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

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Short Title

TDO ubiquitination controls tryptophanemia

One Sentence Summary

A degron masked by tryptophan triggers TDO ubiquitination to contain tryptophan catabolism

Summary

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 non-catalytic exo-sites, resulting in rapid catabolism of tryptophan. In low tryptophan, the lack of tryptophan binding in the exo-sites destabilizes the tetramer into inactive monomers and dimers, and unmasks a 4-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 non-metabolizable 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.

Significance Statement

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 non-catalytic 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.

Introduction

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 ± 15µM (mean ± 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 8 to 9-fold 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 two hours

(Fig. 1A), while *TDO2* 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 100µM, we observed that the level of TDO protein correlated 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 sacrificed them at different time points. TDO levels in the liver increased within thirty minutes 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 hours in the presence of tryptophan, decreased to 1 hour 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 multi-protein 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, such as a 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 non-metabolizable 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 panels). 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 non-catalytic 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 exo-sites 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 exo-site (28). These results suggested that the binding of tryptophan or alpha-methyl-tryptophan to these exo-sites 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 sacrificed 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 60µM to 20µM (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 exo-sites stabilizes TDO, thereby increasing tryptophan catabolism.

Next, we explored how the absence of tryptophan induces TDO polyubiquitination and degradation. TDO has a tetrameric 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 exo-sites 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 192kDa, 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 96kDa to 48kDa, 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 exo-sites, 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 non-catalytic 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 exo-sites. We therefore deleted a series of residues located either at the interface between the monomers, or in the exo-site. 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 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 98-105 (Fig. 6A). According to the crystal structure, these residues are included in the TDO exo-site 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 200-213 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 200-213 deletion did not stabilize the tetrameric conformation (SI Appendix, Fig. S3). Therefore, amino acids 200-213 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 208-211, which constitute the core of the exo-site, stabilized TDO in the absence of tryptophan, while mutation of other residues, such as 204-206, did not (Fig. 6D). Together, these results suggest that the exo-site of each TDO subunit contains a degron, with sequence $WLER_{208\text{-}211}$, 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 post-translational 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 non-catalytic exo-sites 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 exo-sites might retard proteasome-mediated degradation, the authors did not describe this mechanism in molecular terms.

Our results provide novel molecular insights into this process, by showing that tryptophan binding to exo-sites stabilizes the homo-tetrameric 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 exo-sites contain a 4-amino acid degron that targets TDO for degradation by the proteasome. This degron, with sequence $WLER_{208\text{-}211}$, is masked by the binding of tryptophan in the exo-sites, 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 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, also called SCF, 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 post-translational 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 ±15µM) 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). 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

Cells lines

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. HEK293 cell line was purchased from ATCC in 2014 (ATCC-CRL-1573). HEK293 hTDO cl119 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), non-essential amino acids (0.55mM L-arginine, 0.24mM L-asparagine, 1.5mM glutamine) and antibiotics (100µg/ml streptomycin, 100IU/ml penicillin). To analyze TDO stability, cells were incubated in IMDM medium without L-tryptophan (ME130013L1, Life Technologies) complemented with non-essential amino acids (0.55mM L-arginine, 0.24mM L-asparagine, 1.5mM 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 80µM or 500µM, previously dissolved in PBS) was obtained from Sigma Aldrich (T-0254). Cycloheximide (50µg/ml, dimethyl sulfoxide [DMSO]) was bought from Cell signaling (#2112S). Bortezomib (1µM, DMSO) was obtained from Santa-Cruz Biotechnology (sc-217785). MLN7243 (10µM, DMSO) and MLN4924 or pevonedistat (5µg/ml, DMSO) were obtained from Active Biochem (A-1384 and A-1139, respectively). A838241 (now commercialized by Ambeed) (10µM, DMSO) was provided by Patrick T. Gunning under the name "Compound1" and was produced by Takeda (35). Alpha-methyl-DL-tryptophan (500µM, PBS or methanol 50%, racemic mixture) was ordered from Sigma (M8377) and from Angene Chemical (#153-91-3). 680C91 (33µM, 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 3ml of IMDM medium containing 80µM tryptophan (#21980-032 from Life Technologies)

complemented with 10% FBS (#7524 from Sigma) and non-essential amino acids (0.55mM L-arginine, 0.24mM L-asparagine, 1.5mM glutamine). The next day, cells were washed once with PBS and incubated in 3ml of fresh medium containing 80µM of tryptophan. This step is essential to increase and stabilize TDO protein with tryptophan before starting the experiments. Exactly 16h 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 2ml of IMDM medium without L-tryptophan, complemented with non-essential amino acids (0.55mM L-arginine, 0.24mM L-asparagine, 1.5mM glutamine) without serum. This medium was supplemented with 80µM tryptophan (T-0254 from Sigma) or PBS depending on the experimental conditions. To analyze TDO half-life, 50µg/ml cycloheximide were 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. 1*E,* 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. 1*C* and *D* and Fig. 5*A*), A172 cells were cultured in medium containing tryptophan for one week until the exhaustion of tryptophan to start the experiment with a low level of TDO. Then, 6 x 10^5 A172 cells for each condition were incubated in IMDM medium containing 80µM of tryptophan complemented with serum and non-essential amino acids. 680C91 or alpha-methyl-tryptophan were directly added to the medium (see references in Compounds section). Cells were harvested by scraping after 6, 12, 24, 48, 72 and 96 hours. 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 x 10^5) were cultured in IMDM medium containing 80µM of tryptophan in normoxia (20% oxygen) or in hypoxia (1% oxygen) in Whitley H35 Hypoxystation for 24h.

Dominant negative forms of cullin RING ubiquitin ligases

HEK293 hTDO cl119 cells (5 x 10^6) that constitutively express TDO2 were plated and cultured in IMDM medium containing 80µM of 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, Thermofisher) following their instructions (20µg of DNA with 30µl of Turbofect). To stabilize the TDO protein in transfected cells, medium for transfection was complemented with 1mM of 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 80µM of tryptophan or PBS and 50µg/ml of cycloheximide. TDO stability was evaluated by harvesting the cells after 1, 2 or 4 hours 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 ThermoFisher (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% NP40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) supplemented with cOmplete Protease Inhibitor Cocktail (#000000011697498001, Sigma). Lysates were homogenized by passing solutions through a 30G syringe and if required by sonicating or incubating them at 100°C for 15min. To remove cellular debris, samples were then centrifuged at 9,500g for 10 minutes. Protein concentrations were measured by the bicinchoninic acid assay (BCA) (#23225, Pierce). Then, proteins (15 or 20µg) were heated at 70°C for 10 minutes with NuPAGE LDS (lithium dodecyl sulfate) Sample Buffer (NP0007, ThermoFisher) and NuPAGE Sample Reducing Agent (NP0009, ThermoFisher). Proteins were then separated by electrophoresis on denaturing Nu-PAGE 4-12% 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, ThermoFisher). Samples were transferred on nitrocellulose membrane (IB23001, ThermoFisher) by using the iBlot Dry Blotting System (ThermoFisher). The transfer program was: 1 minute at 20 V, 4 minutes at 23 V and 2 minutes 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 (1h 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 1h. 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, ThermoFisher) for 10-15 minutes. The western blot quantification was achieved with ImageJ.

Whole livers were crushed in Pierce radioimmunoprecipitation assay (RIPA) buffer (#89901, ThermoFisher) with Halt Protease and Phosphatase Inhibitor Cocktail (#78446, ThermoFisher) using the TissueLyser LT (Qiagen). Lysates were homogenized with 25G needles and the samples shaken for 30 minutes at 4°C and then centrifuged for 10 minutes at 20,000 g. Protein concentrations were measured by the BCA assay (#23225, Pierce). The samples were heated at 95°C for 5 minutes with a homemade loading buffer 6x (bromophenol blue 0.06% w/v, SDS 12% w/v, glycerol 47%, dithiothreitol (DTT) 9.3% w/v, Tris 60mM pH 6.8). Twenty micrograms of proteins were then separated by electrophoresis on denaturing Nu-PAGE 4%-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 70kDa and incubated with the primary antibodies at room temperature for 2 hours 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 Cocktail (#78446 ThermoFisher) and 1% of n-dodecyl-β-D-maltoside (BN20051, Invitrogen). Tryptophan, alpha-methyl-tryptophan or PBS were added in the lysis buffer at the same concentration than in the culture medium. Samples were then centrifuged at 12,500g for 1h. Protein concentration was evaluated in the supernatant by the BCA assay (#23225, Pierce). Then, proteins (15 or 20µg) were loaded on NativePage Bis-Tris gel 4-16% (Invitrogen, BN2111BX10) with NativePage G250 buffer (BN20041, Invitrogen). Migration was performed at room temperature in pre-cooled buffer as described in the manual of the Native PAGE 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, Thermofisher) by using the iBlot Dry Blotting System (ThermoFisher) with a transfer program of 7 minutes at 20V. To fix the proteins, the membranes were incubated in 8% acetic acid for 15 minutes. 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 ThermoFisher), polyubiquitinated conjugates mouse monoclonal antibody (FK1 from Enzo), FLAG-M2 mouse monoclonal antibody (F1804 from Sigma) were diluted at 1/1,000. Hypoxia-inducible factor 1-alpha (HIF-1α) 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 home made 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 1µg/ml (10). Mouse TDO protein was detected with the mouse anti-TDO clone V at 1µg/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 48h and then force-fed with L-tryptophan 200mg/kg body weight (Sigma, #93659, dissolved in water at pH 3 and heated at 95°C until complete dissolution). Mice were dissected after 30 minutes, 1, 2, and 4 hours (4 mice per group). For gavage with alpha-methyl-DL-tryptophan, mice were starved for 24h and then force-fed with alpha-methyl-DL-tryptophan 500mg/kg body weight (Sigma, #M8377, dissolved in water at pH 3 and heated at 95°C until complete dissolution). Mice were dissected after 30 minutes, 1, 2, 4, 8, 12, and 18 hours (4 mice per group). In both cases, control mice were starved, but not force-fed (Time

0). Around 300 µl 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 3 parts: 2 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 5µm-thick paraffin sections as previously described (36). For TDO detection, the primary mouse-rabbit chimeric anti-TDO monoclonal antibody (mAb) V was diluted at 5µg/ml in immunohistochemistry (IHC) diluent (ADI-950-244-0250, Enzo) and incubated for 1 hour. Secondary staining was performed with EnVision+ HRP goat anti-rabbit Dako antibody (K4003, Agilent).

Gel filtration chromatography

A172 cells (50 x 10^6) for each condition were cultured in IMDM medium-containing 80µM of tryptophan. After exactly 16h, 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 80µM of tryptophan or PBS, 50µg/ml cycloheximide and 1µM bortezomib for 6h. The cells were lysed in native lysis buffer (20mM Tris, 100mM NaCl, 1µM bortezomib and 80µM tryptophan or PBS) with a Dounce. To maintain the TDO conformation, lysates were not frozen and followings step were performed at 4°C. Samples were centrifuged at 11,000g for 20 min to remove the cellular debris, and the supernatant was recovered and centrifuged a second time at 100,000g for 1 hour. Proteins of the supernatant were concentrated and precipitated with 80% (v/v) of a saturated ammonium sulfate solution. Pellets were resuspended in 500µl of lysis buffer. Separation of proteins was performed on Superdex 200 HR 10/30 (Amersham) with a flow rate of 1ml/min and fractions of 500µl were collected. One hundred microliters of each fraction were lyophilized and analyzed by western blot.

TUBE Pull Down

A172 cells (4 x 10^6) were cultured in IMDM medium containing 80µM of tryptophan. The next day, cells were washed once with PBS and incubated in fresh medium containing 80µM tryptophan. After exactly 16h, the cells were washed three times with PBS to remove the tryptophan. The cells

were incubated in IMDM medium without L-tryptophan, complemented with 1µM of bortezomib and 500µM of tryptophan or PBS for 1h, 2h or 4h. An increase in the tryptophan concentration was required to prevent TDO polyubiquitination. A172 cells were lysed in homemade lysis buffer (100mM sodium phosphate buffer pH 7.4, 2mM EDTA, 1% NP40, 100mM N-Ethylmaleimide, 1mM DTT, Halt Protease and phosphatase Inhibitor Cocktail (#78446 ThermoFisher) and 1µM 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 Mads Gyrd-Hansen and analyzed by western blot as described (20). We used 20µg of GST-1xUBA^{Ubq} and 10µM of 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 (R0532, Thermofisher) following the instructions of the manufacturer (4µg of DNA for 6µl of Turbofect). Transfected cells were selected with 10µg/ml blasticidin. TDO stability was evaluated as described in *Analysis of TDO Stability.*

Statistical method

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 (Fig. 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 (Fig. 2A, C and D; Fig. 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, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.*

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Disclosure of Potential Conflicts of Interest

B.J. Van den Eynde is co-founder of iTeos Therapeutics. The other authors declared no potential conflicts of interest.

Data and materials availability

- Supplementary Information is available for this paper.
- All data are available in the manuscript or the supplementary materials.

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Figure Legends

Figure 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 80µM 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 vs without tryptophan (Two-way ANOVA).

(C) A172 cells were incubated in medium containing tryptophan (80µM), and treated or not with TDO inhibitor (TDOi) 680C91 (33µM), 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 72h, until the exhaustion of tryptophan (start). Cells were then incubated for 6h 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.

Figure 2. Tryptophanemia controls liver TDO protein levels

(A) C57BL/6 mice were starved for 48 hours to stabilize the systemic concentration of tryptophan and the level of TDO protein in the liver, before receiving tryptophan 200mg/kg body weight by oral gavage (n=20). Four mice were sacrificed 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) represent the results (Mean + 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 vs 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 show the 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).

Figure 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 (50µg/ml) with or without 80µM 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 0h.
(**B**) TDO stability was evaluated in the presence of cycloheximide (50µg/ml) and proteasome inhibitor, bortezomib (1µM), 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 500µM of tryptophan and with bortezomib (1µM) 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 µM tryptophan for the indicated times, in the presence of cycloheximide (50µg/ml) and MLN7243 (10µM), 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.

Figure 4. Role of Cullin RING ubiquitin ligase 1 (CRL1) in TDO degradation

(**A**) A172 cells were first cultured in medium containing tryptophan (start), and then incubated in medium with or without 80µM tryptophan for the indicated times. Cells were treated with cycloheximide (50µg/ml) and pevonedistat/MLN4924 (5µg/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 cl119) were transiently transfected with 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 (50µg/ml) in the presence or the absence of tryptophan (80µM). Protein levels of TDO and CULDN were evaluated by western blot with an 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.

Figure 5. Stabilization of the TDO tetrameric conformation by tryptophan and alpha-methyl-tryptophan

(**A)** A172 cells were first cultured in medium with tryptophan for one week, until the exhaustion of tryptophan. Cells were then incubated for 6-96h in tryptophan-containing medium (80µM) supplemented or not with alpha-methyl-tryptophan (500µM). TDO protein level was evaluated by western blot. One representative out of three independent experiments is shown on the left panel. The right panels show the kynurenine and tryptophan concentrations measured by HPLC in the supernatants. Mean + SD of triplicates. $*p < 0.05$ for medium with alpha-methyl-tryptophan vs without alpha-methyl-tryptophan (Two-way ANOVA).

(**B)** C57BL/6 mice starved for 24h received alpha-methyl-tryptophan 500mg/kg body weight by oral gavage (n=32). Four mice were sacrificed 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 vs initial situation (Unpaired t-test with Welch correction). One representative out of three independent experiments is shown.

(**C**) Systemic concentrations of tryptophan, kynurenine and alpha-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 µM tryptophan for 6 h, in the presence of cycloheximide (50µg/ml) and bortezomib (1µM). 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 (80µM), and then incubated for the indicated times in tryptophan-free medium with or without alpha-methyl-tryptophan (500µM), in the presence of cycloheximide (50µg/ml) and bortezomib (1µM). 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.

Figure 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 exo-site (TDO Δ200-213 or TDO Δ98-105). Cells were cultured initially in tryptophan-containing medium (start) and then incubated in medium with or without 80µM tryptophan for the indicated times. The left panel shows TDO protein levels evaluated by western blot and normalized to actin. The right panel shows the kynurenine and tryptophan concentrations measured by HPLC in the supernatants of the same cells cultured for 30h in tryptophan containing medium. Mean + SD of pentaplicates. ****$p <$ 0.0001 for TDO mutated proteins vs TDO WT (Unpaired t-test with Welch correction).

(**B**) Schematic structure of the TDO tetramer (PDB file ID: 5TIA) (28). Each monomer is labeled with a different color. The inset shows tryptophan binding to an exo-site. The purple, blue and red colors in the 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 exo-site.

(**C**) HEK293 cells transfected with TDO mutated by deletion of residues in the catalytic site (TDO Δ169-187) were tested as in (A).

(**D**) HEK293 cells transfected with TDO mutated by alanine substitutions in the exo-site (TDO mut208-11 or TDO mut204-206) were tested as in (A).

All these experiments were performed independently three times.

Figure 1

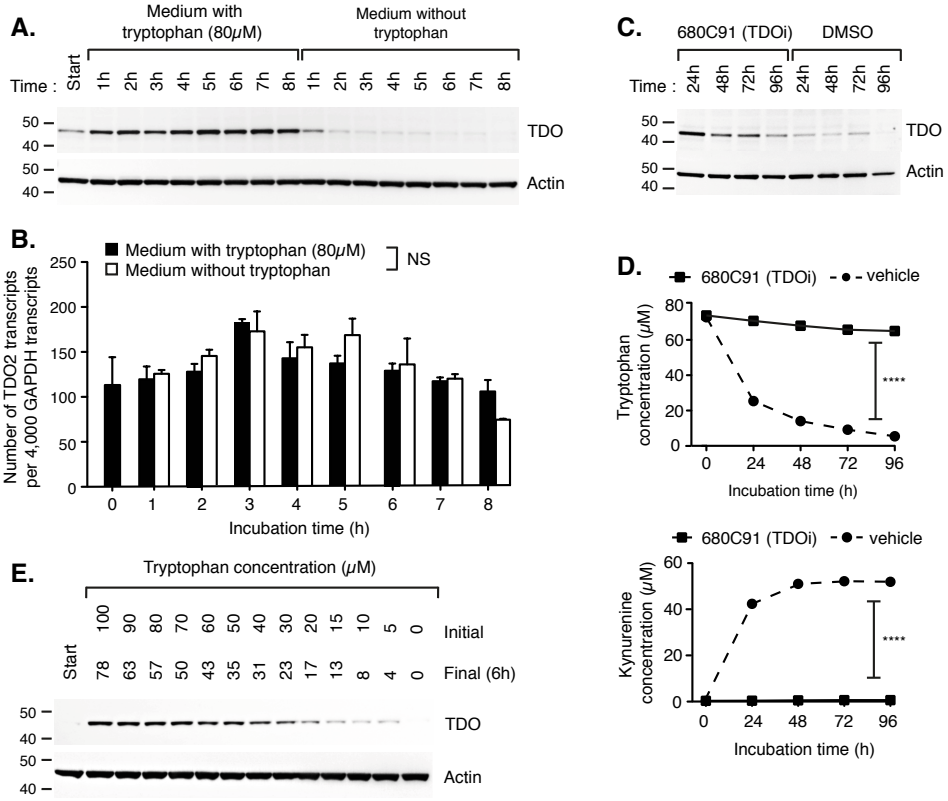

A.
Medium with tryptophan (80μM)
Medium without tryptophan
Time :
Start
1h 2h 3h 4h 5h 6h 7h 8h 1h 2h 3h 4h 5h 6h 7h 8h
50
40
TDO
Actin
B.
Medium with tryptophan (80μM)
Medium without tryptophan
NS
Number of TDO2 transcripts per 4,000 GAPDH transcripts
Incubation time (h)
C.
680C91 (TDOi)
DMSO
24h 48h 72h 96h 24h 48h 72h 96h
D.
680C91 (TDOi)
vehicle
Tryptophan concentration (μM)
****
Kynurenine concentration (μM)
E.
Tryptophan concentration (μM)
100 90 80 70 60 50 40 30 20 15 10 5 0
Initial
78 63 57 50 43 35 31 23 17 13 8 4 0
Final (6h)

Figure 2

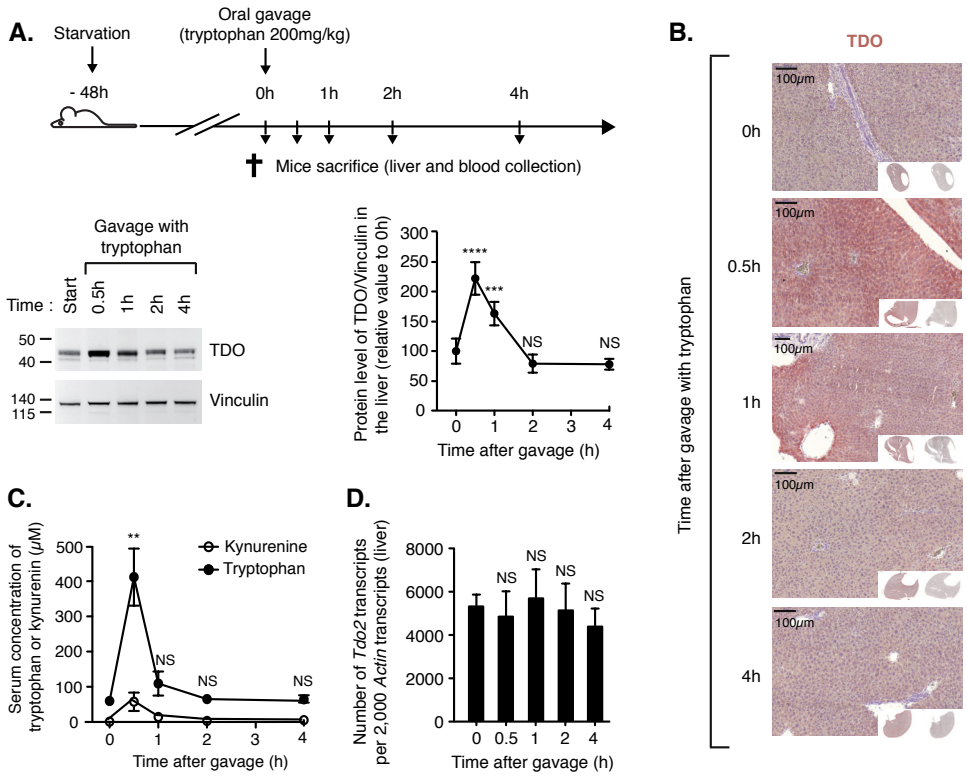

A.
Starvation
Oral gavage
(tryptophan 200mg/kg)
- 48h
0h
1h
2h
4h
Mice sacrifice (liver and blood collection)
Gavage with tryptophan
Time :
Start
0.5h
1h
2h
4h
50
40
TDO
140
115
Vinculin
Protein level of TDO/Vinculin in the liver (relative value to 0h)
Time after gavage (h)
B.
TDO
Time after gavage with tryptophan
0h
0.5h
1h
2h
4h
100μm
C.
Serum concentration of tryptophan or kynurenin (μM)
Kynurenine
Tryptophan
Time after gavage (h)
D.
Number of Tdo2 transcripts per 2,000 Actin transcripts (liver)
Time after gavage (h)

Figure 3

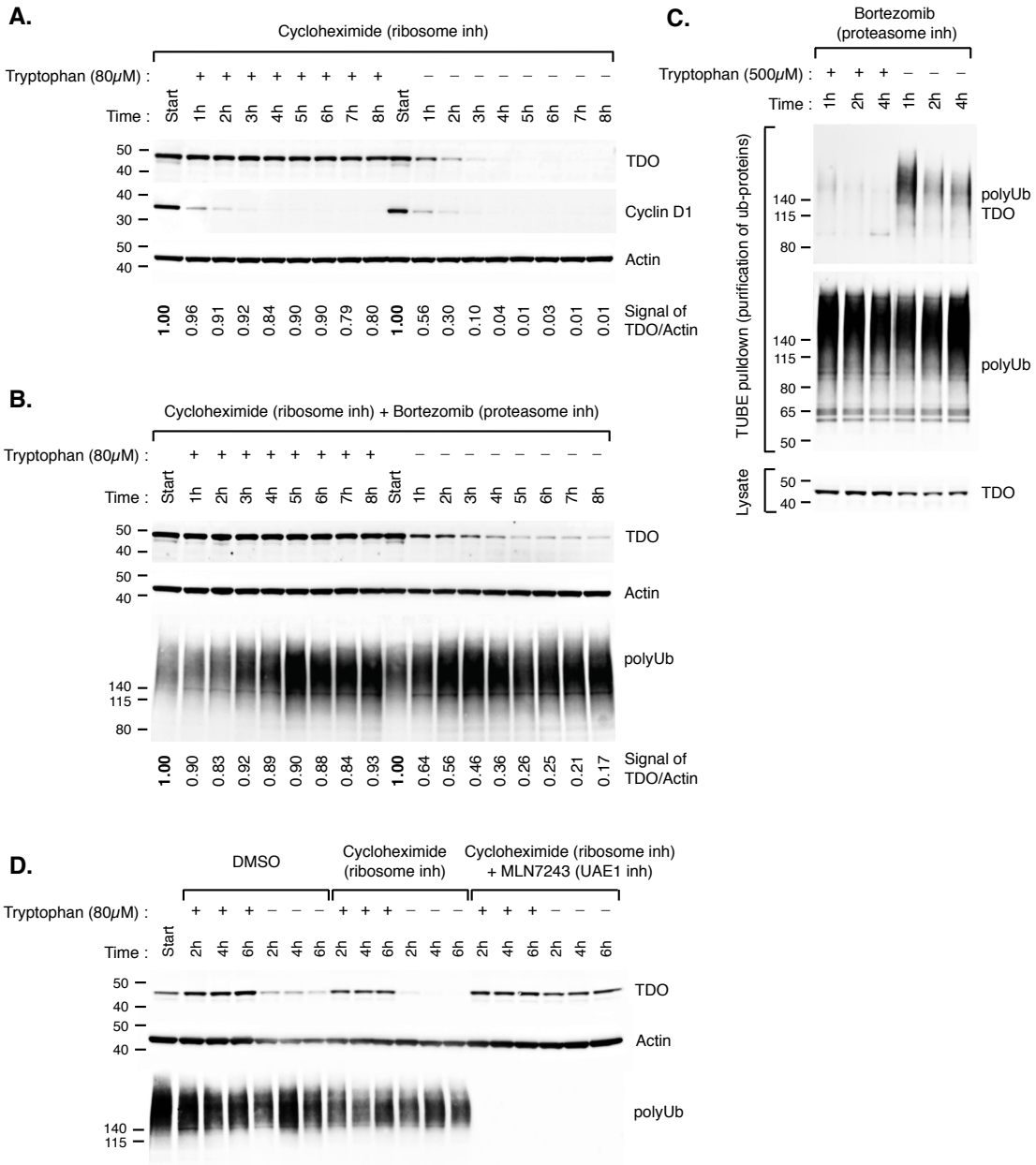

A.
Cycloheximide (ribosome inh)
Tryptophan (80μM) :
Time :
Start
1h
2h
3h
4h
5h
6h
7h
8h
TDO
Cyclin D1
Actin
Signal of TDO/Actin
1.00 0.96 0.91 0.92 0.84 0.90 0.90 0.79 0.80 1.00 0.56 0.30 0.10 0.04 0.01 0.03 0.01 0.01
B.
Cycloheximide (ribosome inh) + Bortezomib (proteasome inh)
Tryptophan (80μM) :
Time :
TDO
Actin
polyUb
Signal of TDO/Actin
1.00 0.90 0.83 0.92 0.89 0.90 0.88 0.84 0.93 1.00 0.64 0.56 0.46 0.36 0.26 0.25 0.21 0.17
C.
Bortezomib (proteasome inh)
Tryptophan (500μM) :
Time :
TUBE pulldown (purification of ub-proteins)
polyUb
TDO
polyUb
Lysate
TDO
D.
DMSO
Cycloheximide (ribosome inh)
Cycloheximide (ribosome inh) + MLN7243 (UAE1 inh)
Tryptophan (80μM) :
Time :
TDO
Actin
polyUb

Figure 4

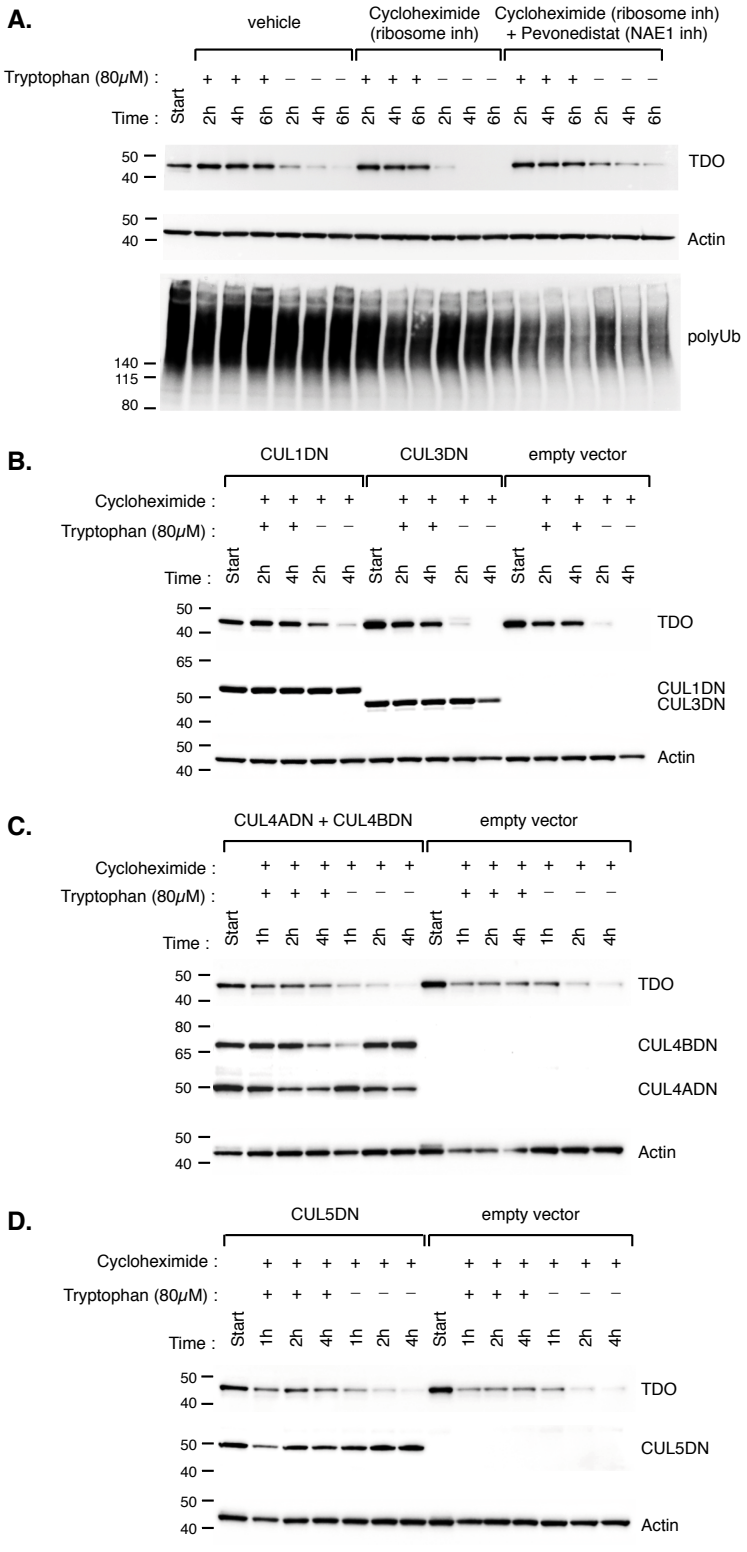

A.
vehicle
Cycloheximide (ribosome inh)
Cycloheximide (ribosome inh) + Pevonedistat (NAE1 inh)
Tryptophan (80μM) :
Time :
Start
2h
4h
6h
TDO
Actin
polyUb
B.
CUL1DN
CUL3DN
empty vector
Cycloheximide :
Tryptophan (80μM) :
Time :
TDO
CUL1DN
CUL3DN
Actin
C.
CUL4ADN + CUL4BDN
empty vector
Cycloheximide :
Tryptophan (80μM) :
Time :
1h
2h
4h
TDO
CUL4BDN
CUL4ADN
Actin
D.
CUL5DN
empty vector
Cycloheximide :
Tryptophan (80μM) :
Time :
TDO
CUL5DN
Actin

Figure 5

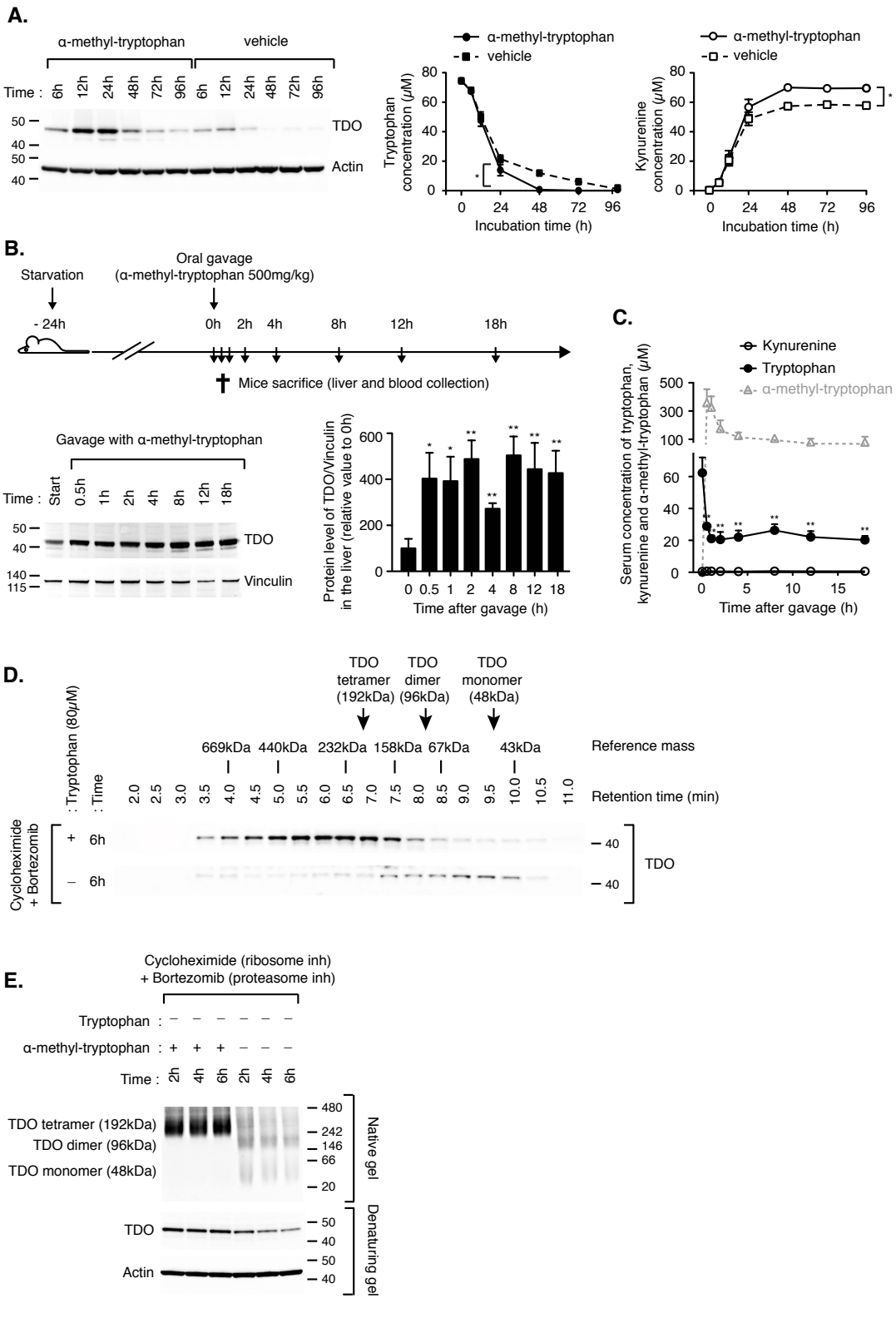
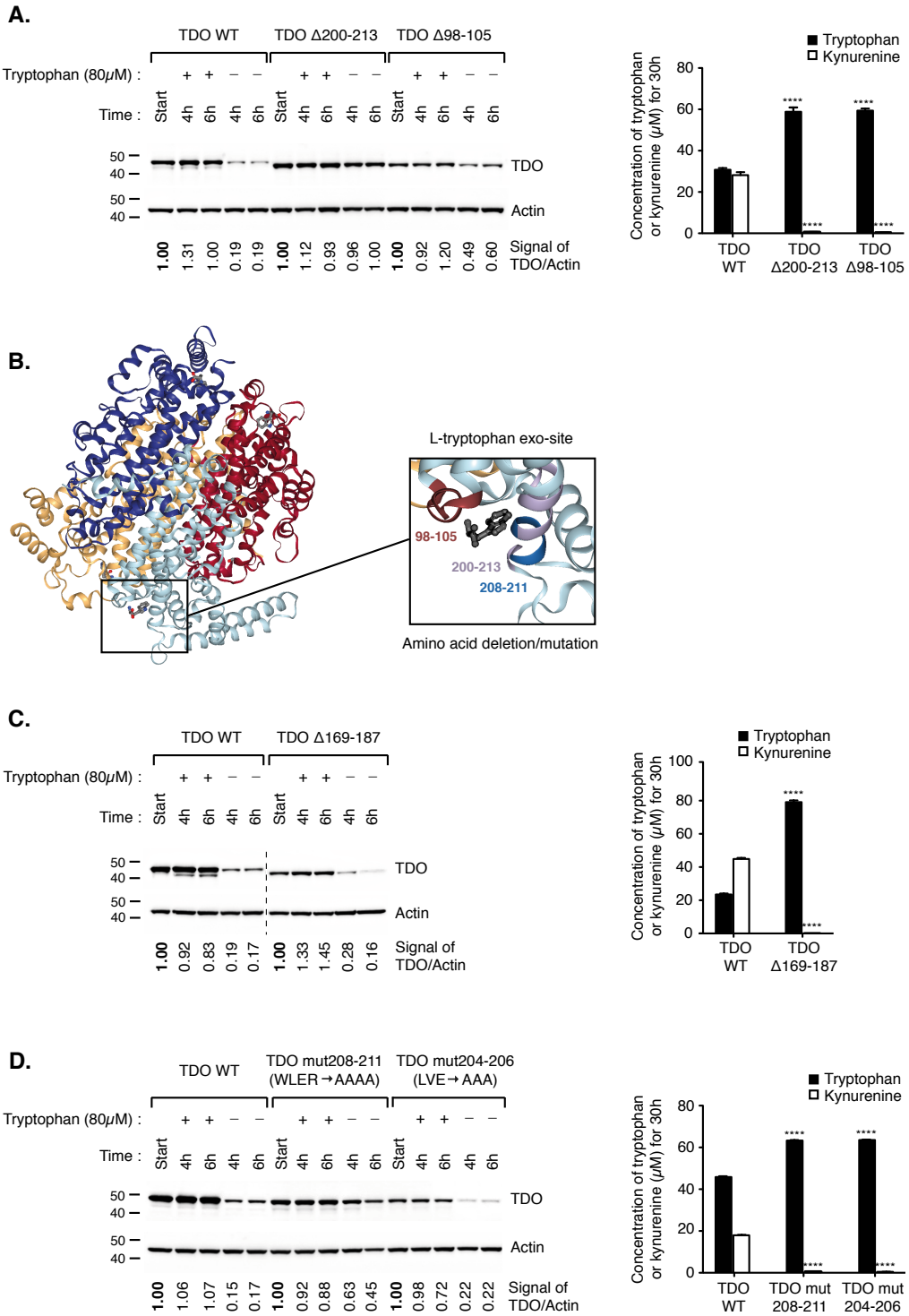

A.
α-methyl-tryptophan
vehicle
Time : 6h 12h 24h 48h 72h 96h 6h 12h 24h 48h 72h 96h
50
40
50
40
TDO
Actin
Tryptophan concentration (μM)
Incubation time (h)
Kynurenine concentration (μM)
Incubation time (h)
B.
Starvation
- 24h
Oral gavage
(α-methyl-tryptophan 500mg/kg)
0h 2h 4h 8h 12h 18h
Mice sacrifice (liver and blood collection)
Gavage with α-methyl-tryptophan
Time : Start 0.5h 1h 2h 4h 8h 12h 18h
50
40
TDO
140
115
Vinculin
Protein level of TDO/Vinculin in the liver (relative value to 0h)
Time after gavage (h)
C.
Kynurenine
Tryptophan
α-methyl-tryptophan
Serum concentration of tryptophan, kynurenine and α-methyl-tryptophan (μM)
Time after gavage (h)
D.
TDO tetramer (192kDa)
TDO dimer (96kDa)
TDO monomer (48kDa)
669kDa 440kDa 232kDa 158kDa 67kDa 43kDa
Reference mass
: Tryptophan (80μM)
: Time
2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0 10.5 11.0
Retention time (min)
Cycloheximide + Bortezomib
+ 6h
– 6h
40
40
TDO
E.
Cycloheximide (ribosome inh)
+ Bortezomib (proteasome inh)
Tryptophan : – – – – – –
α-methyl-tryptophan : + + + – – –
Time : 2h 4h 6h 2h 4h 6h
TDO tetramer (192kDa)
TDO dimer (96kDa)
TDO monomer (48kDa)
480
242
146
66
20
Native gel
TDO
Actin
50
40
50
40
Denaturing gel

Figure 6


A.
TDO WT
TDO Δ200-213
TDO Δ98-105
Tryptophan (80μM) :
Time :
Start
4h
6h
TDO
Actin
Signal of TDO/Actin
1.00 1.31 1.00 0.19 0.19
1.00 1.12 0.93 0.96 1.00
1.00 0.92 1.20 0.49 0.60
Tryptophan
Kynurenine
Concentration of tryptophan or kynurenine (μM) for 30h
TDO WT
TDO Δ200-213
TDO Δ98-105
B.
L-tryptophan exo-site
98-105
200-213
208-211
Amino acid deletion/mutation
C.
TDO WT
TDO Δ169-187
1.00 0.92 0.83 0.19 0.17
1.00 1.33 1.45 0.28 0.16
D.
TDO WT
TDO mut208-211 (WLER→AAAA)
TDO mut204-206 (LVE→AAA)
1.00 1.06 1.07 0.15 0.17
1.00 0.92 0.88 0.63 0.45
1.00 0.98 0.72 0.22 0.22
TDO mut 208-211
TDO mut 204-206

Supplementary Figures and Tables

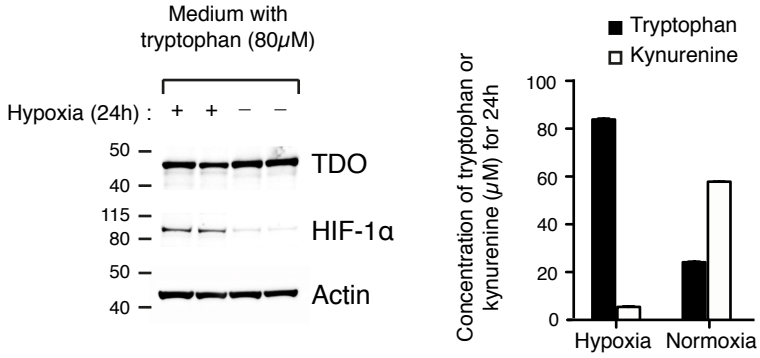


Figure S1. Hypoxia does not decrease TDO protein levels, while it reduces TDO catalytic activity.
A172 cells cultured in tryptophan-containing medium (80μM) were exposed to normoxia (20% oxygen) or hypoxia (1% oxygen) for 24h. TDO and HIF1α protein levels were analyzed by western blot. Stabilization of HIF1α confirmed effective hypoxic conditions. One representative experiment out of two independent is shown. The right panel illustrates the expected reduction of TDO catalytic activity in hypoxic conditions, with the measurement of tryptophan and kynurenine in the 24h supernatant of A172 cells.

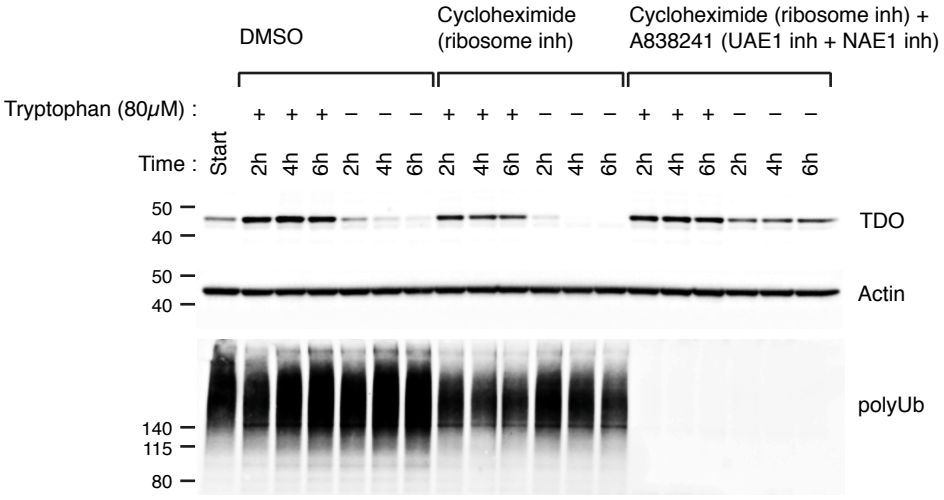


Figure S2 (related to Fig. 3D). Blocking polyubiquitination prevents TDO degradation in the absence of tryptophan.
A172 cells initially grown in tryptophan-containing medium (start) were then incubated in medium with or without 80μM tryptophan, in the presence of 50μg/ml cycloheximide and 10μM A838241, an inhibitor of E1 ubiquitin ligases and NEDD8 activating enzymes*. Western blots were performed to analyze the level of TDO protein and of polyubiquitinated proteins, as a control for E1 ubiquitin ligases inhibition. One representative experiment out of three independent is shown.

* Lukkarila, J. L. et al. Identification of NAE Inhibitors Exhibiting Potent Activity in Leukemia Cells: Exploring the Structural Determinants of NAE Specificity. ACS medicinal chemistry letters 2, 577-582, doi:10.1021/ml2000615 (2011).

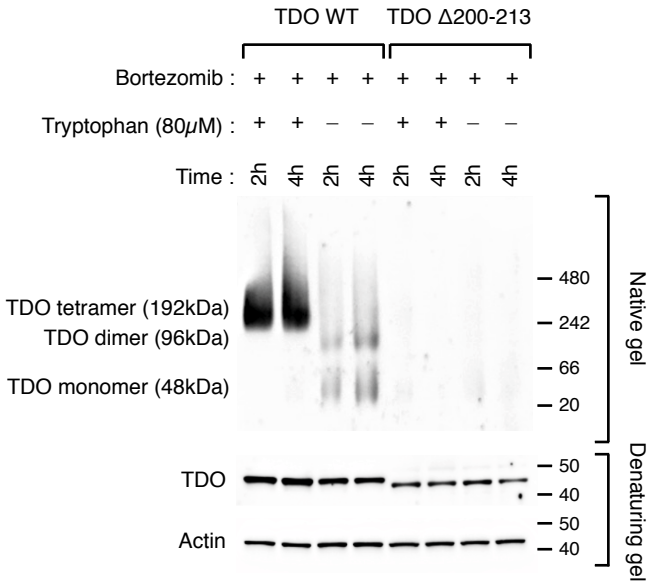


Figure S3 (related to Fig. 6). Deleting residues 200-213 in the exosite does not stabilize the tetrameric conformation of TDO.
HEK293 cells stably transfected with expression vectors encoding TDO WT or TDO Δ200-213 were incubated in medium with or without 80μM tryptophan in the presence of bortezomib (1μM). TDO molecular mass was evaluated by performing a western blot analysis in native (top) or denaturing conditions (bottom). One representative experiment out of three independent is shown.

Table S1 (related to Fig. 3 and 4).
Inhibition of the ubiquitin-proteasome system increases TDO half-life in the absence of tryptophan

Inhibitors	**Cycloheximide**	**Cycloheximide Bortezomib**	**Cycloheximide MLN7243**	**Cycloheximide Pevonedistat/MLN4924**
Targets	**Ribosomes**	**Ribosomes Proteasomes**	**Ribosomes Ubiquitin activating E1**	**Ribosomes NEDD8 activating enzymes**
Medium with tryptophan Half-life (± SD)	> 8h	> 8h	> 6h	> 6h
Medium without tryptophan Half-life (± SD)	1h22 (±13) min	2h04 (±18) min	> 6h	2h02 (±33) min
Related figure	Fig 3A	Fig 3B	Fig 3D	Fig 4A
Number of experiments quantified	2	2	3	3

The table shows the half-life of TDO in A172 cells incubated in medium with or without tryptophan and treated or not with proteasome and ubiquitin ligase inhibitors from Fig 3 and 4. Signals of TDO/Actin were quantified from kinetic degradations and related to the value at the time 0h. Data were plotted by experiments and half-life values represent the mean of half-lives from independent experiments, as indicated.

Table S2 (related to Fig. 4B-D).
Transfection of the dominant-negative CUL1 increases TDO half-life in the absence of tryptophan

Vector (dominant negative)	**Empty vector**	**CUL1DN**	**CUL3DN**	**CUL4ADN CUL4BDN**	**CUL5DN**
Medium with tryptophan Half-life (± SD)	> 4h	> 4h	> 4h	> 4h	> 4h
Medium without tryptophan Half-life (± SD)	47 (±7) min	1h29 (±23) min	46 (±9) min	39 min	49 min
Related figure	Fig 4B	Fig 4B	Fig 4B	Fig 4C	Fig 4D
Number of experiments quantified	2	2	2	1	1

The table shows the half-life of TDO in HEK293 cells stably transfected with *TDO2* (HEK293 hTDO cl19) transiently transfected with dominant negative cullins incubated in medium with or without tryptophan (in presence of cycloheximide). Signals of TDO/Actin were quantified from kinetic degradations and related to the value at the time 0h. Data were plotted by experiments and half-life values represent the mean of half-lives from independent experiments or the value of a single experiment, as indicated. For Fig 4C and 4D, the experiment was repeated twice, but only one experiment could be quantified.

Supplementary Information

Conditions for qPCR

RT-qPCR were performed using the following primers:
human *TDO2* (Forward: CATGGCTGGAAAGAACTC, Reverse: CTGAAGTGCTCTGTATGAC, Probe: TTTAGAGCCACATGGATTTAACTTCTGGG); human *GAPDH* (Forward: TCAACGACCACTTTGTCAAGC, Reverse: CCAGGGGTCTTACTCCTTGG, Probe: CCTGGTATGACAACGAATTTGGCTACAGC); mouse *Tdo2* (Forward: GTATCTATGGAGGACAATGAAG, Reverse: GATGAATAGGTGCTCGTCATG, Probe: CCTCCTTTGCTGGCTCTGTTTACACC); mouse *β-Actin* (Forward: CTCTGGCTCCTAGCACCATGAAG, Reverse: GCTGGAAGGTGGACAGTGAG, Probe: ATCGGTGGCTCCATCCTGGC). The probes were labeled with 5' FAM and 3'TAMRA. Standard curves were added for m*Tdo2* and m*β-Actin.* Cycling conditions were usually: denaturation at 95°C for 15 sec, and hybridation and elongation at 60°C for 1min, except for m*Tdo2* (3 min at 95°C, then 40 cycles of 10 sec at 95°C and 1 min at 60°C), and for m*β-Actin* (3 min at 95°C, then 40 cycles of 3 sec at 95°C and 30 sec at 60°C).

Construction of plasmids producing deleted and mutated TDO proteins

Deletions were introduced into the ORF of *TDO2* by using PCR with two divergent oligonucleotides corresponding to either border of the deleted sequence. The oligonucleotides (100pmoles of each) were first phosphorylated with 5U of T4 polynucleotide kinase (NEB) in 10µl of T4 DNA ligation buffer (NEB) for 30 min at 37°C followed by 20 min at 65°C to inactivate the enzyme. These primers (0.5µM each) were then used to amplify the recombinant vector (10ng) in 50µl of Phusion GC buffer (Finnzyme) with 1U of Phusion DNA polymerase (Finnzyme). PCR conditions were 1 cycle at 98°C for 30 sec, 30 cycles at 98°C for 10 sec, at a temperature corresponding to the lower Tm + 2°C for 30 sec and at 72°C for 4 min and finally 1 cycle at 72°C for 10 min. 5µl of the PCR reaction were separated in an agarose-ethidium bromide gel where we usually observed a bright PCR product of 7.1kb. The PCR products were purified on size-exclusion chromatography columns. One µl (about 40ng) of PCR product was then incubated with 5U of T4 DNA ligase in 50µl of T4 DNA ligation buffer (NEB) for 1h30 at 37°C to circularize the vector. One twentieth (2.5µl) of the ligation reaction was used to transform XL1-Blue competent bacteria (Agilent Technologies). Plasmid DNA was purified from 3-5 colonies and sequenced. Deletions were found in most of the

sequenced plasmids. Oligonucleotides used to introduce the deletions: human TDO del200-213 (Forward: GGTTTAGAGCCACATGGATTTAAC, Reverse: CTTTTCCTGCTCAGATTTAAGTAGC); human TDO del98-105 (Forward: AGGAACATGCTTAAGGTTGTTTCTCG, Reverse: AAAGATCTCTCGAACAGAATCCAAC); human TDO del169-187 (Forward: GAAAATGAACTGCTACTTAAATCTGAG, Reverse: AAGAACACCTATCTTGTTTTCTAATAGTC). Point mutations were introduced into the ORF of *TDO2* with the QuikChange II site-directed mutagenesis kit (Agilent Technologies) following the protocol of the manufacturer. Oligonucleotides used to introduce the mutations: human TDO mut208-211 (Forward: GTGGAGGCAGCGGCGGCAGCAACTCCAGGTTTAGAGCCACATGG, Reverse: CCATGTGGCTCTAAACCTGGAGTTGCTGCCGCCGCTGCCTCCAC); human TDO mut204-206 (Forward: CACTTCTGGAAGCAGCGGCGGCATGGCTGGAAAGAACTCCAGG, Reverse: CCTGGAGTTCTTTCCAGCCATGCCGCCGCTGCTTCCAGAAGTG).