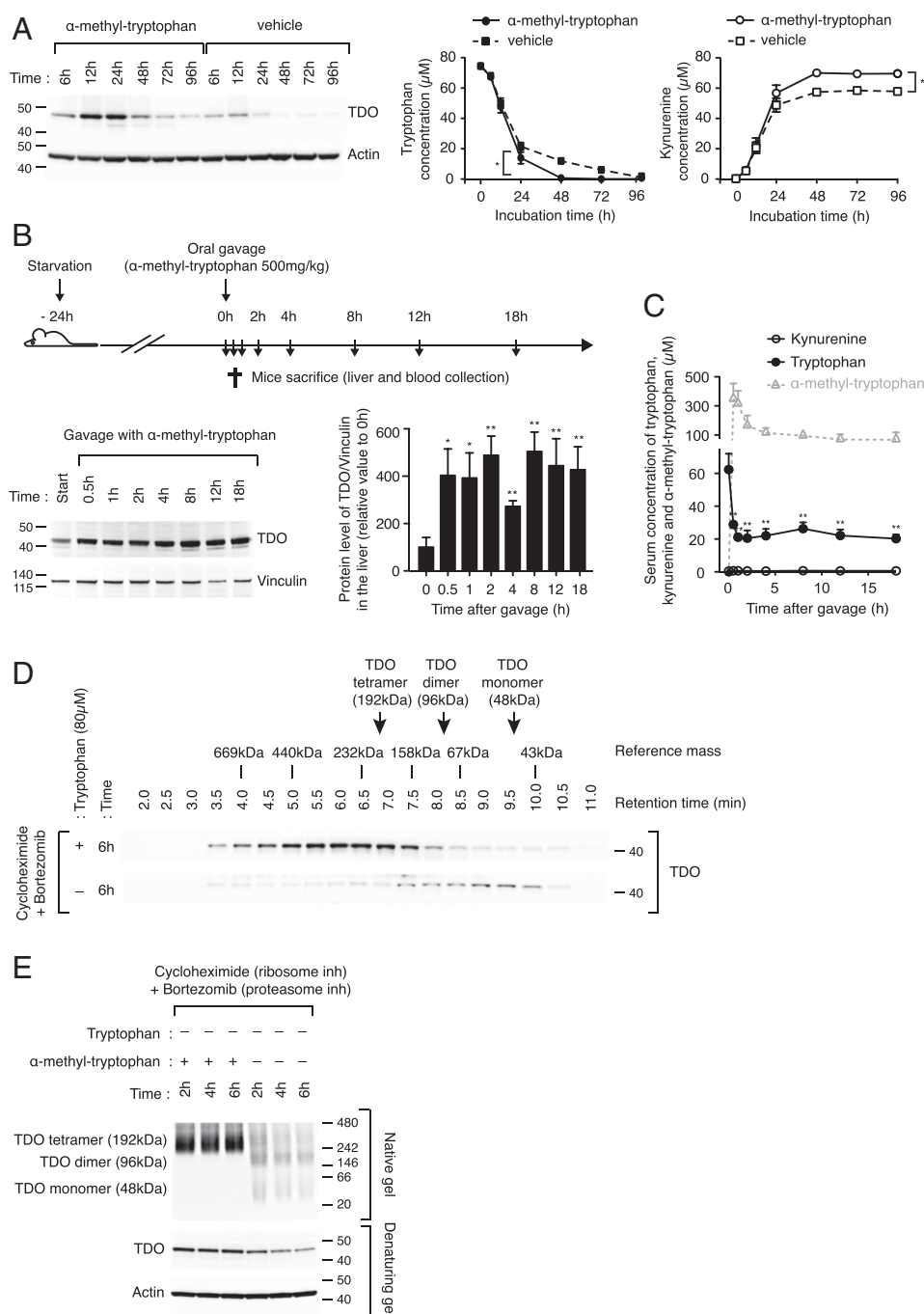


Fig. 5. Stabilization of the TDO tetrameric conformation by tryptophan and α -methyl-tryptophan. (A) A172 cells were first cultured in medium with tryptophan for 1 wk until the exhaustion of tryptophan. Cells were then incubated for 6 to 96 h in tryptophan-containing medium (80 μ M) supplemented or not with α -methyl-tryptophan (500 μ M). TDO protein level was evaluated by western blot. One representative out of three independent experiments is shown on *Left*. *Right* shows the kynurenine and tryptophan concentrations measured by HPLC in the supernatants. Mean + SD of triplicates. * p < 0.05 for medium with α -methyl-tryptophan versus without α -methyl-tryptophan (two-way ANOVA). (B) C57BL/6 mice starved for 24 h received α -methyl-tryptophan 500 mg/kg body weight by oral gavage (n = 32). Four mice were killed at each indicated time point to evaluate TDO protein levels in the liver by western blot. One representative mouse of four is illustrated for each time point (*Left*), and the graph (*Right*) represents the results (mean + SD) of four mice for each time point. * P < 0.05; ** P < 0.01 for each time point versus initial situation (unpaired t test with Welch correction). One representative out of three independent experiments is shown. (C) Systemic concentrations of tryptophan, kynurenine, and α -methyl-tryptophan were analyzed by HPLC in the sera of mice from B. Statistical analyses were performed as in B. (D) A172 cells were initially cultured in tryptophan-containing medium and then incubated with or without 80 μ M tryptophan for 6 h in the presence of cycloheximide (50 μ g/mL) and bortezomib (1 μ M). Proteins were separated according to their mass by gel filtration chromatography in native conditions. Chromatography fractions were then analyzed by western blot in denaturing conditions. Retention time of reference proteins by chromatography allowed an estimation of the TDO molecular mass. One representative experiment out of two is shown. (E) A172 tumor cells were initially cultured in tryptophan-containing medium (80 μ M) and then incubated for the indicated times in tryptophan-free medium with or without α -methyl-tryptophan (500 μ M) in the presence of cycloheximide (50 μ g/mL) and bortezomib (1 μ M). TDO molecular mass was evaluated by performing a western blot analysis in native (*Top*) or denaturing conditions (*Bottom*). One representative out of three independent experiments is shown.

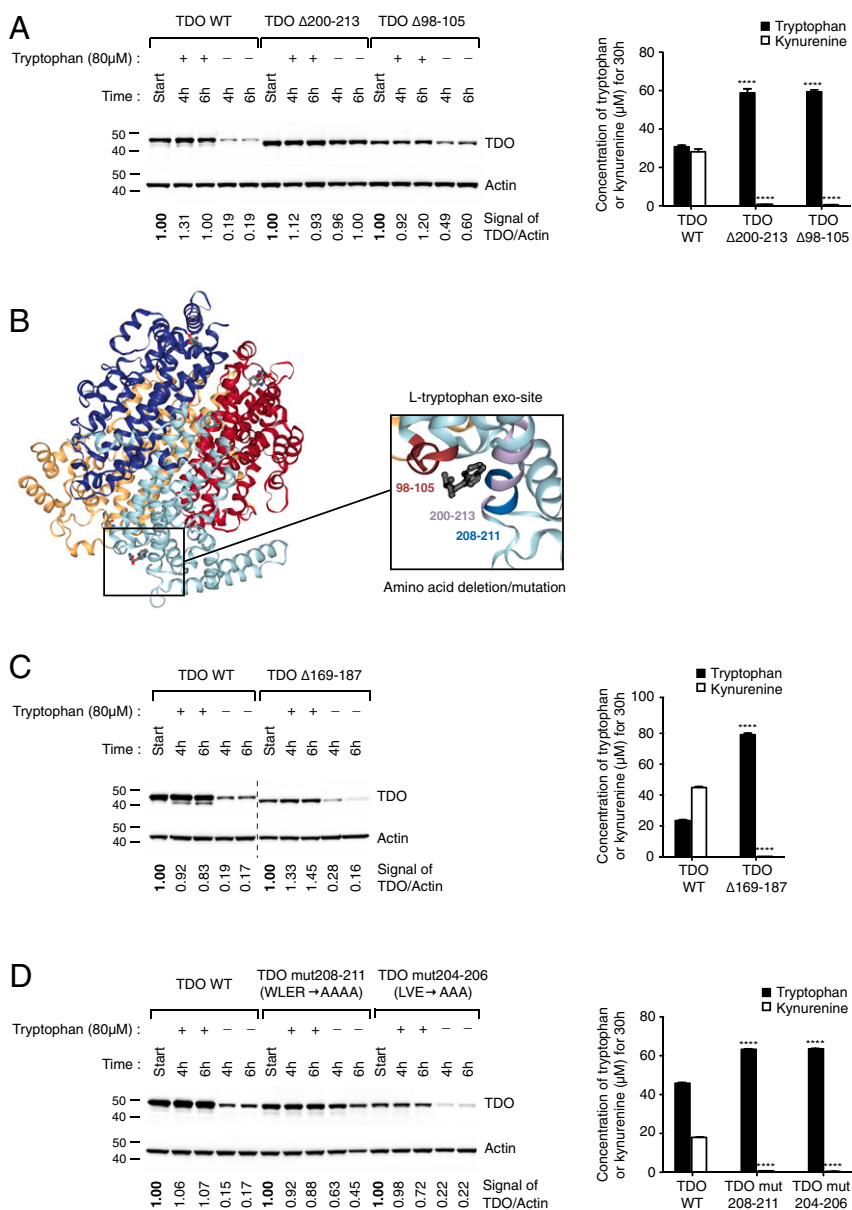


Fig. 6. Localization of the tryptophan-binding TDO degron. (A) HEK293 cells were stably transfected with expression vectors encoding either wild-type TDO (TDO WT) or mutant TDO with deletion of residues forming the tryptophan exosite (TDO Δ200 to 213 or TDO Δ98 to 105). Cells were cultured initially in tryptophan-containing medium (start) and then incubated in medium with or without 80 μM tryptophan for the indicated times. (Left) TDO protein levels evaluated by western blot and normalized to actin. (Right) Kynurenine and tryptophan concentrations measured by HPLC in the supernatants of the same cells cultured for 30 h in tryptophan-containing medium. Mean + SD of pentaplicates. **** $P < 0.0001$ for TDO-mutated proteins versus TDO WT (unpaired t test with Welch correction). (B) Schematic structure of the TDO tetramer (Protein Data Bank file ID: 5TIA) (28). Each monomer is labeled with a different color. (Inset) Tryptophan binding to an exosite. The purple, blue, and red colors in *Inset* identify amino acids whose mutation stabilizes TDO in the absence of tryptophan. These amino acids represent the likely degron that is masked by tryptophan binding to the exosite. (C) HEK293 cells transfected with TDO mutated by deletion of residues in the catalytic site (TDO Δ169 to 187) were tested as in A. (D) HEK293 cells transfected with TDO mutated by alanine substitutions in the exosite (TDO mut208-11 or TDO mut204-206) were tested as in A. All these experiments were performed independently three times.

absence—by recombinant E3 ubiquitin ligases Ubc7/gp78, Ubc7/Hrd1, and UbcH5a/CHIP/Hsc70/Hsp40 (28). However, these E3 ligases are unlikely to play a physiological role in living cells. Although they belong to the RING family of E3 ligases, they are not part of the cullin RING subfamily of E3 ligases, which are the only RING E3 ligases to require neddylation to be activated (23, 24). We observed that TDO degradation in the absence of tryptophan was prevented in cells treated with neddylation-inhibitor pevonedistat. Therefore, the E3 ubiquitin ligases proposed by Lewis-Ballester appear not to be involved in TDO degradation in the absence of tryptophan. Our results with dominant-negative cullins

rather indicate that the SKP1-CUL1-F-box E3 ligase is responsible for TDO ubiquitination in the absence of tryptophan. The identity of the F-box protein involved in this molecular complex remains to be defined.

Another innovative aspect of our work is to show the *in vivo* relevance of this posttranslational control of TDO stability in the liver, which allows to maintain stable tryptophan levels in the whole body despite high variations in dietary supply. Maintaining tryptophanemia in a physiological concentration range (60 ± 15 μM) is essential to general homeostasis in vertebrates: first, because tryptophan is a precursor of serotonin, a neurotransmitter

important for mood control, and of the kynurenine family of neuroactive compounds (2) and second, because tryptophan and its kynurenine derivatives play an important role in immune regulation: tryptophan conversion into kynurenine locally suppresses T lymphocyte responses, contributing to the lack of immune rejection of fetal tissues and the resistance of tumors to immune control (11, 17, 30, 31). In mice and humans, genetic TDO deficiency causes hypertryptophanemia and hyperserotoninemia (9, 15, 16). In mice, this resulted in reduced anxiety and increased neurogenesis (9). It also improved tumor rejection and the efficacy of immune checkpoint inhibitors (16). Hypertryptophanemia could be normalized by reducing tryptophan supply in the diet (16).

The mechanism described here could explain the well-established observation of increased TDO activity by tryptophan (27, 32) and may represent a general system for the control of blood levels of essential amino acids. In support of this proposal is the observation that cysteine dioxygenase was degraded by the proteasome in the absence of cysteine, even though the structural and molecular mechanism involved in this case remains to be defined (33, 34). Our results also provide a framework to manipulate tryptophanemia, using TDO inhibitors to increase it and TDO stabilizers like alpha-methyl-tryptophan to reduce it. This could be of medical interest for the management of mood disorders, autoimmune disorders, and cancer.

Materials and Methods

Cell Lines. The human glioblastoma A172 cell line (ATCC-CRL-1620) was obtained from Antisense Pharma GmbH. Authentication of cells was performed in November 2019 by short tandem profiling (Promega Powerplex HS 16). Cells were certified as mycoplasma free in November 2019. The HEK293 cell line was purchased from ATCC in 2014 (ATCC-CRL-1573). The HEK293 hTDO c119 cell line derived from HEK293-EBNA cells stably transfected with pEF6/V5-His expression vector encoding human TDO, as described (17).

Culture Medium. A172 and HEK293 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (#21980-032 from Life Technologies) complemented with 10% fetal bovine serum (FBS) (#7524, Sigma), nonessential amino acids (0.55 mM L-arginine, 0.24 mM L-asparagine, 1.5 mM glutamine), and antibiotics (100 µg/mL streptomycin, 100 IU/mL penicillin). To analyze TDO stability, cells were incubated in IMDM medium without L-tryptophan (ME130013L1, Life Technologies) complemented with nonessential amino acids (0.55 mM L-arginine, 0.24 mM L-asparagine, 1.5 mM glutamine) but without serum and antibiotics. This medium was supplemented with tryptophan (T-0254, Sigma) or phosphate-buffered saline (PBS) depending on the experimental conditions.

Compounds and Inhibitors for Cell Culture. Following list includes inhibitor origins, their final concentrations in cell medium and the solvent of the stock solution. L-tryptophan (used at 80 µM or 500 µM, previously dissolved in PBS) was obtained from Sigma-Aldrich (T-0254). Cycloheximide (50 µg/mL, dimethyl sulfoxide [DMSO]) was bought from Cell Signaling (#21125). Bortezomib (1 µM, DMSO) was obtained from Santa Cruz Biotechnology (sc-217785). MLN7243 (10 µM, DMSO) and MLN4924 or pevonedistat (5 µg/mL, DMSO) were obtained from Active Biochem (A-1384 and A-1139, respectively). A838241 (now commercialized by Ambeed) (10 µM, DMSO) was provided by Patrick T. Gunning under the name "Compound 1" and was produced by Takeda (35). Alpha-methyl-DL-tryptophan (500 µM, PBS or methanol 50%, racemic mixture) was ordered from Sigma (M8377) and from Angene Chemical (#153-91-3). 680C91 (33 µM, DMSO) was bought from Sigma (SML0287).

Analysis of TDO Stability. A172 cells (6×10^5) or HEK293 cells (10^6) for each condition were plated and initially cultured in 3 mL IMDM medium containing 80 µM tryptophan (#21980-032 from Life Technologies) complemented with 10% FBS (#7524 from Sigma) and nonessential amino acids (0.55 mM L-arginine, 0.24 mM L-asparagine, and 1.5 mM glutamine). The next day, cells were washed three times with PBS and incubated in 3 mL fresh medium containing 80 µM tryptophan. This step is essential to increase and stabilize TDO protein with tryptophan before starting the experiments. Exactly 16 h later, cells were washed three times with PBS to remove the last traces of tryptophan. An aliquot was harvested to evaluate the abundance of TDO at time 0 (starting point). The rest of the culture was incubated in 2 mL of IMDM medium without L-tryptophan, complemented with nonessential amino acids (0.55 mM L-arginine, 0.24 mM L-asparagine, 1.5 mM glutamine) without serum. This

medium was supplemented with 80 µM tryptophan (T-0254 from Sigma) or PBS depending on the experimental conditions. To analyze TDO half-life, 50 µg/mL cycloheximide was directly diluted in the medium. Other inhibitors (*Compounds and Inhibitors for Cell Culture*) used to study TDO stability were added at the same time. TDO stability was evaluated by harvesting the cells at different time points. Cells were directly scraped and frozen to avoid adding tryptophan with trypsin and medium. For Fig. 1E, A172 cells were incubated in IMDM for 72 h until complete tryptophan exhaustion, and used without further incubation in fresh medium (starting point). For Fig. 1C and D and Fig. 5A, A172 cells were cultured in medium containing tryptophan for 1 wk until the exhaustion of tryptophan to start the experiment with a low level of TDO. Then, 6×10^5 A172 cells for each condition were incubated in IMDM medium containing 80 µM tryptophan complemented with serum and non-essential amino acids. 680C91 or alpha-methyl-tryptophan were directly added to the medium (see *Compounds and Inhibitors for Cell Culture*). Cells were harvested by scraping after 6, 12, 24, 48, 72, and 96 h. In parallel, tryptophan and kynurenine concentrations were analyzed in the supernatant by high performance liquid chromatography (HPLC).

Tryptophan and Kynurenine Dosage by HPLC. Cell culture supernatant or mouse serum was analyzed by HPLC as described (17). Time 0 for cell lines corresponds to initial concentrations of tryptophan and kynurenine in medium alone.

Hypoxia. A172 cells (6×10^5) were cultured in IMDM medium containing 80 µM tryptophan in normoxia (20% oxygen) or in hypoxia (1% oxygen) in Whitley H35 Hypoxystation for 24 h.

Dominant-Negative Forms of CLRs. HEK293 hTDO c119 cells (5×10^6) that constitutively express TDO2 were plated and cultured in IMDM medium containing 80 µM tryptophan, as described for A172 cells. The next day, the cells were transiently transfected with pcDNA3 vectors encoding the dominant-negative forms of cullins with a FLAG-tag generated by the group of Wade Harper (26) and obtained from Addgene (#15818-15823). For transfection, we used Turbofect transfection reagent (R0532, Thermo Fisher) following their instructions (20 µg DNA with 30 µl Turbofect). To stabilize the TDO protein in transfected cells, medium for transfection was complemented with 1 mM tryptophan (degradation of tryptophan is particularly fast in transfected cells). Twenty-four hours after the transfection, the cells were washed with PBS to remove tryptophan and separated in five equal fractions. A first aliquot was harvested to evaluate the abundance of TDO at time 0 (starting point). The rest of the culture was incubated in IMDM medium without L-tryptophan supplemented with 80 µM tryptophan or PBS and 50 µg/mL cycloheximide. TDO stability was evaluated by harvesting the cells after 1, 2, or 4 h of incubation.

RT-qPCR. Total RNA was extracted from cells with the NucleoSpin RNA kit from Macherey Nagel (#740955) and was retrotranscribed to complementary DNA (cDNA) by using the RevertAid kit from Thermo Fisher (K1691). For mouse experiments, livers were crushed in the lysis buffer of the RNA extraction kit using a TissueLyser LT (Qiagen). qPCR experiments were performed with the Takyon Rox probe core kit dTTP from Eurogentec (UF-RPCT-C0201) in a StepOnePlus thermal cycler (Applied Biosystems).

PCR conditions are provided in *SI Appendix, Supplementary Information*.

Western Blot (Denaturing Gel). Cells were lysed in homemade lysis buffer (0.5% Nonidet P-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) supplemented with cComplete Protease Inhibitor Mixture (#000000011697498001, Sigma). Lysates were homogenized by passing solutions through a 30G syringe and, if required, by sonicating or incubating them at 100 °C for 15 min. To remove cellular debris, samples were then centrifuged at 9,500 g for 10 min. Protein concentrations were measured by the bicinchoninic acid assay (BCA) (#23225, Pierce). Then, proteins (15 or 20 µg) were heated at 70 °C for 10 min with NuPAGE LDS (lithium dodecyl sulfate) Sample Buffer (NP0007, Thermo Fisher) and NuPAGE Sample Reducing Agent (NP0009, Thermo Fisher). Proteins were then separated by electrophoresis on denaturing NuPAGE 4 to 12% Bis-Tris gels (NP0321BOX and WG1402Box, Novex) in 3-(N-morpholino)propanesulfonic acid (MOPS) running buffer (NP0001-02, Novex) supplemented with NuPage antioxidant at 1/1,000 (NP0005, Thermo Fisher). Samples were transferred on nitrocellulose membrane (IB23001, Thermo Fisher) by using the iBlot Dry Blotting System (Thermo Fisher). The transfer program was the following: 1 min at 20 V, 4 min at 23 V, and 2 min at 25 V. Nitrocellulose membranes were blocked in PBS or Tris-buffered saline (TBS) containing 0.1% Tween and 5% milk and then probed with the primary antibodies in PBS/TBS with 0.1% Tween and

5% BSA at 4 °C overnight (1 h at room temperature for anti- β -actin). After washing, membranes were then probed with secondary antibodies conjugated with horseradish peroxidase in the same buffer at room temperature for 1 h. Proteins were revealed with the chemiluminescent SuperSignal West Pico substrate (#34578, Pierce) and, if required, with the Femto substrate (#34096, Pierce). Pictures were captured with the Fusion FX camera from Vilbert Lourmat. The different proteins were stained on the same membrane, which was stripped with Restore PLUS Western Blot Stripping Buffer (#46430, Thermo Fisher) for 10 to 15 min. The western blot quantification was achieved with ImageJ.

Whole livers were crushed in Pierce radioimmunoprecipitation assay (RIPA) buffer (#89901, Thermo Fisher) with Halt Protease and Phosphatase Inhibitor Mixture (#78446, Thermo Fisher) using the TissueLyser LT (Qiagen). Lysates were homogenized with 25-G needles, and the samples shaken for 30 min at 4 °C and then centrifuged for 10 min at 20,000 g. Protein concentrations were measured by the BCA assay (#23225, Pierce). The samples were heated at 95 °C for 5 min with a homemade loading buffer 6 $\times$ (bromophenol blue 0.06% wt/vol, SDS 12% wt/vol, glycerol 47%, dithiothreitol (DTT) 9.3% wt/vol, and Tris 60 mM pH 6.8). Twenty micrograms of proteins were then separated by electrophoresis on denaturing NuPAGE 4 to 12% Bis-Tris gels (NP0321BOX and WG1402Box, Novex) in MOPS SDS Running Buffer (NP0001-02, Novex). Transfer and blocking steps were performed as described above for cell lines. Nitrocellulose membranes were cut in two pieces around 70 kDa and incubated with the primary antibodies at room temperature for 2 h and, after washing, with secondary antibodies for 1 h.

Western Blot (Native Gel). To prevent protein denaturation, all the following steps were performed at 4 °C and without SDS. Cells were lysed in non-denaturing lysis buffer composed of NativePAGE sample buffer (BN20032, Invitrogen), Halt Protease and phosphatase Inhibitor Mixture (#78446 Thermo Fisher), and 1% of n-dodecyl- β -D-maltoside (BN20051, Invitrogen). Tryptophan, alpha-methyl-tryptophan, or PBS was added in the lysis buffer at the same concentration than in the culture medium. Samples were then centrifuged at 12,500 g for 1 h. Protein concentration was evaluated in the supernatant by the BCA assay (#23225, Pierce). Then, proteins (15 or 20 μ g) were loaded on NativePage Bis-Tris gel 4 to 16% (Invitrogen, BN2111BX10) with NativePage G250 buffer (BN20041, Invitrogen). Migration was performed at room temperature in precooled buffer as described in the manual of the NativePAGE Novex Bis-Tris Gel from Life Technology (MAN0000557). We used the system of the two cathode buffers. Samples were transferred on polyvinylidene difluoride (PVDF) membranes (IB401001, Thermo Fisher) by using the iBlot Dry Blotting System (Thermo Fisher) with a transfer program of 7 min at 20 V. To fix the proteins, the membranes were incubated in 8% acetic acid for 15 min. Then, to remove the coomassie blue, the membranes were briefly dipped in methanol (100%). Blocking and next steps were performed like for the denaturing western blot.

Antibodies for Western Blot. Cyclin D1 mouse monoclonal antibody (DCS-6 MA124750 from Thermo Fisher), polyubiquitinated conjugates mouse monoclonal antibody (FK1 from Enzo), and FLAG-M2 mouse monoclonal antibody (F1804 from Sigma) were diluted at 1/1,000. Hypoxia-inducible factor 1- α (HIF-1 α) rabbit polyclonal antibody (#10006421 from Cayman) was used at 1/2,000. Actin mouse monoclonal antibody (A5441 from Sigma) and vinculin mouse monoclonal antibody (hVIN-1, V9131 from Sigma-Aldrich) were diluted at 1/10,000. Three antibodies were used to detect TDO by western blot. Specificity of the TDO homemade antibodies was previously validated on human cells or tissues and mouse livers. Human TDO protein was revealed with the TDO mouse monoclonal antibody clone III (homemade) at 1 μ g/mL (10). Mouse TDO protein was detected with the mouse anti-TDO clone V at 1 μ g/mL (homemade) (10). To analyze the polyubiquitination and the native conformation of TDO, we used the TDO rabbit polyclonal antibody (HPA039611 from Sigma) at 1/1,000. Anti-rabbit horseradish peroxidase (HRP)-linked IgG antibody (#7074 from Cell Signaling) and anti-mouse HRP-linked IgG antibody (#405306 from BioLegend) were used at 1/2,500. Anti-mouse HRP-linked IgG antibody (HAF007 from R&D Systems) and anti-mouse HRP-linked IgM antibody (sc-2973 Santa Cruz Biotechnology) were used at 1/5,000 (excepted for actin: 1/15,000).

Mice. C57BL/6J Ola Hsd mice were purchased from Envigo. All mice were bred under specific pathogen-free conditions and handled according to national guidelines for animal care.

Gavage and Tissue Preparation. For gavage with L-tryptophan, mice were starved for 48 h and then force-fed with L-tryptophan 200 mg/kg body weight (Sigma, #93659, dissolved in water at pH 3 and heated at 95 °C until

complete dissolution). Mice were dissected after 30 min, 1, 2, and 4 h (four mice per group). For gavage with alpha-methyl-DL-tryptophan, mice were starved for 24 h and then force-fed with alpha-methyl-DL-tryptophan 500 mg/kg body weight (Sigma, #M8377, dissolved in water at pH 3 and heated at 95 °C until complete dissolution). Mice were dissected after 30 min, 1, 2, 4, 8, 12, and 18 h (four mice per group). In both cases, control mice were starved but not force-fed (Time 0). Around 300 μ L of blood were collected by retro-orbital bleeding; after blood coagulation at room temperature, the serum was retrieved after centrifugation. The livers were dissected immediately after bleeding. Each liver was cut in three parts: two pieces were frozen for RNA or protein extraction, and the third piece was fixed with 4% formaldehyde (overnight at 4 °C) and embedded in paraffin using the Vacuum Infiltration Processor (Tissue-Tek).

Immunohistochemistry Using Chimeric Mouse–Rabbit mAb V. To detect TDO on mouse tissues, we replaced the Fc portion of the TDO clone V antibody (10) with that of a rabbit IgG to avoid detection of endogenous immunoglobulins with the secondary antibodies. For details on the construction of the chimeric antibody, see Hoffmann et al. (36). Immunostaining was performed on 5- μ m-thick paraffin sections as previously described (36). For TDO detection, the primary mouse–rabbit chimeric anti-TDO monoclonal antibody (mAb) V was diluted at 5 μ g/mL in immunohistochemistry (IHC) diluent (ADI-950-244-0250, Enzo) and incubated for 1 h. Secondary staining was performed with EnVision+ HRP goat anti-rabbit Dako antibody (K4003, Agilent).

Gel Filtration Chromatography. A172 cells (50×10^6) for each condition were cultured in IMDM medium containing 80 μ M tryptophan. After exactly 16 h, the cells were washed three times with PBS to remove the tryptophan. Then, the cells were incubated in IMDM medium without L-tryptophan, complemented with 80 μ M tryptophan or PBS, 50 μ g/mL cycloheximide, and 1 μ M bortezomib for 6 h. The cells were lysed in native lysis buffer (20 mM Tris, 100 mM NaCl, 1 μ M bortezomib, and 80 μ M tryptophan or PBS) with a Dounce. To maintain the TDO conformation, lysates were not frozen, and following steps were performed at 4 °C. Samples were centrifuged at 11,000 g for 20 min to remove the cellular debris, and the supernatant was recovered and centrifuged a second time at 100,000 g for 1 h. Proteins of the supernatant were concentrated and precipitated with 80% (vol/vol) of a saturated ammonium sulfate solution. Pellets were resuspended in 500 μ L lysis buffer. Separation of proteins was performed on Superdex 200 HR 10/30 (Amersham) with a flow rate of 1 mL/min, and fractions of 500 μ L were collected. One hundred microliters of each fraction were lyophilized and analyzed by western blot.

TUBE Pull Down. A172 cells (4×10^6) were cultured in IMDM medium containing 80 μ M tryptophan. The next day, cells were washed once with PBS and incubated in fresh medium containing 80 μ M tryptophan. After exactly 16 h, the cells were washed three times with PBS to remove the tryptophan. The cells were incubated in IMDM medium without L-tryptophan, complemented with 1 μ M bortezomib and 500 μ M tryptophan or PBS for 1 h, 2 h, or 4 h. An increase in the tryptophan concentration was required to prevent TDO polyubiquitination. A172 cells were lysed in homemade lysis buffer (100 mM sodium phosphate buffer pH 7.4, 2 mM EDTA, 1% Nonidet P-40, 100 mM N-Ethylmaleimide, 1 mM DTT, Halt Protease, Phosphatase Inhibitor Mixture [#78446 Thermo Fisher], and 1 μ M bortezomib) complemented or not with tryptophan according to the cell culture condition. Ubiquitin conjugates were purified using GST-1xUBA^{ubq} ubiquitin affinity reagent provided by the group of M. G.-H. and analyzed by western blot as described (20). We used 20 μ g GST-1xUBA^{ubq} and 10 μ M Glutathione Sepharose 4B beads (17-0756-01, GE Healthcare) for each sample.

Construction and Transfection of Plasmids Producing Deleted and Mutated TDO Proteins. A pEF6/V5-His expression plasmid carrying a 1.7kb *TDO2* cDNA was used as template (17). Mutagenesis protocol is available in [SI Appendix, Supplementary Information](#). HEK293 cells were transfected with the mutated vectors and the Turbofect transfection reagent (T0532, Thermo Fisher) following the instructions of the manufacturer (4 μ g DNA for 6 μ L Turbofect). Transfected cells were selected with 10 μ g/mL blasticidin. TDO stability was evaluated as described in *Analysis of TDO Stability*.

Statistical Methods. Our experiments were performed independently at least three times (except for the gel filtration chromatography and hypoxia experiments: two times). When we compared the same cells in western blot, qPCR, and HPLC experiments, only one representative experiment is shown. Statistical analysis was done with GraphPad 5 software. To compare the *TDO2* mRNA levels in A172 cells cultured with and without tryptophan over time, we applied a two-way ANOVA (Fig. 1B). For the analysis of the 680C91 or alpha-methyl-tryptophan effects on TDO activity, we compared the

tryptophan and kynurenine concentrations over time with and without inhibitor by performing a two-way ANOVA (Figs. 1D and 5A). For mouse experiments, a multiple unpaired *t* test was applied to compare mice over time for TDO2 expression, TDO protein level, and TDO activity (Figs. 2A, C, and D and 5B and C). The Welch correction was applied for samples with unequal variances. The *P* values < 0.05 were considered statistically significant. For all figures, the *P* values are annotated as follows: NS, *P* > 0.05; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

Data Availability. All study data are included in the article and/or supporting information.

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