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Pharmacological induction of hypoxia-inducible transcription factor ARNT attenuates chronic kidney failure

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#equal contribution

Running title: ARNT attenuates organ failure

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Abstract

Progression of chronic kidney disease associated with progressive fibrosis and impaired tubular epithelial regeneration is still an unmet biomedical challenge, because once chronic lesions have manifested, no effective therapies are available as of yet for clinical use. Prompted by various studies across multiple organs demonstrating that preconditioning regimens to induce endogenous regenerative mechanisms protect various organs from later incurring acute injuries, we here aimed to gain insights into the molecular mechanisms underlying successful protection and to explore whether such pathways could be utilized to inhibit progression of chronic organ injury. We identified a protective mechanism that is controlled by the transcription factor ARNT, which effectively inhibits progression of chronic kidney injury by transcriptional induction of ALK3, the principal mediator of anti-fibrotic and pro-regenerative BMP signaling responses. We further report that ARNT expression itself is controlled by the FKBP12/YY1 transcriptional repressor complex, and that disruption of such FKBP12/YY1 complexes by picomolar FK506 at sub-immunosuppressive doses increases ARNT expression, subsequently leading to homodimeric ARNT-induced ALK3 transcription. Direct targeting of FKBP12/YY1 with in vivo-morpholino approaches or small molecule inhibitors including GPI-1046 were equally effective to induce ARNT expression with subsequent activation of ALK3-dependent canonical BMP signaling responses and attenuated chronic organ failure in models of chronic kidney, but also cardiac and liver injuries. In summary, we report an organ protective mechanism, which can be pharmacologically modulated by immunophilin ligands FK506, GPI-1046 or therapeutically targeted by in vivo-morpholino approaches.
**Introduction**

Progression of chronic kidney disease (CKD) towards end-stage renal disease (ESRD) is an unmet biomedical challenge, just like chronic progressive failure of any other parenchymal organ, because effective therapies to target progressive loss of functional parenchymal cells and organ fibrosis are not yet available. While efforts to implement interventional therapeutic regimens to treat already established chronic kidney lesions were all frustrating, it has been known for decades that parenchymal organs including the kidney can be preconditioned to resist later ensuing tissue injuries, preventing progressive loss of functional epithelium and kidney fibrosis (1-3). Based on the organ-spanning effectiveness of preconditioning regimens, we hypothesized that the underlying mechanisms could be utilized to aid the kidney in realizing its endogenous regenerative capacity even after manifestation of chronic kidney lesions.

Among different preconditioning approaches, several independent studies highlighted efficacy of FK506 (synonym Tacrolimus or Fujimycin) administration to protect against acute experimental injuries (4), involving various parenchymal organs including kidney (5), heart (6), liver (7), lung (8), brain (9), spinal cord (10), skin (11), and intestine (12). FK506 is a macrolide calcineurin inhibitor (CNI) that elicits immunosuppression by complexing with distinct FK506-binding proteins (FKBPs) to attenuate NFAT- and NFκB-dependent transcription of pro-inflammatory cytokines (13). FK506 is commonly used to lower risk of rejection after allogeneic organ transplantation, albeit its clinical utility is limited by its nephrotoxicity (14). While possible reno-protection by FK506 appears counter-intuitive in light of its clinically established nephrotoxicity, effective organ protection was demonstrated at doses far below concentrations required for calcineurin-dependent immunosuppression (picomolar versus nanomolar range) (5-9), suggesting involvement of calcineurin-independent and yet unknown mechanisms. Here, we aimed to elucidate molecular mechanisms underlying such protective activity of low-dose FK506 administration and to explore if this pathway could be utilized to even protect from chronic progressive organ failure.
Results

**Low-dose FK506 protects the kidney from chronic organ injury dependent on enhanced ALK3 signaling.** Based on previous regimens (5-9), we administered low-dose FK506 (0.02, 0.075 and 0.2 mg/kg orally per day) to mice starting one day prior to challenge with UUO (**Figure 1A**). As controls, we also included standard-immunosuppressive dose FK506 (5.0 mg/kg orally per day) and Cyclosporine A (CsA, 10 mg/kg orally per day), an alternative CNI that acts independent of FKBP12 (15, 16).

Histopathological analysis demonstrated that low-dose FK506 reduced both, chronic tubular injury and interstitial fibrosis with an optimum dose of 0.075 and 0.2 mg/kg FK506 per day (**Figure 1B-E** and **Supplementary Figure 1A-C**). In contrast, CsA failed to attenuate tubular injury or interstitial fibrosis (**Figure 1B-E** and **Supplementary Figure 1A-C**), suggesting that observed reno-protective efficacy of low-dose FK506 was mediated by mechanisms independent of calcineurin phosphatase inhibition.

Administration of low-dose FK506 (0.02, 0.075 and 0.2 mg/kg orally per day) resulted in picomolar, sub-immunosuppressive FK506 blood concentrations of 245±73, 452±71 and 535±36 pg/mL, respectively (**Figure 1F**). To gain insights into underlying mechanisms, we next performed gene set enrichment analysis of transcriptional expression datasets for bioactive small molecules (accession number GSE5258) with evidence that FK506 induces expression of genes involved in BMP signaling responses (GO terms GO:0071772 and GO:0071773, **Supplementary Figure 2A** and **Supplementary Table 1**) (17, 18), including the proto-typical type I BMP receptor ALK3 (synonym type I BMP receptor serine/threonine kinase, BMPR1A). Because multiple independent studies demonstrated that administration of Alk3 ligands protected against fibrosis and loss of functional parenchymal cells in various organs including kidney (19-26), we aimed to explore a possible causal link between low-dose FK506, Alk3 and reno-protection. Analysis of UUO-challenged murine kidneys revealed that FK506 specifically induced expression of Alk3, but not of related type I BMP receptor Alk6 (**Figure 2A-C**). FK506-induced Alk3 expression was associated with nuclear pSmad1/5/8 accumulation (correlating with protective efficacy of low-dose FK506, **Figure 2D-F** and **Supplementary Figure 2B**), whereas non-protective CsA failed to induce Alk3 expression and pSmad1/5/8 (correlating with failure of CsA to protect injured kidneys, **Figure 2E,F** and **Supplementary Figure 2B**). Inhibition of canonical BMP signaling responses by administration of dorsomorphin derivate small molecule LDN-193189 (LDN, 3
mg/kg intraperitoneally per day) completely blocked FK506-mediated reno-protection (Figure 2G-I and Supplementary Figure 2C-E), confirming that FK506 elicits protection from chronic organ failure by enhanced Alk3 signaling (27).

YY1 links immunophilin FKBP12 and ALK3 transcription in response to FK506. We next aimed to gain insights into the molecular mechanisms underlying observed increased Alk3 transcription within tubular epithelial cells (TECs) upon low-dose FK506 administration. We exposed murine TECs to different concentrations of FK506 ranging from standard nanomolar dosages used for immunosuppression in transplant patients (2-200 nM) down to picomolar concentrations (0.02-0.2 nM) reflecting FK506 regimens which we had used in our murine studies. FK506 induced optimal Alk3 transcription and pSmad1/5/8 accumulation at concentrations from 0.2 nM to 2 nM, whereas higher concentrations had no further enhancing effects (Figure 3A-C and Supplementary Figure 3A,B). Because FK506 elicits biological function by complexing with distinct FKBPs, we next depleted FKBP family members Fkbp12, Fkbp25, Fkbp38 and Fkbp56 in cultured TECs (Supplementary Figure 3C-F). Whereas knockdown of Fkbp25, Fkbp38 and Fkbp56 did not affect Alk3 transcription, depletion of Fkbp12 (Fkbp12kd) induced Alk3 transcription without further addition of FK506 to culture media (Figure 3D). Because we did not detect direct interaction of Fkbp12 and Alk3 (Figure 3E and Supplementary Figure 3G) (8), and Fkbp12 has no DNA binding properties itself, we hypothesized that Fkbp12 alters Alk3 transcription by interacting with a to-be-determined transcriptional regulator, and that FK506 affects Alk3 expression via interaction with this Fkbp12/transcriptional regulator complex. We identified enrichment of genes regulated by Yin Yang 1 (YY1) in response to FK506 (accession number GSE5258, Supplementary Figure 3H and Supplementary Table 2) (17, 18, 28), an established FKBP12 interacting protein (Supplementary Figure 3I) (29). YY1 is a GLI-Kruppel family member known to regulate transcription based on its interaction with adapter proteins, including FKBPs (30). Co-immunoprecipitation using antibodies to Yy1 (IP: Yy1) revealed direct interaction of Yy1 and Fkbp12 in cultured TECs, while such Fkbp12/Yy1 interaction was not detectable when FK506 was added to culture media (Figure 3F and Supplementary Figure 3J). While in untreated TECs, Fkbp12 and Yy1 were equally present and detection of Fkbp12/Yy1 complexes correlated with low Alk3 transcription,
depletion of Yy1 (Yy1\textsuperscript{kd}) was equally effective in induction of Alk3 transcription and subsequent BMP signaling activation as compared to FKBP12-depleted cells (Fkbp12\textsuperscript{kd}, Figure 3G-I and Supplementary Figure 3K). In addition, supplementation of FK506 to culture media did not additionally induce Alk3 transcription when Yy1 had been depleted (Figure 3G-I), indicating that FK506-mediated Alk3 transcription is primarily dependent on Yy1.

To further substantiate that tubular Yy1 controls transcriptional Alk3 repression in injured kidneys, we generated mice conditionally depleted for Yy1 in TECs (referred as γGT\textsuperscript{cre+};Yy1\textsuperscript{fl/fl}, Supplementary Figures 4A,B). In mice challenged with UUO (Figure 4A), conditional Yy1 depletion in TECs resulted in enhanced Alk3 transcription, protection from tubular injury and renal fibrogenesis (Figure 4B-G). When Yy1 was depleted in TECs, administration of FK506 had no additive effects (Figure 4B-G), indicating that FK506-mediated anti-fibrotic and pro-regenerative efficacy is dependent on presence and modulation of YY1 signaling in TECs.

**ARNT causally links disruption of FKBP12/YY1 complexes to increased ALK3 transcription.** In control experiments in which Cycloheximide (CHX, 10 µg/mL) was added to cell culture media to block protein translation, FK506 failed to induce Alk3 transcription and canonical BMP signaling responses (nuclear pSmad1/5/8, Figure 5A-C). These observations suggested that de novo translation of yet unknown mediator(s) is required to induce Alk3 transcription after release from Fkbp12/Yy1 transcriptional repressor complexes, supported by absence of putative Yy1 binding motifs within the Alk3 proximal promoter region (31). By using an unbiased, array-based approach and computational prediction of putative transcriptional factor binding sites in human TECs (31), we identified 6 candidate transcriptional factors with a binding motif within the ALK3 proximal promoter at least 2-fold induced upon FK506 exposure: AR, ARNT, CEBPB, CREB1, GATA3 and MAX (Figure 5D,E). While qRT-PCR confirmed transcriptional induction of all identified factors upon exposure to FK506 (Figure 5F), only siRNA-mediated depletion of Arnt (Arnt\textsuperscript{kd}) prevented transcriptional Alk3 induction in response to FK506 (Figure 5G and Supplementary Figure 5A-F). Furthermore, we observed robust Arnt induction upon depletion of either Fkbp12 (Fkbp12\textsuperscript{kd}) or Yy1 (Yy1\textsuperscript{kd}, Figure 5H), suggesting Arnt as possible link between Fkbp12/Yy1 complex disruption and enhanced Alk3 transcription. These observations were
further supported by transcriptional expression datasets with robust induction of ARNT and enrichment of ARNT-regulated genes induced in response to FK506 (accession number GSE5258, Supplementary Figure 3H and Supplementary Table 2) (17, 18, 28). To further explore a causal link between Fkbp12/Yy1 complex disruption and increased Alk3 transcription, we performed Arnt ChIP PCR using antibodies to Yy1 for immunoprecipitation (IP: Yy1). ChIP demonstrated binding of Yy1 to its putative motif within the Arnt proximal promoter in cultured TECs that had been maintained in control media without FK506 (with low Alk3 expression levels, Figure 5I). Addition of FK506 to culture media (associated with enhanced Alk3 transcription) reduced Yy1 binding to the Arnt proximal promoter (Figure 5I), associated with transcriptional Arnt induction (Figure 5J). Supplementation of culture media with FK506 did not additionally induce Arnt mRNA levels in experiments in which Arnt had been induced by Fkbp12 (Fkbp12ko) or Yy1 depletion (Yy1ko, Figure 5J), supporting that FK506-mediated Arnt transcription is primarily dependent on Fkbp12/Yy1.

To further substantiate the causal link between Arnt and Alk3 regulation, we next analyzed efficacy of FK506 to enhance Alk3 transcription when Arnt was blocked in cultured TECs (Arntkd). When Arnt induction was depleted, FK506 failed to induce Alk3 transcription (Figure 6A and Supplementary Figure 5B). In addition, transgenic Arnt over-expression (Arntoe) alone was sufficient to specifically induce Alk3 transcription (Figure 6B and Supplementary Figure 5G,H). Generally, ARNT (synonym HIF1β) is a member of the PAS domain family and forms heterodimeric transcription factors with other PAS family members (HIF1α in hypoxia responses and the dioxin receptor AHR in xenobiotic signaling) or homodimers with itself to elicit transcriptional activation (by binding to the E-box core sequence CACGTG) (32). FK506 exposure did not markedly induce pathways involved in hypoxic signaling or drug metabolism (including xenobiotic signaling, Figure 6C,D), and co-immunoprecipitation using antibodies to Arnt (IP: Arnt) did not provide evidence for Arnt/Hif1α or Arnt/Ahr interaction in cultured TECs in response to FK506 (Figure 6E-G). These observations were further confirmed by preserved effectiveness of FK506 or transgenic Arnt over-expression to induce Alk3 transcription when either Hif1α (Hif1αkd) or Ahr (Ahrkd) were depleted (Figure 6H,I and Supplementary Figure 5I-L). To elucidate capacity of Arnt homodimer formation in cultured TECs, we next generated EGFP-tagged (ArntEGFP) and myc-tagged (Arntmyc) Arnt overexpression plasmids and confirmed formation of Arnt
homodimers by co-immunoprecipitation with presence of Arnt\textsuperscript{myc} after pulldown of Arnt\textsuperscript{EGFP} (IP: EGFP) and vice versa (IP: myc, Figure 6J,K). Analysis of endogenous Arnt by native gel electrophoresis revealed presence of Arnt dimers within TECs cultivated under standard conditions and further enhancement upon FK506 exposure (Figure 6L). We did not detect Hif1α, Hif2α or Ahr as constituent of Arnt dimers, further suggesting that transcriptional responses to FK506 in TECs are mediated by homodimerized Arnt (Figure 6L) (32). Alk3 ChIP-PCR revealed increased Arnt binding to its respective homodimer motif upon exposure to FK506 (associated with enhanced Arnt homodimers and enhanced Alk3 transcription, Figure 6M) (33, 34). Transcriptional Alk3 induction was further confirmed by reporter assays, as Arnt over-expression effectively induced Alk3 proximal promoter activity (Alk3\textsuperscript{wt}, Figure 6N). In contrast, Arnt failed to induce Alk3 transcription when the Arnt homodimer target sequence was disrupted (CACGTG to TATATA, Alk3\textsuperscript{mut}, Figure 6N).

These findings in cultured TECs were further confirmed by increased Arnt levels in γGT\textsuperscript{cre+};Yy1\textsuperscript{fl/fl} conditional knockout mice and in mice treated with low-dose FK506 (Figure 7A-E, correlating with enhanced Alk3 transcription and reno-protection). Furthermore, analysis of total kidney lysates by immunoprecipitation, native gel analysis and subsequent immunoblotting revealed enhanced formation of Arnt dimers without interaction with Hif1α, Hif2α or Ahr (Figure 7F), confirming a critical role of Arnt homodimerization.

**Selective modulation of FKBP12/YY1 signaling effectively modulates protective ARNT within chronically injured kidneys.** To elucidate therapeutic implication of our findings, we next selectively modulated constituents of identified Fkbp12/Yy1/Arnt signaling axis in mice challenged with UUO by administration of in vivo-morpholinos (VMO) targeting translational start sites (Supplementary Table 3) (35). As compared to control-VMO targeting a human β-globin intron mutation (36), administration of VMO targeting Fkbp12 (Fkbp12-VMO), Yy1 (Yy1-VMO) or Arnt (Arnt-VMO) effectively reduced intrarenal protein levels of their respective transcript (Figure 8A,B and Supplementary Figure 6A-F). Administration of Fkbp12-VMO, Yy1-VMO or FK506 equally induced intrarenal Arnt, associated with attenuated tubular injury and tubulointerstitial fibrosis (Figure 8A-D and Supplementary Figure 6F-H). Administration of FK506 had no additive protective effects in cohorts of mice that had also received
Fkbp12-VMO or Yy1-VMO (Figure 8A-D and Supplementary Figure 6F-H). In addition, administration of Fkbp12-VMO or Yy1-VMO did not further enhance the effect of FK506 on reno-protection (Figure 8A-D and Supplementary Figure 6F-H), mirroring the effect of genetic Yy1 depletion which we had observed in γGT<sup>cre</sup>;Yy1<sup>fl/fl</sup> conditional knockout mice. In contrast, FK506 failed to protect kidneys from tubular injury and progressive fibrotic disease when intrarenal Arnt induction was depleted (Figure 8A-D and Supplementary Figure 6F-H).

**Selective targeting of FKBP12 by non-immunosuppressive FKBP12 inhibitor GPI-1046 effectively modulates FKBP12/YY1/ARNT signaling and protects from chronic renal failure.** Because our data suggested that picomolar FK506 elicited reno-protective properties independent of calcineurin inhibition, we next explored efficacy of a specific small molecule Fkbp12 inhibitor, 3-(3-pyridyl)-1-propyl-(2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinedine carboxylate (GPI-1046), an FK506 derivate without immunosuppressive properties (37), to modulate intrarenal Fkbp12/Yy1/Arnt/Alk3 signaling and to pre-empt chronic renal injury. Exposure of cultured TECs to GPI-1046 (10 µM) was equally effective in enhancing Arnt and Alk3 transcription (Figure 9A,B). Based on our previous regimen, we next administered GPI-1046 (10 mg/kg subcutaneously per day) one day prior to UUO challenge (Figure 9C) (37). Non-immunosuppressive GPI-1046 effectively induced intrarenal Arnt and Alk3 transcription, BMP signaling responses and protected from tubular injury and renal fibrogenesis (Figure 9D-K), further supporting that specifically immunophilin Fkbp12 is involved in modulating intrarenal Arnt/Alk3 signaling and protection from injury independent of calcineurin modulation or immunosuppressive properties. This is further confirmed by uniform immune cell infiltration in cohorts of mice administered either picomolar FK506 or non-immunosuppressive GPI-1046, not affecting intrarenal NFAT signaling (Supplementary Figure 7A-C). To test if administration of FK506 or GPI-1046 was also effective to attenuate chronic kidney disease progression when administration is initiated after manifestation of kidney injury, we next administered FK506 or GPI-1046 three days after challenging with UUO (0.2 mg/kg or 30 mg/kg orally per day, respectively, Figure 10A). Both, FK506 and small molecule Fkbp12 inhibitor GPI-1046 were equally effective in inducing
intrarenal *Arnt* and *Alk3* transcription, associated with attenuated tubular injury and renal fibrogenesis (Figure 10B-H).

**Selective targeting of FKBP12 protects from chronic injury in the heart and liver.** Effectiveness of FK506 had been documented in various parenchymal organs (4-12), in line with transcriptional *Arnt* induction present in the kidney (confirming previous results), but also in the heart, brain, spinal cord, skin, liver, lung and intestine in response to FK506 or GPI-1046 (0.2 mg/kg or 30 mg/kg orally per day, respectively, Figure 11A,B). Based on robust *Arnt* induction observed in the heart and liver (Supplementary Figure 8A,B), we next analyzed presence of an Fkbp12/Yy1/Arnt/Alk3 signaling axis in rodent models of cardiac fibrosis after continuous minipump delivery of angiotensin II (AT II) and carbon tetrachloride (CCl₄)-induced liver failure (23, 38). Fkbp12 and Yy1 were detectable in chronically injured hearts and livers (Supplementary Figure 9A-F), suggesting that repressive Fkbp12/Yy1 complexes were equally present. To elucidate if identified Fkbp12/Yy1/Arnt/Alk3 signaling axis could be equally targeted to protect from chronic heart failure and fibrosis, we next administered GPI-1046 (10 mg/kg subcutaneously per day) one day prior to administration of AT II (Figure 11C). GPI-1046 was equally effective in induction of Alk3-dependent canonical BMP signaling responses and protection from fibrogenesis in the heart (Figure 11D-K and Supplementary Figure 9G-J). These results were also confirmed in mice challenged with CCl₄ (Figure 12A), GPI-1046 equally induced Alk3-dependent signaling responses and protected from chronic liver failure (Figure 12B-H and Supplementary Figure 9K,L).

**An FKBP12/YY1/ARNT/ALK3 signaling axis translates to humans.** With regard of translational implications of our findings, FKBP12 and YY1 were equally detectable in human pathologies including chronically injured kidneys, livers and lungs as prerequisite of identified organ protection mediated by FK506 (Supplementary Figure 10A-C and Supplementary Table 6). To elucidate whether modulation of an FKBP12/YY1/ARNT/ALK3 signaling axis was not only limited to mice but similarly effective in humans, we next exposed human TECs to FK506 and analyzed efficacy to induce protective ARNT. Exposure of human TEC cultures to previous established picomolar FK506 equally induced *ARNT*
transcription and ALK3 (Figure 13A,B). Because FK506 has been in clinical use for decades to prevent rejection of kidney transplants at high immunosuppressive doses (with well established adverse toxicity likely masking beneficial effects) and not at low doses at which we observed optimal reno-protection in rodents, we focused in our analysis on the induction of ARNT expression and subsequent ALK3-mediated BMP signaling responses kidney allografts matched for comparable kidney function, chronic tubular injury and extent of interstitial fibrosis (Supplementary Figure 11A-F and Supplementary Table 7). Immunolabelling confirmed presence of FKBP12 and YY1 in all allografts (Figure 13C), while enhanced ARNT and ALK3 transcription was present in kidney biopsies of patients on an FK506-based immunosuppressive regimen as compared to CsA (Figure 13C-H).
Discussion

Here, we report a reno-protective mechanism that is controlled by the transcription factor ARNT, which effectively inhibits progression of chronic kidney injury by inducing ALK3 transcription. We further report that ARNT expression itself is controlled by the FKBP12/YY1 transcriptional repressor complex (Supplementary Figure 12A), and that disruption of such FKBP12/YY1 complexes by picomolar FK506 at sub-immunosuppressive doses, small molecule FKBP12 inhibitor GPI-1046, or by direct targeting of FKBP12/YY1 using in vivo-morpholinos increases ARNT levels (Supplementary Figure 12B). Subsequent activation of ALK3-dependent canonical BMP signaling responses by ARNT homodimer formation (independent of HIF1α or AHR) attenuates chronic organ failure in models of chronic kidney, cardiac and liver injuries (Supplementary Figure 12C,D).

Identified FKBP12/YY1/ARNT/ALK3 signaling axis is supported by mining of public expression profiling databases in various organs: in context of the kidney, an inverse correlation between intrarenal ALK3 expression and FKBP12/YY1 is confirmed by several array datasets performed in different renal pathologies (Supplementary Figure 13A-F) (39-44), confirmed in microdissected renal tubules (Supplementary Figure 14A) (45). Furthermore, kidney allografts of patients on FK506-based immunosuppressive regimen displayed enhanced intrarenal ARNT and ALK3 transcription as compared to allografts of patients on CsA (Supplementary Figure 14B,C) (44). Organ protection by the FKBP12/YY1/ARNT/ALK3 signaling axis is in line with extensive literature on two previously unconnected lines of research as several studies highlighted efficacy of low-dose FK506 administration to protect against acute experimental injuries including the kidney, heart and liver (5-7), and multiple pre-clinical studies established beneficial effects of ALK3-mediated BMP signaling in these organs (19-23).

At the mechanistic level, we report that the homodimerization of ARNT is critical to elicit its function in inducing ALK3 transcription. ARNT in general is a member of the PAS domain family which is predominantly known to heterodimerize with other PAS family members to form heterodimeric transcription factors, classically with an α subunit of HIF or the dioxin receptor AHR to mediate hypoxia or xenobiotic responses by targeting genomic E-box motifs (32). A unique function of homodimeric ARNT as compared to heterodimeric ARNT is supported by recent studies that established the
palindromic E-box motif CACGTG as critical binding site for ARNT homodimers, while ARNT heterodimers bind to asymmetric E-box motifs in hypoxic/xenobiotic response elements (33, 34). As ARNT homodimerization potentially provides another therapeutic target, additional research is warranted to explore the underlying mechanisms. In this regard, our studies suggest that the fate decision of homodimerization over heterodimerization is in part regulated by intracellular ARNT levels controlled by FKBP12/YY1 transcriptional repressor complexes. This is supported by array datasets confirming transcriptional ARNT induction when YY1 was depleted (Supplementary Figure 14D,E) (46), and binding of YY1 repressor to the ARNT proximal promoter (47). In addition, clustering of transcriptome array datasets revealed inverse correlation between ARNT and FKBP12/YY1 in multiple organ systems including renal, cardiovascular and digestive tissues, confirming our findings (accession number GSE3526, Supplementary Table 8) (48). In this context, previous reports implicate that activation of an YY1 signaling axis is detectable in renal, cardiac, hepatic and pulmonary pathologies, and YY1 depletion protects from chronic organ failure (49-51).

It is tempting to speculate that modulation of newly identified FKBP12/YY1/ARNT/ALK3 signaling axis may be a promising target in chronic failure of multiple organ systems. In this regard, low-dose FK506 has already entered clinical testing (ClinicalTrials.gov identifier NCT01647945) and shown promise in pulmonary arterial hypertension patients to induce protective BMP signaling responses (52). Observed beneficial efficacy of FK506 was counter-intuitive at first sight, because decades of use as immunosuppressant upon kidney transplantation had revealed its CNI nephrotoxicity, thereby limiting its clinical use (14). However, our studies established that FK506-induced organ protection is independent of CNI activity as it is achieved at picomolar doses far below the nanomolar immunosuppressive regimens. Hence, we provide mechanistic evidence for why protective activity of FK506 in interventional therapeutic regimens has remained elusive thus far. Efficacy of the specific FKBP12 inhibitor GPI-1046 to protect and attenuate disease progression in kidney, heart and liver validated observed efficacy of FK506, and also provided a more specific tool from a translational perspective. Thus, we speculate that pharmacologic modulation of ARNT homodimerization and FKBP12/YY1 complexes may have translational potential for organ protection in the future.
Materials and Methods

Generation of γGT<sup>Cre</sup>;<Yy1<sup>fl/fl</sup> mice. B6;129S4-Yy1<sup>1m2Yshi/J</sup> (referred as Yy1<sup>fl/fl</sup>) mice were obtained from Jackson Laboratory (Bar Harbor, USA), Tg(Ggt1-cre)M3Egn/J (referred as γGT<sup>Cre</sup>) mice were previously described and genetic backgrounds were identical when comparing experimental groups (19).

Unilateral ureteral obstruction (UUN). Eight to twelve weeks-old C57BL/6, γGT<sup>Cre</sup>;<Yy1<sup>fl/fl</sup> and γGT<sup>Cre</sup>;<Yy1<sup>fl/fl</sup> control mice were anesthetized with isoflurane inhalation (2-3%), analgesia was performed by subcutaneous injection of 0.1 mg/kg body weight per day Buprenorphine. The ureter was separated from the surrounding tissues and two ligatures were placed about 5 mm apart in upper two-thirds of the left ureter to obtain reliable obstruction. Mice were sacrificed 3, 7 or 10 days after ureteral obstruction for further analyses, as described before (53).

Angiotensin II (AT II)-induced cardiac hypertrophy and fibrosis. Eight to twelve weeks-old C57BL/6 mice were anesthetized with isoflurane inhalation (2-3%), analgesia was performed by subcutaneous injection of 0.1 mg/kg body weight Buprenorphine per day. Osmotic minipumps (Alzet, Cupertino, USA) were loaded with AT II to continuously deliver 1.44 µg/kg body weight per day and implanted subcutaneously (38). Measurements of blood pressure were performed using a tail cuff system, systolic, diastolic, mean arterial pressure (MAP) and heart rate were recorded and mice were sacrificed 14 days after implantation for further analyses.

Tetrachlormethan (CCl<sub>4</sub>)-induced liver fibrosis. Eight to twelve weeks-old C57BL/6 mice were intraperitoneally injected with 0.25 (first injection), 0.5 (second injection) and 1 mL/kg body weight CCl<sub>4</sub> (25% v/v dissolved in sterile oil) twice a week (23). Mice were sacrificed after 42 days for further analyses.

Cell culture. HK–2 (ATTC, Manassas, USA) are immortalized proximal tubule epithelial cells derived from normal adult human kidney. The murine proximal tubular epithelial cell line MCT was generated from the renal cortex of SJL mice (54). All cells were routinely tested negative for the presence of mycoplasma contamination. None of the cell lines used in this manuscript is listed in the ICLAC and NCBI Biosample database of misidentified cell lines. Cells were cultured in Dulbecco’s modified Eagle’s
(DMEM, Gibco, Carlsbad, USA) medium supplemented with 100 g/mL penicillin, 100 g/mL streptomycin and 10% heat–inactivated fetal bovine serum (FBS, Sigma, St. Louis, USA) at 37°C in 5% CO₂.

Further material and method details are listed in Supplemental Experimental Procedures.

**Statistical analysis.** The numbers of individual mice and independent experiments are indicated in the corresponding figure legends. For single group comparison, two-tailed Student’s t test was used to determine statistical significance. One-way ANOVA with Bonferroni post-hoc analysis was used for multiple comparisons of samples to determine statistical significance. Linear regression was performed comparing indicated pairs of parameters, \( r^2 \) and values of \( p \) are indicated in the corresponding graphs. Statistical significance was calculated with Prism 5 software (GraphPad, La Jolla, USA) and defined as values of \( p<0.05 \), indicated as * \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \) or **** \( p<0.0001 \).

**Study approval.** All animal studies had been carried out with the approval of the Landesamt für Verbraucherschutz und Lebendmittelsicherheit (LAVES, Oldenburg, Germany) and the University Medical Center Göttingen (UMG, Göttingen, Germany). The use of parts of human specimens for research purposes was approved by the Ethics Committee of the University Medical Center Göttingen (UMG, Göttingen, Germany). All patients consented to the use for research purposes, and all samples were deidentified. A pathologist diagnosed for the presence of disease, and clinical data are presented in Supplemental Table 6 and 7.
Author contributions

BT and DT performed, designed and supervised experiments, collected and analyzed data, generated the figures and co-wrote the manuscript. GN, FK, GR, AK and TL performed experiments and collected data. SH and GAM provided human specimens. MZ conceived the project, designed and supervised experiments, analyzed data, and co-wrote the manuscript. EMZ, GAM and RK edited the manuscript.

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Statement of competing financial interests

All authors declare no competing financial interests.
References


Figure 1. Low-dose FK506 protects the kidney from chronic organ injury. (A) Mice were challenged with UUO and treated with either vehicle buffer, FK506 (0.02, 0.075, 0.2, 5.0 mg/kg orally per day, respectively) or CsA (10 mg/kg orally per day) starting one day prior of surgery. (B) The panels show representative photomicrographs of periodic acid Schiff's-stained (PAS) kidney sections (scale bars 50 µm) and sections immunolabelled with primary antibodies against Collagen-1 (scale bars 25 µm). (C) Tubular damage at day 10 after ureteral obstruction was semi-quantitatively scored using PAS-stained kidney sections (0=healthy, 1=mild, 2=moderate, 3=severe, n=6/group, data are presented as means±s.d., **p<0.01, ****p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (D) The graph summarizes average means of relative tubulointerstitial fibrosis 3, 7 and 10 days after ureteral obstruction (n=6/group, data are presented as means±s.d., ****p<0.0001, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (E) In mice receiving FK506, areas positive for Collagen-1 was assessed (n=6/group, data are presented as means±s.d., ****p<0.0001, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (F) FK506 concentrations were measured in whole blood samples of UUO mice receiving either vehicle buffer or FK506 (0.02, 0.075, 0.2 mg/kg orally per day, respectively) using colorimetric ELISA measurements compared to standards (n=6/group, data are presented as aligned dot plots with means).
Figure 2. FK506-mediated renoprotection depends on ALK3-dependent signaling responses. (A,B) Mice were challenged with UUO and treated with either vehicle buffer, indicated concentrations of FK506 or CsA starting one day prior of surgery. Analyzed by qRT-PCR 10 days after ureteral obstruction, the bar graphs reflect relative mRNA expression levels of type I BMP receptors Alk3 and Alk6 (n=3/group, data are presented as means±s.d., ** p<0.01, **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (C-F) Analyzed by immunoblotting of total kidney lysates and immunostaining, type I BMP receptor Alk3 (scale bars 25 µm) and pSmad1/5/8 (scale bars 25 µm) was assessed (n=6/group, data are presented as means±s.d., ** p<0.01, **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (G-I) Mice were challenged with UUO and treated with either vehicle buffer or low-dose FK506 (0.2 mg/kg orally per day) when specifically canonical pSmad1/5/8-dependent ALK3 signaling transduction was pharmacologically blocked with small molecule LDN-193189 (LDN, 3 mg/kg intraperitoneally per day), the panels show representative photomicroographs of sections immunolabelled with primary antibodies against pSmad1/5/8 (scale bars 25 µm) and MTS-stained fibrotic kidney sections (scale bars 50 µm, n=6/group, data are presented as means±s.d., * p<0.05, **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis).
Figure 3. FK506 disrupts an FKBP12/YY1 transcriptional repressor complex. (A) TECs were exposed to vehicle, indicated concentrations of FK506 (0.02, 0.2, 2, 20, 200 nM, respectively) or equimolar Cyclosporine A (CsA, 10 nM), mRNA expression levels of Alk3 was analyzed by qRT-PCR (n=3 independent experiments, data are presented as means±s.d., ** p<0.01, **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (B,C) Representative photomicrographs of phosphorylated Smad1/5/8 complex (pSmad1/5/8) immunostainings overlayed with differential interference contrast (DIC, scale bars 25 µm) are shown (n=3 independent experiments, data are presented as means±s.d., **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (D) As analyzed by qRT-PCR, Alk3 mRNA expression levels in TECs were analyzed after siRNA-mediated knockdown of Fkbp12 (Fkbp12kd), Fkbp25 (Fkbp25kd), Fkbp38 (Fkbp38kd) or Fkbp56 (Fkbp56kd, n=3 independent experiments, data are presented as means±s.d., * *** p<0.001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (E) As analyzed by co-immunoprecipitation after Alk3 pull-down (IP: Alk3), direct interaction between Fkbp12 and Alk3 was assessed. (F) As analyzed by co-immunoprecipitation after Yy1 pull-down (IP: Yy1), direct interaction between Yy1 and Fkbp12 was assessed. (G) Alk3 mRNA expression levels were assessed by qRT-PCR after knockdown of either Yy1 (Yy1kd) or Fkbp12 (Fkbp12kd) and exposure to FK506 (n=3 independent experiments, data are presented as means±s.d., ** p<0.01, # no significance, values of p were calculated using Student’s t test). (H,I) Representative photomicrographs of pSmad1/5/8 immunostainings overlayed with differential interference contrast (DIC, scale bars 25 µm) are shown (n=3 independent experiments, data are presented as means±s.d., ** p<0.01, # no significance, values of p were calculated using Student’s t test).
Figure 4. FK506-mediated protection is dependent on presence and modulation of YY1 signaling in TECs. (A) Mice conditionally depleted for YY1 in TECs (γGTcre−;Yy1fl/fl) and corresponding littermate controls (γGTcre−;Yy1fl/fl) were challenged with UUO and treated with either vehicle buffer or FK506 (0.2 mg/kg orally per day) starting one day prior of surgery. (B) Alk3 mRNA expression levels were analyzed by qRT-PCR (n=3/group, data are presented as means±s.d., *** p<0.001, **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (C-G) Representative photomicrographs of immunostainings for Alk3 (scale bars 25 µm), PAS-stained fibrotic kidney sections (scale bars 50 µm), MTS (scale bars 50 µm) and Collagen-1 (scale bars 25 µm) in mice challenged with UUO are shown (n=3/group, data are presented as means±s.d., * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis).
Figure 5. ARNT causally links disruption of FKBP12/YY1 complexes to enhanced ALK3 transcription. (A) TECs were exposed to cycloheximide (CHX) one hour prior FK506 incubation, Alk3 mRNA expression was assessed by qRT-PCR (n=3 independent experiments, data are presented as mean±s.d., **** **p<0.001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (B,C) Immunostaining of pSmad1/5/8 overlayed with differential interference contrast (DIC, scale bars 25 µm, n=3 independent experiments, data are presented as mean±s.d., **** **p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (D,E) FK506-mediated transcriptional network alterations, log base 2 fold changes (log₂ FC) are shown by heat map. (F) Validation by qRT-PCR upon FK506 exposure (n=3 independent experiments, data are presented as mean±s.d., *** p<0.001, **** p<0.0001, # no significance, values of p were calculated using Student’s t test in comparison to DMSO-treated control cells). (G) Alk3 mRNA levels were analyzed by qRT-PCR (n=3 independent experiments, data are presented as mean±s.d., ** p<0.01, *** p<0.001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (H) As assessed by qRT-PCR, Arnt mRNA expression levels are shown (n=3 independent experiments, data are presented as mean±s.d., *** p<0.001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (J) Binding of Yy1 to the Arnt proximal promoter was analyzed by chromatin immunoprecipitation (ChIP) after Yy1 pull-down (IP: Yy1, n=3 technical replicates, data are presented as mean±s.d., ** p<0.01, *** p<0.001, # no significance, values of p were calculated using Student’s t test).
Figure 6. ARNT targets a palindromic E-box motif specific for ARNT homodimers required for ALK3 transcription. (A) Alk3 mRNA was assessed by qRT-PCR after depletion of Arnt (Arnt\textsuperscript{kd}, n=3 independent experiments, data are presented as means±s.d. **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (B) Alk3 mRNA expression levels after Arnt over-expression (Arnt\textsuperscript{oe}) are shown (n=3 independent experiments, data are presented as means±s.d., **** p<0.0001, values of p were calculated using Student's t test). (C) Hypoxic signaling and drug metabolism. (E-G) Analyzed by co-immunoprecipitation (CoIP) using antibodies to Arnt (IP: Arnt), Arnt/Hif1α and Arnt/Ahr interactions were assessed. (H) Efficacy of FK506 or Arnt over-expression to induce Alk3 mRNA expression levels in cultured TECs depleted for HIF1α (Hif1α\textsuperscript{kd}) or AHR (Ahr\textsuperscript{kd}, n=3 independent experiments, data are presented as means±s.d., *** p<0.001, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (J, K) Analyzed by CoIP, homodimer formation was assessed in cultured TECs after EGFP-tagged (Arnt\textsuperscript{EGFP}) and myc-tagged (Arnt\textsuperscript{myc}) ARNT overexpression and pulldown of Arnt-EGFP (IP: EGFP) or Arnt-myc (IP: myc). (L) Dimer formation of Arnt/Hif1α, Arnt/Hif2α and Arnt/Ahr was assessed by native gel electrophoresis. (M) Binding of Arnt to the Alk3 proximal promoter was analyzed by ChIP and subsequent target PCR after Arnt pull-down (IP: Arnt, n=3 technical replicates, data are presented as means±s.d., ** p<0.01, *** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (N) Analyzed by reporter assays, Alk3 proximal promoter activity was assessed in presence (Alk3\textsuperscript{wt}) or absence (Alk3\textsuperscript{mut}, CACGTG to TATATA) of the palindromic E-box motif (n=5 independent experiments, data are presented as means±s.d., * p<0.05, # no significance, values of p were calculated using Student’s t test).
Figure 7. Evidence for Arnt homodimer formation in mice treated with FK506. (A,B) Representative kidney sections of γGT<sup>cre<sup>+</sup></sup>;Yy1<sup>fl/fl</sup> and γGT<sup>cre<sup>+</sup></sup>;Yy1<sup>fl/fl</sup> control mice immunolabelled with primary antibodies against Arnt (scale bars 25 µm) are shown (n=3/group, data are presented as means±s.d., **p<0.01, ***p<0.001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (C-E) Arnt protein levels were analyzed by immunoblotting and immunostaining (scale bars 25 µm, n=6/group, data are presented as means±s.d., ***p<0.001, ****p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (F) Dimer formation of Arnt/Arnt, Arnt/Hif1α, Arnt/Hif2α and Arnt/Ahr in total kidney lysates was assessed by native gel electrophoresis after Arnt pulldown.
Figure 8. Selective modulation of FKBP12/YY1 effectively modulates protective ARNT within chronically injured kidneys. (A-D) Mice were treated daily with either intraperitoneal administration of control in vivo-morpholinos (control-VMO), in vivo-morpholinos targeting translational start site of Fkbp12 (Fkbp12-VMO), Yy1 (Yy1-VMO) or Arnt (Arnt-VMO) starting two days prior of surgery and orally treated with either vehicle buffer or FK506 (0.2 mg/kg orally per day) starting one day prior of surgery. Representative photomicrographs of kidney sections labelled for Arnt (scale bars 25 µm), MTS (scale bars 50 µm) and Collagen-1 (scale bars 25 µm) are shown (n=6/group, data are presented as means±s.d., **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis).
Figure 9. Selective targeting of FKBP12 by GPI-1046 effectively protects from chronic renal failure. (A,B) TECs were exposed to vehicle, FK506 (200 pM) or GPI-1046 (10 µM), relative Arnt and Alk3 mRNA expression levels were analyzed by qRT-PCR (n=3 independent experiments, data are presented as means±s.d., * p<0.05, **** p<0.0001, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (C) Mice were challenged with UUO and treated with either vehicle buffer or GPI-1046 (10 mg/kg subcutaneously per day) starting one day prior of surgery. (D,E) Analyzed by qRT-PCR 10 days after ureteral obstruction, intrarenal Arnt and Alk3 mRNA expression levels are shown (n=4/group, data are presented as means±s.d., *** p<0.001, **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (F-K) Representative photomicrographs of immunostainings for Alk3 (scale bars 25 µm), pSmad1/5/8 (scale bars 25 µm), PAS-stained fibrotic kidney sections (scale bars 50 µm), MTS (scale bars 50 µm), Collagen-1 (scale bars 25 µm) are shown (n=6/group, data are presented as means±s.d., * p<0.05, **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis).
Figure 10. Selective targeting of FKBP12 by GPI-1046 protects from already established fibrotic lesions. (A) Mice were challenged with UUO and administered either vehicle buffer, FK506 (0.2 mg/kg orally per day) or GPI-1046 (30 mg/kg orally per day) starting three days after UUO surgery. (B,C) Analyzed by qRT-PCR, intrarenal Arnt and Alk3 mRNA expression levels were assessed (n=4/group, data are presented as means±s.d., * p<0.05, **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (D-H) Representative photomicrographs of immunostainings for Alk3 (scale bars 25 µm), PAS-stained fibrotic kidney sections (scale bars 50 µm), MTS (scale bars 50 µm) and Collagen-1 (scale bars 25 µm) are shown (n=6/group, data are presented as means±s.d., ** p<0.01, *** p<0.001, **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis).
Figure 11. Pharmacological modulation of an FKBP12/YY1/ARNT/ALK3 signaling axis protects from chronic heart failure. (A,B) Analyzed by qRT-PCR, Arnt mRNA expression levels are shown in response to FK506 (0.2 mg/kg orally per day) or GPI-1046 (30 mg/kg orally per day, n=3/group, data are presented as means±s.d., * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis in comparison to vehicle-treated control mice). (C) Mice were challenged with AT II delivered by osmotic minipumps, vehicle buffer or GPI-1046 (10 mg/kg subcutaneously per day) were administered starting one day prior. (D-I) Representative photomicrographs of immunostainings for Alk3 (scale bars 25 µm), pSmad1/5/8 (scale bars 25 µm), MTS (scale bars 50 µm), Collagen-1 (scale bars 25 µm) and αSMA (scale bars 25 µm) in mice challenged with AT II are shown (n=6/group, data are presented as means±s.d., **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (J,K) Arnt and Alk3 mRNA expression levels were analyzed by qRT-PCR (n=4/group, data are presented as means±s.d., **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis).
Figure 12. Pharmacological modulation of an FKBP12/YY1/ARNT/ALK3 signaling axis protects from chronic liver failure. (A) Mice were challenged with intraperitoneal injections of CCl4, vehicle buffer or GPI-1046 (10 mg/kg subcutaneously per day) were administered starting one day prior. (B-H) Representative photomicrographs of immunostainings for Alk3 (scale bars 25 μm), pSmad1/5/8 (scale bars 25 μm), MTS-stained fibrotic kidney sections (scale bars 50 μm), Sirius red (scale bars 50 μm), Collagen-1 (scale bars 25 μm) and aSMA (scale bars 25 μm) in mice challenged with CCl4 are shown (n=5-7/group, data are presented as means±s.d., ** p<0.01, *** p<0.001, **** p<0.0001, # no significance, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis).
Figure 13. An FKBP12/YY1/ARNT signaling axis translates to humans. (A) Human TEC cultures were exposed to vehicle or indicated concentrations of FK506. ARNT mRNA expression was analyzed by qRT-PCR (n=3 independent experiments). (B) Analyzed by SDS-PAGE and subsequent immunoblotting, ALK3 was assessed in response to FK506. (C-H) In a small cohort of kidney transplant recipients with comparable histological patterns and immunosuppressive regimens based on CsA or FK506, kidney sections immunolabelled with primary antibodies against FKBP12 (scale bars 25 µm), YY1 (scale bars 25 µm), ARNT (scale bars 50 µm), ALK3 (scale bars 100 µm), and pSmad1/5/8 (scale bars 25 µm) are shown (measurements were done in 10 visual fields, data are presented as mean±s.d., *** p<0.001, **** p<0.0001, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis). (G,H) ARNT and ALK3 mRNA expression levels were assessed by qRT-PCR (measurements were done in technical triplicates, data are presented as mean±s.d., ** p<0.01, **** p<0.0001, values of p were calculated using one-way ANOVA with Bonferroni post-hoc analysis).