The Journal of Physiology

Prolyl-4-hydroxylases 2 and 3 control erythropoietin production in renin-expressing cells of mouse kidneys

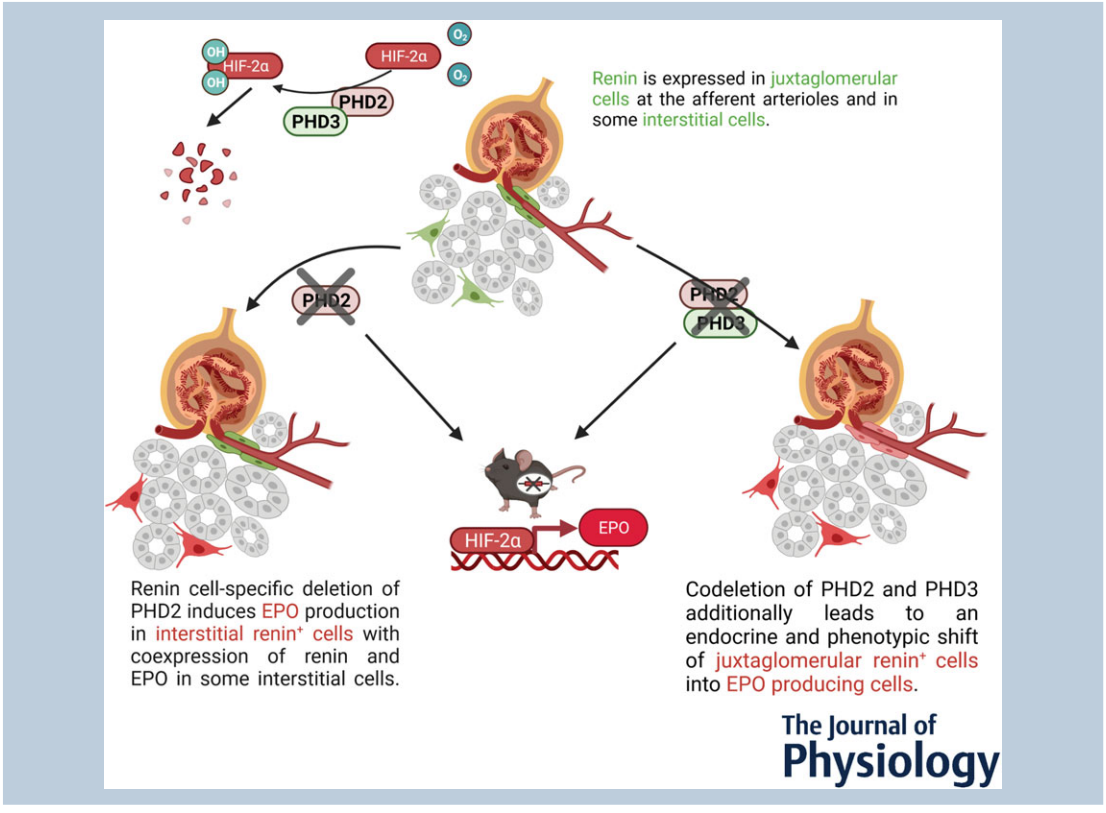
Katharina A. E. Broeker[1], Michaela A. A. Fuchs[1], Julia Schrankl[1], Claudia Lehrmann[2], Gunnar Schley[3], Vladimir T. Todorov[4], Christian Hugo[4], Charlotte Wagner[1] and Armin Kurtz[1]

[1]*Institute of Physiology, University of Regensburg, Regensburg, Germany*
[2]*Institute of Physiology II, University of Regensburg, Regensburg, Germany*
[3]*Department of Nephrology and Hypertension, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany*
[4]*Division of Nephrology, Department of Internal Medicine III, University Hospital Carl Gustav Carus, Dresden, Germany*

Edited by: Kim Barrett & Helle Praetorius

Linked articles: This article is highlighted in a Perspectives article by Fandrey. To read this article, visit https://doi.org/10.1113/JP282675.

The peer review history is available in the Supporting Information section of this article (https://doi.org/10.1113/10.1113/JP282615#support-information-section).



Katharina Broeker is a post doctoral fellow at the Institute of Physiology at the University of Regensburg. She started working in the field of erythropoietin research with her PhD, investigating the different cell types of potential renal EPO-producing cells. She has since been interested in the mechanisms that can induce EPO production in different cells and in investigating the fate of EPO-producing cells in damaged kidneys. In the future her research will be focused on the endocrine plasticity of renal interstitial cells and on characterizing these cells along with learning more about their physiological functions in health and disease.

DOI: 10.1113/JP282615

Abstract Activation of the hypoxia-signalling pathway induced by deletion of the ubiquitin-ligase von Hippel–Lindau protein causes an endocrine shift of renin-producing cells to erythropoietin (EPO)-expressing cells. However, the underlying mechanisms have not yet been investigated. Since oxygen-regulated stability of hypoxia-inducible transcription factors relevant for EPO expression is dependent on the activity of prolyl-4-hydroxylases (PHD) 2 and 3, this study aimed to determine the relevance of different PHD isoforms for the EPO expression in renin-producing cells *in vivo*. For this purpose, mice with inducible renin cell-specific deletions of different PHD isoforms were analysed. Our study shows that there are two subgroups of renal renin-expressing cells, juxtaglomerular renin$^+$ cells and platelet-derived growth factor receptor-β^+ interstitial renin$^+$ cells. These interstitial renin$^+$ cells belong to the cell pool of native EPO-producing cells and are able to express EPO and renin in parallel. In contrast, co-deletion of PHD2 and PHD3, but not PHD2 deletion alone, induces EPO expression in juxtaglomerular and hyperplastic renin$^+$ cells and downregulates renin expression. A strong basal PHD3 expression in juxtaglomerular renin$^+$ cells seems to prevent the hypoxia-inducible transcription factor-2-dependent phenotype shift into EPO cells. In summary, PHDs seem important for the stabilization of the juxtaglomerular renin cell phenotype. Moreover, these findings reveal tubulointerstitial cells as a novel site of renal renin expression and suggest a high endocrine plasticity of these cells. Our data concerning the distinct expression patterns and functions of PHD2 and PHD3 provide new insights into the regulation of renin-producing cells and highlight the need for selective PHD inhibitors.

(Received 17 November 2021; accepted after revision 29 November 2021; first published online 4 December 2021)
Corresponding author K. A. E. Broeker: Institute of Physiology, University of Regensburg, Universitätsstraße 31, D-93053 Regensburg, Germany. Email: katharina.broeker@ur.de

Abstract figure legend In addition to the typical juxtaglomerular renin-producing cells, interstitial cells expressing renin can be found. Renin cell-specific deletion of PHD2 induces EPO production in these interstitial renin$^+$ cells. Codeletion of PHD2/PHD3 additionally leads to a phenotypic shift of juxtaglomerular renin$^+$ cells into EPO producing cells. Created with BioRender.com®.

Key points

- Renal renin-expressing cells can be clearly distinguished into two subgroups, the typical juxtaglomerular renin-producing cells and interstitial renin$^+$ cells.
- Interstitial renin$^+$ cells belong to the cell pool of native erythropoietin (EPO)-producing cells, show a fast EPO response to acute hypoxia-inducible factor-2 (HIF-2) stabilization and are able to express EPO and renin in parallel.
- Only co-deletion of the prolyl-4-hydroxylases (PHD) 2 and 3, but not PHD2 deletion alone, induces EPO expression in juxtaglomerular renin$^+$ cells.
- Chronic HIF-2 stabilization in juxtaglomerular renin-expressing cells leads to their phenotypic shift into EPO-producing cells.
- A strong basal PHD3 expression in juxtaglomerular renin$^+$ cells seems to prevent a HIF-2-dependent phenotype shift into EPO cells suggesting PHD3 fulfils a stabilizer function for the juxtaglomerular renin cell phenotype.

Introduction

Maintenance of blood oxygen levels as well as salt and blood pressure homeostasis is a central endocrine function of the kidneys. These functions are mediated by the hormone erythropoietin (EPO), which regulates erythropoiesis, and by the protease renin, which regulates the activity of the renin–angiotensin–aldosterone system controlling blood pressure and salt homeostasis. Erythropoietin is produced and constitutively secreted by a few peritubular interstitial cells (Bachmann *et al.* 1993; Maxwell *et al.* 1993), regulated by oxygen delivery to the peritubular interstitium (Koury *et al.* 1989; Eckardt *et al.* 1993) and its gene transcription is triggered by the

transcription factor hypoxia-inducible factor-2 (HIF-2) (Scortegagna *et al.* 2003; Paliege *et al.* 2010). Renin is produced, stored and secreted by specialized vascular smooth muscle cells in the terminal juxtaglomerular segments of afferent arterioles (Gomez *et al.* 1989), regulated by blood pressure, salt content in the tubular lumen and sympathetic nerve activity (Skott & Briggs, 1987; Graham *et al.* 1990; Holmer *et al.* 1997). EPO- and renin-producing cells share some similarities in spite of their different localizations and functions. Both cell types derive from the FoxD1$^+$ renal mesenchymal progenitor population (Li *et al.* 2014). Regulation of EPO and of renin production occurs mainly by recruitment of additional cells rather than by a gradual regulation on a single cell level (Koury *et al.* 1989; Berka *et al.* 1990; Eckardt *et al.* 1993; Machura *et al.* 2012). Thus increasing interstitial tissue hypoxia leads to a recruitment of EPO-expressing cells in the interstitium spreading from the cortico-medullary junction throughout the whole cortex and outer medulla (Eckardt *et al.* 1993; Broeker *et al.* 2020). Hypotension or salt deficiency lead to a recruitment of juxtaglomerular renin-expressing cells along the afferent arterioles and expanding into the perivascular and periglomerular interstitium of the cortex leading to renin cell hyperplasia (Cantin *et al.* 1977; Sequeira López *et al.* 2004). Over the past years evidence has been provided that erythropoietin and renin production might be related more closely than previously thought. A recent study reported that at least a subpopulation of tubulointerstitial cells might be capable of expressing renin in states of severe anaemia (Miyauchi *et al.* 2021), which is commonly expected to drive interstitial EPO expression. Recently, we found that cell type-specific deletion of the ubiquitin-ligase von Hippel–Lindau protein (VHL), which causes a stabilization of the hypoxia-inducible transcription factors, led to a HIF-2-dependent endocrine shift of juxtaglomerular renin-producing cells. These cells stopped producing renin and instead started to express EPO (Kurt *et al.* 2013, 2015; Gerl *et al.* 2015). The mechanisms underlying this endocrine shift are still not clear, as loss of VHL may affect multiple pathways in addition to HIF-stabilization. Moreover, the observed endocrine shift has not yet been reported for other situations leading to tissue hypoxia such as arterial hypoxia or anaemia.

The hypoxia-signalling pathway relevant for EPO gene regulation is dependent on the activity of prolyl-4-hydroxylases (PHD) which convey the oxygen sensitivity of HIFs (Schofield & Ratcliffe, 2004; Kaelin & Ratcliffe, 2008). For HIF-triggered EPO gene expression in the kidneys, PHD2 and PHD3 are considered the relevant isoforms (Minamishima *et al.* 2008; Takeda *et al.* 2008; Kobayashi *et al.* 2017). Since these PHD isoforms might be differentially expressed among cell types, we were interested in evaluating the role of these PHDs for the endocrine function of renin-producing cells. In this context we aimed to analyse not only the typical juxtaglomerular renin$^+$ cells but also periglomerular hyperplastic renin$^+$ cells, which can develop, for example, in situations of insufficient aldosterone production (Makhanova *et al.* 2006). For analysing the basal PHD expression pattern in juxtaglomerular and hyperplastic renin$^+$ cells, RNAscope was performed. After renin cell-specific deletion of PHDs, the localization and number of EPO$^+$ cells were analysed. Moreover, renin expression in response to PHD2 and/or PHD3 deletion was determined.

Methods

Ethical approval

All animal experiments were performed according to the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and the animal ethics checklist of this journal (Grundy, 2015). All experiments were approved by the local ethics committee and in compliance with the guidelines of the animal facility of the University of Regensburg.

Animals

Animals were housed under specific pathogen-free conditions. Mice were kept on a controlled 12 h light and 12 h dark cycle and a controlled level of temperature and humidity. They were maintained on standard rodent chow (0.6% NaCl; Ssniff, Soest, Germany) with free access to tap water.

Experimental groups consisted of males and females in similar numbers (age 14–16 weeks; genetic background C57/Bl6J). All animals were euthanized by dislocation of the neck after sedation (ketamine, 80 mg/kg body weight (BW); xylazine 10 mg/kg BW, intraperitoneally).

To specifically target PHD or VHL expression in renin-producing cells, mice expressing a reverse tetracycline-dependent *trans* activator from the mouse renin gene locus (mRen-rtTAm2) (Starke *et al.* 2015) combined with a Cre recombinase controlled by the Tet-on system (LC1-Cre) (Schönig *et al.* 2002) were used, referred to as iRen$^{Cre/+}$ mice.

iRen$^{Cre/+}$ PHD2^{ff}, iRen$^{Cre/+}$ PHD3^{ff}, iRen$^{Cre/+}$ PHD2^{ff} PHD3^{ff} and iRen$^{Cre/+}$ Vhl^{ff} mice were generated by cross-breeding iRen$^{Cre/+}$ mice and mice with loxP-flanked PHD2 (Franke *et al.* 2013; Singh *et al.* 2013) and/or PHD3 (Takeda *et al.* 2006) or VHL (JAX stock no. 012933; The Jackson Laboratory, Bar Harbor, ME, USA) (Haase *et al.* 2001) alleles.

Furthermore, mice lacking aldosterone synthase (Aldo-KO mice), an established mouse model with

Table 1. Primer sequences used for genotyping

Genotype	Sequence (5′ to 3′), fwd	Sequence (5′ to 3′), rev	Sequence (5′ to 3′), rev2
PHD2 flox	CGCATCTTCCATCTCCATTT	CTCACTGACCTACGCCGTGT	GGCAGTGATAACAGGTGCAA
PHD3 flox	ATGGCCGCTGTATCACCTGTAT	CCACGTTAACTCTAGAGCCACTGA	
Vhl flox	CTAGGCACCGAGCTTAGAGGTTTGCG	CTGACTTCCACTGATGCTTGTCACAG	
LC1-Cre	GAAATGTCCGTTCGGTTGGCAGAAGC	CCAAAACCGTGATGGAATGGAACAACA	
mRen-rtTAm2	CCCACTTCTGAGACAAGC	GGTCAAAGTCGTCAAGGG	
Aldo	ACCAGAGCCCAAATGTCTCA	CTGATGCATCTCCAGGTGTA	CTGAACTTGTGGCCGTTTAC
mT/mG	CTCTGCTGCCTCCTGGCTTCT	CGAGGCGGATCACAAGCAATA	TCAATGGGCGGGGGTCGTT

juxtaglomerular renin cell hyperplasia, were used in this study (Lee *et al.* 2005; Makhanova *et al.* 2006). iRen$^{Cre/+}$ Aldo-KO PHD2^{ff}, iRen$^{Cre/+}$ Aldo-KO PHD3^{ff}, iRen$^{Cre/+}$ Aldo-KO PHD2^{ff} PHD3^{ff} and iRen$^{Cre/+}$ Aldo-KO Vhl^{ff} mice were generated by crossbreeding iRen$^{Cre/+}$ mice with the aldosterone synthase-deficient strain and mice carrying the respective loxP-flanked PHD and VHL alleles.

For lineage tracing of renin-producing cells, iRen$^{Cre/+}$ mT/mG and iRen$^{Cre/+}$ Aldo-KO mT/mG mice were used. The membrane-Tomato/membrane-green fluorescent protein (GFP) (mT/mG; JAX stock no. 007676) (Muzumdar *et al.* 2007) reporter mice express GFP in all cells with Cre recombinase activity.

Genotyping was performed using the primers listed in Table 1. Littermates negative for the Cre construct were used as controls.

Induction of Cre activity

iRen$^{Cre/+}$ activity was induced at an age of 8 weeks by giving the mice doxycycline hyclate via drinking water *ad libitum* for 21 days (2 g doxycycline/l, 5% sucrose, protected from light, exchanged every 3–4 days; AppliChem GmbH, Darmstadt, Germany), followed by a 3-week period without doxycycline.

ICA injection

The stabilization of HIFs was achieved with a single bolus injection of the unspecific prolyl-4-hydroxylase inhibitor 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido) acetate (ICA) (40 mg/kg BW) or vehicle (phosphate-buffered saline; PBS) intraperitoneally, as described in previous studies (Schley *et al.* 2012; Wang *et al.* 2012*b*; Gerl *et al.* 2016). Mice were analysed 4 h after the injection.

In situ hybridization via RNAscope

Target mRNAs were detected with the RNAscope® Multiplex Fluorescent v2 kit (Advanced Cell Diagnostics ACD, Hayward, CA, USA). The *in situ* hybridization was performed according to the manufacturer's instructions (Wang *et al.* 2012*a*). Kidney tissue was perfusion-fixed with 40 ml of 10% neutral buffered formalin solution, dehydrated in an ascending ethanol and isopropanol series and embedded in paraffin. Hybridization signals were detected on 5 μm tissue sections using Opal fluorophores 480, 570, and 690 (Akoya Biosciences, Marlborough, MA, USA) in Tyramide Signal Amplification (TSA) buffer. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) included in the Multiplex Fluorescent v2 kit. Slices were mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA) and stored at 4°C until analysed. To ensure interpretable results, positive and negative controls were routinely enclosed and evaluated. RNAscope probes used are listed in Table 2.

Table 2. RNAscope probes used for *in situ* hybridization

RNAscope® probe	Cat No.
EGFP-C2	400281-C2
Mm-Egln1 (PHD2)	315491
Mm-Egln3 (PHD3)	434931
Mm-Epo	315501
Mm-Epo-C2	315501-C2
Mm-Gja5 (connexin 40)	518041
Mm-Nt5e (CD73)	437951
Mm-Pdgfrb	411381
Mm-Pdgfrb-C2	411381-C2
Mm-Renin-C3	433461-C3
2.5 Duplex positive control probe -Mm	321651
2-plex negative control probe	320751

Cell counting

For cell counting ImageJ software was used. Cells were counted using the tool 'cell counter notice' on three transverse sections per kidney and the mean values were calculated. In total six animals per genotype were

analysed. Counting was performed by two persons independently, without knowledge of the genotype or treatment of the mice.

Immunofluorescence/immunohistochemistry

For immunofluorescence staining, kidney tissue was perfusion fixed with 3% paraformaldehyde, dehydrated in an ascending methanol and isopropanol series and embedded in paraffin. Five-micrometre sections of paraffin-embedded kidneys were blocked with 10% horse serum/1% BSA in PBS and incubated with goat anti-renin (AF4277, R&D Systems, Minneapolis, MN, USA), chicken anti-GFP (ab13970, Abcam, Cambridge, UK) and rabbit anti-platelet-derived growth factor receptor (PDGFR)-β (ab32570, Abcam) antibodies at 4°C overnight. After three washes with BSA/PBS, sections were incubated with Cy2, Cy3 and Cy5 secondary antibodies (Dianova, Hamburg, Germany) and mounted with Glycergel (Agilent, Waldbronn, Germany). HIF-2α and HIF-1α (polyclonal rabbit anti-HIF-1α; dilution of 1:10,000; Cayman Chemicals, Ann Arbor, MI, USA) stainings were performed on 3 μm paraffin-embedded kidney sections using the signal amplification system (CSA) from DAKO (Glostrup, Denmark) as described elsewhere (Schley *et al*. 2012, 2019; Buchholz *et al*. 2014).

Microscopy

All micrographs were captured with an Axio Observer.Z1 Microscope (Zeiss, Jena, Germany) and a Plan-Apochromat ×20/0.8 objective, a ×1 tube lens and an Apotome.2 system. The Colibri7 (Zeiss) was used as the light source. Chromogenic images were taken with the Axiocam 305 colour, fluorescence images with an Axiocam 506mono. Excitation wavelength used were 630, 567, 475 and 385 nm and emissions were detected at 673, 561, 500 and 465 nm, respectively. For detail images, 5–15 z-stacked images were combined for maximum projection. Overviews of whole kidney sections were generated by combination of tiles taken at ×20 magnification. Images represented in the same figure were captured with the same light intensities and exposure times.

Determination of mRNA expression by real-time PCR

Total RNA was isolated from kidneys using acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski & Sacchi, 1987) and quantified by photometer. For cDNA synthesis via Moloney murine leukaemia virus reverse transcriptase (Thermo Fisher Scientific), 1 μg of the isolated RNA was used. mRNA expression abundance was quantified by real-time PCR using the LightCycler SYBR Green I Master Kit and the LightCycler 480 Instrument (Roche Diagnostics, Mannheim, Germany). mRNA expression data were normalized to ribosomal protein L32 (RPL32). Table 3 lists primer sequences.

Table 3. Primer sequences used for qPCR

Genes	Sequence (5′–3′)	Product size (bp)
RPL32	fwd: TTAAGCGAAACTGGCGGAAAC rev: TTGTTGCTCCCATAACCGATG	100
Akr1b7	fwd: CCACTGGCCACAGGGATT rev: TTTGCCTTTATTGTCTTTGGGTAA	61
EPO	fwd: AATGGAGGTGGAAGAACAGG rev: ACCCGAAGCAGTGAAGTGA	174
Renin	fwd: ATGAAGGGGGTGTCTGTGGGGTC rev: ATGTCGGGGAGGGTGGGCACCTG	194

Abbreviations: Akr1b7, aldo-keto reductase family 1, member B7; EPO, erythropoietin; RPL32, ribosomal protein L32.

Determination of haematocrit values, plasma EPO concentrations and plasma renin concentrations

Blood samples were taken from tail vein into heparin-coated capillary tubes (Kabe Labortechnik GmbH, Nümbrecht, Germany). Haematocrit values were determined after centrifugation (4 min, 14,354 g, room temperature). Plasma samples were stored at −80°C prior to use. Plasma EPO concentration was determined using the Quantikine Mouse EPO ELISA kit (R&D Systems), according to the manufacturer's protocol. Plasma renin concentration was analysed using the DuoSet Mouse Renin 1 Kit (R&D Systems) according to the manufacturer's protocol.

Statistical analyses

All data are presented as means ± SD. Statistical significance was determined by one-way ANOVA with Tukey's correction or unpaired Student's *t*-test, two-tailed as stated in the Results section. *P*-values and group size are stated in the Results section. The data were analysed using GraphPad Prism9 (GraphPad Software, San Diego, CA, USA).

Results

Only deletion of PHD2 or co-deletion of PHD2 and PHD3, but not PHD3 deletion alone, induces EPO production in renin+ cells

To study the functional role of different PHD isoforms in renin-expressing cells, PHD2 and/or PHD3 was deleted

Table 4. Haematocrit values and number of EPO mRNA-expressing cells per transverse kidney section of mice with cell type-specific deletions of PHD isoforms or VHL deletion

	iRen$^{Cre/+}$				
	Control	PHD2^{ff}	PHD3^{ff}	PHD2^{ff} PHD3^{ff}	Vhl^{ff}
Hct (%)	49.4 ± 2.3 (*n* = 13)	73.3 ± 9.7* (*n* = 11)	49.8 ± 1.7 (*n* = 11)	72.9 ± 6.7* (*n* = 11)	75.4 ± 4.8* (*n* = 11)
EPO^{+} cells per transverse kidney section					
Interstitial	11.2 ± 4.8	183.5 ± 74.3*	9.8 ± 5.8	212.5 ± 52.3*	203.5 ± 65.6*
Juxtaglomerular	none	none	none	68.0 ± 18.7	74.0 ± 17.0

Values are means ± SD. $*P < 0.0001$ *vs*. control animals. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test. For the determination of EPO cell numbers, three transverse kidney sections per animal were analysed and the mean values calculated; in total six animals per genotype were analysed and values are means ± SD of six animals per group.

under the control of the renin gene promotor in adult mouse kidneys.

Deletion of PHD2 in renin^{+} cells upregulated renal EPO mRNA abundance and plasma EPO concentrations by about 5-fold compared to controls. The mice became polycythaemic with haematocrit values of about 73%.

Deletion of PHD3 exerted no effect on renal EPO mRNA abundance, plasma EPO concentrations and haematocrit values (Fig. 1, Table 4).

Co-deletion of PHD2 and PHD3 increased EPO mRNA abundance and plasma EPO concentrations to numerically higher levels than PHD2 deletion alone (6-fold increase compared to controls; Fig. 1). The mice showed haematocrit values of about 73% (Table 4). For comparison, animals with inducible deletion of the ubiquitin ligase von Hippel–Lindau protein (VHL) were analysed. VHL deletion prevents the proteasomal degradation of HIF-2α and therefore induces EPO production. EPO expression, plasma EPO concentrations and haematocrit levels in VHL deficient mice were increased to a similar degree as the co-deletion of PHD2 and PHD3 (Fig. 1, Table 4).

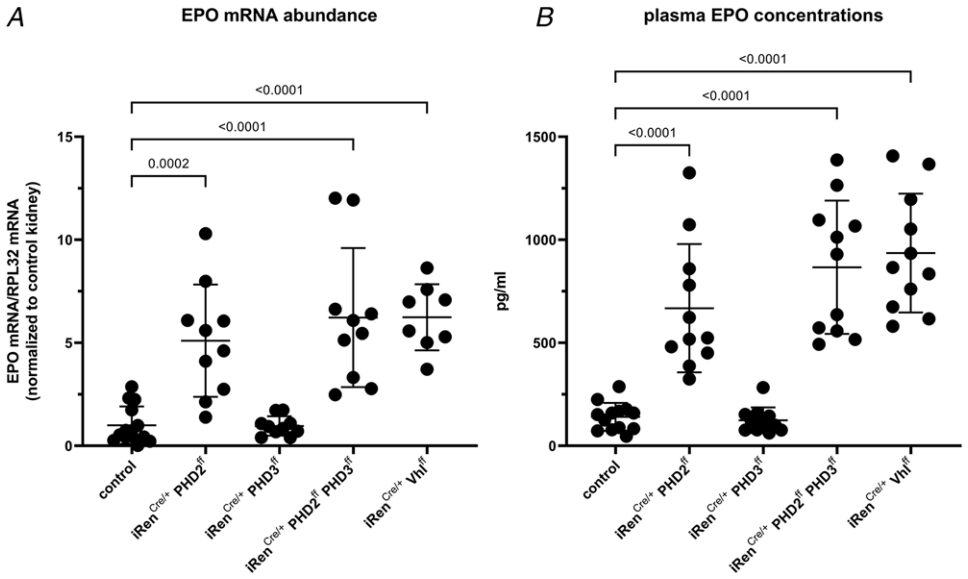


Figure 1. EPO mRNA abundance (*A*) and plasma EPO concentrations (*B*) in mice with conditional deletion of different PHD isoforms or VHL in renin-expressing cells
Deletion of PHD2 in renin-expressing cells significantly increased renal EPO mRNA expression and plasma EPO concentrations compared to control animals. Conditional deletion of PHD3 had no effect on renal EPO expression. Deletion of both PHD2 and PHD3, under control of the renin gene promotor, led to a further increase of renal EPO mRNA abundance and plasma EPO levels compared to PHD2 deletion alone. VHL deletion had the same effect on the expression of EPO as the combined deletion of PHD2 and PHD3. Data are means ± SD of ≥8 mice per genotype. *P*-values are stated above the respective line. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test.

Interstitial but not juxtaglomerular renin$^+$ cells express EPO after PHD2 deletion. Juxtaglomerular renin$^+$ cells undergo an endocrine shift after PHD2 and PHD3 co-deletion with upregulation of EPO and downregulation of renin expression

Inducible deletion of PHD2 in renin$^+$ cells led to an increase of EPO-expressing cells from a mean of 11 to about 184 cells per transverse kidney section (Table 4, Fig. 2). In control animals, few native EPO$^+$ cells are located in the interstitium along the cortico-medullary border. Interestingly after PHD2 deletion, the additional EPO-expressing cells were detected only throughout the interstitium of the cortex and outer medulla but not in juxtaglomerular regions that are the typical sites of renin production (Fig. 3).

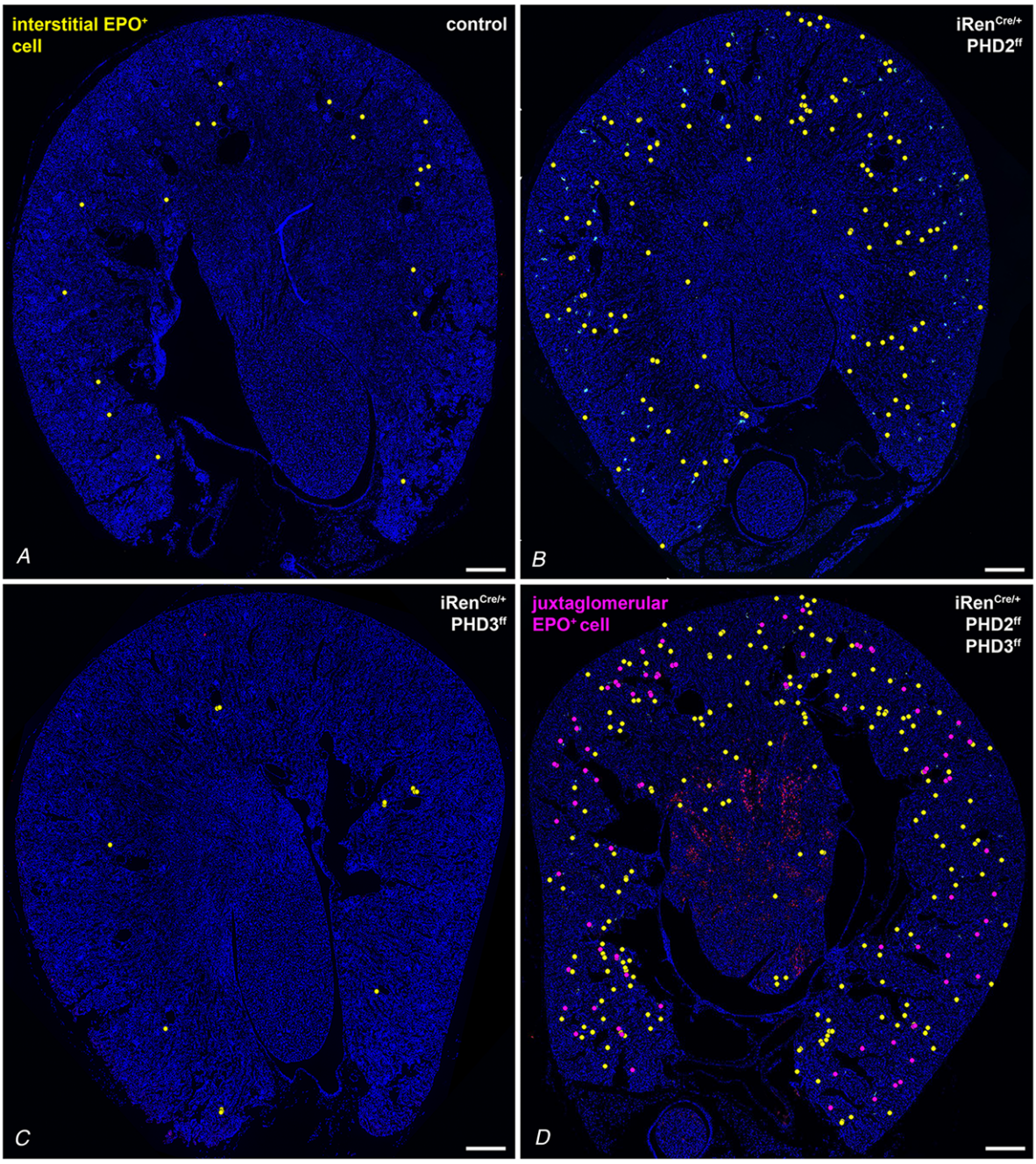


Figure 2. RNAscope for EPO mRNA on whole kidney sections of wild-type mice and mice with cell-specific deletion of PHD isoforms in renin-expressing cells
EPO mRNA-expressing cells are highlighted with coloured circles. Kidneys of wild-type and iRen$^{Cre/+}$ PHD3^{ff} animals showed only a few interstitial EPO$^+$ cells (yellow circles) per transverse kidney section (*A* and *C*).

Deletion of PHD2 in renin-expressing cells raised the number of interstitial EPO$^+$ cells to a mean of 184 per kidney section (*B*). Additional deletion of PHD3 induced EPO mRNA expression in formerly renin-expressing juxtaglomerular cells at the glomerular pole (pink circles, *D*). The number of interstitial EPO-expressing cells was not further increased compared to iRen$^{Cre/+}$ PHD2^{ff} animals. Nuclei were counterstained with DAPI (blue). Scale bars: 500 μm.

We therefore investigated the extent to which interstitial cells actively express renin in wild-type mouse kidneys. RNAscope for renin and PDGFR-β mRNA, which is the classical marker for interstitial cells, confirmed the existence of renin-expressing cells in the interstitium (239.4 $\pm$ 90.4 interstitial renin$^+$ cells per transverse kidney section; $n = 10$; Fig. 4). Renin cell lineage tracing via immunofluorescence on kidney sections of iRen$^{Cre/+}$ mT/mG mice further confirmed the activity of the renin gene promotor in interstitial cells (207.1 $\pm$ 67.3 interstitial GFP$^+$ cells per transverse kidney section; $n = 8$). However, we could not detect renin immunoreactivity in GFP$^+$ tubulointerstitial cells (Fig. 5).

PDGFR-β^+ interstitial cells include all potential EPO-expressing cells of the kidneys. Thus, we examined if these cells are in principle able to express EPO and renin at the same time after iRen$^{Cre/+}$ driven deletion of PHD2. RNAscope for EPO and renin mRNA revealed that about 12.6% of EPO$^+$ interstitial cells coexpressed renin mRNA ($n = 6$; Fig. 6*A*). Moreover, we analysed renin lineage induced GFP mRNA expression via RNAscope. This revealed that the EPO$^+$ cells in iRen$^{Cre/+}$ PHD2^{ff} mT/mG animals coexpressed GFP mRNA labelling these cells as interstitial renin lineage cells (Fig. 6*B*).

Deletion of PHD3 in renin$^+$ cells did not induce EPO expression and exerted no effect on the number of interstitial EPO cells compared to control animals (Table 4, Figs 2 and 3*C*).

After PHD2 and PHD3 co-deletion in renin$^+$ cells, we observed EPO induction not only in interstitial cells but also in juxtaglomerular renin$^+$ cells. The number of induced interstitial EPO$^+$ cells did not differ from mice with only PHD2 deletion. However, additional EPO mRNA expression was detected in about 70 juxtaglomerular renin$^+$ cells per transverse kidney section. Renin mRNA expression was strongly downregulated in these cells. Only some juxtaglomerular cells coexpressed renin and EPO mRNA (Table 4, Figs 2 and 3*D*). Former juxtaglomerular renin-producing cells were

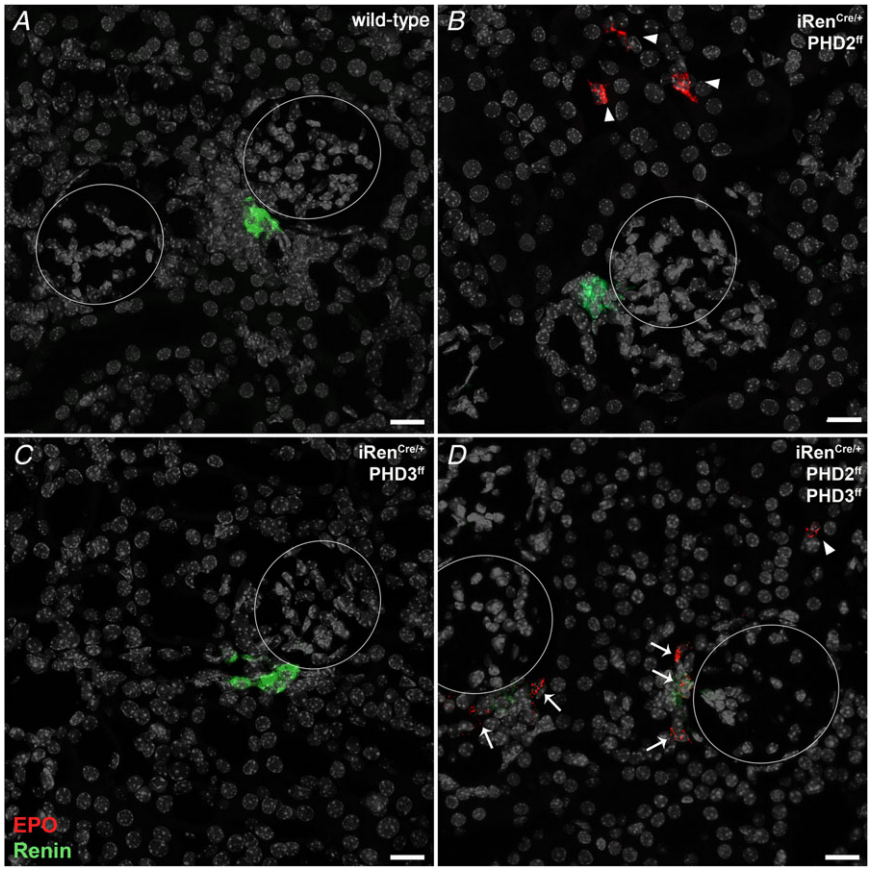


Figure 3. RNAscope for EPO (red) and renin (green) mRNA on kidney sections of wild-type mice and mice with cell-specific deletions of PHD isoforms in renin-expressing cells
Circles indicate glomeruli. Nuclei were counterstained with DAPI (grey). *A*, wild-type mice showed robust renin expression in the typical juxtaglomerular position. *B*, deletion of PHD2 under control of the renin promotor induced EPO mRNA expression exclusively in interstitial cells throughout the renal cortex and outer medulla (arrowheads). Renin mRNA expression at the glomerular pole was unchanged. *C*, deletion of PHD3 alone did not alter EPO or renin expression. *D*, co-deletion of PHD2 and PHD3 in renin-producing cells induced interstitial EPO expression (arrowhead) and led to an endocrine shift of the juxtaglomerular renin-producing cells with a significant downregulation of renin mRNA expression and *de novo* expression of EPO mRNA (arrows). Scale bars: 20 μm.

identified using renin lineage induced GFP expression (Fig. 7).

Results for VHL-deficient mice mimicked the findings obtained in mice after PHD2 and PHD3 co-deletion (Table 4).

Interstitial renin$^+$ cells express PHD2, whereas juxtaglomerular renin$^+$ cells coexpress PHD2 and PHD3

To investigate the differential induction of EPO expression in juxtaglomerular and interstitial renin$^+$

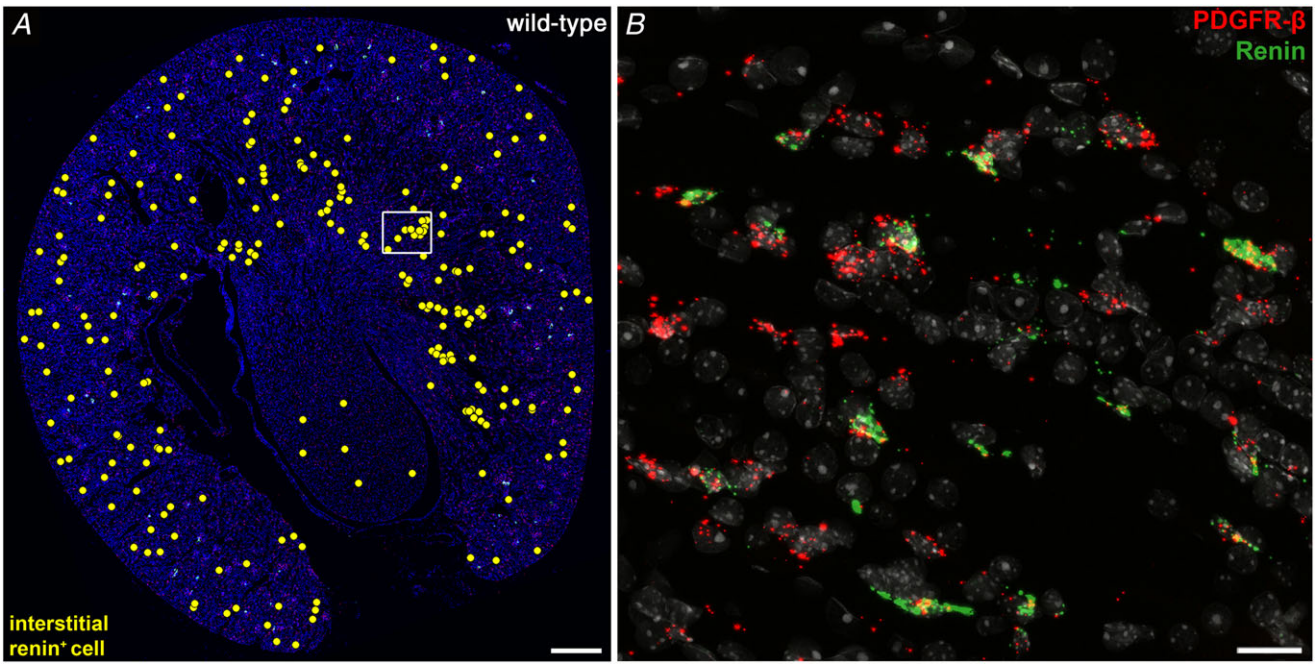


Figure 4. Co-hybridization of renin and PDGFR-β mRNA on a kidney section of a wild-type mouse
A, overview of a transverse kidney section showed about 239 interstitial renin$^+$ cells per kidney section throughout the kidney cortex and the outer medulla (highlighted with yellow circles). Nuclei were counterstained with DAPI (blue). Scale bar: 500 μm. *B*, high magnification detail of the co-hybridization of renin (green) and PDGFR-β (red) mRNA as a marker for interstitial cells. Colocalization of both mRNAs confirmed that some interstitial PDGFR-β^+ cells express renin. Nuclei were counterstained with DAPI (grey). Scale bar: 20 μm.

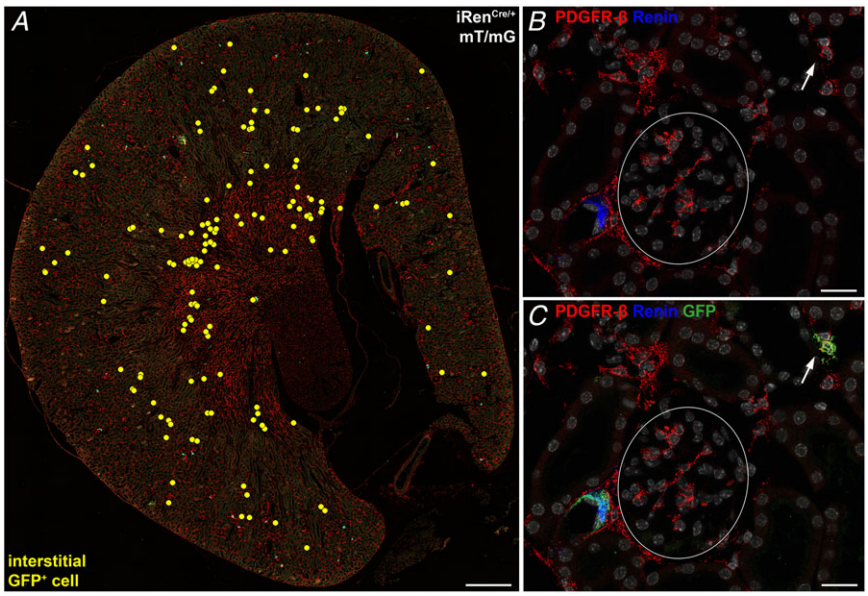


Figure 5. Lineage tracing of renin cells using renin gene promotor-driven GFP expression
A, interstitial GFP$^+$ cells are highlighted with yellow circles on a kidney section of an iRen$^{Cre/+}$ mT/mG reporter mouse tracing the renin cell lineage with a double immunofluorescence staining for GFP and PDGFR-β. GFP expression showed that the renin promotor was active in these interstitial cells during doxycycline treatment. Scale bar: 500 μm. *B* and *C*, detail of immunofluorescence staining for PDGFR-β (red), renin (blue) and GFP (green). Interstitial renin lineage cells coexpressed PDGFR-β and GFP (arrow), but showed no immunoreactivity for renin. Juxtaglomerular cells were positive for renin and GFP. Nuclei were counterstained with DAPI (grey). Circles indicate glomeruli. Scale bars: 20 μm.

cells, we analysed the expression pattern for PHD2 and PHD3. PHD2 mRNA was widely distributed in many cells across all kidney zones, including interstitial, mesangial and tubular cells (Fig. 8). In contrast, PHD3 mRNA was expressed by fewer cells. Prominent PHD3 RNAscope signals were observed in juxtaglomerular renin-producing cells. These juxtaglomerular renin-expressing cells also expressed PHD2 (Fig. 8, top). Triple *in situ* hybridization for renin, PDGFR-β and either PHD2 or PHD3 mRNA showed that almost all interstitial renin$^+$ cells expressed PHD2, but only a few expressed PHD3 (Fig. 8, bottom).

EPO production in hyperplastic renin$^+$ cells is regulated like in native juxtaglomerular renin$^+$ cells

Some situations like low blood pressure or chronic salt deficiency lead to an increased production of renin through modulation of the renin–angiotensin–aldosterone system. Additional renin is produced by the reversible recruitment of renin-expressing cells leading to renin cell hyperplasia at the glomerular pole. Renin cell hyperplasia comprises retrograde induction of renin expression in cells of the afferent arterioles and more prominently induction of renin in periglomerular–perivascular cells.

We aimed to investigate whether hyperplastic renin$^+$ cells showed a similar ability to produce EPO after deletion of different PHD isoforms as we observed in native renin$^+$ cells. In aldosterone synthase-deficient mice (Aldo-KO mice), chronic renin–angiotensin–aldosterone system dysregulation led to robust renin cell hyperplasia (Fig. 9).

Thus, we used Aldo-KO mice to study the expression of PHDs in hyperplastic renin$^+$ cells. Compared to wild-type mice, renin mRNA levels and plasma renin concentrations were 13- and 15-fold elevated in Aldo-KO mice (see also Fig. 12).

Hyperplastic juxtaglomerular renin$^+$ cells showed a clear expression of PHD3 mRNA, similar to juxtaglomerular renin$^+$ cells of wild-type mice. Hyperplastic renin$^+$ cells also expressed PHD2 mRNA albeit to a lesser extent compared to the expression of PHD3 (Fig. 9).

We generated mouse models with inducible deletion of different PHD isoforms under control of the renin promotor in mice with Aldo-KO background (iRen$^{Cre/+}$ Aldo-KO PHD2^{ff} mice, iRen$^{Cre/+}$ Aldo-KO PHD3^{ff} mice and iRen$^{Cre/+}$ Aldo-KO PHD2^{ff} PHD3^{ff} mice). As in juxtaglomerular renin$^+$ cells, lack of PHD3 in hyperplastic renin$^+$ cells had no effect on EPO expression in these cells (Fig. 10*C*).

iRen$^{Cre/+}$-induced PHD2 deletion in Aldo-KO mice stimulated EPO expression to a similar extent as in mice with intact aldosterone synthase. Again, induction of EPO gene expression was exclusively observed in about 200 interstitial cells of the cortex and outer medulla, but not in juxtaglomerular hyperplastic cells (Tables 4 and 5, Fig. 10). In accordance with this finding was the observation that deletion of PHD2 only marginally stabilized HIF-2α in hyperplastic renin$^+$ cells (Fig. 11*B*).

Inducible co-deletion of PHD2 and PHD3 in Aldo-KO mice led again to an endocrine shift of juxtaglomerular and hyperplastic renin$^+$ cells as observed in iRen$^{Cre/+}$ PHD2^{ff} PHD3^{ff} mice with an induction of EPO expression and a parallel downregulation of renin. EPO mRNA abundance and plasma EPO concentrations increased to significantly higher levels than for PHD2 deletion alone (Fig. 10). In those kidneys, EPO expression was detected in both interstitial and juxtaglomerular hyperplastic cells (Fig. 10*D*). HIF-2α immunoreactivity was readily detectable in hyperplastic renin$^+$ cells after PHD2

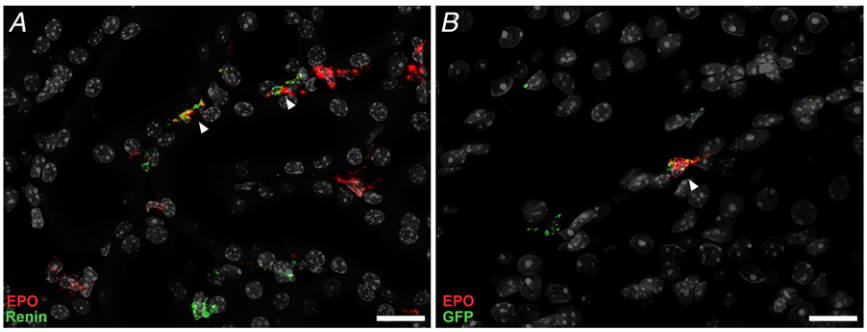


Figure 6. Co-RNAscopes for EPO and renin mRNA or EPO and GFP mRNA on iRen$^{Cre/+}$ PHD2^{ff} mT/mG kidney sections
Nuclei were counterstained with DAPI (grey). *A*, after deletion of PHD2 in renin$^+$ cells, some interstitial renin$^+$ cells (green) coexpressed EPO mRNA (red, arrowheads). The overlap of renin and EPO mRNA was detected in about 12.6% of all interstitial EPO$^+$ cells on kidney sections of iRen$^{Cre/+}$ PHD2^{ff} mT/mG mice. *B*, former interstitial renin-expressing cells were detected via GFP expression using iRen$^{Cre/+}$ PHD2^{ff} mT/mG reporter mice. These mice expressed GFP in all cells in which the Cre recombinase was active. Co-hybridization of EPO (red) and GFP (green) mRNA confirmed that interstitial EPO-expressing cells were renin lineage cells (arrowhead). Scale bars: 20 μm.

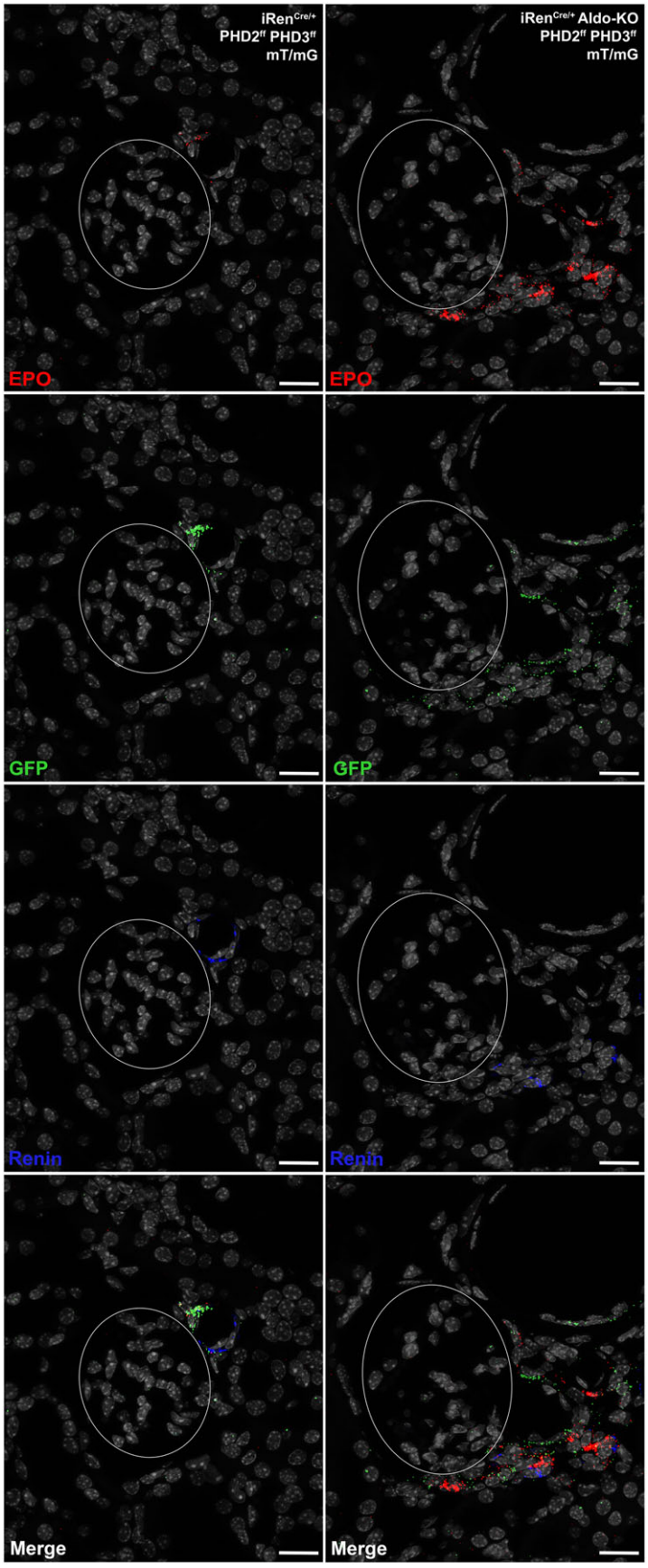


Figure 7. Co-RNAscopes for EPO (red), GFP (green) and renin (blue) mRNA on kidney sections of iRen$^{Cre/+}$ PHD2^{ff} PHD3^{ff} mT/mG mice or iRen$^{Cre/+}$ Aldo-KO PHD2^{ff} PHD3^{ff} mT/mG mice
Nuclei were counterstained with DAPI (grey). Circles indicate glomeruli. Left column: after co-deletion of PHD2 and PHD3 in renin-expressing cells, juxtaglomerular renin^{+} cells underwent an endocrine shift. The cells started to express EPO, whilst renin expression was strongly downregulated. Former renin-producing cells could be identified by iRen$^{Cre/+}$ induced GFP expression using mT/mG reporter mice for lineage tracing. Right column: co-deletion of PHD2 and PHD3 in hyperplastic renin^{+} cells also led to an endocrine shift of these cells. They expressed EPO, whilst renin expression was significantly downregulated. Former renin-producing cells could be identified by iRen$^{Cre/+}$ induced GFP expression using mT/mG reporter mice for lineage tracing. Only a few hyperplastic cells expressed EPO and renin simultaneously. Scale bars: 20 μm.

Table 5. Haematocrit values and number of EPO-expressing cells per transverse kidney section of Aldo-KO mice with different renin cell-specific deletions of PHD isoforms or VHL

	iRen$^{Cre/+}$ Aldo-KO				
	Control	PHD2^{ff}	PHD3^{ff}	PHD2^{ff} PHD3^{ff}	Vhl^{ff}
Hct (%)	50.6 ± 2.4 (n = 13)	82.0 ± 6.8* (n = 11)	50.6 ± 3.1 (n = 11)	83.1 ± 5.9* (n = 11)	83.3 ± 5.1* (n = 9)
EPO$^+$ cells per transverse kidney section					
Interstitial	11.0 ± 8.0	188.2 ± 45.2*	9.5 ± 8.7	212.7 ± 81.5*	221.3 ± 58.9*
Juxtaglomerular	none	none	none	227.0 ± 87.3	231.8 ± 56.5

Values are means ± SD. *P < 0.0001 *vs.* control animals. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test. For the determination of EPO cell numbers, three transverse kidney sections per animal were analysed and the mean values calculated; in total six animals per genotype were analysed and values are means ± SD of six animals per group.

and PHD3 co-deletion (Fig. 11*C*). In contrast to the upregulation of EPO, renin expression was strongly downregulated in the hyperplastic cells. Coexpression of renin and EPO mRNA was only rarely observed in juxtaglomerular hyperplastic cells (Figs 10*D* and 7).

Co-deletion of PHD2 and PHD3 in Aldo-KO mice increased EPO expression to the same degree as the stabilization of HIF-2 through deletion of VHL (Table 5).

Additionally, HIF-1α stabilization was analysed in the different genotypes. A positive nuclear staining could only be observed in juxtaglomerular hyperplastic cells and some interstitial cells after PHD2 and PHD3 co-deletion (Fig. 11*D*–*F*).

PHD2 and PHD3 co-deletion leads to a phenotypic shift of juxtaglomerular renin$^+$ cells

Since the RNAscope analysis suggested an endocrine shift with a strong downregulation of juxtagomerular and hyperplastic renin expression in mice with an induction of EPO in these cells, we further analysed renin expression. Kidney renin mRNA abundance and plasma renin concentrations were significantly reduced in non-Aldo-KO and Aldo-KO mice with PHD2 and PHD3 co-deletion compared to the respective control mice (Fig. 12). VHL deletion led to similar decreases in renin expression and plasma renin concentrations as co-deletion of PHD2 and PHD3. PHD2 deletion alone did not change renin production.

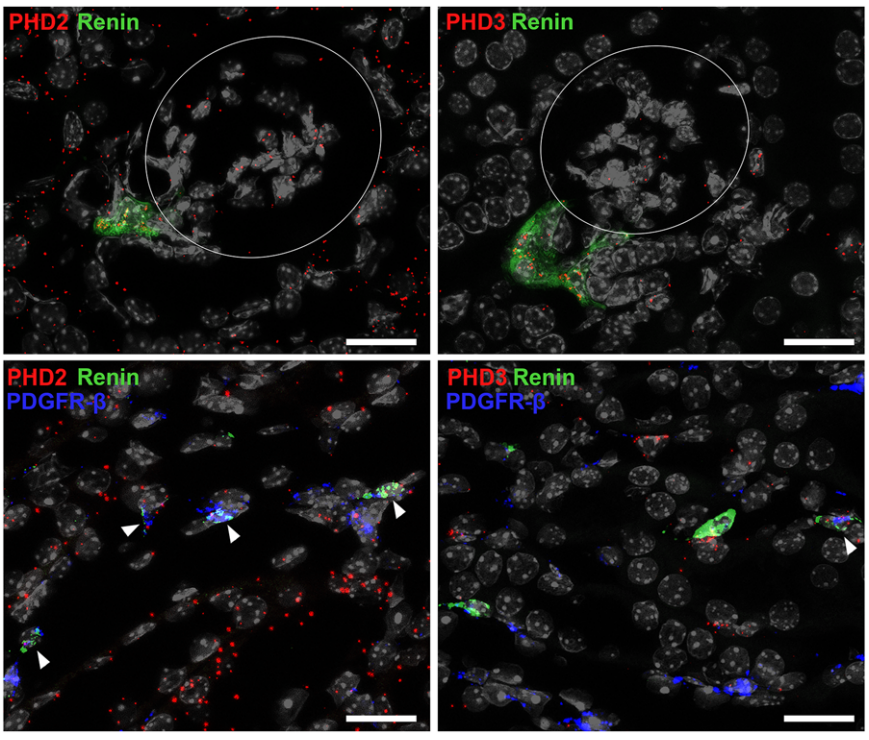


Figure 8. Expression of PHD2 and PHD3 mRNA in juxtaglomerular renin-expressing cells and interstitial renin$^+$ cells detected on kidney sections of wild-type mice
Nuclei were counterstained with DAPI (grey). Top: juxtaglomerular renin$^+$ cells (green) expressed PHD2 and PHD3 (red). Circles indicate glomeruli. Bottom: triple *in situ* hybridization for renin (green), PDGFR-β (blue) and PHD2 (red, left) showed the expression of PHD2 in almost all interstitial renin$^+$ cells (arrowheads). In contrast, PHD3 (red, right) was only expressed in a few interstitial renin$^+$ cells. Scale bars: 20 μm.

Due to the observed endocrine shift of juxtaglomerular renin$^+$ cells after co-deletion of PHD2 and PHD3 we wondered if other markers that are typically expressed by juxtaglomerular and hyperplastic renin$^+$ cells were also affected by this shift. Therefore, we analysed the expression of connexin-40 (Cx40) and aldo-keto reductase family 1 member B7 (Akr1b7). Triple RNAscope for EPO, renin and Cx40 mRNA revealed that after PHD2 and PHD3 co-deletion hyperplastic cells expressing EPO no longer expressed Cx40 mRNA (Fig. 13). Akr1b7 mRNA abundance was reduced to about 45% in kidneys of iRen$^{Cre/+}$ Aldo-KO PHD2^{ff} PHD3^{ff} mice compared to controls (0.45 $\pm$ 0.22 *vs.* 1.00 $\pm$ 0.54; $n \geq 8$ animals per group; means $\pm$ SD; $P = 0.0124$, unpaired *t*-test, two-tailed). Furthermore, we analysed CD73 expression as a typical marker of interstitial cells and native EPO-producing cells. Juxtaglomerular and hyperplastic renin-producing cells did not coexpress CD73. After the endocrine shift CD73 and EPO coexpression was detectable in former renin-producing cells of iRen$^{Cre/+}$ Aldo-KO PHD2^{ff} PHD3^{ff} mice (Fig. 13).

Short-term stabilization of HIF-2α does not induce EPO expression in juxtaglomerular renin$^+$ cells

To obtain a better understanding of the endocrine shift of juxtaglomerular and hyperplastic renin$^+$ cells into EPO-producing cells, a single dose of the PHD inhibitor 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido)-acetate (ICA) was injected into wild-type and Aldo-KO mice intraperitoneally. Four hours after injection of ICA, EPO mRNA abundance and plasma EPO concentrations were significantly increased (Table 6). Short-term PHD inhibition with ICA induced EPO gene expression exclusively in PDGFR-β^+ interstitial cells (Fig. 14*E*). About 10% of EPO$^+$ cells coexpressed renin mRNA ($n = 6$; Fig. 14). In juxtaglomerular renin$^+$ cells, ICA application did not stimulate EPO mRNA expression (Fig. 14*E*).

Since PHD3 expression was prominent in juxtaglomerular renin$^+$ cells and the endocrine shift was only observed after co-deletion of PHD2 and PHD3, we examined if PHD3 protects juxtaglomerular renin$^+$ cells from EPO expression. Therefore, iRen$^{Cre/+}$ PHD3^{ff} and iRen$^{Cre/+}$ Aldo-KO PHD3^{ff} mice were injected with ICA. The overall effect of ICA on EPO expression, however, was not different between mice with PHD3 deficiency in renin$^+$ cells and mice with intact PHD3 expression (Table 6, Fig. 15).

HIF staining showed a strong stabilization of HIF-2α in interstitial cells of the cortex and outer medulla after ICA injection in control mice and mice with PHD3 deletion. Moreover, HIF-2α positive nuclear staining could be observed in juxtaglomerular cells which was

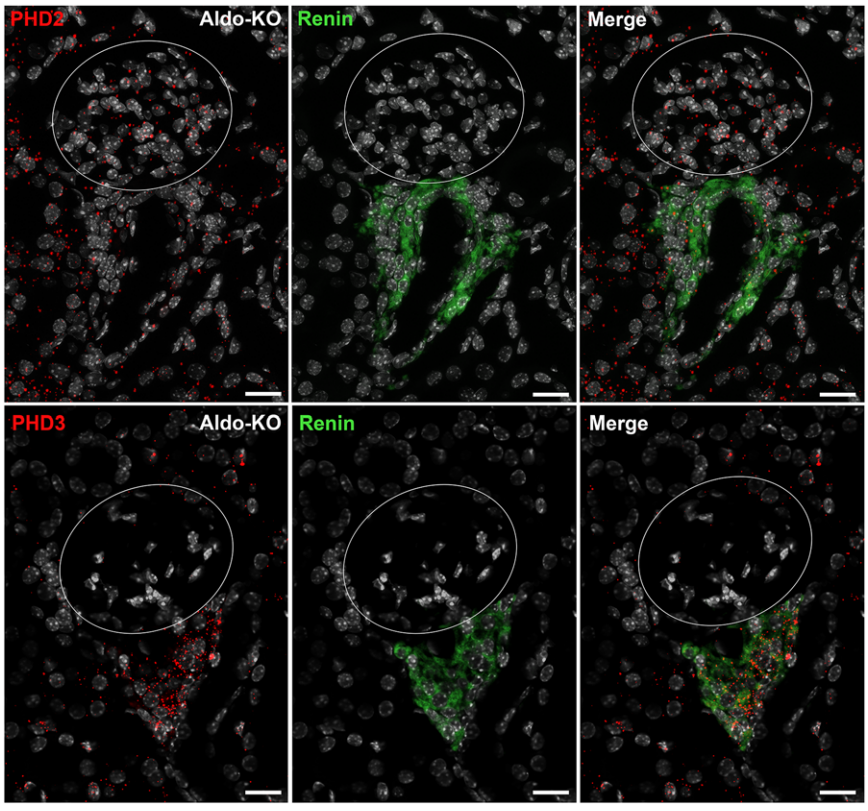
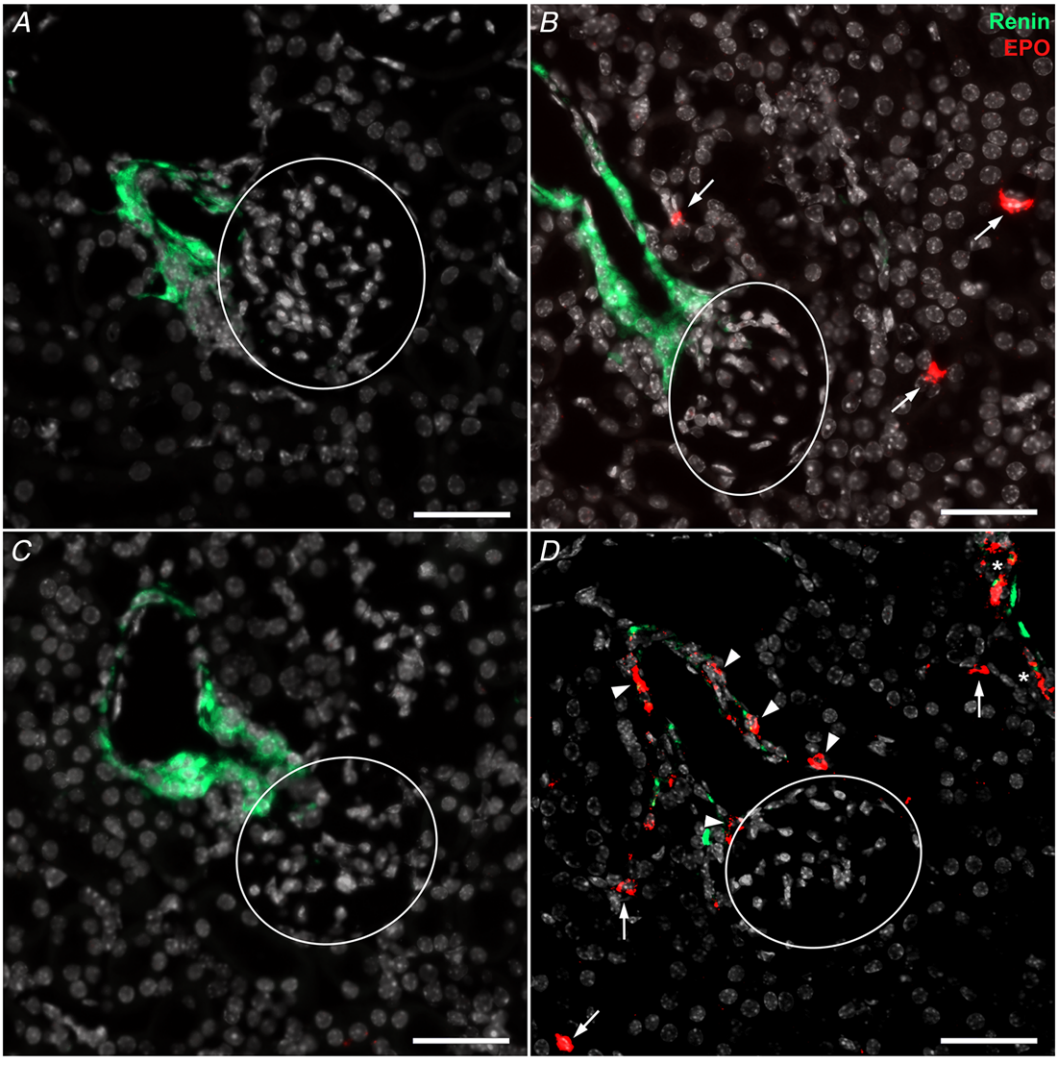


Figure 9. Details of PHD2 and PHD3 mRNA expression in hyperplastic renin$^+$ cells of aldosterone synthase deficient (Aldo-KO) mice
Nuclei were counterstained with DAPI (grey). Circles indicate glomeruli. Details of co-RNAscopes for PHD2 (red; top) or PHD3 (red; bottom) and renin (green) mRNA on kidney sections of Aldo-KO mice. Channel merge on the right shows expression of both PHD2 and PHD3 in hyperplastic renin$^+$ cells. PHD3 seems to be the predominant PHD isoform in hyperplastic renin$^+$ cells. Scale bars: 20 μm.



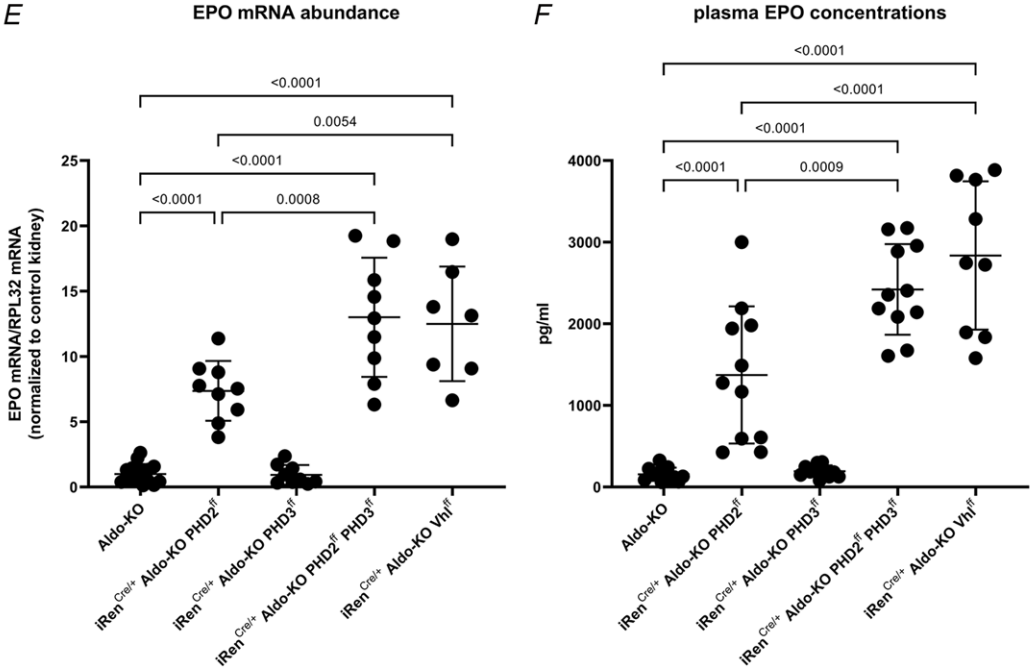


Figure 10. Cortical details showing co-RNAscopes for EPO (red) and renin (green) mRNA on kidney sections of Aldo-KO mice with different PHD deletions in renin-producing cells, as well as renal EPO mRNA abundance and plasma EPO concentrations of these mice
A, in Aldo-KO mice juxtaglomerular renin cell hyperplasia could be detected. There was no EPO expression detectable in the outer cortex. Only a few interstitial EPO-producing cells were located along the cortico-medullary border comparable to wild-type mice. *B*, iRen$^{Cre/+}$ Aldo-KO PHD2^{ff} mice showed an elevated number of interstitial EPO-expressing cells (arrows) throughout the cortex and outer medulla compared to Aldo-KO control animals. Juxtaglomerular hyperplastic renin$^+$ cells did not express EPO mRNA. *C*, PHD3 deletion had no effect on the EPO expression in iRen$^{Cre/+}$ Aldo-KO PHD3^{ff} mice. *D*, in iRen$^{Cre/+}$ Aldo-KO PHD2^{ff} PHD3^{ff} mice not only was the number of interstitial EPO-expressing cells (arrows) increased, but also juxtaglomerular hyperplastic renin-producing cells started to express EPO (arrowheads). Moreover, former renin-producing cells recruited along vessels also expressed EPO (asterisks). Meanwhile, renin mRNA expression was strongly downregulated in cells with induced EPO expression. Circles indicate glomeruli. Nuclei were counterstained with DAPI (grey). Scale bars: 50 μm. *E* and *F*, EPO mRNA abundance (*E*) and plasma EPO concentrations (*F*) in mice with Aldo-KO background and conditional deletion of different PHD isoforms or VHL in renin-expressing cells. Deletion of PHD2 significantly increased renal EPO mRNA expression and plasma EPO concentrations. PHD3 deletion alone had no effect on renal EPO expression. Deletion of both PHD2 and PHD3 significantly increased renal EPO mRNA and plasma levels compared to PHD2 deletion alone. Deletion of VHL had the same effect on the expression of EPO as the combined deletion of PHD2 and PHD3. Data are means ± SD of ≥7 mice per genotype. *P*-values are stated above the respective line. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test.

more pronounced in iRen$^{Cre/+}$ Aldo-KO PHD3^{ff} mice. In contrast, HIF-1α was stabilized in several tubular cells in the cortex and outer medulla and in cells in the inner stripe of the outer medulla and the papillary region due to the prolyl-4-hydroxylase inhibition (Fig. 15). Apparently, short-term HIF-2α stabilization with ICA was sufficient to trigger EPO expression in interstitial renin$^+$ cells but not in juxtaglomerular renin$^+$ cells irrespective of PHD3 activity.

Discussion

This study aimed to determine the relevance of the prolyl-4-hydroxylase isoforms 2 and 3 for the control of EPO production in renin-producing cells. To investigate the specific role of PHD2 and PHD3 in renin$^+$ cells, we analysed mouse models with cell type-specific deletions for one or both PHD isoforms. The experiments were performed using mice with and without hyperplastic renin$^+$ cells due to aldosterone synthase knockout.

An intriguing finding of our study was that renin-expressing cells can be clearly differentiated into typical juxtaglomerular renin-producing cells and interstitial renin$^+$ cells. RNAscope revealed that interstitial cells actively expressed renin mRNA. These cells were also positive for PDGFR-β, a typical marker for interstitial fibroblasts and pericytes (Fig. 4). Moreover, lineage tracing confirmed the renin promotor activity using a GFP reporter mouse. In contrast, renin protein was not detectable by immunofluorescence in these interstitial cells (Fig. 5). Therefore, we speculate that the cells produce prorenin which is constitutively released (Pratt *et al.* 1988; Berka *et al.* 1992). The functional relevance of such a postulated prorenin release is not yet clear and requires further consideration.

Previously, it has been shown that all native EPO-producing cells belong to the PDGFR-β^+ fibroblast-like cell population (Gerl *et al.* 2016). Therefore, it is conceivable that tubulointerstitial renin-expressing cells belong to the pool of potential EPO producers in the kidney. Indeed, interstitial renin$^+$ cells contributed to the EPO production during acute HIF-2 stabilization induced by the short-term application of the prolyl-4-hydroxylase inhibitor ICA (4 h). About 10% of all interstitial EPO-producing cells coexpressed renin after ICA injection (Fig. 14). This is in good accordance with a recent study showing interstitial renin and EPO coexpression in anaemic mice (Miyauchi *et al.* 2021).

This coexpression of EPO and renin was also observed during chronic HIF-2 stabilization in renin-expressing cells through deletion of PHD2. Interestingly, PHD2 deletion did not induce EPO in juxtaglomerular and hyperplastic renin$^+$ cells, which are the typical site of renal renin production. Instead, EPO expression was exclusively induced in interstitial cells throughout the cortex and outer medulla. iRen$^{Cre/+}$ (Aldo-KO) PHD2^{ff} mice showed a significant increase in EPO mRNA expression and plasma EPO concentrations leading to polycythaemia (Figs 1 and 10). Previous studies also reported that deletion of PHD2 in interstitial cells of the kidneys causes strong and sustained EPO expression (Kobayashi *et al.* 2016; Souma *et al.* 2016).

Chronic HIF-2 stabilization in renin-expressing cells through co-deletion of PHD2 and PHD3 led to a further enhancement of renal EPO production, compared to PHD2 deletion alone. Although HIF-1 was also stabilized after PHD2 and PHD3 co-deletion, previous data already confirmed HIF-2 as the isoform regulating EPO in stromal cells of the kidney (Kurt *et al.* 2015; Gerl *et al.* 2017). Like in mice with only PHD2 deficiency in renin$^+$ cells, about 200 interstitial cells produced EPO. Additionally, EPO

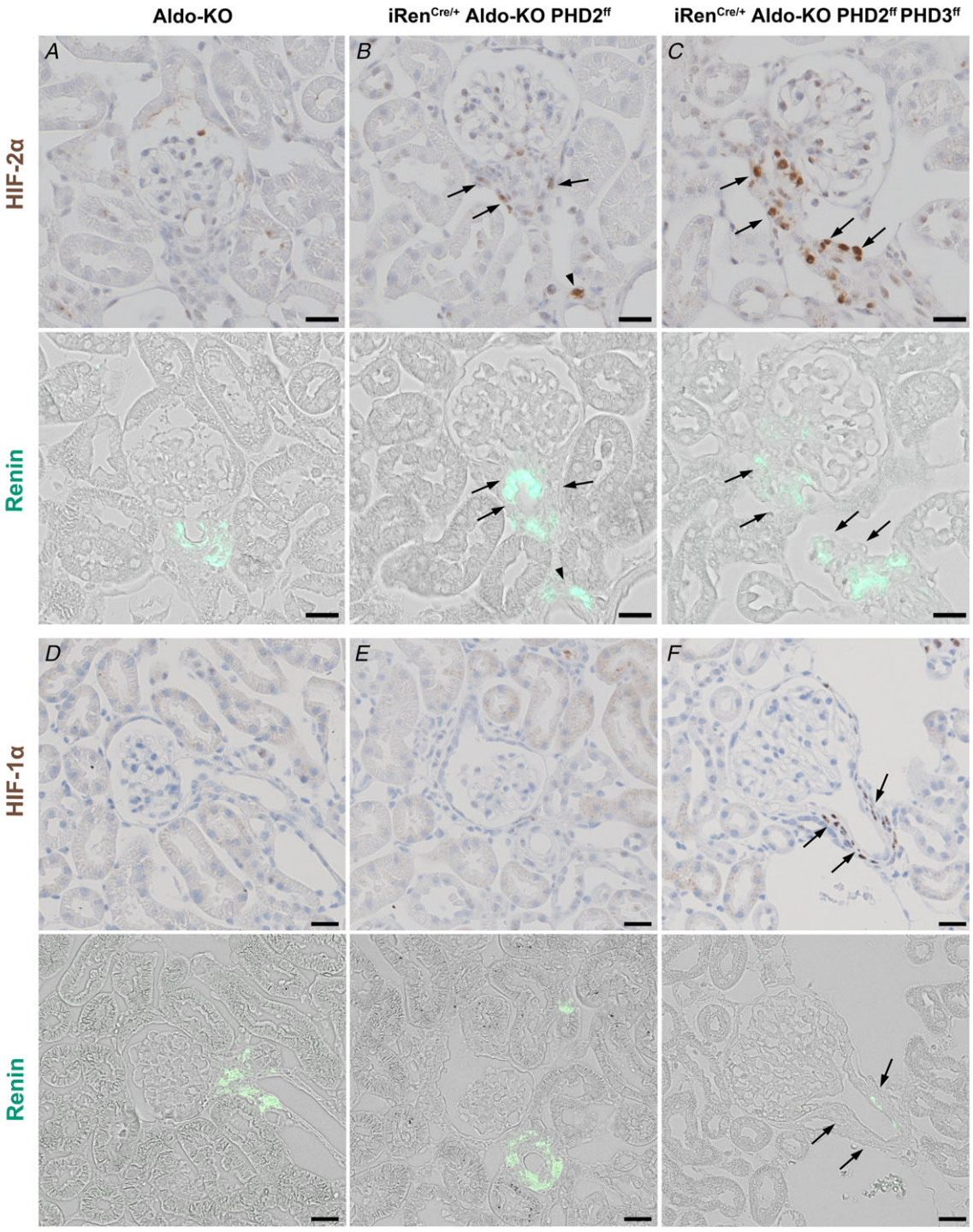


Figure 11. Consecutive kidney sections showing HIF-2α (brown, *A–C*) or HIF-1α (brown, *D–F*) and renin immunoreactivity (green) in Aldo-KO mice (*A* and *D*), iRen$^{Cre/+}$ Aldo-KO PHD2^{ff} mice (*B* and *E*) and iRen$^{Cre/+}$ Aldo-KO PHD2^{ff} PHD3^{ff} mice (*C* and *F*)

Nuclei of HIF-stained kidney sections were counterstained with haematoxylin. Scale bars: 20 μm. *A* and *D*, on kidney sections of Aldo-KO mice neither HIF-2α nor HIF-1α positive nuclei could be detected in hyperplastic renin cell fields. *B* and *E*, after PHD2 deletion sporadic weak HIF-2α signals were detectable in juxtaglomerular renin cell fields (arrows). Stronger HIF-2α immunoreactivity was observed only in some interstitial cells (arrowhead). However, PHD2 deletion was not sufficient to stabilize HIF-1α either in juxtaglomerular or interstitial cells. *C* and *F*, after PHD2 and PHD3 co-deletion distinct HIF-2α and HIF-1α nuclear staining was detected in juxtaglomerular renin-producing cells (arrows). Renin staining was weaker in these cells.

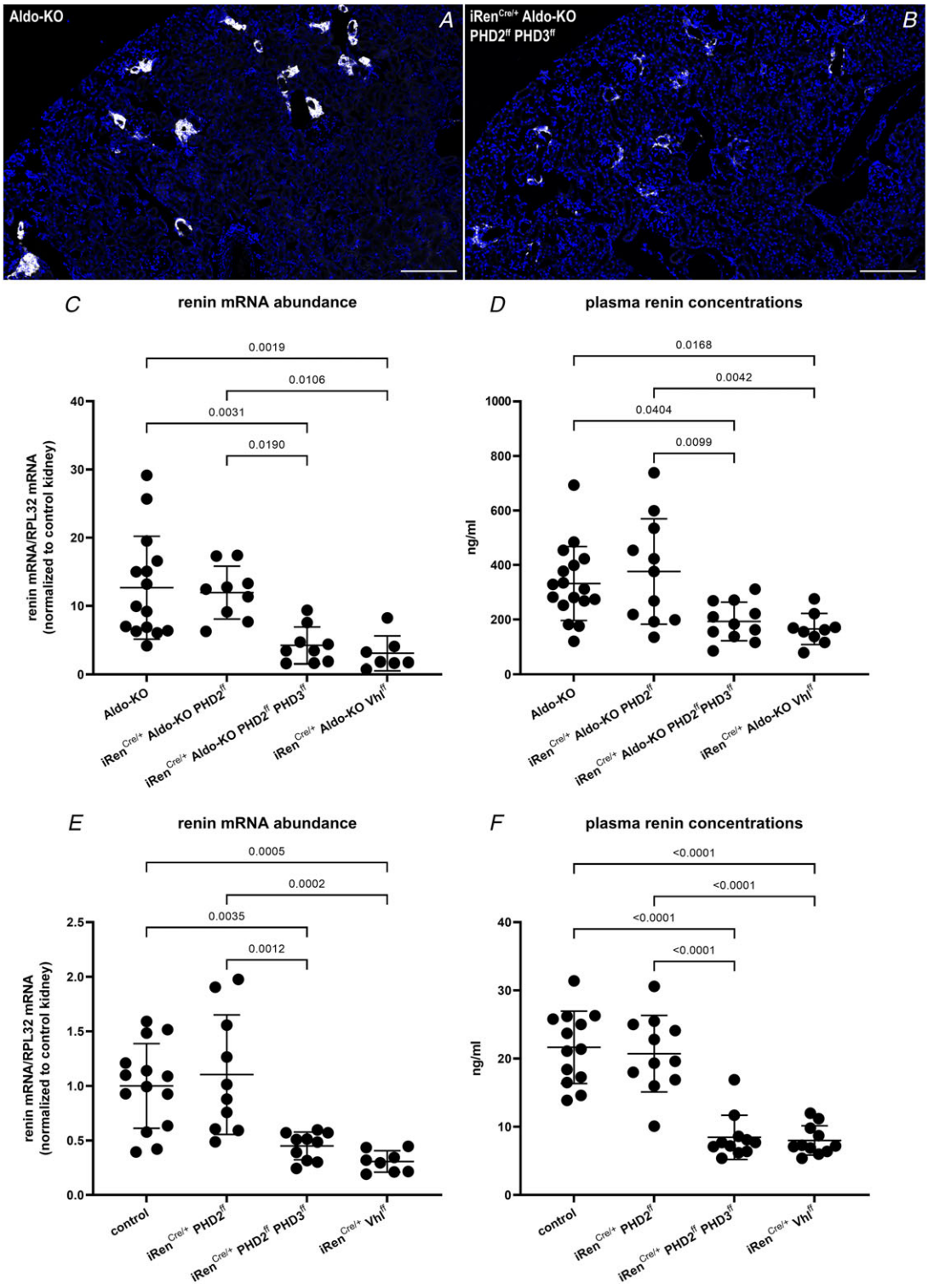


Figure 12. RNAscope showing hyperplastic renin expression as well as renal renin mRNA abundance and plasma renin concentrations of mice used for this study
A and *B*, overview of renin mRNA expression detected by RNAscope (white signal) in the kidney cortex of Aldo-KO mice (*A*) and Aldo-KO mice with co-deletion of PHD2 and PHD3 (*B*). Renin RNAscope signals were noticeably weaker after PHD2 and PHD3 co-deletion. Nuclei were counterstained with DAPI (blue). Scale bars: 200 μm. *C–F*, renin mRNA abundance (*C* and *E*) and plasma renin concentrations (*D* and *F*) in mice with deletions of different PHD isoforms or VHL in mice with or without Aldo-KO background. Data are means ± SD of ≥8 mice per genotype. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test.

expression was induced in juxtaglomerular and hyperplastic renin$^+$ cells after PHD2 and PHD3 co-deletion. Since the juxtaglomerular cells per kidney section were fewer in number compared to interstitial EPO-expressing cells in mice with intact aldosterone synthase, the increase in EPO mRNA expression and plasma EPO levels in iRen$^{Cre/+}$ PHD2^{ff} PHD3^{ff} mice was not significant compared to iRen$^{Cre/+}$ PHD2^{ff} mice. However, the additional increase in plasma EPO concentrations from PHD2 deletion to PHD2 and PHD3 co-deletion was still substantial since it corresponded approximately to basal plasma EPO levels in wild-type mice. Parallel to the upregulation of EPO in juxtaglomerular cells, renin expression was significantly downregulated in these cells. Contrary to interstitial renin$^+$ cells, coexpression of EPO and renin was only rarely detected in juxtaglomerular and hyperplastic renin$^+$ cells (Figs 3 and 10). These findings suggest that juxtaglomerular and hyperplastic renin$^+$ cells undergo an endocrine shift after PHD2 and PHD3 co-deletion meaning these cells switch from renin production to EPO expression.

This shift in endocrine function due to genetic HIF-2 stabilization could not be reproduced by short term (4 h) application of the PHD inhibitor ICA even with previous deletion of PHD3 in renin$^+$ cells (Fig. 15). Based on these findings, we would hypothesize that EPO induction in juxtaglomerular renin$^+$ cells requires cell reprogramming due to prolonged HIF-2 stabilization. Indeed, during chronic HIF-2 stabilization through PHD2 and PHD3 co-deletion, the typical renin cell markers connexin 40 and Akr1b7 were downregulated along with renin in juxtaglomerular cells (Kurtz *et al.* 2007; Brunskill

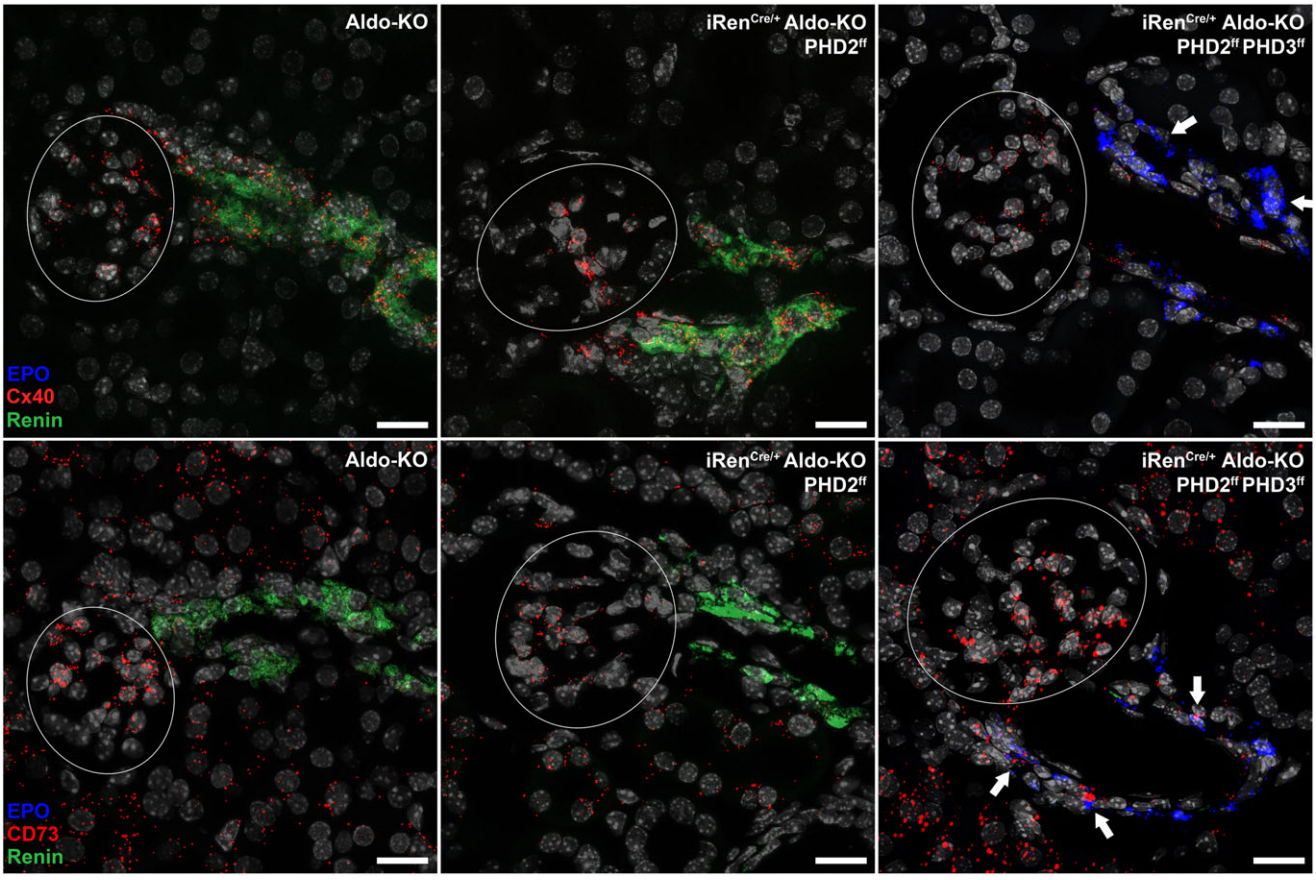


Figure 13. Cortical details showing co-RNAscopes for EPO, renin and Cx40 (top) or CD73 (bottom) mRNA on kidney sections of Aldo-KO mice and Aldo-KO mice with different PHD deletions in renin-producing cells
Circles indicate glomeruli. Nuclei were counterstained with DAPI (grey). Top: hyperplastic renin-producing cells (green) coexpressed Cx40 (red) as a typical marker in Aldo-KO mice and iRen$^{Cre/+}$ Aldo-KO PHD2^{ff} mice. After PHD2 and PHD3 co-deletion an endocrine shift in juxtaglomerular renin$^+$ cells could be observed. Renin mRNA expression was strongly downregulated whereas EPO mRNA (blue) expression was upregulated in these cells. In parallel, the former hyperplastic renin$^+$ cells no longer coexpressed Cx40 (arrows). Bottom: hyperplastic renin-producing cells (green) did not coexpress CD73 (red), which is a typical marker for interstitial fibroblasts. After the endocrine shift CD73 and EPO (blue) coexpression was detectable in former renin-producing cells of iRen$^{Cre/+}$ Aldo-KO PHD2^{ff} PHD3^{ff} mice (arrows). Scale bars: 20 μm.

Table 6. EPO mRNA abundance and plasma EPO concentrations under basal conditions and 4 h after ICA injection

	Control (basal)	Control (4 h ICA)	iRen$^{Cre/+}$ (Aldo-KO) PHD3^{ff} (4 h ICA)
EPO mRNA/RPL32 mRNA (normalized to control kidneys)	1.0 ± 0.6	101.3 ± 69.0 ($P = 0.0133$)	96.0 ± 74.8 ($P = 0.0190$)
Plasma-EPO (pg/ml)	184.5 ± 64.4	12152.1 ± 3356.1 ($P = 0.0001$)	12270.7 ± 6295.2 ($P < 0.0001$)

Values are means ± SD of seven animals per group. Statistical significance compared to control was determined by one-way ANOVA with Tukey's multiple comparisons test.

et al. 2011). On the other hand expression of CD73, which is a typical marker for interstitial fibroblasts and native EPO-producing cells (Broeker *et al*. 2020), was upregulated. These findings indicate not only an endocrine shift but also a phenotypic transformation of these former renin$^+$ cells (Fig. 13). This is in good accordance with our previous findings in mice with VHL deletion in renin$^+$ cells showing that long term stabilization of HIF-2 is required to induce a metaplastic phenotype shift of juxtaglomerular cells to a cell type more

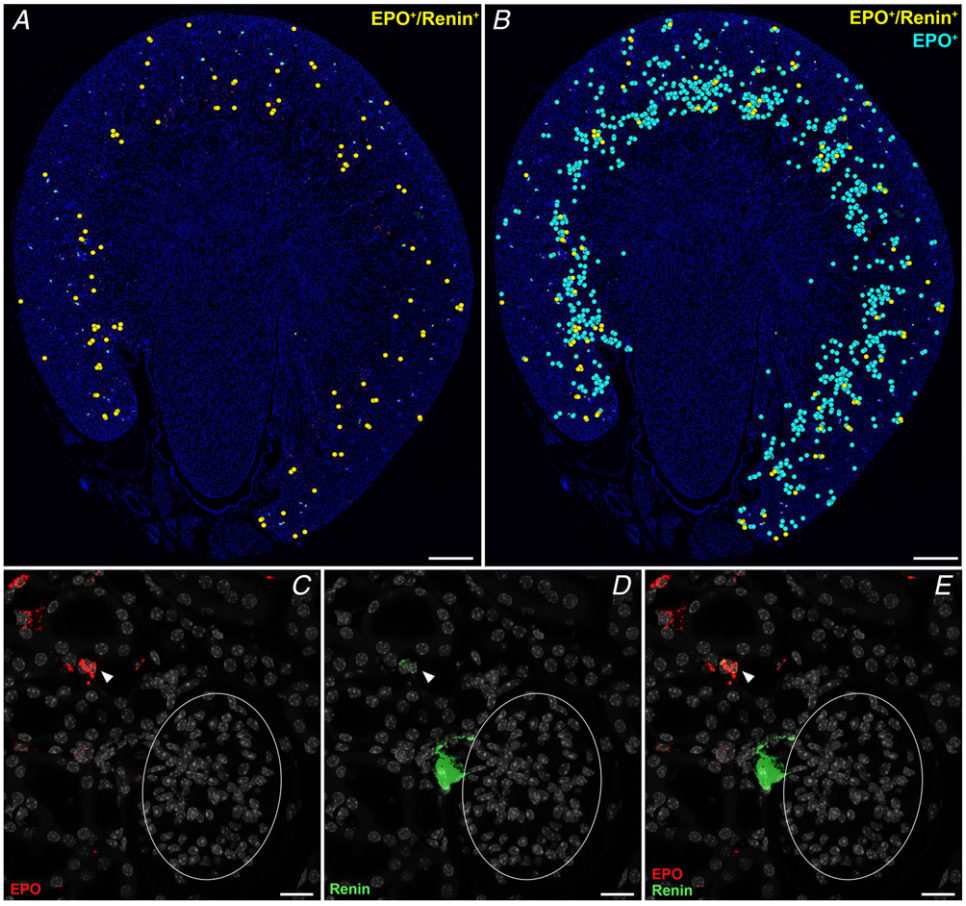


Figure 14. EPO and renin mRNA expression on kidney sections of wild-type mice injected with the prolyl-4-hydroxylase inhibitor 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido)-acetate (ICA) for 4 h
A and *B*, cells simultaneously expressing EPO and renin mRNA after ICA injection are highlighted with yellow circles (*A*) on a transverse kidney section; cells expressing only EPO mRNA were highlighted in light blue to give an impression of the overall induction of EPO expression (*B*). Nuclei were counterstained with DAPI (blue). Scale bars: 500 μm. *C–E*, high resolution images of the renal cortex show EPO mRNA (red) in interstitial cells, but not in juxtaglomerular renin$^+$ cells (green). Coexpression of renin and EPO was detectable in some interstitial cells (arrowhead). Nuclei were counterstained with DAPI (grey). Circles indicate glomeruli. Scale bars: 20 μm.

similar to interstitial cells (Kurt *et al.* 2013, 2015; Gerl *et al.* 2015).

Similar observations were recently reported for brain pericytes in which only co-deletion of PHD2 and PHD3 but not deletions of single PHDs induced EPO gene expression. Moreover, only PHD2 and PHD3 co-deletion but not PHD2 deletion alone led to an expansion of the capillary network in the brain, again suggesting a cellular phenotype shift, which was HIF-2 dependent (Urrutia *et al.* 2016, 2021). Studies regarding tumour cells also showed that chronic HIF-2 stabilization can induce a more dedifferentiated phenotype shift (Nilsson *et al.* 2005; Covello *et al.* 2006). Due to the direct contact of juxtaglomerular renin cells with the arterial blood, it is

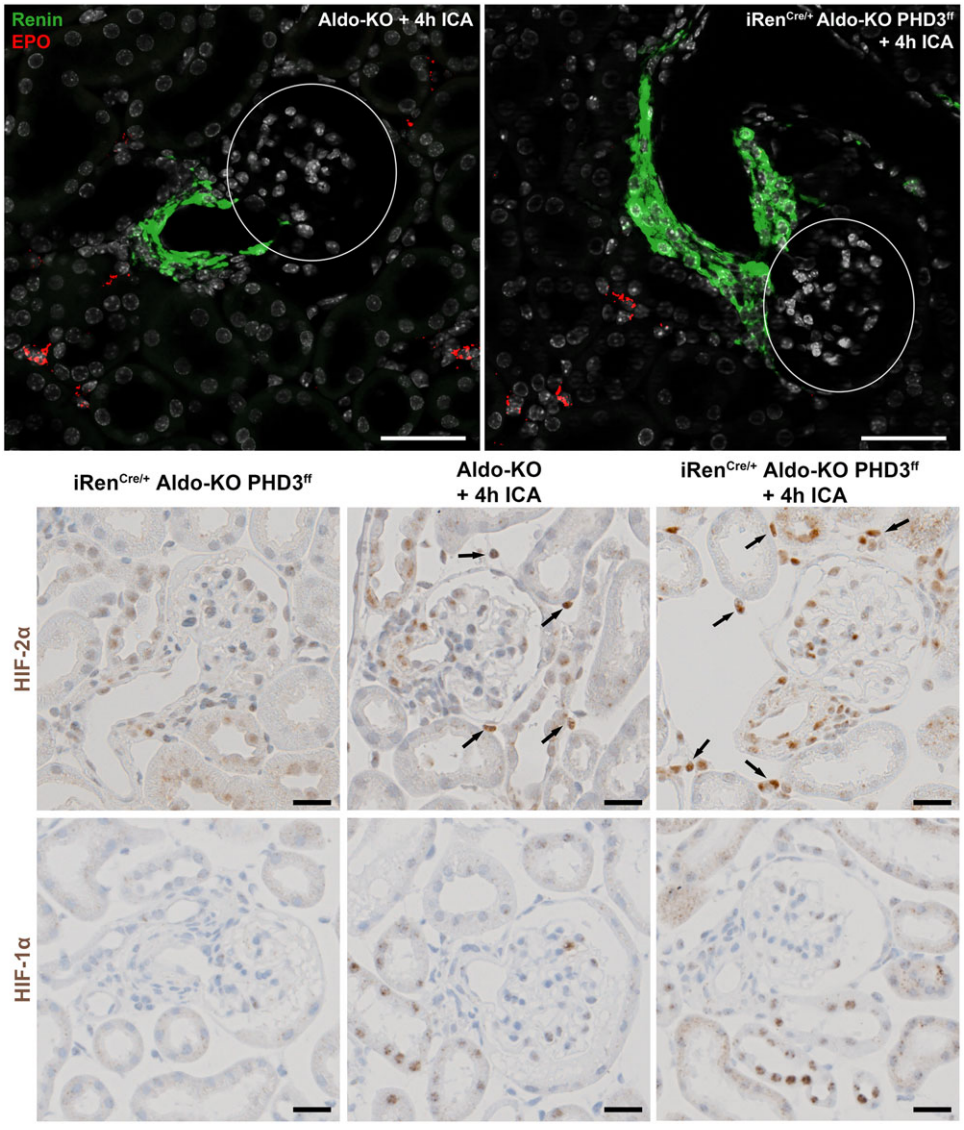


Figure 15. Cortical details showing co-RNAscope for EPO and renin mRNA as well as immunohistochemistry for HIF-2α and HIF-1α on kidney sections of Aldo-KO mice and iRen$^{Cre/+}$ Aldo-KO PHD3^{ff} mice after injection with the prolyl-4-hydroxylase-inhibitor 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido)-acetate (ICA) for 4 h

Top: ICA treatment induced EPO (red) mRNA expression exclusively in interstitial cells of Aldo-KO mice (left). Even if PHD3 had been deleted in renin^{+} (green) cells previous to ICA injection, EPO expression still could be detected only in interstitial cells and not in juxtaglomerular renin-producing cells. Nuclei were counterstained with DAPI (grey). Circles indicate glomeruli. Scale bars: 50 μm. Middle: strong nuclear HIF-2α staining (brown) could be detected in interstitial cells (arrows) of Aldo-KO mice and iRen$^{Cre/+}$ Aldo-KO PHD3^{ff} mice after ICA injection. HIF-2α stabilization could also be observed in juxtaglomerular cells which was more pronounced in iRen$^{Cre/+}$ Aldo-KO PHD3^{ff} mice. Scale bars: 20 μm. Bottom: HIF-1α (brown) was stabilized in several tubular cells due to the prolyl-4-hydroxylase inhibition. Scale bars: 20 μm.

unlikely that long-term stabilization of HIF-2α occurs under physiological conditions in these cells. However, the effects of long-term PHD inhibitor treatment on juxtaglomerular renin cells need to be taken into consideration and further investigated.

Co-deletion of PHD2 and PHD3 produced very similar effects as the deletion of the ubiquitin-ligase von Hippel–Lindau protein (Table 5) (Kurt *et al*. 2013; Gerl *et al*. 2015). This led us to conclude that among the family of prolyl-4-hydroxylases, PHD2 and PHD3 are the isoforms relevant for the HIF-2 mediated control of EPO expression in renin$^+$ cells. PHD1 seems less relevant for the regulation of EPO production in renin$^+$ cells (Minamishima *et al*. 2008; Kobayashi *et al*. 2016).

Our findings concerning the basal expression pattern for PHD2 and PHD3 were in good accordance with previous studies (Lieb *et al*. 2002; Berra et al. 2003; Appelhoff *et al*. 2004; Schödel *et al*. 2009). We investigated the distinct PHD isoform pattern for the two subpopulations of renin-expressing cells and could detect a differential expression for PHD2 and PHD3, which might explain their different EPO-producing capabilities. PHD2 has a widespread basal expression in most kidney cells and all kidney zones, including all renin-expressing cells. The distribution of PHD3 on the other hand was more distinct. However, native juxtaglomerular and hyperplastic renin-producing cells showed strong mRNA signals for PHD3 (Figs 5 and 6). Regarding interstitial renin$^+$ cells, unlike PHD2, only a few were positive for PHD3 mRNA expression (Fig. 5). This lack of PHD3 in interstitial renin$^+$ cells could explain their ability to produce EPO due to PHD2 deletion alone. Native juxtaglomerular and hyperplastic renin-producing cells displayed a high basal expression of PHD3 that could compensate for a loss of PHD2 activity and therefore prevent EPO expression.

Our finding that only co-deletion of PHD2 and PHD3 led to an induction of EPO expression in juxtaglomerular cells would suggest that the basal PHD3 expression in these cells inhibits HIF-2 stabilization and therefore prevents the phenotypic shift into EPO-producing cells. It seems feasible that PHD3 prevents EPO expression in renin-producing cells also in states of renal hypoxia and preserves renin expression. This would explain why under (patho-)physiological situations EPO$^+$ cells are found only in the renal interstitium. In line with this, it is known that during extended periods of hypoxia, PHD3 is the isoform which is able to retain most of its function. Previous studies have also reported a cooperative role for PHD3 in addition to PHD2 in modulating the HIF response (Stiehl *et al*. 2006; Ginouvès *et al*. 2008; Minamishima *et al*. 2009; Jaakkola & Rantanen, 2013).

Therefore, we speculate that the basal expression of PHD3 modifies the sensitivity of more specialized cells for oxygen-regulated HIF activity and thus EPO expression that is centrally mediated by PHD2 activity (Minamishima *et al*. 2008, 2009; Takeda *et al*. 2008). This concept is supported by the incomplete HIF-2α stabilization observed in juxtaglomerular renin-producing cells after PHD2 deletion (Fig. 11), preventing EPO induction and the endocrine shift in these cells. Co-deletion of PHD2 and PHD3 in renin$^+$ cells, however, led to a stabilization of HIF-2α accompanied by a strongly downregulated renin expression and upregulated EPO expression. In line with this, deletion of only PHD3 in renin$^+$ cells had no effect on renal EPO expression.

In summary, our data show that PHD2 and PHD3 are the essential PHD isoforms for HIF signalling in renin-expressing cells. Furthermore, we detected that a subpopulation of PDGFR-β^+ interstitial cells expresses the renin gene, but does not store renin. These interstitial renin$^+$ cells belong to the cell pool of native EPO-producing cells, show a fast EPO response to HIF-2 stabilization and are able to express EPO and renin in parallel. In contrast to the interstitial renin$^+$ cells, only co-deletion of PHD2 and PHD3, but not PHD2 deletion alone, induced EPO expression in juxtaglomerular and hyperplastic renin$^+$ cells. The strong basal PHD3 expression in juxtaglomerular renin$^+$ cells seems to prevent a HIF-2-dependent phenotype shift into EPO cells prerequisite to EPO expression. Consequently, PHD3 seems to fulfil a stabilizer function for the juxtaglomerular renin cell phenotype in regard to hypoxia signalling. Our findings concerning the distinct expression patterns and functions of the PHD isoforms 2 and 3 in renin$^+$ cells provide new insights into the regulation of renal renin-producing cells and highlight the need for selective PHD inhibitors. Moreover, these findings suggest a high endocrine plasticity of tubulointerstitial cells and emphasize the need for further research into the different functions of renal interstitial cells.

References

Appelhoff RJ, Tian Y-M, Raval RR, Turley H, Harris AL, Pugh CW, Ratcliffe PJ & Gleadle JM (2004). Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. *J Biol Chem* **279**, 38458–38465.

Bachmann S, Le Hir M & Eckardt KU (1993). Co-localization of erythropoietin mRNA and ecto-5′-nucleotidase immunoreactivity in peritubular cells of rat renal cortex indicates that fibroblasts produce erythropoietin. *J Histochem Cytochem* **41**, 335–341.

Berka JL, Alcorn D, Coghlan JP, Fernley RT, Morgan TO, Ryan GB, Skinner SL & Weaver DA (1990). Granular juxtaglomerular cells and prorenin synthesis in mice treated with enalapril. *J Hypertens* **8**, 229–238.

Berka JLA, Alcorn D, Ryan GB & Skinner SL (1992). Renin processing studied by immunogold localization of prorenin and renin in granular juxtaglomerular cells in mice treated with enalapril. *Cell Tissue Res* **268**, 141–148.

Berra E, Benizri E, Ginouvès A, Volmat V, Roux D & Pouysségur J (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. *EMBO J* **22**, 4082–4090.

Broeker KAE, Fuchs MAA, Schrankl J, Kurt B, Nolan KA, Wenger RH, Kramann R, Wagner C & Kurtz A (2020). Different subpopulations of kidney interstitial cells produce erythropoietin and factors supporting tissue oxygenation in response to hypoxia in vivo. *Kidney Int* **98**, 918–931.

Brunskill EW, Sequeira-Lopez MLS, Pentz ES, Lin E, Yu J, Aronow BJ, Potter SS & Gomez RA (2011). Genes that confer the identity of the renin cell. *J Am Soc Nephrol* **22**, 2213–2225.

Buchholz B, Schley G, Faria D, Kroening S, Willam C, Schreiber R, Klanke B, Burzlaff N, Jantsch J, Kunzelmann K & Eckardt K-U (2014). Hypoxia-inducible factor-1α causes renal cyst expansion through calcium-activated chloride secretion. *J Am Soc Nephrol* **25**, 465–474.

Cantin M, Araujo-Nascimento MD, Benchimol S & Desormeaux Y (1977). Metaplasia of smooth muscle cells into juxtaglomerular cells in the juxtaglomerular apparatus, arteries, and arterioles of the ischemic (endocrine) kidney. An ultrastructural-cytochemical and autoradiographic study. *Am J Pathol* **87**, 581–602.

Chomczynski P & Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**, 156–159.

Covello KL, Kehler J, Yu H, Gordan JD, Arsham AM, Hu C-J, Labosky PA, Simon MC & Keith B (2006). HIF-2α regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes Dev* **20**, 557–570.

Eckardt K-U, Koury ST, Tan CC, Schuster SJ, Kaissling B, Ratcliffe PJ & Kurtz A (1993). Distribution of erythropoietin producing cells in rat kidneys during hypoxic hypoxia. *Kidney Int* **43**, 815–823.

Franke K, Kalucka J, Mamlouk S, Singh RP, Muschter A, Weidemann A, Iyengar V, Jahn S, Wieczorek K, Geiger K, Muders M, Sykes AM, Poitz DM, Ripich T, Otto T, Bergmann S, Breier G, Baretton G, Fong GH, Greaves DR, Bornstein S, Chavakis T, Fandrey J, Gassmann M & Wielockx B (2013). HIF-1α is a protective factor in conditional PHD2-deficient mice suffering from severe HIF-2α-induced excessive erythropoiesis. *Blood* **121**, 1436–1445.

Gerl K, Miquerol L, Todorov VT, Hugo CPM, Adams RH, Kurtz A & Kurt B (2015). Inducible glomerular erythropoietin production in the adult kidney. *Kidney Int* **88**, 1345–1355.

Gerl K, Nolan KA, Karger C, Fuchs M, Wenger RH, Stolt CC, Willam C, Kurtz A & Kurt B (2016). Erythropoietin production by PDGFR-β+ cells. *Pflugers Arch* **468**, 1479–1487.

Gerl K, Steppan D, Fuchs M, Wagner C, Willam C, Kurtz A & Kurt B (2017). Activation of hypoxia signaling in stromal progenitors impairs kidney development. *Am J Pathol* **187**, 1496–1511.

Ginouvès A, Ilc K, Macías N, Pouysségur J & Berra E (2008). PHDs overactivation during chronic hypoxia "desensitizes" HIFalpha and protects cells from necrosis. *Proc Natl Acad Sci U S A* **105**, 4745–4750.

Gomez RA, Lynch KR, Sturgill BC, Elwood JP, Chevalier RL, Carey RM & Peach MJ (1989). Distribution of renin mRNA and its protein in the developing kidney. *Am J Physiol* **257**, F850–F858.

Graham PC, Stewart HVA, Downie I & Lindop GBM (1990). The distribution of renin-containing cells in kidneys with renal artery stenosis—an immunocytochemical study. *Histopathology* **16**, 347–355.

Grundy D (2015). Principles and standards for reporting animal experiments in *The Journal of Physiology* and *Experimental Physiology*. *J Physiol* **593**, 2547–2549.

Haase VH, Glickman JN, Socolovsky M & Jaenisch R (2001). Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. *Proc Natl Acad Sci U S A* **98**, 1583–1588.

Holmer SR, Kaissling B, Putnik K, Pfeifer M, Krämer BK, Riegger GA & Kurtz A (1997). Beta-adrenergic stimulation of renin expression in vivo. *J Hypertens* **15**, 1471–1479.

Jaakkola PM & Rantanen K (2013). The regulation, localization, and functions of oxygen-sensing prolyl hydroxylase PHD3. *Biol Chem* **394**, 449–457.

Kaelin WG & Ratcliffe PJ (2008). Oxygen sensing by metazoans: the central role of the hif hydroxylase pathway. *Mol Cell* **30**, 393–402.

Kobayashi H, Liu J, Urrutia AA, Burmakin M, Ishii K, Rajan M, Davidoff O, Saifudeen Z & Haase VH (2017). Hypoxia-inducible factor prolyl-4-hydroxylation in FOXD1 lineage cells is essential for normal kidney development. *Kidney Int* **92**, 1370–1383.

Kobayashi H, Liu Q, Binns TC, Urrutia AA, Davidoff O, Kapitsinou PP, Pfaff AS, Olauson H, Wernerson A, Fogo AB, Fong G-H, Gross KW & Haase VH (2016). Distinct subpopulations of FOXD1 stroma-derived cells regulate renal erythropoietin. *J Clin Invest* **126**, 1926–1938.

Koury ST, Koury MJ, Bondurant MC, Caro J & Graber SE (1989). Quantitation of erythropoietin-producing cells in kidneys of mice by in situ hybridization: correlation with hematocrit, renal erythropoietin mRNA, and serum erythropoietin concentration. *Blood* **74**, 645–651.

Kurt B, Gerl K, Karger C, Schwarzensteiner I & Kurtz A (2015). Chronic hypoxia-inducible transcription factor-2 activation stably transforms juxtaglomerular renin cells into fibroblast-like cells in vivo. *J Am Soc Nephrol* **26**, 587–596.

Kurt B, Paliege A, Willam C, Schwarzensteiner I, Schucht K, Neymeyer H, Sequeira-Lopez MLS, Bachmann S, Gomez RA, Eckardt K-U & Kurtz A (2013). Deletion of von Hippel–Lindau protein converts renin-producing cells into erythropoietin-producing cells. *J Am Soc Nephrol* **24**, 433–444.

Kurtz L, Schweda F, de Wit C, Kriz W, Witzgall R, Warth R, Sauter A, Kurtz A & Wagner C (2007). Lack of connexin 40 causes displacement of renin-producing cells from afferent arterioles to the extraglomerular mesangium. *J Am Soc Nephrol* **18**, 1103–1111.

Lee G, Makhanova N, Caron K, Lopez MLS, Gomez RA, Smithies O & Kim H-S (2005). Homeostatic responses in the adrenal cortex to the absence of aldosterone in mice. *Endocrinology* **146**, 2650–2656.

Li W, Hartwig S & Rosenblum ND (2014). Developmental origins and functions of stromal cells in the normal and diseased mammalian kidney. *Dev Dyn* **243**, 853–863.

Lieb ME, Menzies K, Moschella MC, Ni R & Taubman MB (2002). Mammalian *EGLN* genes have distinct patterns of mRNA expression and regulation. *Biochem Cell Biol* **80**, 421–426.

Machura K, Neubauer B, Steppan D, Kettl R, Groβ A & Kurtz A (2012). Role of blood pressure in mediating the influence of salt intake on renin expression in the kidney. *Am J Physiol Renal Physiol* **302**, F1278–F1285.

Makhanova N, Lee G, Takahashi N, Sequeira Lopez ML, Gomez RA, Kim H-S & Smithies O (2006). Kidney function in mice lacking aldosterone. *Am J Physiol Renal Physiol* **290**, F61–F69.

Maxwell PH, Osmond MK, Pugh CW, Heryet A, Nicholls LG, Tan CC, Doe BG, Ferguson DJ, Johnson MH & Ratcliffe PJ (1993). Identification of the renal erythropoietin-producing cells using transgenic mice. *Kidney Int* **44**, 1149–1162.

Minamishima YA, Moslehi J, Bardeesy N, Cullen D, Bronson RT & Kaelin WG (2008). Somatic inactivation of the PHD2 prolyl hydroxylase causes polycythemia and congestive heart failure. *Blood* **111**, 3236–3244.

Minamishima YA, Moslehi J, Padera RF, Bronson RT, Liao R & Kaelin WG (2009). A feedback loop involving the Phd3 prolyl hydroxylase tunes the mammalian hypoxic response in vivo. *Mol Cell Biol* **29**, 5729–5741.

Miyauchi K, Nakai T, Saito S, Yamamoto T, Sato K, Kato K, Nezu M, Miyazaki M, Ito S, Tamamoto M & Suzuki N (2021). Renal interstitial fibroblasts coproduce erythropoietin and renin under anaemic conditions. *EBioMedicine* **64**, 103209.

Muzumdar MD, Tasic B, Miyamichi K, Li L & Luo L (2007). A global double-fluorescent Cre reporter mouse. *Genesis* **45**, 593–605.

Nilsson H, Jögi A, Beckman S, Harris AL, Poellinger L & Påhlman S (2005). HIF-2α expression in human fetal paraganglia and neuroblastoma: relation to sympathetic differentiation, glucose deficiency, and hypoxia. *Exp Cell Res* **303**, 447–456.

Paliege A, Rosenberger C, Bondke A, Sciesielski L, Shina A, Heyman SN, Flippin LA, Arend M, Klaus SJ & Bachmann S (2010). Hypoxia-inducible factor-2α-expressing interstitial fibroblasts are the only renal cells that express erythropoietin under hypoxia-inducible factor stabilization. *Kidney Int* **77**, 312–318.

Pratt RE, Flynn JA, Hobart PM, Paul M & Dzau VJ (1988). Different secretory pathways of renin from mouse cells transfected with the human renin gene. *J Biol Chem* **263**, 3137–3141.

Schley G, Klanke B, Kalucka J, Schatz V, Daniel C, Mayer M, Goppelt-Struebe M, Herrmann M, Thorsteinsdottir M, Palsson R, Beneke A, Katschinski DM, Burzlaff N, Eckardt K-U, Weidemann A, Jantsch J & Willam C (2019). Mononuclear phagocytes orchestrate prolyl hydroxylase inhibition-mediated renoprotection in chronic tubulointerstitial nephritis. *Kidney Int* **96**, 378–396.

Schley G, Klanke B, Schödel J, Kröning S, Türkoglu G, Beyer A, Hagos Y, Amann K, Burckhardt BC, Burzlaff N, Eckardt K-U & Willam C (2012). Selective stabilization of HIF-1α in renal tubular cells by 2-oxoglutarate analogues. *Am J Pathol* **181**, 1595–1606.

Schödel J, Klanke B, Weidemann A, Buchholz B, Bernhardt W, Bertog M, Amann K, Korbmacher C, Wiesener M, Warnecke C, Kurtz A, Eckardt K-U & Willam C (2009). HIF-prolyl hydroxylases in the rat kidney: physiologic expression patterns and regulation in acute kidney injury. *Am J Pathol* **174**, 1663–1674.

Schofield CJ & Ratcliffe PJ (2004). Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol* **5**, 343–354.

Schönig K, Schwenk F, Rajewsky K & Bujard H (2002). Stringent doxycycline dependent control of CRE recombinase in vivo. *Nucleic Acids Res* **30**, e134.

Scortegagna M, Morris MA, Oktay Y, Bennett M & Garcia JA (2003). The HIF family member EPAS1/HIF-2α is required for normal hematopoiesis in mice. *Blood* **102**, 1634–1640.

Sequeira López MLS, Pentz ES, Nomasa T, Smithies O & Gomez RA (2004). Renin cells are precursors for multiple cell types that switch to the renin phenotype when homeostasis is threatened. *Dev Cell* **6**, 719–728.

Singh RP, Franke K, Kalucka J, Mamlouk S, Muschter A, Gembarska A, Grinenko T, Willam C, Naumann R, Anastassiadis K, Stewart AF, Bornstein S, Chavakis T, Breier G, Waskow C & Wielockx B (2013). HIF prolyl hydroxylase 2 (PHD2) is a critical regulator of hematopoietic stem cell maintenance during steady-state and stress. *Blood* **121**, 5158–5166.

Skott O & Briggs JP (1987). Direct demonstration of macula densa-mediated renin secretion. *Science* **237**, 1618–1620.

Souma T, Nezu M, Nakano D, Yamazaki S, Hirano I, Sekine H, Dan T, Takeda K, Fong G-H, Nishiyama A, Ito S, Miyata T, Yamamoto M & Suzuki N (2016). Erythropoietin synthesis in renal myofibroblasts is restored by activation of hypoxia signaling. *J Am Soc Nephrol* **27**, 428–438.

Starke C, Betz H, Hickmann L, Lachmann P, Neubauer B, Kopp JB, Sequeira-Lopez MLS, Gomez RA, Hohenstein B, Todorov VT & Hugo CPM (2015). Renin lineage cells repopulate the glomerular mesangium after injury. *J Am Soc Nephrol* **26**, 48–54.

Stiehl DP, Wirthner R, Köditz J, Spielmann P, Camenisch G & Wenger RH (2006). Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels: evidence for an autoregulatory oxygen-sensing system. *J Biol Chem* **281**, 23482–23491.

Takeda K, Aguila HL, Parikh NS, Li X, Lamothe K, Duan L-J, Takeda H, Lee FS & Fong G-H (2008). Regulation of adult erythropoiesis by prolyl hydroxylase domain proteins. *Blood* **111**, 3229–3235.

Takeda K, Ho VC, Takeda H, Duan L-J, Nagy A & Fong G-H (2006). Placental but not heart defects are associated with elevated hypoxia-inducible factor α levels in mice lacking prolyl hydroxylase domain protein 2. *Mol Cell Biol* **26**, 8336–8346.

Urrutia AA, Afzal A, Nelson J, Davidoff O, Gross KW & Haase VH (2016). Prolyl-4-hydroxylase 2 and 3 coregulate murine erythropoietin in brain pericytes. *Blood* **128**, 2550–2560.

Urrutia AA, Guan N, Mesa-Ciller C, Afzal A, Davidoff O & Haase VH (2021). Inactivation of HIF-prolyl 4-hydroxylases 1, 2 and 3 in NG2-expressing cells induces HIF2-mediated neurovascular expansion independent of erythropoietin. *Acta Physiol* **231**, e13547.

Wang F, Flanagan J, Su N, Wang L-C, Bui S, Nielson A, Wu X, Vo H-T, Ma X-J & Luo Y (2012*a*). RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J Mol Diagn* **14**, 22–29.

Wang Z, Schley G, Türkoglu G, Burzlaff N, Amann KU, Willam C, Eckardt K-U & Bernhardt WM (2012*b*). The protective effect of prolyl-hydroxylase inhibition against renal ischaemia requires application prior to ischaemia but is superior to EPO treatment. *Nephrol Dial Transplant* **27**, 929–936.

Additional information

Data availability statement

All data supporting our results are included in the manuscript.

Competing interests

The authors declare that they have no conflicts of interest.

Author contributions

K.A.E.B. and A.K. conceived and designed the research studies, analysed and interpreted data, wrote the manuscript, and made the figures. K.A.E.B., M.A.A.F., J.S. and G.S. performed experiments and acquired and analysed data. C.L. performed experiments. V.T.T. and C.H. analysed data, contributed mouse lines, and reviewed the manuscript. C.W. reviewed the manuscript. All authors edited the manuscript. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This work was supported by grants from the German Research Foundation (Ku 859/15-2 and SFB 1350, project number 387509280; TO 679/3-1, HU 600/8-1, HU 600/11-1, HU 600/12-1).

Acknowledgements

The authors would like to thank Susanne Fink, Sandra Kohl, Katharina Krieger and Brigitte Rogge for their expert technical assistance. The authors would like to thank Prof. Dr Ben Wielockx for kindly providing the PHD2 and PHD3 flox mice used for this study.

Open Access funding enabled and organized by Projekt DEAL.

Keywords

erythropoietin, hypoxia signalling, phenotype shift, prolyl-4-hydroxylases, renal interstitial cells, renin

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

Peer Review History
Statistical Summary Document