Influence of prolyl hydroxylases on polycystin-1 degradation under normoxic conditions in polycystic kidney disease

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Abstract

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is caused by a genetic disorder in the genes that encode for polycystin-1 (PC1) and polycystin-2 (PC2), which are plasma membrane-spanning proteins. The disease causes development of renal cysts and, subsequently, renal failure in the late middle age. Affected individuals rely mainly on kidney transplantation or dialysis for treatment.

Since PC1 and PC2 are involved in a substantial amount of signaling mechanisms, an aberrant function of these proteins contributes to ADPKD. A 30 kDa fragment (PC1-p30) that is cleaved off PC1 in the cytoplasm has been found to be involved in the regulation of transcription factors. PC1-p30 is rapidly degraded in normal oxygen conditions (normoxia) but is stabilized in a low oxygen environment (hypoxia), similar to the transcription factor HIF-1α. This transcription factor gets hydroxylated on its proline residues by proline hydroxylases (PHDs), which is a signal for ubiquitination and subsequent degradation. The goal of this thesis research was to elucidate whether PC1-p30 gets post-translationally modified by PHDs and whether proline hydroxylation ultimately causes ubiquitination and degradation of PC1-p30 by the proteasome. MDCK-cells that stably express PC1-p30 with doxycycline induction were either treated with $CoCl_2$ (inhibits PHDs and therefore, mimics hypoxic conditions) or MG132 (inhibits the proteasome). PC1-p30 was immunoprecipitated and the hydroxylated as well as mono- and polyubiquitinated version of PC1-p30 were detected via Western-Blot. Additionally, the interaction of PC1-p30 with the one of the isoforms of PHDs was tested. Via immunofluorescence, co-localization of PC1-p30 with PHD3 was demonstrated.

The findings of this thesis suggest that after cleavage off PC1 in the cytoplasm, PC1-p30 is hydroxylated by PHD3 and subsequently ubiquitinated by pVHL and the E3 ubiquitin ligase, which results in degradation by the proteasome. The obtained results give a better understanding of the post-translational modification of PC1-p30. Future experiments will give insight into which proline residues are modified and what other isoforms of proline hydroxylases are involved in the degradation of PC1-p30.

Table of Contents

1 Introduction

1.1 Autosomal Dominant Polycystic Kidney Disease

Autosomal Dominant Polycystic Kidney Disease (ADPKD), or in short, Polycystic Kidney Disease (PKD), is a genetically inherited disease. It is a relatively common genetic disorder and one of the most common causes of renal failure. Estimations of affected individuals lie between 1 in 400 and 1 in 1000. PKD predominantly alters renal tissue and results in the growth of cysts in the kidneys (Figure 1). Formation of cysts can also occur in different organs including the liver, pancreas, or heart. As PKD progresses, it ultimately causes renal failure in the late middle age [1–3].

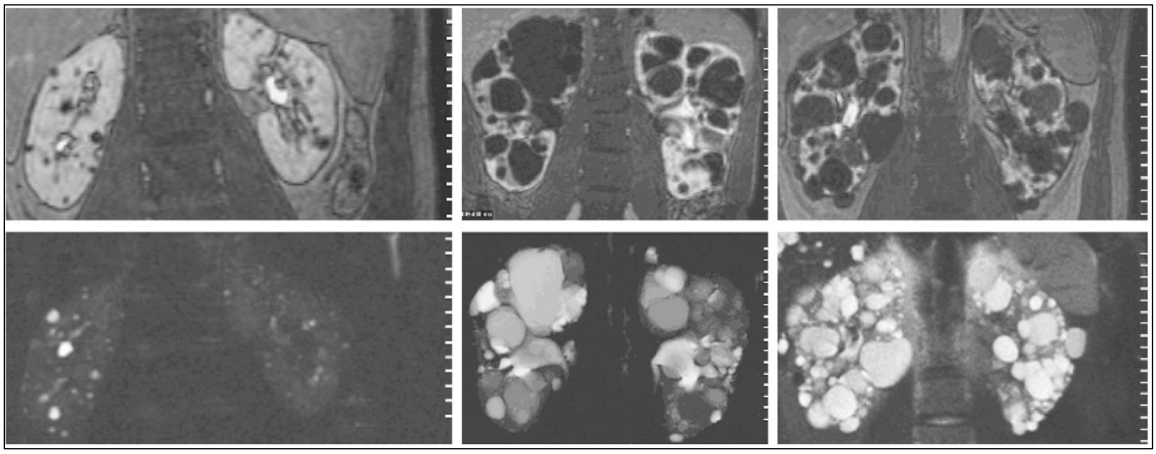


Figure 1: MRI scans from different patients in different stages of ADPKD. From left to right, progression of the disease and formation of cysts (black areas) can be observed. In further developed cystic kidneys, more cysts grow and older cysts gain volume. [2]

Until recently, kidney transplantation and dialysis were the only treatment options for PKD. The first alternative treatment that the Food and Drug Administration (FDA) approved is a drug called JYNARQUE^{TM} (tolvaptan). It is orally administered and prolongs the disease progression. Therefore, a kidney transplantation or dialysis are required in a much later age compared to untreated patients [2, 4].

It has been found that mutations in either of the two genes *PKD1* and *PKD2* are responsible for the disease, whereas 85% of PKD patients carry a mutation in the *PKD1*

gene, the remaining in the latter. *PKD1* and *PKD2* encode for the proteins polycystin-1 (PC1) and polycystin-2 (PC2), which are plasma membrane-spanning proteins that interact with each other and contribute to cellular functions such as regulation of intracellular Ca^{2+} concentration or cell proliferation/differentiation [2, 5].

In terms of disease progression and severity, there are differences that depend on the affected gene. Mutations in *PKD1* have been shown to have a higher impact. There is also evidence that suggests a dosage dependency of dysfunctional PC1. This means that there are less cysts growing and cysts emerge at a later age when the percentage of fully functional polycystin-1 is higher [3].

1.2 Polycystin-1 and polycystin-2

Both PC1 and PC2 are plasma membrane-spanning proteins that show homology in some of their transmembrane regions. PC1 is the larger protein with a size of approximately 450 to 460 kDa. It acts as a receptor protein that belongs to the GPCR family and is considered as an atypical GPCR. The expression of PC1 occurs in renal tubule epithelial cells as well as in several other tissues. It is proposed that PC1 is being activated at the G protein-coupled receptor proteolytic site (GPS domain, Figure 2), which is found in the N-terminal region. The N-terminus of PC1 is extracellular, the C-terminus locates in the cytoplasm. Activation of PC1 at the GPS domain results into autocleavage, but the two termini remain non-covalently attached. There are also smaller C-terminal cleavage products that vary in size. Cleavage of these fragments is induced by mechanical stimuli, polycystin-2 or γ-secretase. These fragments can be transferred to different locations in the cell and are involved in several pathways, where one possibility is the activation of transcription factors (e.g. STAT3 and STAT6). Signaling pathways that can be activated by polycystins are being categorized into: G protein signaling, calcium signaling, cAMP signaling, mTOR, and more. Mutation of one of the two polycystins therefore alters the natural functionality of these pathways. Dysfunctional signaling pathways as well as metabolic changes contribute to the progression of PKD [1, 5–10].

One model of PC1 activation is the interaction with PC2. This interaction presumably occurs at their C-terminal sites as well as at extracellular loops and results in the formation of a heterodimeric complex. By this interaction, Ca^{2+} concentration in the cytoplasm is

regulated by the nonselective cation channel PC2, which belongs to the transient receptor potential (TRP) ion channel family [1, 5, 6].

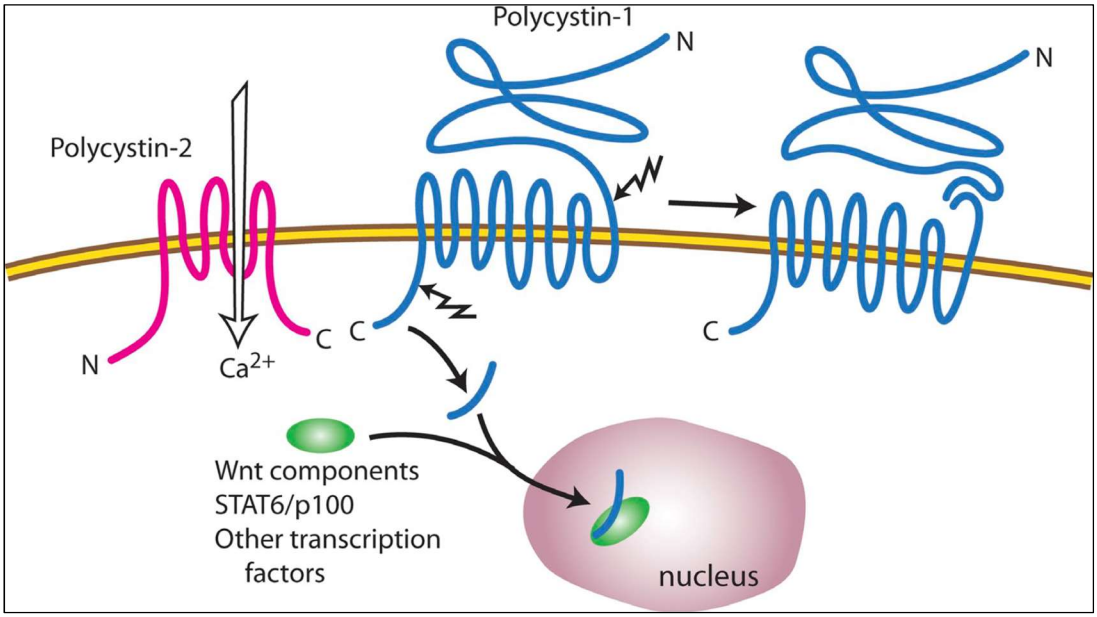


Figure 2: A simplified model of the polycystin-1-polycystin-2 complex that shows cleavage of the N-terminal and C-terminal end of PC1 (at the GPS domain – uppermost black arrow) and the activation of PC2 as a calcium channel. When PC1 is activated, the result is the cleavage of different sized C-terminal fragments. These fragments are transported to different sites within the cell and activate cellular functions and pathways by binding and activating transcription factors or other targets. [6]

PC1 and PC2 can occur at different locations in the cell and have various functions. The PC1-PC2 complex was found to localize in the membrane of the ER, the plasma membrane, in exosomes and in primary cilia. Studies also show that the PC1-PC2 complex is involved in cell-cell and cell-matrix interactions as well as in mechanosensory and chemosensory pathways. PC2 carries out channel functions at the plasma membrane and primary cilia where calcium influx into the cell is regulated dependent on fluid flow. It was discovered that the mutation of one of the two *PKD* genes affects cilia function and that aberrant cilia are one of the main causes for the disease progression of polycystic kidney disease [1, 5, 6, 8, 9].

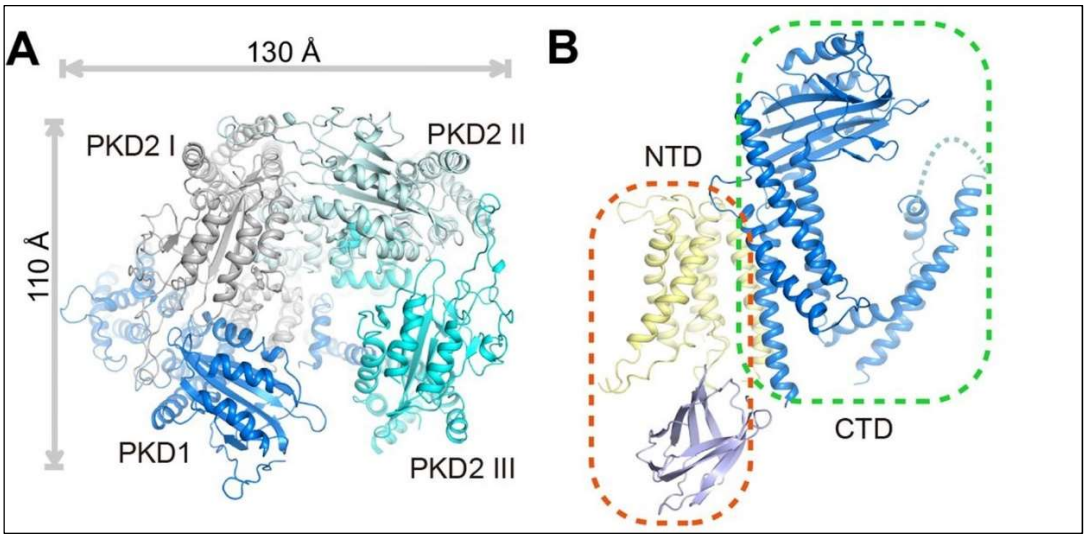


Figure 3: The 3D model that was determined by cryogenic electron microscopy yields a detailed view of the PC1-PC2 interaction (panel A) and polycystin-1 (panel B). Based on this model, the mechanisms of the polycystins can be resolved and thus, a more clear understanding of ADPKD can be gained. [11]

At which sites PC1 and PC2 interact and how they assemble in the plasma membrane has at least been partially resolved. The 3D structure of the PC1-PC2 complex was analyzed in a previous study, in which cryogenic electron microscopy was used to determine the assembly of the two polycystins upon interaction as well as the 3D structure of polycystin-1 (Figure 3). This finding could be essential when it comes to elucidating a detailed disease mechanism. With the knowledge of which mutations disturb a functional PC1-PC2 interaction, the chance of finding novel treatment options is increased [11].

2 Research background and objectives

2.1 HIF-1α and pVHL

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that regulates the expression of HIF target genes (Table 1) that contribute to the adaption of low oxygen in the cell (hypoxic conditions/hypoxia). The regulation of genes requires two subunits of HIF-1, HIF-1α and HIF-1β. The latter subunit is constitutively expressed, HIF-1α is stabilized by hypoxia, but becomes rapidly degraded in the presence of normal oxygen conditions (normoxic conditions/normoxia) [12, 13].

Table 1: A brief overview some of the genes that are targeted by HIF-1α and their corresponding functions upon activation. Erythropoiesis, cell proliferation and energy metabolism are only a portion of affected physiological responses when oxygen levels in the cell is altered. [13]

Examples of target genes	Physiological responses
Erythropoietin, Transferrin	Erythropoiesis and iron metabolism
IGF-1, TGFβ_3	Cell proliferation and viability
Glucose transporters, Pyruvate kinase	Energy metabolism

HIF-1α undergoes various post-translational regulations, such as hydroxylation, ubiquitination, acetylation or phosphorylation. For degradation of HIF-1α in normoxic conditions, it is being hydroxylated at its proline residues and subsequently ubiquitinated by the von Hippel-Lindau protein (pVHL). Physical interaction between HIF-1α and pVHL has been demonstrated in previous experiments. pVHL has a β-domain, which recognizes and binds to the oxygen-dependent degradation domain (ODDD) of HIF-1α. Consequently, the E3 ubiquitin ligase complex targets HIF-1α and a polyubiquitin chain is covalently attached. Ubiquitination of HIF-1α is a signal for its degradation by the proteasome [12–14].

Ubiquitination is a crucial feature of every cell to mark proteins for either translocation or degradation, dependent on the amount of ubiquitin molecules that are being linked by the ubiquitin ligase. In general terms, when a protein is monoubiquitinated, it is a signal for translocation. If polyubiquitination occurs, the protein is further degraded by the proteasome. An example of monoubiquitinated proteins are syntaxins, which interact with SNARE proteins to mediate cell membrane fusion [15, 16].

2.2 Prolyl hydroxylases

As a post-translational modification of HIF-1α, hydroxylation occurs at two proline residues to mark it for degradation. In previous studies, the mutation of the two concerned proline residues resulted in accumulation of HIF-1α. The enzyme that catalyzes the hydroxylation of HIF-1α is called proline hydroxylase or referred to as proline hydroxylase domain (PHD). Optionally, it can be named Egg-laying nine (EGLN). There are three isoforms of proline hydroxylases, PHD1, PHD2 and PHD3 (EGLN1, EGLN2, and EGLN3). The required co-factors and substrates for the hydroxylation of proline residues are α-ketoglutarate, molecular oxygen, iron (II) and ascorbate. The latter two compounds serve as co-factors. Side products of the proline to hydroxy-proline reaction are succinate and CO_2. Prolyl hydroxylases have a variety of hydroxylation targets and are therefore involved in multiple regulation mechanisms. For instance, PHD3 was shown to promote apoptosis [12, 17].

It has been demonstrated that all three isoforms of PHDs are responsible for the hydroxylation of HIF-1α. In contrast, only PHD3 has the most targets that are non-HIF-related. Also, PHD3 turns out to be the most efficient one when it comes to hydroxylation of certain proline residues on HIF-1α and can also hydroxylate targets when exposed to hypoxic conditions. PHD3 is expressed in many different tissues, including neurons and muscle tissue. Within the cell, it can be detected either in the cytoplasm or in the nucleus and assembles itself in aggregates with other proteins under normoxic conditions. For instance, p62 (or sequestome 1) has been shown to physically interact with PHD3. p62 is involved in many pathways and cellular responses, such as inflammatory responses. It is supposed that p62 aggregates with PHD3 under normoxic conditions and promotes its degradation. Therefore, PHD3 is downregulated in normoxia. In contrast, the co-localization of PHD3 with p62 is reversed in hypoxic conditions and thus, leads to higher levels of PHD3 [18, 19].

2.3 PC1-p30

One cleaved product of the C-terminus of PC1 is a 30 kDa large fragment, named PC1-p30, which normally undergoes rapid proteasomal degradation. It was shown that PC1-p30, or p30, is being cleaved off the C-terminal end of polycystin-1 in the cytoplasm and is targeting various locations in the cell. One target is the nucleus, where p30 is involved in the activation of transcription factors. In ADPKD, p30 is overexpressed and therefore alters cellular functions in a negative manner. It has already been demonstrated that p30 has an impact on the development of renal cysts. In order to gain a better understanding of ADPKD and of potential therapeutic strategies, it is important to understand how p30 is regulated and what targets and functions it has [7, 20–22].

A hallmark of ADPKD is the high proliferation rate of cyst-lining cells and a metabolic shift in which aerobic glycolysis is preferentially used to produce energy. This limits their rate of respiration in mitochondria, which indicates that ADPKD cyst-lining cells require a relatively low amount of molecular oxygen. This effect is called Warburg effect and was first observed in cancer cells. It was shown that hypoxia is a distinguishing feature in cystic renal tissue and that this especially appears in cells that line the cysts. This pericystic hypoxia stabilizes HIFs due to the inhibition of PHDs. It is widely hypothesized which molecular mechanisms cause these metabolic changes. One reason on the altered metabolism could be the impact of the polycystins on mitochondrial function. Ca^{2+} release into the cytoplasm and the uptake of Ca^{2+} by mitochondria activates enzymes involved in oxidative phosphorylation. In ADPKD, this regulation is impaired [8, 23, 24].

2.4 Research objectives and approach

As mentioned before, PHDs require oxygen to hydroxylate proline residues. The hypothesis of this thesis research is that PC1-p30 is hydroxylated by proline hydroxylases under normoxic conditions and is subsequently degraded by the proteasome (Figure 4). Furthermore, hypoxia in renal cysts could cause inhibition of PHDs and therefore upregulation of PC1-p30, which then leads to the activation of transcription factors and other cellular effects that are predominant in ADPKD. It is essential to resolve the mechanisms behind PC1-p30 stabilization and possible targets that PC1-p30 has so that potential approaches for ADPKD therapy can be found.

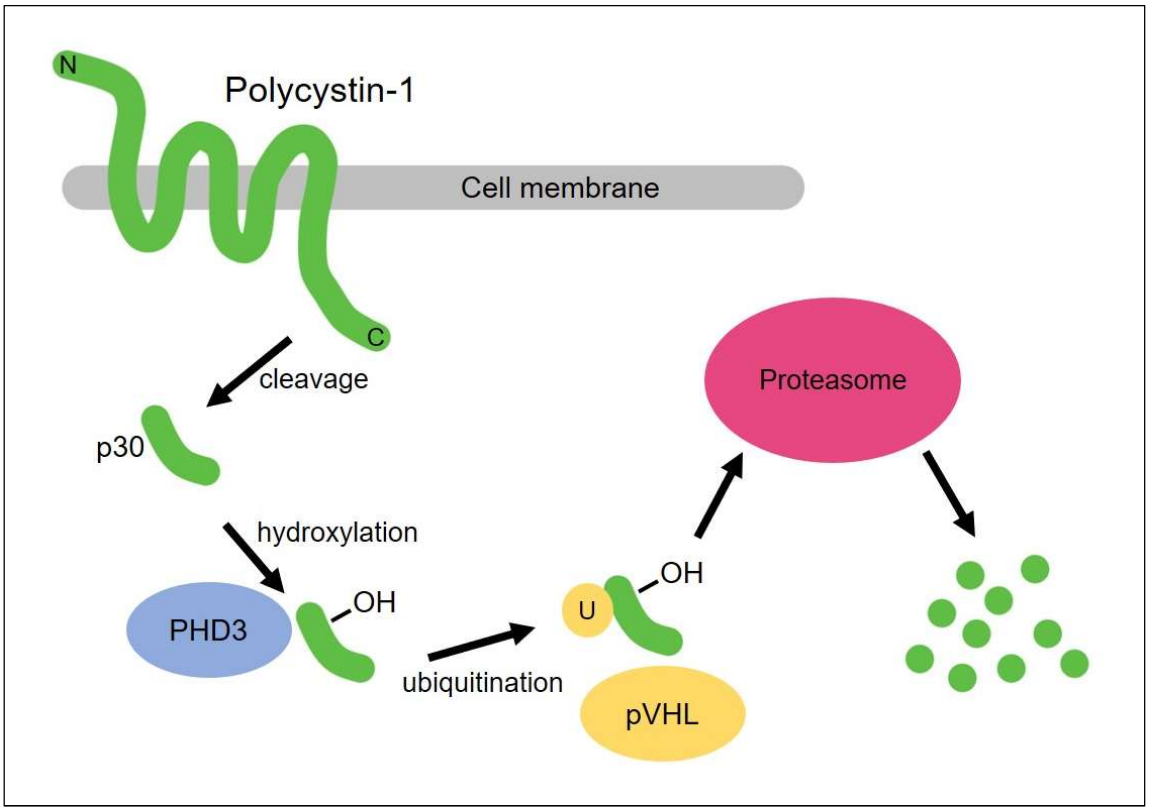


Figure 4: Simplified model of the proposed pathway and modifications that the 30 kDa sized C-terminal fragment of PC1 (p30) undergoes after it is cleaved off PC1. Under normoxic conditions, its proline residues are recognized by proline hydroxylases (presumably PHD3) and subsequently ubiquitinated by pVHL. Ubiquitinated p30 is processed by the proteasome and therefore degrades.

Since it is both demonstrated that PC1-p30 is upregulated in ADPKD [22] and that hypoxia is a typical appearance in cystic kidneys [24], the two reagents MG132 and $CoCl_2$ play a critical role in the conducted experiments. The main experiment involves the upregulation of PC1-p30 by MG132, which is a peptide aldehyde and inhibits the 26S proteasome. $CoCl_2$ is used as a substance that mimics hypoxic conditions, which has already been demonstrated by the upregulation of HIF-1α in LOVO cells [25, 26].

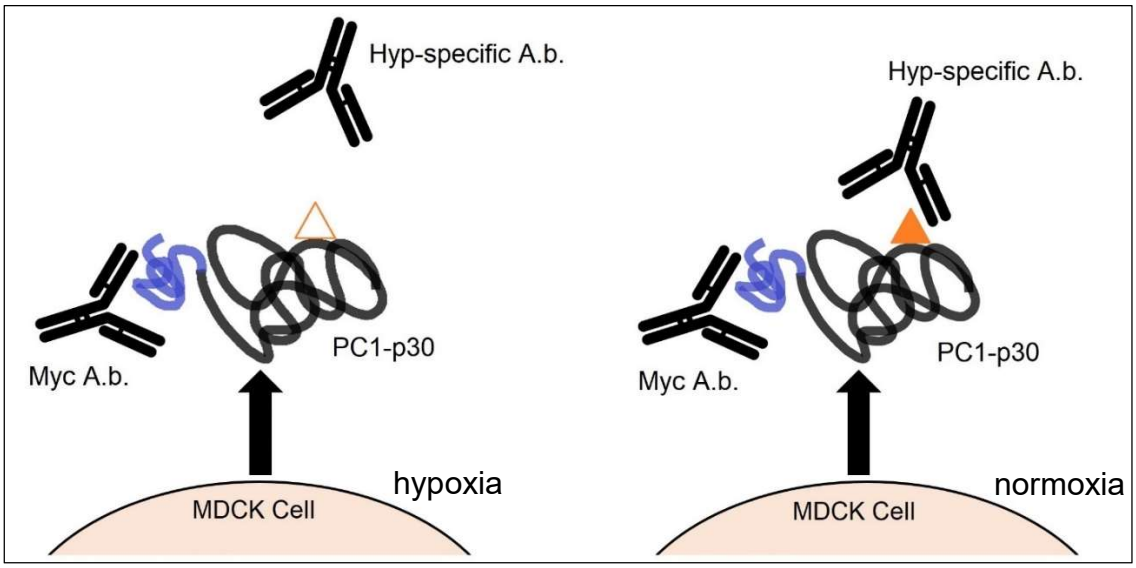


Figure 5: Schematic illustration of the experiment, in which normoxic conditions and upregulation should yield a sufficient amount of hydroxylated PC1-p30 for the detection of hydroxylated proline (symbolized by a filled, orange triangle) with a hydroxyproline-specific antibody. In contrast, hypoxic conditions inhibit PHDs and proline residues of PC1-p30 remain un-hydroxylated (unfilled, orange triangle). Therefore, the hyp-specific antibody is not able to bind, and no signal can be measured.

To detect the hydroxylated version of proline residues on PC1-p30 by PHDs, MDCK-p30 expressing cells are treated with MG132 to accumulate a high amount of PC1-p30 and to detect hydroxyproline with a hydroxyproline-specific antibody. $CoCl_2$ treatment is used to mimic hypoxia and therefore to prevent hydroxylation of p30 on its proline residues by PHDs and thus, to upregulate it. To purify and to concentrate the amount of p30, a myc-tag specific antibody is utilized to pull down myc-tagged p30 via an immunoprecipitation. The samples are analyzed via Western Blot (Figure 5).

To gain a better understanding of how p30 is regulated and to obtain a better picture of all the involved mechanisms around p30, ubiquitination of p30 is tested by probing immunoprecipitated PC1-p30 with a ubiquitin-specific antibody. Treating MDCK-cells with MG132 should not only lead to upregulation of p30 alone, but also of its ubiquitinated version, which would normally lead to the degradation by the proteasome. Samples are also analyzed for PHD3 and HIF-1α in order to test their upregulation by $CoCl_2$ treatment (mimicking hypoxia) in the p30 expressing MDCK cells.

For supporting the hypothesis that p30 is hydroxylated by and physically interacts with PHD3, an immunofluorescence experiment is established. MDCK-p30 cells are

transfected with a PHD3 encoding plasmid and then stained with a fluorescence-labeled antibody. Co-localization of p30 and PHD3 will be visualized by fluorescence microscopy.

3 Materials and Methods

3.1 Utilized cells

Previously transfected Madin-Darby Canine Kidney (MDCK) cells (Figure 6) were used for most of the experiments (MDCK-p30, running number A099, clone number 3). A PC1-p30 expressing construct was stably transfected into the cells. PC1-p30 expression was utilized by a doxycycline (DOX) inducible promoter. DOX was added in a concentration of 50 ng/mL 36 hours prior to lysing the cells. The expression construct included a myc-tag on PC1-p30. The tag enables p30 to be easier immunoprecipitated as well as detected.

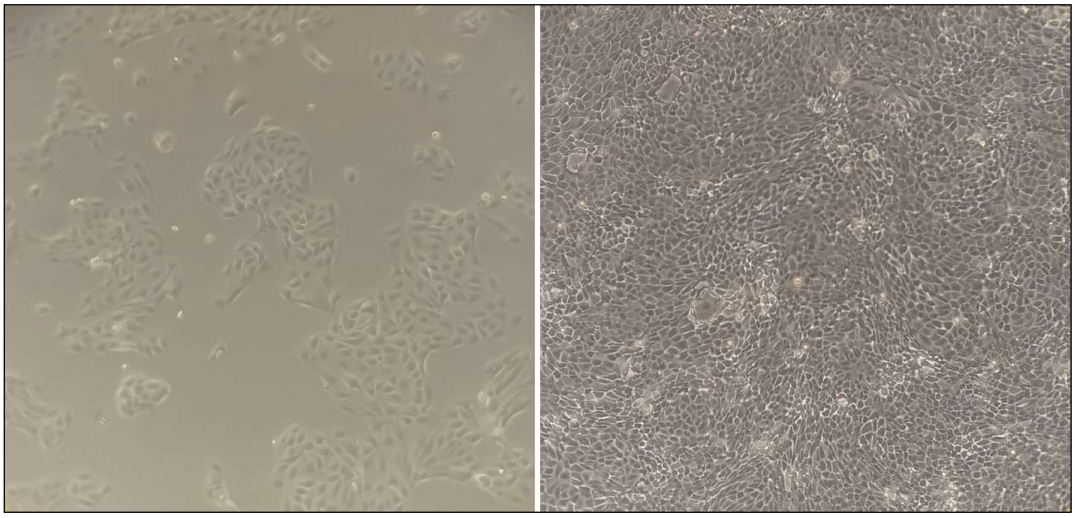


Figure 6: Stably transfected (DOX inducible myc-tagged PC1-p30) MDCK cells were used for the entirety of the experiments. In the left panel, cells exhibit sub-confluency (approximately 50%). In the right panel, cells have reached full confluency.

The expression system of PC1-p30 is a Tet-On system (Figure 7). In the Tet-On system, doxycycline (a derivative of tetracycline) is used to activate a protein called reverse tetracycline-controlled transactivator (rtTA), which is constitutively expressed. When rtTA is activated by binding to DOX, it binds to the Tet response element (TRE). By this activation, the target gene is expressed [27].

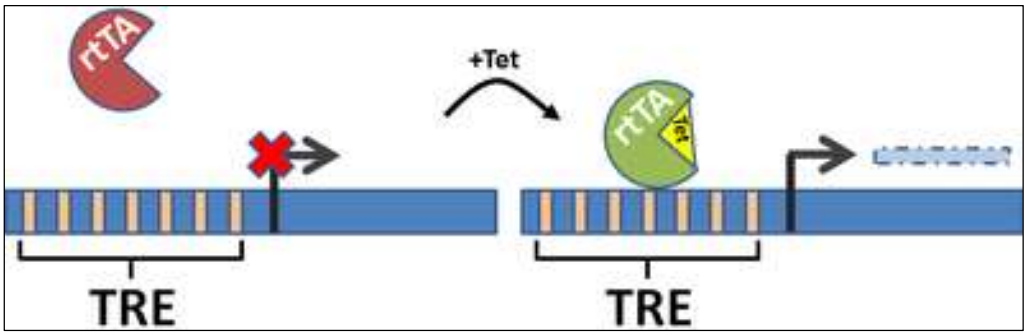


Figure 7: Illustration of the used Tet-On system in the MDCK-p30 cell line. Doxycycline (DOX) is used instead of Tetracycline (Tet) for the expression of the PC1-p30 protein fragment. Once DOX is added to the cells (in a concentration of 50 ng/mL), rtTA is activated and binds to TRE. As a consequence, PC1-p30 will be expressed. [27]

3.2 Handling cell culture

MDCK-p30 cells were cultivated in Minimal Essential Medium (MEM) with 1 % Penicillin-Streptomycin (Pen/Strep) antibiotic, 1 % L-glutamine (L-GLN) and 5 % Fetal Bovine Serum (FBS) (Table 2). MEM, L-GLN and Pen/Strep were obtained from *Mediatech, Inc*. The used cell culture dishes for cell cultivation were *Falcon*® 10 cm tissue culture dishes from *CORNING* (treated with vacuum gas plasma).

Table 2: MDCK-p30 cells were cultivated in medium that contains the listed components, plus Fetal Bovine Serum. L-GLN and Pen/Strep were diluted in a 1:100 ratio, FBS was diluted 1:20.

Medium component	Details
Minimal Essential Medium Eagle (MEM)	Contains Earle's salts
L-Glutamine	200 mM solution
Penicillin Streptomycin	100x solution

MDCK-cells were cultivated in a *HERA cell* incubator from *HERAEUS* in a 5% CO_2 environment and at 37 °C. Maintenance of MDCK-p30 clones was implemented by feeding the cells with 10 mL of MEM containing FBS, L-GLN and Pen/Strep (full MEM). Every 72

hours, the medium was swapped with fresh medium to prevent depletion of nutrients, serum-ingredients, etc.

Cells were only split into a new passage number when multiple plates with cells were required for the experiments. Before cells underwent the splitting procedure, the medium was aspirated from the dish and 1x Dulbecco's Phosphate Buffer Saline (DPBS) solution (*Mediatech, Inc.*) was added. The dish was gently rocked for a couple of seconds to wash the cells and the buffer solution was aspirated. The washing step was repeated a second time before 2 mL of 0.25 % trypsin solution (*Mediatech, Inc.*) was added. The dish was incubated for 10 minutes in the cell culture incubator to trypsinize the cells completely from the cell culture dish. 8 mL of full MEM was added to the dish and remaining cell clumps were resuspended by pipetting the solution up and down. The suspension was transferred into a centrifuge tube and cells were spun down. The medium was aspirated to remove the trypsin from the cells and cells were resuspended in 10 mL fresh full MEM. For most of the experiments, a 1:10 splitting ratio was used to yield a confluency of 80 % after 3 days. For a 1:10 ratio, 1 mL of cell suspension was added to 9 mL of full MEM in a 10 cm dish. MDCK-cell confluency was observed after every 24 hours under the inverted microscope.

Feeding and splitting cells was done under sterile conditions in a class II biological safety cabinet. Before full MEM was used, it was pre-heated in a 37 °C water bath. In the following, a list of the essential materials for cell culture maintenance and splitting procedure is shown:

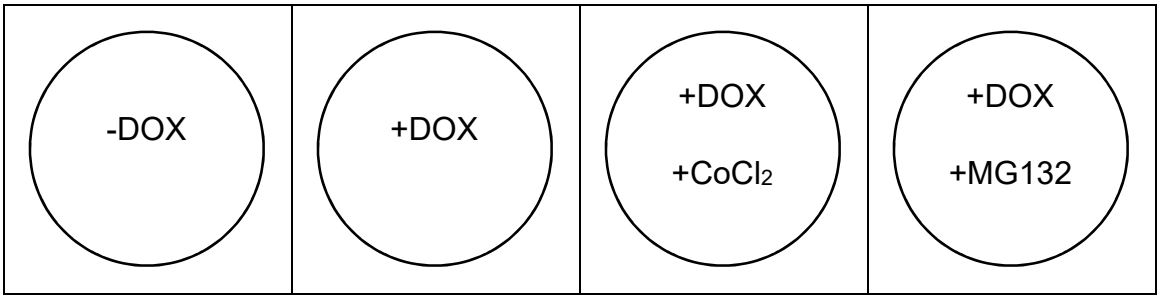
- Minimal Essential Medium (with 5 % FBS, 1 % L-GLN, 1 % Pen/Strep)
- 0.25 % trypsin solution (containing 0.1 % EDTA)
- Phosphate buffered saline (PBS) solution
- Cell culture dishes
- Cell culture incubator
- Class II biological safety cabinet
- Vacuum aspirator
- Inverted microscope

3.3 Inducing and treating cells

Induction of the myc-tagged PC1-p30 was performed with a DOX concentration of 50 ng/mL. The existing medium in each dish was replaced by 8 mL of the DOX containing medium

when the cell confluency reached 80 %. Cells were then incubated for 24 hours (preDOXing).

Table 3: Schematic overview of the different treatment groups of MDCK-p30 cells. Cells were either DOX induced or not (negative control). To upregulate p30, treatment with either $CoCl_2$ (200 µM) or MG132 (2 µM) was implemented for 12 hours.



After preDOXing, cells were treated with either $CoCl_2$ (200 µM) or MG132 (2 µM) for 12 hours before cell lysis. Both reagents were added to the DOX containing medium in the dishes, therefore, a continuous induction by DOX during the treatment was ensured. Table 3 shows a schematic of the resulting groups. DOX and MG132 were obtained from the existing stock solutions. A fresh $CoCl_2$ stock solution was prepared shortly before the treatment.

3.4 Lysing cells

3.4.1 Lysing cells for immunoprecipitation

After DOXing and treating the MDCK-p30 cells with MG132 and $CoCl_2$, dishes were put on ice and the medium was aspirated. The SDS containing buffer for cell lysis contains:

- 0.5 % SDS
- 50 mM NaCl
- 5 mM EDTA
- 50 mM TRIS-HCl, pH adjusted to 7.4

To prevent degradation of PC1-p30 in the cell lysate by various proteases, protease inhibitor (PI) cocktail and PMSF were added to the SDS cell lysis buffer. The PI cocktail was added

in a 1:1000 dilution and PMSF in a 1:100 dilution (working concentration 1 mM). The cell lysis buffer was kept on ice and 500 µL ice cold buffer was added to each dish and distributed on the whole dish by gently swirling and rocking the dish. MDCK cells were scraped off each dish with the reverse side of a micropipette tip (P1000). The cells were transferred into microcentrifuge tubes, boiled for 5 minutes and put on ice to cool down the lysate. Cells were then sheared with a sonicator for 10 seconds with an output of 2 and immediately put on ice. The cell shearing process was repeated one more time.

500 µL of dilution buffer was added to each tube. The dilution buffer contains:

- 2.5 % Triton X-100
- 50 mM NaCl
- 5 mM EDTA
- 50 mM TRIS-HCl, pH adjusted to 7.4

Insoluble material was spun down in the cold room (4 °C) for 5 minutes at full speed. The supernatant was transferred into new microcentrifuge tubes and the pellet was discarded. At this point, freezing the lysate was possible before continuation of the experiment.

For the first group of samples, 50 µL of the lysate was removed and mixed with 2x SDS sample buffer as well as 10x DTT in the appropriate ratios and then boiled for 5 minutes and frozen at -20 °C. This group of samples is called “Input”. As a preparatory step before the immunoprecipitation, CL-2B beads (from *Amersham Biosciences*, lot # 310212) were used to pre-clear the cell lysates. Beads were washed with wash buffer 4 times. The wash buffer contains:

- 0.5 % Triton X-100
- 50 mM NaCl
- 5 mM EDTA
- 50 mM TRIS-HCl, pH adjusted to 7.4

Washing the beads was done by adding 1 mL wash buffer, resuspending beads, centrifuging beads down with a few 100 rpm and discarding the supernatant. After the last wash step, a 50 % slurry of beads and wash buffer was made. To each tube with lysate, 50 µL of the resin slurry was added and tubes were rotated at 4 °C for 30 minutes. This step was repeated

once more by spinning beads down (a few 100 rpm) and transferring the supernatant into new tubes. Again, 50 µL of CL-2B slurry was added, suspension was rotated at 4 °C for 30 minutes and after centrifugation at a few 100 rpm, the supernatant was transferred into new tubes.

3.4.2 Lysing cells for non-IP experiments

For preliminary experiments, MDCK-cells were cultivated and treated in the same manner as in chapter 3.2 and 3.3. As described in 3.4.1, 500 µL of SDS lysis buffer (containing PIs and PMSF) was added to the precooled and aspirated cell culture dishes. The cells were scraped off the dishes and the cell suspensions were collected in microcentrifuge tubes. Cells were boiled, sonicated and dilution buffer was added (see 3.4.1). After spinning down all the insoluble materials (cold room, 5 minutes and high speed), the entire supernatant was transferred into new tubes and 2x SDS sample buffer together with 10x DTT was added in the according dilution range. Samples were boiled for 5 minutes and loaded on an SDS-PAGE gel for analysis. The remaining cell lysate was frozen at -20 °C.

3.5 Immunoprecipitation

The pre-cleared cell lysate obtained from the previous step was used for the IP procedure. 2 µL of the mouse anti-myc tag antibody (9E10 ascites, obtained from *Bio World*) was added to each sample. To guarantee sufficient binding to the myc-tag on p30, the antibody incubation of the lysate was performed by rotation overnight at 4 °C. In the meantime, the resin for the pull-down procedure was prepared. Protein G agarose beads (Protein G Sepharose™ 4 Fast Flow from *GE Healthcare*, lot # 10244349) were washed with 1 mL buffer 3 times. Briefly, washing buffer was added, beads were resuspended, spun down at a few 100 rpm and the supernatant was discarded. Washing buffer that is equal to the volume of beads was added to create a 50 % slurry of protein G beads.

After the overnight incubation with the myc-tag antibody, 50 µL of the protein G resin slurry was added. Samples were incubated for 2.5 hours at 4 °C with rotation. The beads were spun down (only a few 100 rpm required) and 50 µL of the supernatant was collected in new microcentrifuge tubes. 2x SDS sample buffer and 10x DTT were added in the new tubes accordingly to the volume and samples were boiled for 5 minutes and then frozen at -20 °C. This sample group is called “After IP”.

The remaining supernatant was collected (approximately 1 mL) and frozen at -20 °C. The pellet of beads was washed with wash buffer 3 times (1 mL). The beads were resuspended in wash buffer and spun down at a few 100 rpm. After the last wash step, most of the supernatant was removed and discarded with a micropipette. A syringe needle (30 Gauge) was used to aspirate the remaining supernatant until protein G beads were almost dry. 50 µL 2x SDS sample buffer and 5 µL 10x DTT was added to the beads and the samples were boiled for 5 min. The samples were frozen at -20 °C for continuing at a later point. Alternatively, the IP samples were kept on ice while the other samples (Input and After IP) were thawed for loading on an SDS-PAGE gel. Before running the samples on the gel, they were centrifuged to spin down any insoluble materials.

3.5.1 Principle of Immunoprecipitation

Immunoprecipitation (IP) is a process of “pulling down” a target protein or even multiple proteins from samples of cell lysates, depending on the type of buffer used for lysing the cells. When cells are lysed under denaturing conditions with an SDS containing buffer, interactions between proteins are destroyed and therefore only the target protein is being pulled down. If a mild lysis buffer (e.g. containing Triton X-100 instead of SDS) is used, protein-protein interactions are retained. With this method, proteins that interact with the target protein are being co-pulled down and protein-protein interactions can be determined via Western Blot.

Aside from which lysis method is used, it is also important to select the appropriate resin (or beads) and a suitable antibody for the pull-down procedure. The IP antibody ideally binds strongly and highly specific to the target antigen. The antibody could either be specific to a naturally occurring antigen in the cell or a tag (e.g. myc, HA, His) that is fused to a stably expressed target protein. Depending on the IgG antibody species used, either protein A or protein G coupled Sepharose is used as an IP resin. Protein A or G bind to the Fc region of the antibody. Thus, the antibody-epitope complex is captured on the resin and multiple wash steps are performed to purify the sample [28].

3.6 SDS-PAGE and Western blot

12% in-house made SDS-PAGE gels were used for running the Input, After IP and IP samples. 5 µL of PageRuler^{TM} Plus Prestained Protein Ladder from *Thermo Scientific* was

used as a marker. 10 µL of the samples were loaded on the gel and the gel was run at 100 V, either for 90 minutes or until the sample buffer stain reached the bottom of the gel. As a running buffer, 1x TGS was used.

The proteins were transferred to a nitrocellulose membrane via a wet transfer. A 1x TG buffer (containing 20% methanol) was used for the transfer. The protein transfer was performed at 100 V for 105 minutes. The transfer apparatus was either kept in a container with ice or in the cold room (4 °C). The nitrocellulose membrane was then stained for approximately 5 minutes with a Ponceau S stain solution, which had the following components:

- 0.5 % Ponceau S
- 1 % glacial acetic acid

After staining, the membrane was rinsed with milliQ H_2O twice before a picture was taken with the Azure™ c300 imager from *azure biosystems* in the Azure cSeries Acquisition Software. Membranes that were later probed with a ubiquitin-specific antibody were boiled for 10 minutes in milliQ H_2O. Membranes were blocked in a 1x TBST buffer with either 5 % dry milk or 5 % BSA. A 5 % BSA containing buffer was used for membranes that were later probed with hydroxyproline-specific antibody or probed for any other low abundance/endogenous proteins. The blocking procedure was performed for 1 hour at room temperature. Incubation with the primary and secondary antibodies was utilized in the same solution that was used for blocking. The immunoblotting procedure included incubation with the corresponding primary antibodies overnight at 4 °C or 1.5 hours at room temperature in sealed plastic wraps (to reduce the volume of the used antibodies). Before secondary antibody incubation, the membranes were rinsed with 1x TBST twice and then washed with 1x TBST on an orbital shaker for 10 minutes three times. The membranes were subsequently incubated with the respective secondary antibody for 1.5 hours at room temperature and then rinsed twice and washed with 1x TBST for 10 min on an orbital shaker three times.

In Table 4, a full list of the used primary and secondary antibodies used for Western-Blot with their respective dilution ratio can be obtained.

Table 4: Shown are all used primary and secondary antibodies with their dilution ratios. The used blocking solution (either BSA or dry milk) is shown with each primary antibody. The same blocking solution was used with their respective secondary antibody. Additionally, the company the antibody was ordered from and the batch/lot number can be obtained. (*Inventory running number – Weimbs Lab)

1° antibody	Details	RN*
rabbit anti-PC1 (against C-terminus), 1:1,000	– in 5 % dry milk – in-house antibody	266
rabbit anti-HIF-1α polyclonal (against C-terminus), 1:200	– in 5 % BSA – obtained from *Cayman Chemical Company* – batch# 0484536-1	586
rabbit anti-hydroxyproline polyclonal, 1:500	– in 5 % BSA – obtained from *Abcam* – lot# GR3210175-2	582
rabbit anti-PHD3 polyclonal (against C-terminus), 1:1,000	– in 5 % dry milk – obtained from *Novus Biologicals* – lot C-1	651
mouse anti-myc tag 9E10 ascites, 1:1,000	– In 5 % dry milk – Obtained from *Bio World*	407
rabbit anti-ubiquitin polyclonal, 1:500	– in 5 % BSA – obtained from *upstate cell signaling solution* – lot# 22476	358
mouse anti-ubiquitin monoclonal, 1:500	– in 5 % BSA – obtained from *Santa Cruz Biotechnology* – lot#: D0115	326
2° antibody	**Details**	**RN***
goat anti-rabbit polyclonal, 1:10,000	– HRP conjugated – Obtained from *Jackson ImmunoResearch Laboratories, Inc.*	18
goat anti-mouse polyclonal (light chain specific), 1:10,000	– HRP conjugated – Obtained from *Jackson ImmunoResearch Laboratories, Inc*	72
goat anti-mouse polyclonal, 1:10,000	– HRP conjugated – Obtained from *Jackson ImmunoResearch Laboratories, Inc.*	20

To detect the probing on the nitrocellulose membrane, ECL solution was used as a substrate for the horseradish peroxidase that is linked to secondary antibodies. The substrate solution was made by mixing an equal amount (approximately 200 µL per membrane) of the two ECL reagents:

- Detection Reagent 1: Peroxide Solution (*Thermo Scientific*)
- Detection Reagent 2: Luminol Enhancer Solution (*Thermo Scientific*)

Once the ECL solution was mixed, it was distributed on the membrane and images were taken with the Azure™ c300 imager from *azure biosystems* in the Azure cSeries Acquisition Software. For band images, the chemiluminescence mode was used. For marker pictures, the visible light mode was used.

3.7 Immunofluorescence

3.7.1 Transient transfection of MDCK-p30 cells

As a preparation for immunofluorescence (IF) experiments, round coverslips (No 1.5) were sterilized. The coverslips were dipped in 100 % ethanol, flamed briefly and placed into the wells of a 12 well cell cultivation plate from *Corning Incorporated*.

MDCK-cells were dislodged with a 0.25 % trypsin solution and resuspended in 10 mL full MEM (see chapter 3.2 for detailed splitting procedure). The concentration of cells in suspension was determined by mixing a small volume (approximately 100 µL) of the suspension with a 0.4 % trypan blue solution (in PBS) in a 1:2 ratio. The trypan blue solution was obtained from *Mediatech, Inc*. 10 µL of the stained cells were pipetted into the two chambers of a Countess™ cell counting chamber slide from *Invitrogen™* and cells were counted with the Countess™ II Automated Cell Counter from *life technologies*. An appropriate amount of MDCK cell suspension was diluted in fresh full MEM (to achieve a cell concentration of approximately 25,000 cells/mL) and 2 mL of MEM was added to each well in the plate. Therefore, 50,000 cells per well were plated. To reach an ideal sub-confluency for DNA transfection, cells were incubated for 24 to 48 hours and medium was replaced by either MEM or DOX-containing MEM (50 ng/mL) prior to transfection.

Per well, 200 µL of MEM that contained 1 % L-GLN and 1 % Pen/Strep (no FBS), was mixed with 2 µg of the pEGFP-N1-PHD3 plasmid in a 1.5 mL microcentrifuge tube and

briefly vortexed. The plasmid was a gift from Eric Metzen (Addgene plasmid #21402). For positive transfection-controls, cells were transfected with 2 µg of an EGFP encoding plasmid (pEGFP-N1). TurboFect^{TM} Transfection Reagent from *Thermo Fisher Scientific* was briefly vortexed and 6 µL of the transfection reagent was added to the DNA mixture, which was again briefly vortexed and then incubated at room temperature for 20 minutes. The DNA/reagent mixture was added drop-wise to the wells and the 12 well plate was gently rocked. The cells were then incubated for 24 to 48 hours to provide sufficient time for DNA uptake and protein expression.

3.7.2 Fixing and antibody staining

Before staining with a myc-tag antibody for indirect IF, MDCK cells had to be fixed on the coverslips. Therefore, the medium was aspirated and 1.5 mL of 4 % formaldehyde was added to each well. The 12 well plate was gently rocked on an orbital shaker for 25 minutes at room temperature. The formaldehyde solution was aspirated and the cover slips were washed twice with 1.5 mL of 1x TBS. 1 mL of quench solution (a fresh solution was made from 1 M stock solutions of NH_4Cl and Glycine before each experiment) was added to each well after TBS was removed. Incubation was performed with gentle shaking at room temperature for 10 minutes. The quench solution consisted of:

- 75 mM NH_4Cl
- 20 mM Glycine
- 1x TBS

The cover slips were washed 3 times in 1x TBS. For the blocking step, 1 mL of blocking/permeabilization solution was added to each well and the plate was incubated at 37° C for 1 hour. The blocking/permeabilization solution contained:

- 2 % BSA
- 0.2 % Triton X-100
- 1x TBS

While the coverslips were blocked, the primary antibody solution was prepared (50 µL for each coverslip). A mouse-anti myc (monoclonal, 9B11, obtained from *Cell Signaling*

Technology, Inc., lot #7, Inventory running #211) antibody was diluted 1:8000 in the blocking/permeabilization solution. Also, the humidifying chamber was prepared. Paper towels were placed in a plastic tray and soaked with milliQ H_2O. After blocking, the coverslips were placed on parafilm (lid of 12 well plate as a support underneath) in the humidifying chamber and 50 µL of the primary antibody solution was pipetted on top of the corresponding coverslips. The plastic tray was covered with a lid and the humidifying chamber was incubated for 1.5 hours at 37 °C.

The coverslips were placed back in the 12 well plate and washed with cell wash solution, which contained:

- 0.7 % fish skin gelatin
- 0.05 % Triton X-100
- 1x TBS

The cell washing step was performed by adding 1.5 mL of the cell washing solution to each well, gently shaking the plate for 5 minutes at room temperature on an orbital shaker and aspirating the solution. This step was repeated 3 more times, the cell washing solution was replaced by fresh solution after each step. While the cells were washed, the secondary antibody solution was prepared in blocking/permeabilization solution. A donkey anti-mouse (polyclonal, conjugated with DyLight 594 fluorescent dye, obtained from *Jackson ImmunoResearch Laboratories, Inc.*, Inventory running #94) secondary antibody was diluted 1:1,000. 50 µL per coverslip were prepared.

The same humidifying chamber as explained beforehand was prepared. The coverslips were placed on parafilm and 50 µL of the secondary antibody solution was added to each coverslip. The incubation was performed for 1 hour at 37 °C. For the cell washing procedure, the coverslips were washed 4 times in the same manner as after the primary antibody incubation and then rinsed twice with 1x TBS.

The cells were post-fixed by adding 1.5 mL of 4 % formaldehyde to each well and by gently shaking the plate for 10 minutes at room temperature. Then, the coverslips were rinsed twice with 1x TBS. ProLong™ Gold antifade reagent with DAPI was used to mount the cover slips on glass slides. A small drop of mounting medium was placed on each glass slide. The coverslips were removed from the 12 well plate and most of the remaining solution was gently dried with a Kimwipe. The coverslips were placed upside down on the mounting

medium and the medium was hardened for 24 hours. Since the mounting medium already contains DAPI staining, no additional nuclei staining was required. The remaining buffer components were rinsed off the glass slides with milliQ H_2O to prevent crystal formation.

3.7.3 Microscopy and imaging

Pictures were taken with the *Olympus* IX81 inverted fluorescence microscope. For capturing pictures in 60X or 100X magnification, immersion oil was applied to the coverslips. The used software for image capturing was Micro Manager 1.4. DAPI, FITC and Texas Red channels were used to capture each single fluorescence staining of the cells or respective tagged proteins. All three channel pictures were merged in Adobe Photoshop CC 2018 to analyze for co-localization.

3.7.4 Principle of IF

Similar to Western Blot, IF relies on immunodetecting target proteins (either natural structure or tag) with specific antibodies. The difference lies in the conjugated molecule. IF requires antibodies that are linked to a fluorophore, which is excited by a certain wavelength and then emits light with another distinct wavelength. In contrast to Western Blot, IF staining visualizes where in the cell the antibody binds to. Therefore, antigens can be traced and localized to distinct compartments or organelles in the cell. Additionally, protein-protein interactions can be determined (co-staining) when the two target proteins are detected by antibodies with different fluorescent dyes. The cell structure in IF is being retained by fixing the cells with formaldehyde [29].

There are two different ways of detecting antigens in IF, direct or indirect. With direct IF staining, the antigen is detected by a primary antibody that is already conjugated to the fluorophore. When indirect staining is applied, an additional incubation step with a secondary antibody is performed. In this case, the secondary antibody is conjugated to the fluorophore and binds specifically to the Fc region of the primary antibody [29].

4 Results

4.1 Ponceau S stain for cell lysis and loading control

As a cell lysis and loading control, reversible Ponceau S staining was used in all experiments. Figure 8 shows a stained nitrocellulose membrane after 5 minutes of incubation with Ponceau S staining solution and rinsing the membrane twice with milliQ H_2O.

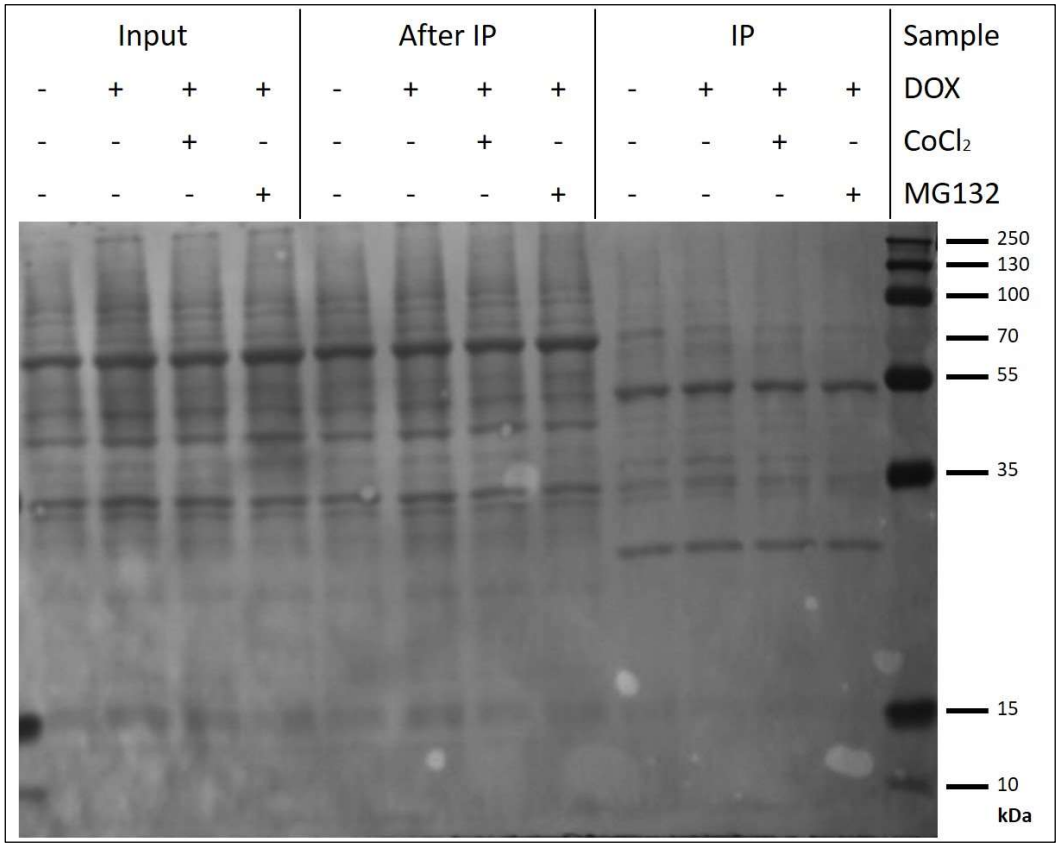


Figure 8: A nitrocellulose membrane is shown after transfer and before immunoblotting. The membrane was stained with Ponceau S solution. Input and After IP samples represent protein bands of a MDCK cell lysate. IP samples show less protein content. Two strong bands in the IP samples represent the heavy (below 55 kDa) and light chain (below 35 kDa) of the pull-down antibody (anti-myc tag).

The IP samples show a much higher purity compared to either Input or After IP, indicated by less intense bands. The only intense bands in the IP lanes result from the light and heavy chain of the antibody that was used for the pull-down procedure. Once an image

of the stained membrane was captured, the membrane was rinsed with milliQ H_2O once more. The remaining Ponceau S stain was automatically washed off the membrane during the blocking step.

4.2 Immunoprecipitation of PC1-p30

As a basis for the experiment, a sufficient pull-down of PC1-p30 had to be established. Therefore, MDCK-p30 cells were induced with DOX (50 ng/mL) and treated with $CoCl_2$ (200 µM) or MG132 (2 µM) as described in the Materials and Methods section. For the immunoprecipitation, a myc-tag specific antibody (9E10, mouse) and protein G Sepharose resin were used. Figure 9 demonstrates the outcome of a PC1-p30 pull-down on a Western Blot.

Figure 9: All three sample groups were loaded on an SDS-PAGE gel and transferred on a nitrocellulose membrane via wet transfer. The membrane was probed for PC1-p30 with the in-house PC1CT antibody in a 1:1,000 dilution (5 % dry milk in 1x TBST) and the respective secondary antibody (1:10,000). Based on horse radish peroxidase detection with ECL substrate solution, imaging was performed to visualize bands. The Input group (which represents cell lysate before IP) shows a relatively low amount of PC1-p30 on a slightly lower molecular weight than 35 kDa. Only slight bands were detected in the After IP samples and strong bands can be observed in the IP samples. Compared to untreated cells, an increased amount of p30 was detected by treatment with either $CoCl_2$ or MG132.

All three sample groups (Input, After IP and IP) were compared by Western Blot (probed with in-house PC1CT antibody). The detection of bands was performed by evenly distributing the ECL reagent on the membrane. A much stronger p30 signal was detected in the IP group compared to the cell lysate. Almost no PC1-p30 was measured in the

supernatant of IP (After IP). The chemiluminescence picture of the bands was overlayed with the marker picture in Adobe Photoshop CC to determine the band sizes. Visualizing bands on nitrocellulose membranes was done in the same fashion for all experiments.

4.3 Probing for HIF-1α and PHD3

As a positive control for the inhibition of PHDs and the resulting upregulation of HIF-1α under hypoxia (mimicked by $CoCl_2$-treatment), MDCK-p30 Input samples (equivalent to cell lysates) were run on a gel, transferred to a nitrocellulose membrane, and probed with a commercial rabbit anti-HIF-1α antibody (Figure 10).

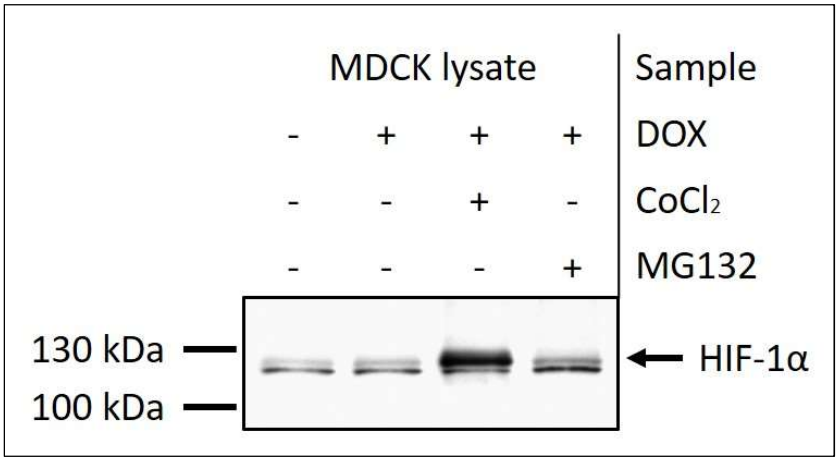


Figure 10: Result of the for HIF-1α probed nitrocellulose membrane. Treatment with $CoCl_2$ yielded a band in the molecular weight range of approximately 120 kDa. Only slight bands are visible in the remaining lanes. Unspecific bands appeared in a short distance under the HIF-1α positive bands. Probing with the primary antibody (rabbit anti-HIF-1α) was done in a 1:200 dilution. The blocking buffer was 1x TBST containing 5 % BSA for 1° and 2° antibody.

Probing and imaging the nitrocellulose membrane yielded a band that appears at a molecular weight of approximately 120 kDa. Only a low amount of HIF-1α was detectable in the -DOX, +DOX and +DOX/+MG132 lanes.

In a different experiment, also cell lysates (Input) were run on a gel and analyzed for the upregulation of PHD3. The lower part of the nitrocellulose membrane (~25 kDa area) was probed with the rabbit anti-PHD3 antibody (Figure 11).

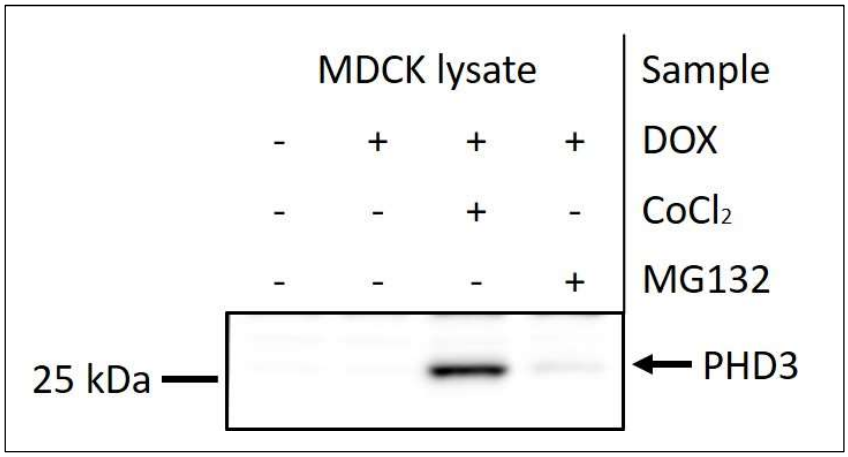


Figure 11: Probing the lower part of the nitrocellulose membrane of Input samples yielded a detectable band of approximately 25 kDa in the $CoCl_2$ treated lane. Almost no signal was detected in the remaining lanes. Probing was performed with the rabbit anti-PHD3 antibody, diluted 1:1,000 in 1x TBST containing 5% dry milk. The same buffer was used for secondary antibody incubation.

Analysis of the MDCK cell lysate sample group resulted into the detection of a visible band that has a molecular weight of >25 kDa in the $CoCl_2$ lane.

4.4 Detection of hydroxyproline

Besides an established immunoprecipitation and working positive controls (HIF-1α, PHD3) for mimicking hypoxic conditions, the detection of hydroxylated PC1-p30 depicts the major endeavor of this thesis. By using an antibody that reacts specifically towards hydroxyproline, a band was detected at the expected molecular weight of PC1-p30 (Figure 12). The band slightly underneath 35 kDa appears much stronger than in the other lanes. In the -DOX and +DOX lanes, only a slight signal was detected. A subtle band can be observed in the $CoCl_2$ lane.

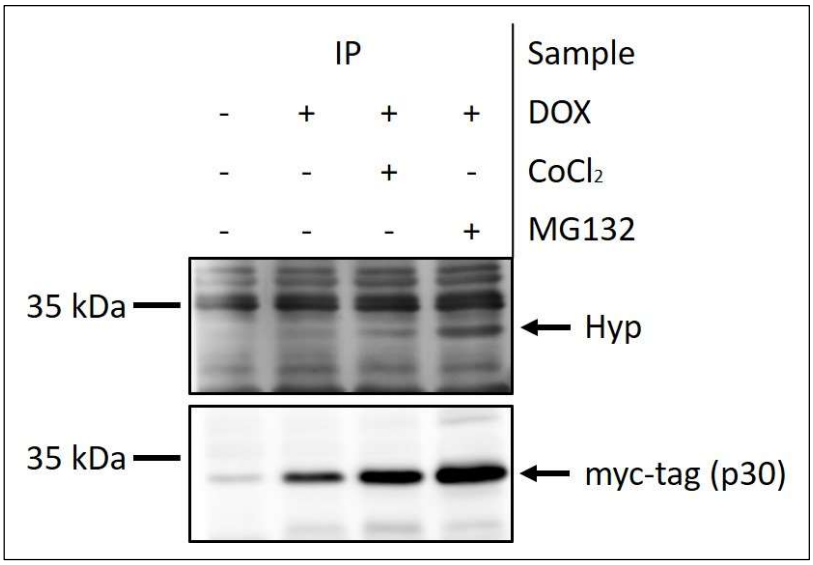


Figure 12: Probing the IP section of the nitrocellulose membrane resulted into the detection of high background and many unspecific bands, but also a relatively strong band at the proposed height of PC1-p30 (upper segment). The lower segment of the figure represents the same piece of the membrane, but re-robed with a myc-tag specific antibody. The initial probing with the hydroxyproline antibody was performed with a 1:500 dilution in 1x TBST that contains 5 % BSA. For the re-probing step, the myc-tag antibody was diluted 1:1,000 in 1x TBST with 5 % dry milk.

To confirm the position of the detected hydroxyproline band, the nitrocellulose membrane was washed with 1x TBST after ECL imaging and re-probed with the mouse anti-myc antibody (which was also used for pull-down). Re-probing included blocking, primary and secondary antibody incubation as described in chapter 3.6. The re-probing resulted in detectable PC1-p30 at the same position as the hydroxyproline band.

4.5 Detection of ubiquitin

4.5.1 Comparison of ubiquitin antibodies

To identify a suitable antibody for the probing of IP samples to detect ubiquitinated PC1-p30, MDCK cell lysates were run on a gel and probed with 2 different anti-ubiquitin antibodies (Table 4). Before the blocking step, the nitrocellulose membranes were boiled for 10 minutes in milliQ H_2O.

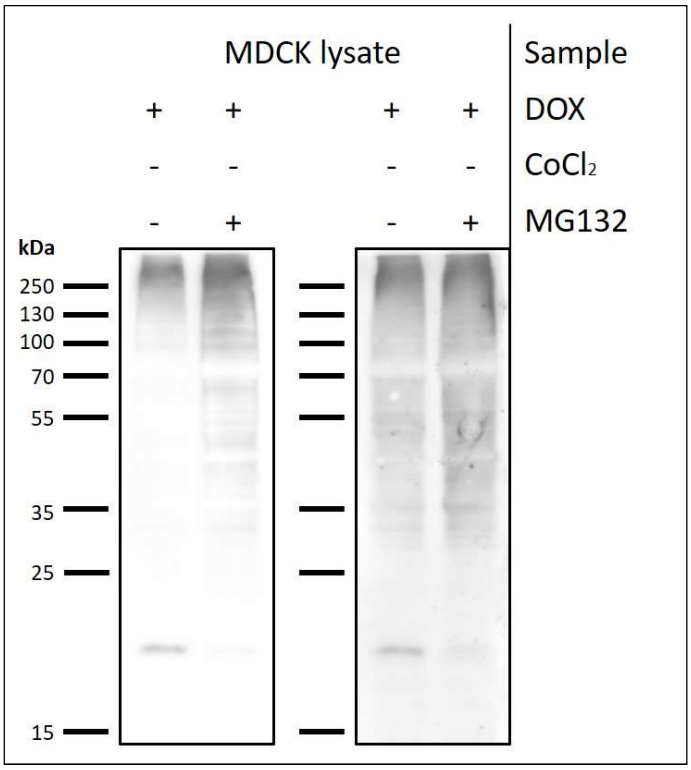


Figure 13: MDCK cell lysates were tested for antibody specificity via Western Blot. The left nitrocellulose membrane was probed with the rabbit anti-ubiquitin antibody and the right membrane with the mouse anti-ubiquitin antibody (Table 4) in a 1:500 dilution. Blocking and immunoblotting was performed in 1x TBST containing 5 % BSA. A more distinct smear resulted from probing with the rabbit antibody (left membrane).

Figure 13 shows the outcome of the antibody comparison. Two of the four standard samples of MDCK cell lysates were used (only DOX induced and DOX + MG132 treated cells) to test the antibodies for specificity. Probing with the mouse anti-ubiquitin antibody resulted in a more extensive smear in the left lane, which represents the cell lysate of MDCK cells that were solely treated with DOX. A clearer difference can be observed on the membrane that was probed with the rabbit antibody.

4.5.2 Mono- and polyubiquitinated PC1-p30

Based on the result of the previous chapter (4.5.1), the rabbit anti-ubiquitin primary antibody was used for the detection of ubiquitination of PC1-p30. Therefore, IP samples were loaded on a gel and transferred on a nitrocellulose membrane. Again, the membrane was boiled in milliQ H_2O for 10 minutes and then immunoblotted with the appropriate 1° and 2° antibody. An image was taken after adding the ECL reagent (Figure 14).

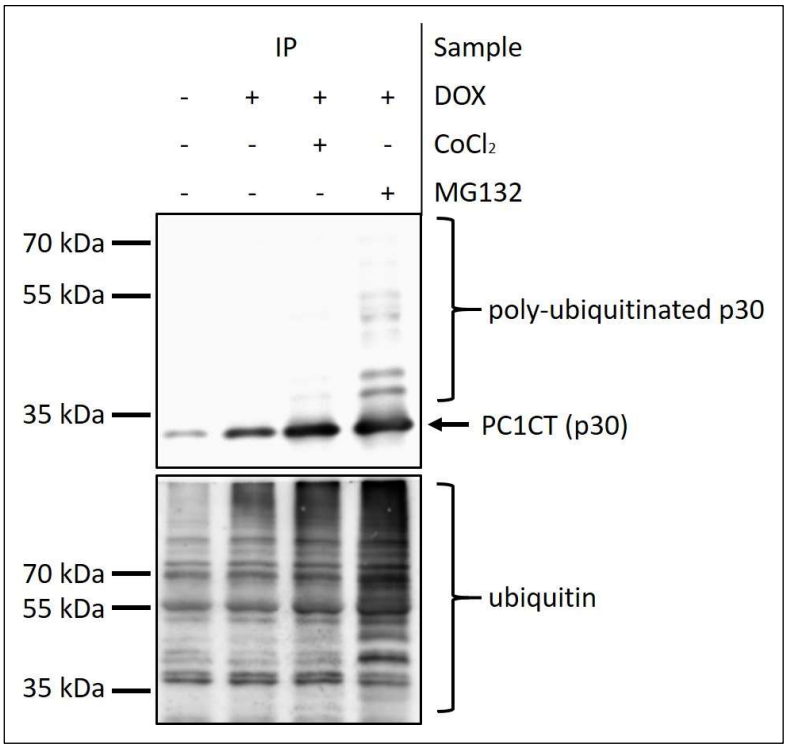


Figure 14: IP samples were loaded on different membranes and probed with two different antibodies. The upper membrane shows the result of immunoblotting with the in-house rabbit anti-PC1CT antibody. Blotting was performed in 5 % dry milk 1x TBST solution with the antibody in a 1:1,000 dilution ratio. Distinct bands were detected above the p30 band in the molecular weight range of 35 to >55 kDa. The lower membrane was probed 1:500 with the rabbit anti-ubiquitin antibody in 5 % BSA 1x TBST solution. The strongest smear appeared when cells were treated with MG132, a high background can be observed in the other sample lanes.

A second membrane with IP samples was probed with the PC1CT specific antibody to detect p30 and thus, to detect any band shifts that could represent ubiquitinated versions of p30. In addition to the expected PC1-p30 band at a molecular weight of ~30 kDa, more bands appeared in the range between 35 and >55 kDa. Even though a strong smear emerged when IP samples were probed for ubiquitin, distinct bands appeared between 35 and 55 kDa compared to the samples that were either untreated or only treated with $CoCl_2$.

4.6 Indirect IF staining of MDCK-p30 cells

An EGFP encoding plasmid was used to confirm whether the used transfection reagent (TurboFect™) with the applied transfection ratio (reagent to DNA) was appropriate for

MDCK-cell transfection and whether the transfection efficiency was sufficient. Figure 15 shows cells in a 60X magnification that express EGFP.

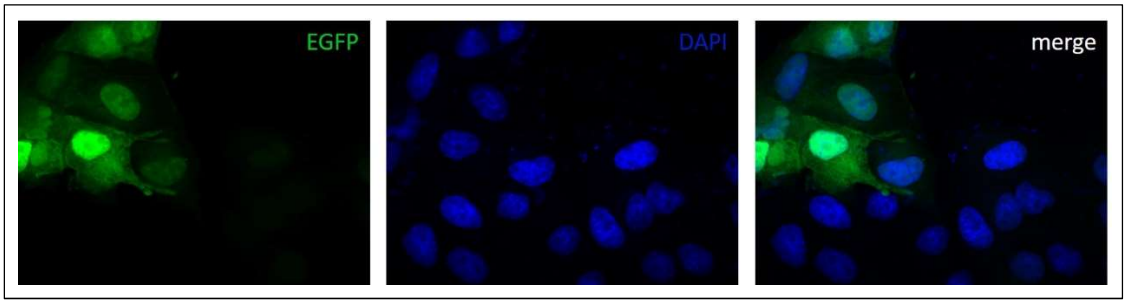


Figure 15: MDCK-p30 cells were transiently transfected with an EGFP encoding plasmid. This served as a positive control for successful transfection. The cells were observed under the inverted fluorescence microscope with 60X magnification. The FITC channel was used for excitation, which resulted in green fluorescence of EGFP positive cells. The DAPI channel was used for imaging nuclei. The two resulting pictures were merged via an RGB overlay in Adobe Photoshop CC.

To test for the interaction of PHD3 with PC1-p30, MDCK-p30 cells were transiently transfected with a PHD3 encoding plasmid (EGFP tagged) and induced with DOX (50 ng/mL) for the expression of p30. Myc-tagged p30 was stained with a mouse anti-myc primary and with a donkey anti-mouse secondary antibody (DyLight 594 fluorescent dye conjugated). Since PHD3 was already tagged with EGFP, no second staining was required. Figure 16 shows the resulting fluorescence microscopy images that were taken in 100X magnification.

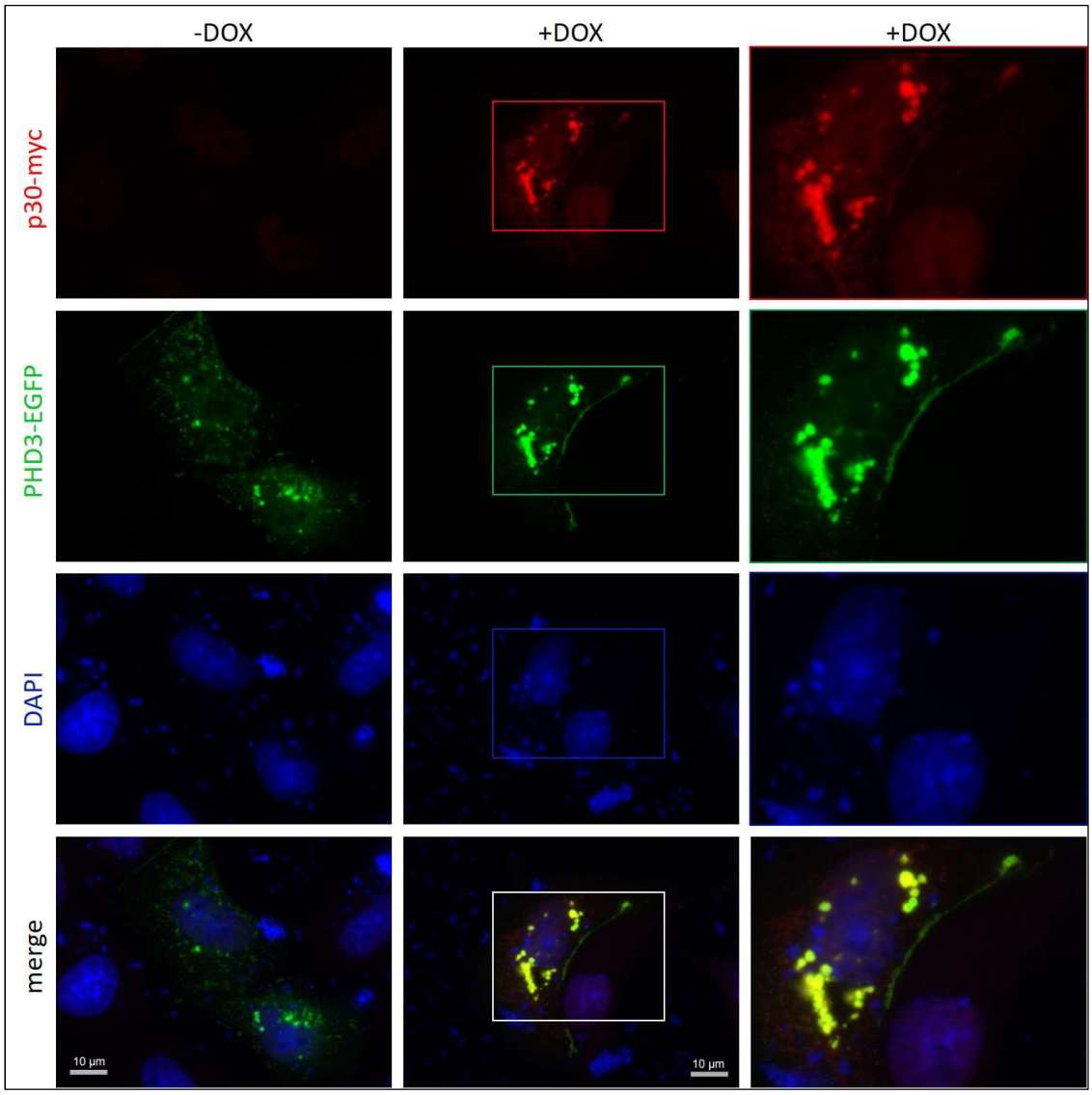


Figure 16: MDCK-p30 cells were either DOX induced or were left untreated. After reaching sub-confluency, the cells were transiently transfected with a PHD3-EGFP encoding plasmid. After cell fixation, myc-tagged p30 was stained with the mouse anti-myc antibody in a 1:8,000 dilution ratio. The secondary DyLight conjugated antibody was used in a 1:1,000 dilution. The p30 antibody staining was visualized by excitation in the Texas Red channel under the inverted fluorescence microscope. PHD3-EGFP and nuclei were visualized with the FITC and DAPI channel, respectively. The pictures were captured with 100X magnification. All three images were merged via RGB overlay in Photoshop. The right column represents zoomed in panels of the marked areas in the middle column. DOX induced cells showed p30 and PHD3 localization around the nucleus on the same positions.

Untreated MDCK-p30 cells that were stained for p30-myc showed no red fluorescing spots in virtually all PHD3 positive cells (cells with green fluorescence spots). DOX induction

caused cells that were PHD3 positive to accumulate p30 that is visible by red fluorescing spots. Overlaying of the red, green and blue channel resulted into yellow spots, indicating that p30-myc and PHD3-EGFP occurred at the same location in all cells that transiently expressed PHD3-EGFP.

5 Discussion

5.1 PC1-p30 pull-down efficiency

Based on the result of the PC1-p30 immunoprecipitation and subsequent analysis by Western Blot (Figure 9), an enrichment of the stably expressed p30 protein fragment was achieved. One indication of a successful pull-down is that the bands in the After IP sample lanes almost disappeared (only a low signal can be observed). The After IP samples represent the supernatant of cell lysate after the samples were incubated with the antibody and the protein G resin. One essential part of an efficient pull-down represents optimal binding efficiency between the anti-myc antibody and the myc-tagged protein fragment as well as between the Fc region of the IP antibody and the protein G beads. The results show that the binding efficiency was therefore enough to remove a substantial amount of PC1-p30 from the crude MDCK cell lysate.

The second critical part of an immunoprecipitation is the amount of target protein that remains bound to the resin while the washing steps are being performed. Comparing the amount of PC1-p30 between the Input and IP samples shows that a purification of PC1-p30 of about 10-fold was achieved (based on an estimation of the band intensities of the MG132 lanes). To estimate an ideal amplification factor of the IP, the following rough calculation will be used:

- 50 µL of Input sample was removed from 1000 µL lysate
- About 5 µL of that sample was loaded on an SDS-PAGE gel (ignoring the added amount of sample buffer)
- -> This represents approximately 0.5 % of the entire lysate
- 10 µL of about 50 µL IP sample was used per lane
- -> This represents approximately 20 % of the entire sample
- --> Therefore, the purification of PC1-p30 in an ideal IP would be 40-fold (0.5 % to 20 % of analyzed sample)

The discrepancy between the reached and ideal concentration (approximately 10-fold versus 40-fold) can have several reasons. One reason for the loss of the target protein could be protein loss during the washing steps of the protein G resin. Extensive washing could have resuspended a portion of PC1-p30 in the washing buffer. Since three washing

steps were performed, a high amount of protein could have been lost by this. This could be prevented by optimizing the components of the washing buffer or by conducting less washing steps. However, a loss via washing can only be assumed. To determine how much and if PC1-p30 was washed off the resin, it would have been required to collect the supernatant after each washing step and subject it to a Western Blot analysis.

Additionally, it is essential that almost the entire protein is detached from the beads by the boiling step in SDS sample buffer. After the washing steps, the buffer was removed by a syringe needle and sample buffer was added before the samples were boiled for 5 minutes. If some of the protein remains on the beads after boiling, then a longer boiling time should be considered for a higher PC1-p30 signal in the IP samples.

Another important factor is the stability of the target protein. The amount of immunoprecipitated PC1-p30 could be significantly reduced if the protein becomes degraded by proteases in the cell lysate. Since protease inhibitors were added (protease inhibitor cocktail and the serine protease inhibitor PMSF) to the lysis buffer even before the cells were lysed, most of the proteases were inhibited and p30 was therefore stabilized in the samples. In addition, all buffers that were used for the IP contained 5 mM EDTA. EDTA is known to inhibit metalloproteases. Therefore, it served as an additional factor to stabilize p30 [30, 31].

5.2 Stabilization of p30, HIF-1α and PHD3

For a stabilization of PC1-p30, MDCK cells that were induced by doxycycline were treated with the hypoxia-mimicking substance $CoCl_2$ and with the proteasome inhibitor MG132. In comparison to untreated cells, a significant upregulation was achieved. MG132 treatment had a higher impact on the prevention of p30 degradation and therefore, yielded a higher amount of stabilized p30 (Figure 9). Also, the $CoCl_2$ treatment resulted into a higher concentration of p30 compared to -DOX and +DOX treated cells, verifying the stabilization of p30 under hypoxic conditions.

HIF-1α was significantly upregulated by treatment with $CoCl_2$ (Figure 10). Even though the detected band of approximately 120 kDa on the for HIF-1α probed membrane appears to be larger than the expected molecular weight of HIF-1α, the results still demonstrate that HIF-1α is stabilized under hypoxia in MDCK cells. Phosphorylation causes the increase of the molecular weight of HIF-1α and therefore the shift on a Western Blot, as

described in detail in a previous study. Also, according to the manufacturer's website of the antibody, a band of 120 kDa can be considered as HIF-1α [32, 33].

The theoretical molecular weight of PHD3 according to the antibody manufacturer website is expected to be 27 kDa. This is in accordance with the detected band that appears when MDCK cells were treated with $CoCl_2$ (Figure 11). Therefore, a significant stabilization of the prolyl hydroxylase PHD3 was measured and confirms previous findings in different studies. Thus, it also serves as another piece of evidence around the mechanisms that are related to PC1-p30 degradation under normoxia. Despite the high concentration of PHD3, p30 remains upregulated due to the lack of oxygen and thus, the missing hydroxylation by PHD3 [19, 34].

5.3 Proline hydroxylation of PC1-p30

To detect whether PC1-p30 is being hydroxylated by prolyl hydroxylases, a relatively efficient pull-down of p30 and a high concentration of p30 due to its upregulation by MG132 are required. These two goals were achieved, even though the pull-down efficiency could have been higher. Reasons of a decreased IP efficiency are explained in chapter 5.1.

Another important factor of this kind of detection is the specificity of the antibody. Considering the low specificity of the hydroxyproline antibody used in the experiments, the obtained result (Figure 12) displays a band that is indicative of hydroxylation of PC1-p30. Despite the high background, it was feasible to detect a band at the molecular weight of p30. This was verified by re-probing the same membrane with a highly specific antibody that is reactive towards the myc-tag of the stably expressed PC1-p30 fragment in MDCK cells. A comparison between the two bands shows that they emerged at the same position in the membrane.

A band was also detected in the $CoCl_2$ lane. Under hypoxic conditions, PHDs are normally inhibited and therefore no hydroxylation occurs due to the lack of molecular oxygen. The appearance of the band could have several reasons. First, it has already been shown that PHDs can retain their functionality under hypoxic conditions. Second, PHD was shown to be abundant in hypoxic conditions (Figure 11). Therefore, only a portion of hydroxylated PC1-p30 could already yield a band [35].

The hydroxyproline antibody might not be specific enough to distinguish between unmodified proline residues and hydroxylated proline residues, or different hydroxylated versions of amino acids. Antigens are required to have a minimum molecular weight of about 8 to 10 kDa. When haptens are used for creating an immune response, it is also possible to produce antibodies that react toward smaller compounds than 8 kDa. In this case, the small substance is linked to a large carrier protein. The used antibody (obtained from Abcam) was produced by creating an immunogenic reaction towards a synthetic hydroxyproline peptide that is conjugated to bovine serum albumin (BSA). Therefore, detection of hydroxyproline on proteins other than BSA might not be the most ideal way for yielding a very high signal [36].

A similar experiment was conducted earlier, when HEK cells that overexpressed PC1 and PC2 were analyzed for proline hydroxylation on the C-terminal fragment of PC1. The findings here (Figure 12) and in the previous study suggest that proline hydroxylation occurs at the C-terminal fragment of PC1 (p30) and that PHDs catalyze this reaction under normoxia [9].

5.4 Ubiquitination of PC1-p30

As a preliminary experiment, two different ubiquitin antibodies were tested for their specificity. Untreated cells were compared with MG132 treated cells (both groups were DOX induced) and the latter ones are expected to show an intense smear when probed for ubiquitin due to the inhibition of the proteasome. Comparison between the two lanes of untreated cells (Figure 13, left lanes in both panels) was taken into consideration for the selection of the antibody for the main experiment. Therefore, the rabbit anti-ubiquitin antibody was used for the detection of ubiquitinated PC1-p30.

Probing of IP samples with the selected antibody resulted in a strong smear in the MG132 lane (Figure 14). There, at least two distinct bands appeared in a molecular weight range of 35 to 55 kDa. These two bands are not visible in the other lanes despite the high background and smear that emerged. When probed with the PC1CT antibody, bands around the same molecular weight appeared. Also, additional bands with a higher molecular weight are visible in the MG132 lane.

Based on the molecular weight of single ubiquitin molecules (approximately 8 to 9 kDa), the detected bands on the membrane probed for ubiquitin and the band shifts of p30

on the membrane probed for PC1CT suggest that ubiquitinated versions of PC1-p30 appear in MDCK cells and that inhibition of the proteasome by MG132 prevents its degradation. pVHL as part of the E3 ubiquitin ligase complex attaches ubiquitin covalently to p30 and this leads to its degradation. The appearance of multiple bands indicates that different lengths of ubiquitin chains are attached to p30. A monoubiquitinated version of p30 seems also be part of the modified p30 pool and would mean that this version is marked for relocation within the cell instead for its degradation. Further experiments might reveal a more detailed insight into this [13, 16].

5.5 PHD3 co-localizes with PC1-p30

As an additional experiment to determine which isoform of the 3 proline hydroxylases interacts with p30 and consequently hydroxylases its proline residues, MDCK-p30 cells were transiently transfected with a PHD3 encoding plasmid. Since the expressed PHD3 was already tagged with EGFP, only p30-myc indirect staining was required. Based on the obtained result (Figure 16), co-localization of transiently expressed PHD3 and stably expressed p30 occurred (indicated by yellow spots in overlaid image). Since this interaction occurs under forced conditions, a similar experiment in which endogenous PHD3 and p30 are being stained would be of interest. However, the outcome of this experiment indicates that PHD3 and p30 physically interact. The hypothesized molecular mechanism (Figure 4) is supported by this. Further research will elucidate which of the PHDs is mainly responsible for p30 degradation.

5.6 Influence factors on results and troubleshooting

Throughout the research project, alterations in terms of the PC1-p30 expression levels were observed. One challenge was to yield an upregulation of p30 that displays a sufficient amount for maximum proline hydroxylation of p30 and thus, detecting hydroxyproline with the available antibody (general issues with the used hyp-antibody are already explained in chapter 5.3). Since the pull-down procedure was kept consistent, the most varying parameters presumably occurred on the side of cultivating the MDCK cells.

Mammalian cells in cell culture require a high amount of energy. MDCK cells undergo mitosis every 24 hours (until full confluency). Therefore, they need energy for the

synthesis of large biomolecules such as DNA and proteins. This is why L-glutamine is one essential part of the medium. The cells use L-glutamine as a precursor for the production of other amino acids and also utilize the nitrogen found in the L-glutamine. The challenge with L-glutamine in cell culture is the low stability. When dissolved in liquid medium, it breaks down into ammonium and pyroglutamate within a relatively short period of time. Since L-glutamine is being readily incorporated into the metabolism in the cell but becomes less concentrated in the medium over time, big differences in terms of protein expression occur compared between cells that are fed with fresh and older medium. Prolyl hydroxylation of p30 and other proteins strongly depend on α-ketoglutarate, which is one requirement for the enzymatic function of prolyl hydroxylases. Depletion of L-glutamine could potentially influence the outcome of the experiment in a negative manner since the amount of available α-ketoglutarate in the TCA cycle might strongly depend on the amount of L-glutamine. As a solution, low amounts of full-medium were made to ensure that the medium was used up within a short period of time. The difference of L-glutamine concentration between experiments was minimized, but not entirely prevented [37].

The second crucial component of the cell medium was fetal bovine serum (FBS). FBS is used as a source of different essential factors for cell growth. It contains different sized proteins and peptides that are responsible for stimulating growth and other vital functions to maintain mammalian cells. Additional components are vitamins, hormones and trace elements, which give FBS a high complexity. However, concentrations of the serum contents vary from batch to batch and might therefore prevent the cells from growing under consistent conditions. Since FBS is obtained from fetal bovines, there is a high risk of contamination of the end-product by mycoplasma, viruses, and more. These contaminations pose a potential risk when it comes to undesired changes of results [38, 39].

5.7 Conclusions

Taken together, the obtained results lead to several conclusions and yield an improved understanding of how the 30 kDa fragment of PC1 is modified post-translationally after its cleavage off the C terminus. In the same manner as PHDs target HIF-1α, prolyl hydroxylases recognize proline residues on PC1-p30 and hydroxylate them under normoxic conditions. Based on the finding that PHD3 shows the most targets apart from HIFs [18] and the detected co-localization via IF, it is suggested that PHD3 hydroxylates p30. Whether PHD3 alone promotes the degradation of p30 and whether PHD1 and PHD2 also participate in that

process, has to be tested in future experiments. Despite its downregulation in normoxia, enough PHD3 is available to catalyze the reaction. This means that hypoxia hinders PHDs to modify proline residues at least to a certain extent. This protection causes p30 to be stabilized.

Furthermore, the results indicate that due to proline hydroxylation, pVHL targets p30 and causes it to be ultimately ubiquitinated. Different shifts in the molecular weight of p30 suggest that mono- and polyubiquitinated versions of p30 exist. The proteasome recognizes ubiquitinated p30 and therefore degrades it. Whether solely polyubiquitination causes degradation of p30, remains unsolved. Further experiments might give clearer insight into this.

6 Outlook

6.1 Further research questions

Based on the gained knowledge, subsequent experiments that involve the investigation of PC1-p30 might be performed. It would be of interest to determine how many and which proline residues are hydroxylated under normal oxygen conditions. This question could be resolved by introducing different mutations into the DNA sequence of PC1-p30. Mutation of the proline residues could prevent PHDs to mark p30 for proteasomal degradation and this could cause p30 to be upregulated in normoxic conditions without treatment of either $CoCl_2$ or MG132.

Another interesting question is which of the three proline hydroxylase isoforms are responsible for proline hydroxylation on p30. One experiment could contain MDCK-p30 cells that are transfected with either a PHD1 or PHD2 encoding plasmid. Indirect immunofluorescence staining of myc-tagged p30 and either PHD1 or PHD2 could give more insight into co-localization of these isoforms with p30. Another approach would be to silence the different PHD genes and to see which gene inactivation results into p30 stabilization.

Aside from the interaction with PHDs and pVHL, it would be of interest to identify other proteins or targets of PC1-p30. Since it has been demonstrated that p62 forms aggregates with PHD3 and therefore causes its degradation [19], the question of whether p62 affects degradation of p30 in the same manner emerges. One way of resolving that question would be performing a co-immunoprecipitation of p62 with p30 with a subsequent Western Blot. A p62/p30 interaction could be shown by detecting p62 in the pull-down samples. A second way is to conduct an IF experiment. By transfecting MDCK-p30 cells with a p62-encoding plasmid and co-staining p30 and p62, co-localization could be demonstrated. With these two methods, further molecular targets of p30 might be detected.

6.2 Relevance of results

It is important to describe the molecular mechanisms that PC1-p30 is involved in and more importantly, how PC1-p30 is stabilized in ADPKD. Based on the research of this thesis and by conducting further experiments, a more detailed picture of PC1-p30 stabilization and degradation will be obtained. A potential therapy might be to reverse the aberrant

upregulation of p30 or preventing it from initiating pathways that are normally inactive in healthy tissue that exhibits normoxic conditions. These and other approaches could eventually lead to decelerate the formation of renal cysts or even to halt the progression of ADPKD.

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List of Figures

List of Tables

List of Abbreviations

ADPKD	Autosomal Dominant Polycystic Kidney Disease
PKD	Polycystic Kidney Disease
PC1	polycystin-1
PC2	polycystin-2
GPCR	G-protein-coupled receptor
GPS	G protein-coupled receptor proteolytic site
STAT	signal transducer and activator of transcription
mTOR	mammalian target of rapamycin
cAMP	cyclic adenosine monophosphate
TRP	transient receptor potential
HIF	hypoxia-inducible factor
IGF-1	insulin like growth factor 1
$TGF\beta_3$	transforming growth factor beta 3
pVHL	von Hippel-Lindau protein
ODDD	oxygen-dependent degradation domain
SNARE	soluble N-ethylmaleimide-sensitive factor activating protein receptor
EGLN	egg-laying nine
PHD	prolyl hydroxylase domain
hyp	hydroxyproline
p30 / PC1-p30	30 kDa fragment of the C terminal PC1 tail
p62	sequestome 1
MDCK	Madin-Darby Canine Kidney
DOX	doxycycline

Tet	tetracycline
rtTA	reverse tetracycline-controlled transactivator
TRE	Tet response element
MEM	Minimal Essential Medium
Pen/Strep	Penicillin/Streptomycin
FBS	fetal bovine serum
DPBS	Dulbecco’s Phosphate Buffer Saline
IP	immunoprecipitation
IF	immunofluorescence
PI	protease inhibitor