Cysteine S-glutathionylation Promotes Stability and Activation of the Hippo Downstream Efector Transcriptional Co-activator with PDZ-binding Motif (TAZ)

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ABSTRACT

TAZ (transcriptional co-activator with PDZ-binding motif) and YAP (Yes-associated protein) are critical transcriptional co-activators downstream of the hippo pathway involved in the regulation of organ size, tissue regeneration, proliferation and apoptosis. Recent studies suggested common and distinct functions of TAZ and YAP and their diverse impact under several pathological conditions. Here, we report differential regulation of TAZ and YAP in response to oxidative stress. Hydrogen peroxide (H₂O₂) exposure leads to increased stability and activation of TAZ but not of YAP. H₂O₂ induces reversible S-glutathionylation at conserved cysteine residues within TAZ. We further demonstrate that TAZ S-glutathionylation is critical for reactive oxygen species (ROS) mediated TAZ-dependent TEAD transactivation. Lysophosphatidic acid (LPA), a physiological activator of YAP and TAZ, induces ROS elevation and subsequently TAZ S-glutathionylation which promotes TAZ mediated target gene expression. TAZ expression is essential for renal homeostasis in mice and we identify basal TAZ S-glutathionylation in murine kidney lysates, which is elevated during ischemia/reperfusion (I/R) injury in vivo. This induced nuclear localization of TAZ and increased expression of connective tissue growth factor (CTGF). These results describe a novel mechanism by which ROS sustains total cellular levels of TAZ. This preferential regulation suggests TAZ to be a redox sensor of the hippo pathway.

Introduction:

The hippo signaling pathway has been under intense scrutiny due to its conserved ability to regulate organ size and cell proliferation (1). The canonical hippo pathway revolves around the
kinase cascade of Mst1/2, Lats1/2 and their coactivators Sav1 and Mob which leads to phosphorylation of transcriptional co-activator with a PDZ-binding motif (TAZ) and Yes Associated protein (YAP). Both TAZ and YAP are transcriptional co-activators and downstream effectors of the hippo pathway. Stability of these proteins depends on Lats1/2 phosphorylation of TAZ at S89 (S127 in YAP) leading to 14-3-3 binding and cytoplasmic retention. Furthermore, phosphorylation of Ser 314 of TAZ and Ser 381 of YAP primes them for subsequent casein kinase 1 mediated ubiquitination and proteasomal degradation (2). In the absence of Lats1/2 activity both TAZ and YAP function as transcriptional co-activators and initiate gene expression promoting proliferation and cell survival by binding to transcription factors such as TEAD or RUNX2 (3). Thereby TAZ and YAP modulate the expression of a variety of target genes (2,4). Recently, it has been shown that G protein coupled receptor (GPCR) ligands, lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) can lead to dephosphorylation and promote nuclear localization of YAP and TAZ (5). Both TAZ and YAP have been shown to be upregulated in various cancers and involved in epithelial-mesenchymal transition (EMT) leading to metastasis (6,7).

Despite overlapping functions of both TAZ and YAP, recent evidence suggests that they have distinct physiological roles. Knockout of Yap in mice leads to embryonic lethality (8), whereas Taz knockout mice are viable but develop cystic kidney disease and lung emphysema (9,10). On the protein level TAZ and YAP display approximately 50% sequence similarity including the conserved phosphorylation residues. They have many common, but also few distinct protein-protein interaction partners that are essential for their stability and function (11). Interestingly, three conserved cysteine residues are only present in TAZ highlighting its potential redox regulation.

The human proteome contains approximately 200,000 cysteine residues making them one of the least commonly used amino acids (12). This implies evolutionary importance of cysteine content, which correlates with the degree of organism’s biological complexity (13). Homeostasis between cellular oxidation and reduction reactions plays a critical role in signal transduction. Reactive oxygen species (ROS) are in general regarded as cytotoxic, mutagenic and inducers of oxidative stress. However, several studies implicate the role or ROS in stimulation or inhibition of cell proliferation, apoptosis and cell senescence (14). Post-translational modifications of cysteines can directly influence these processes. S-glutathionylation is a well characterized protein modification: An oxidized glutathione (GSSH) forms a disulfide linkage with the reactive thiol group of cysteine resulting in protein-glutathione complex. Mild oxidative stress upon elevated superoxide (O$_2^-$) or hydrogen peroxide (H$_2$O$_2$) can induce S-glutathionylation at cysteine residues, which can lead to alteration of protein function (15-17).

In this study, we demonstrate that TAZ is s-glutathionylated at residue C261 and C315 and C358 in vitro. Circumstantial s-glutathionylation of TAZ may lead to activation of its transcriptional activity and could function as a redox sensor of the hippo pathway. We propose a
previously unidentified signaling pathway wherein ROS can directly modulate TAZ activity.

**Experimental Procedures**

**Cell Culture**

HEK 293T cells were cultured in Dulbecco’s Minimum Essential Media (GIBCO) supplemented with 10% fetal calf serum (FCS). Transient transfection was performed using Lipofectamine (Invitrogen) or the calcium phosphate method. Cells were treated with indicated concentrations of hydrogen peroxide (Sigma-Aldrich), Lysophosphatidic acid (TOCRIS) for 1 or 6 hours and harvested for protein or RNA preparations. Cells were incubated with antioxidants reduced glutathione (Sigma-Aldrich), N-acetylcysteine (Sigma-Aldrich) or the NOX inhibitors Apocynin (Sigma-Aldrich) and diphenyleneiodonium (Sigma-Aldrich) as indicated. NIH3T3 TAZ flp-in stable cell lines were cultured in Dulbecco’s Minimum Essential Media (GIBCO) supplemented with 10% fetal calf serum (FCS) in the presence of 200 µg/ml hygromycin (Invitrogen).

**Plasmids and Antibodies**

Murine TAZ (NP_598545.2) and YAP (NP_033560.1) cDNA was provided by M. Yaffe (Massachusetts Institute of Technology, Boston, MA). TAZ cysteine mutants were generated using site directed mutagenesis to mutate the cysteine residues to alanine in positions 261, 315, and 358 in murine FLAG.TAZ pcDNA6 (Invitrogen). The mutant constructs were verified by sequencing and restriction digestion. The FLAG.EPS plasmid was used as control protein. Antibodies were purchased from Sigma-Aldrich (anti-FLAG/M2 (cat.F3165), Cell Signaling Technology (anti-YAP/TAZ (cat.8418), anti TAZ (cat.4883), anti YAP (cat.4912), anti-phospho YAP/TAZ (cat.13008) anti β tubulin (cat.2128) and anti–β actin (cat.3700), Virogen (anti-GSH cat.101-A-100), Millipore (anti-V5 cat.AB3792)

**Reverse Transcription and Quantitative Real Time PCR (qPCR) Analysis**

One microgram of total RNA from HEK 293T cells was used to generate cDNA templates for reverse transcriptase PCR. The first strand cDNA synthesis was performed using a hexamer (Applied Biosystems) and Superscript II reverse transcriptase (Applied Biosystems). The first strand cDNA products were further diluted 1:1 and used as qPCR templates. The SYBR Green-based qPCR analysis was carried out in the real time PCR system (7900HT; Applied Biosystems). Duplicate reactions were carried out for each sample. All samples were normalized by the expression level of the housekeeping gene β-actin. Primer sequences used in the qPCR assays were:

- hCYR61fp 5´ACCTCGCATCTTATACAACC, rp 5´TTCTTTCACAAGGCAGCCTC
- 5´GAACCTCGAGCAAGAGATGG 3´ rp5´AGGTCCTACTGAGATGGATGG 3´
- 5´GCCGTTGGAGATGACCTT-3´ rp5´AGGTCCTACTGAGATGGATGG 3´
- 5´TGCTTGTGTTCTGAGATCGGTGAT 3´ and mYAP fp 5´

TaqMan assays (Applied Biosystems) were used to evaluate CTGF (Hs00170014_m1) and beta actin (4326315E) levels.
Immunoprecipitation

HEK 293T cells were transiently transfected using the calcium phosphate method, and the total amount of DNA was always adjusted with empty pcDNA6 (Invitrogen). For TAZ and NOX4 expression studies, plasmids were co-transfected in the ratio 5:1. The following day, cells were harvested with ice-cold PBS. The harvested cells were lysed in a 1% Triton X-100 buffer (1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM NaF, 15 mM Na3PO4, 2 mM Na3VO4, 25 mM NEM, and complete protease inhibitors [PIM complete; Roche]) for 15 min on ice. After centrifugation at 15,000 g for 15 min at 4°C and ultracentrifugation at 100,000 g for 30 min at 4°C, the supernatant was incubated at 4°C for 1 h with the anti-FLAG (M2) covalently coupled to agarose beads (Sigma-Aldrich) or with 1 μg of the appropriate first antibody and 30 μl protein G sepharose beads (GE Healthcare). Before the addition of antibodies, a small aliquot of each supernatant was preserved and diluted with 2× SDS-PAGE sample buffer for later Western blot analysis (lysate). The beads were washed extensively with lysis buffer, and bound proteins were resolved by SDS-PAGE, blotted on to polyvinylidene fluoride membranes. Visualization was performed by infra-red detection using donkey anti mouse IRDye 680 or donkey anti rabbit IRDye 800 in the Odyssey Infrared Imaging System (LI-COR Biosciences). Quantification was performed with the Licor Image Studio™ analysis software provided in a linear dynamic range.

Luciferase assays

The luciferase reporter plasmid (pGBD-Hyg-Luc) was transfected together with an activator plasmid (pGal4-TEAD), pGL4.74 (Promega) for normalization, and the indicated expression plasmids (TAZ, YAP, NOX4 and control empty pcDNA6) into HEK 293T cells in a 96-well format using Lipofectamine 2000 (Invitrogen) as a transfection reagent. The total amount of DNA was always adjusted with empty pcDNA6. Renilla luciferase and firefly luciferase activities were measured by using a reporter assay system (Dual Luciferase; Promega) in a luminometer (Mithras LB 940; Berthold) at 6 or 24 h post treatment. Transfections and measurements were performed in triplicates for each single experiment, and each experiment was repeated at least three times. The remaining lysates were pooled in 2X laemmli and TAZ expression was determined by immunoblotting. Error bars shown in the figures represent SEM. P-values were calculated using two-tailed unpaired Student’s t-test.

Measurement of Intracellular ROS

2 x 10^4 HEK 293T cells were seeded in optical bottomed 96 well plates (Nunc) in complete DMEM. 16 hours later cells were challenged with H2O2 or LPA for 1 hour. Cells were loaded either with 10 μM H2DCFDA (Ex.488nm/Em.520nm; Sigma-Aldrich) or 10 μM DHE (Ex.510nm/Em.595nm; Sigma-Aldrich) for 30 minutes at 37°C. Cells were gently rinsed in HBSS once, and fluorescence intensity was immediately quantified in multimode plate reader (Perkin Elmer).
Ischemia reperfusion injury

We used a rodent warm ischemia reperfusion model. The surgical procedure was approved by the “Landesamt für Natur, Umwelt und Verbraucherschutz NRW” (LANUV 84-02.04.2013.A158). Male 10 to 12 weeks old C57BL/6J mice were anaesthetized with a Ketavet / Xylazin injection i.p. The following steps were conducted on a heat pad to preserve the physiological body temperature of the experimental animals. After cessation of reflexes a median laparotomy was carried out. The right kidney pedicle was mobilized and clamped for 30 minutes with an atraumatic micro-vascular clamp. Successful ischemia was confirmed by discoloration of the kidney. During ischemia the abdomen was covered with gauze soaked with normal saline. At the end of ischemia, the clamp was released and the kidney was controlled for visual signs of reperfusion. 5 µM of Hoechst 33342 and dhiydroethidine (DHE) was injected via retro orbital injection. Five minutes after reperfusion, two-photon imaging of the intact kidney was performed in Leica (TCS SP8 MP-OPO) in 20X water immersion objective as previously described (18). Animals were sacrificed by cervical dislocation and the right ischemic and left unharmed kidney was harvested in PBS for protein extraction. 500µg of total protein extract was used for immunoprecipitation experiments.

Immunohistochemistry

Tissue sections were deparaffinized by 2 changes of xylene for 5 minutes each; slides were then rehydrated in 2 changes of 100% ethanol for 3 minutes, followed by 95% and 70% ethanol for 1 minute, and rinsed in TBS. Sections were boiled in sodium citrate buffer (10 mM sodium citrate, pH 6.0) for 20 minutes. Slides were allowed to cool and endogenous peroxidase activity was quenched using 3% H2O2 in methanol. Sections were pretreated with unconjugated avidin followed by biotin treatment (Vector laboratories, SP2001). Slides were then incubated with primary anti YAP (1:500) or anti TAZ (V386) (1:500) at 4°C overnight. Slides were washed three times with TBS, followed by incubation with biotinylated donkey anti-rabbit IgG antibody diluted secondary (1:500) for 1 hour. After final washing, slides were incubated with ABC reagent (Vector labs) for 30 minutes. Finally, DAB reagent (Jackson labs) was added to the sections for 5 minutes and counterstained with hematoxylin. Sections were dehydrated and mounted with histomount. The slides were then scanned with automated slidescanner (Leica) and images were quantified using positive pixel count feature in Imagescope (Leica).

Confocal Imaging

NIH-3T3 TAZ or YAP GFP flp-in cells were grown on µ-Dish 35 mm, high Glass Bottom dishes (Ibidi). Cells were then exposed to 250µM H2O2 or vehicle control (PBS) for 1 hour at 37°C. Cells were washed once in complete media and fluorescent images were captured at 488-nm excitation using the Zeiss LSM710 META confocal imaging system with a x40/1.3 NA oil objective at room temperature. Images were analyzed and quantified using ImageJ (NIH).
Statistics

All statistical comparisons between groups were analyzed using 2-tailed Student’s t test. Differences in means among multiple groups were analyzed using one-way ANOVA with the Bonferroni post-test unless otherwise indicated. P values less than 0.05 were considered significant in all analyses. The data were computed using GraphPad Prism version 5.0

Results

H$_2$O$_2$ elevates total protein levels of TAZ and increases TAZ/TEAD activity

A previous study observed increased levels of TAZ but not YAP upon stimulation with the GPCR agonist lysophosphatidic acid (LPA) suggesting that TAZ might be differently regulated than YAP (5). It has been well characterized that LPA induces ROS via PLC/PKC/NOX pathway (19,20). Hence, we hypothesized that ROS could specifically augment total TAZ levels. To assess whether ROS modulates TAZ/YAP expression, we first investigated the role of exogenous hydrogen peroxide (H$_2$O$_2$) in HEK 293 cells transiently expressing either wild-type YAP or TAZ. HEK 293T cells exposed to increasing concentrations of H$_2$O$_2$ (0, 50 100, 200 µM) for 1 hour showed a dose dependent increase in intracellular ROS levels as determined by dichlorodihydrofluorescein (DCF) staining in live cells which was inhibited by pre-incubating cells with antioxidant reduced glutathione (GSH) (Fig. 1A). In this model we observed an increase in total TAZ (Fig. 1B) whereas YAP was significantly degraded (Fig. 1C). To validate whether H$_2$O$_2$ differentially alters TAZ and YAP stability, we pretreated cells expressing TAZ and YAP with cycloheximide (CHX) followed by exposure H$_2$O$_2$ for 1 hour. We observed that total YAP levels were decreased whereas TAZ levels remained stable under these conditions (Fig. 1D). YAP and TAZ are essential co-activators of the TEAD family of transcription factors. To determine whether H$_2$O$_2$ influences transactivation of YAP/TAZ we performed TEAD reporter gene assay as previously described (21). Interestingly, TAZ/TEAD4 transactivation principally remained unaltered following 6 hour incubation with a slight, but significant increase at 50 µM H$_2$O$_2$ (Fig. 1E) whereas there was a significant decrease of YAP/TEAD4 transactivation when exposed to H$_2$O$_2$ (Fig. 1F). NAD(P)H oxidase 4 (NOX4) serves as the endogenous source of H$_2$O$_2$ in cellular systems (22) and has been implicated in several murine renal disease models (23). Hence, we investigated whether NOX4 derived H$_2$O$_2$ could alter TAZ/YAP/TEAD activity in a similar manner. NOX4 co-expression significantly upregulates TAZ/TEAD activity whereas YAP/TEAD activity was diminished consistent with our findings using exogenous H$_2$O$_2$ (Fig. 1G). In addition, increasing amounts of exogenously expressed NOX4 decreased YAP expression without affecting TAZ (Fig. 1H), which is again congruent with H$_2$O$_2$ treatment (Fig. 1B/C). Similar results were obtained using NOX1, NOX3 and p22-phox overexpression constructs (data not shown).

H$_2$O$_2$ did not significantly alter TAZ/TEAD interaction but YAP/TEAD4 interaction was considerably diminished (Fig. 1 I-J). To
substantiate the role of H2O2 induced TAZ/YAP target gene expression we quantified mRNA expression of CTGF which is a *bona fide* target gene for both TAZ and YAP (2). H2O2 exposure increased CTGF expression in a dose dependent manner in cells expressing TAZ/TEAD4 constructs which were attenuated by pre-treating cells with antioxidant reduced glutathione (GSH) (Fig. 1K). However, YAP failed to induce CTGF expression above the basal levels despite H2O2 exposure (Fig. 1L). Next, we generated stable cell lines expressing wild-type GFP tagged TAZ and YAP at low expression levels in NIH-3T3 Flp-in cells as previously described (11). NIH-3T3 Flp-in cells have the advantage of single copy genomic integration. TAZ GFP wild-type showed increased accumulation and nuclear localization upon 1 hour H2O2 exposure as compared to YAP GFP wild-type (Fig. 1M) confirming that ROS could differentially regulate TAZ and YAP.

**H2O2 induces s-glutathionylation in TAZ at three cysteine residues**

TAZ and YAP are transcriptional co-activators downstream of the hippo pathway and well characterized in several pathophysiological models (24). Comparing the amino acid sequences of mouse proteins reveals that TAZ has 3 evolutionarily conserved cysteine residues (C261, C315 and C358) whereas YAP has none. Of the three cysteine residues in TAZ C315 is evolutionarily conserved from lower organisms to higher mammals (Fig. 2A). We speculated that the cysteine residues in TAZ could undergo H2O2 induced s-glutathionylation and that this could be responsible for the H2O2 effect on TAZ expression and activation. Oxidative stress can directly modify cysteine residues leading to formation of intramolecular disulfide bonds. Exposing recombinant His TAZ protein with 100 µM H2O2 for 30 min did not alter protein mobility under non reducing conditions suggesting that oxidant stress did not directly modify TAZ (Fig. 2B). HEK 293T cells were co-transfected with wild-type TAZ and YAP. 100 µM H2O2 induced s-glutathionylation in TAZ but not YAP as detected in precipitates with an antibody directed against GSH. TAZ s-glutathionylation was partially reversed by addition of 1 mM dithiothreitol (DTT) to cells 30 minutes post H2O2 exposure (Fig. 2C). H2O2 induced TAZ s-glutathionylation in a dose dependent manner which was inhibited by pre-treating cells with antioxidants such as reduced glutathione (GSH) or N-acetylcysteine (NAC). Interestingly, there was no detectable phosphorylated TAZ (S89) upon s-glutathionylation (Fig. 2D). Protein s-glutathionylation can occur at single or multiple cysteine residues in a context dependent manner (25). Analysis of different C-A mutants revealed that only the triple mutant lacking all three cysteine residues but not the single or double mutants was negative for s-glutathionylation. These observations suggest that TAZ can undergo s-glutathionylation at all three residues with higher specificity towards the evolutionarily conserved residue C315 (Figure 2 E-I).

**H2O2 mediated activation of TAZ depends on s-glutathionylation at three cysteine residues**

Since all three cysteines undergo s-glutathionylation, we then investigated the sensitivity of TAZ cysteine triple mutant towards H2O2 mediated activation using TEAD reporter
assay. Strikingly, TAZ triple mutant did not show any significant activation following 6 hour exposure of H$_2$O$_2$ when compared to wild type (Fig. 3A). We then characterized the TAZ cysteine mutants in TAZ/TEAD reporter assays. Here, the TAZ C315A mutant evoked a significantly elevated transactivation as compared to the wild-type whereas both the C261A and C358A mutants did not show any significant change (Fig. 3B). Quantitative PCR analysis confirmed elevated expression of TAZ target genes CTGF and CYR61 in HEK 293 cells expressing the TAZ C315A mutant supporting the idea that C315 may enhance TAZ stability and increase expression of target genes (Fig. 3 C-D). In contrast, co-immunoprecipitation experiments demonstrated that affinity towards TEAD4 is not significantly altered in the C-A mutants (Fig. 3E). Interestingly, despite enhanced transactivation of C315A mutant, total protein levels were reduced in these cell lysates, which might be due to nuclear translocation, since expression levels in whole-cell lysates were found to be equal (Fig 3B). Phosphorylation of S89 was increased in TAZ C261A and C261/315/358A mutants (Fig. 3F). 

In summary, these findings indicate that all three cysteine residues play an essential role in ROS mediated activation of TAZ.

**GPCR agonist LPA induces s-glutathionylation of TAZ**

Lysophosphatidic acid (LPA) is a potent phospholipid derived G protein coupled receptor (GPCR) agonist. LPA has been previously demonstrated to induce elevation of superoxide (O$_2^-$) via PLC/PKC/NOP pathway in cancer cell lines (19,20). To corroborate LPA induced ROS elevation, we treated HEK 293T cells with increasing concentrations of LPA for 1 hour and quantified superoxide generation using dihydroethidium (DHE). LPA induces a significant elevation of O$_2^-$ which was mitigated by supplementing with N-acetylcysteine or the pan NOX inhibitor apocynin (Fig. 4A). To elucidate the physiological relevance of TAZ s-glutathionylation we hypothesized that LPA derived ROS could induce TAZ s-glutathionylation in HEK 293T cells. LPA stimulation of HEK 293T cells expressing TAZ wild-type resulted in TAZ s-glutathionylation in a dose dependent manner, which was inhibited by pre-incubation with antioxidants or NOX inhibitor apocynin (Fig. 4B). Since LPA is a physiological agonist of both TAZ and YAP we performed TEAD reporter assay with increasing concentrations of LPA. We observed an increase reporter activity for both TAZ and YAP at 1 µM LPA. However, YAP/TEAD activity is significantly reduced at 5 µM LPA while TAZ/TEAD activity was slightly altered at this concentration suggesting that LPA induced TAZ s-glutathionylation could sustain TAZ/TEAD activity. This is further supported by the finding, that the TAZ cysteine triple mutant is insensitive to LPA treatment (Fig. 4C). This enhanced transactivation of TAZ/TEAD at higher concentrations of LPA might correlate to the increased levels of intracellular ROS. Moreover, LPA induced TAZ target gene expression as demonstrated by qPCR analyses for CTGF and CYR61 even at the high concentration of 5 µM which was reduced by ROS suppression using NOX inhibitors (Apocynin, APO) or antioxidants (n-acetylcysteine, NAC) (Fig. 4 E-F). Taken together, our results indicate that LPA mediated...
NOX-derived ROS promotes s-glutathionylation of TAZ and thus increases TAZ target gene expression predominantly through TAZ.

**TAZ s-glutathionylation in vivo**

Both TAZ and YAP play crucial roles in renal development. YAP knockout mice are embryonic lethal (8) while TAZ knockout mice develop a severe cystic kidney disease (9,10). Conditional knockout models underline the importance of TAZ and YAP in kidney development (26). In our study, immunohistochemical staining reveals higher expression of TAZ in the medullary region in the adult murine kidney when compared to YAP indicating prominent role of TAZ in renal homeostasis (Fig. 5 A-B). To understand whether TAZ s-glutathionylation is a relevant physiological mechanism, we first investigated basal TAZ s-glutathionylation in murine kidney tissue homogenates. Strikingly, co-immunoprecipitation experiments identified basal TAZ s-glutathionylation in renal homogenates (Fig. 5C). Renal ischemia reperfusion (I/R) injury has been well documented to induce oxidative stress by elevation of superoxide and H₂O₂ (27). We propose that oxidative stress could also lead to TAZ s-glutathionylation in kidneys. We induced I/R injury in the left kidney (the right kidney served as control) by clamping the kidney pedicle for 40 minutes followed by 5 minutes reperfusion in mice pre-injected with the DNA marker Hoechst 3342 and superoxide sensitive dye DHE. DHE undergoes oxidation by superoxide which then enhances its DNA binding and fluorescence. Dual photon imaging shows significant increase in superoxide levels as seen by nuclear DHE signal when compared to the control right kidney (Fig 5 D). Total tissue homogenates from the control and I/R kidneys were precipitated using the total YAP/TAZ antibody and probed with anti-GSH antibody under non-reducing conditions. Results indicate a significant increase in TAZ s-glutathionylation upon renal I/R injury. (Fig 5E) Immunohistochemical staining reveals total and nuclear TAZ levels to be increased in the tubular compartments in I/R kidneys whereas YAP levels remain unchanged. Furthermore, CTGF levels were also found to be elevated following I/R injury, indicating the significance of the redox activation of TAZ (Fig 5 F). These results indicate that TAZ s-glutathionylation is an important pathophysiological response upon oxidative stress signaling which could lead to enhanced stability and activation leading to tissue repair.

**Discussion**

Regulation of cellular protein levels, phosphorylation and localization of YAP and TAZ determines the functional output of the hippo pathway. Increased YAP/TAZ protein levels have been well documented during physiological responses such as cell proliferation and wound healing (1) as well as in cystic kidney diseases (28). Previous studies on regulation of YAP and TAZ relies on phosphorylation events in the conserved C-terminal and N-terminal phosphodegron (6,29). However, several studies also indicate that biological responses mediated by these proteins are quite different with tissue and organ specificities (1,30). Despite similarities, outstanding questions such as context-dependent activation and factors which uniquely regulate nuclear and cytoplasmic
localization of TAZ and YAP still remain. It has been previously shown that hippo signaling induces YAP inactivation upon I/R injury in cardiomyocytes (31), but the role of TAZ during oxidative stress is not investigated.

Because of its greater stability in comparison to all other oxidant species, H$_2$O$_2$ is the major source of cellular ROS. Upon basal conditions cellular compartments are maintained in a reduced state, during conditions of metabolic stress ROS accumulates in cells and directly interferes with intracellular pathway or directly damages cellular components via oxidation. However, receptor mediated signaling can lead to subtle elevation of ROS which participates in signal transduction. Our data indicate accumulation of TAZ upon short term exposure to H$_2$O$_2$ or NOX4. This also led to an increased activation of TAZ but not YAP TEAD reporter assays and target gene expression assert a striking difference between the two proteins in response to ROS in vitro. As a defense against ROS mediated oxidation cells use antioxidants to revert to a reduced state. Furthermore, localization studies in stable cell lines expressing TAZ GFP or YAP GFP reveal increased nuclear localization of TAZ and but not YAP upon H$_2$O$_2$ exposure. This complementary response authenticates the differential response of TAZ and YAP towards H$_2$O$_2$ induced oxidative stress. We hypothesized that the noticeable TAZ response to H$_2$O$_2$ could be due to redox regulation of conserved cysteine residues in TAZ.

Here we demonstrate for the first time that TAZ, but not YAP undergoes s-glutathionylation on basal conditions and upon mild oxidative stress in HEK 293T cells. Physiological relevance of s-glutathionylation is further substantiated by reversibility of the process. TAZ s-glutathionylation is reversed by restoring cells to a reduced state either by supplementing with excess antioxidant (reduced GSH) or reducing agent DTT. By mutational analysis, we identify that TAZ undergoes s-glutathionylation at all three cysteine residues (C261, C315 and C358). Interestingly, neither exposure to exogenous H$_2$O$_2$ nor mutation of these cysteine residues altered the TAZ/TEAD4 interaction. S-glutathionylation can lead to a gain or loss and in some cases to no noticeable alterations in protein function (25). In our study H$_2$O$_2$ or LPA induced TAZ s-glutathionylation enhanced the transcriptional activation of TEAD as well as increased target gene expression when compared to YAP. This is consistent to the fact that H$_2$O$_2$ or LPA did not lead to TEAD activation in the TAZ cysteine triple mutant which fails to undergo s-glutathionylation. Phosphorylation of TAZ at S89 is crucial for 14-3-3 binding and cytoplasmic localization (32). Results from immunoprecipitation experiments reveal that glutathionylated TAZ is not phosphorylated at S89 and hence could participate in activation of TEAD upon ROS exposure. The evolutionarily conserved C315 residue lies in the TAZ C terminal phosphodegron hotspot. TAZ C315 could also be the favorable site for s-glutathionylation as the incorporated GSH moiety might interact with surrounding amino acid bases bringing about a structural change in the protein and thereby modulating function.

Previous studies have demonstrated that sequential phosphorylation at TAZ S311 and S314 by LATS and CK1-epsilon is essential for
SCFβ-TrCP mediated TAZ ubiquitylation and degradation. We speculate that TAZ C315A behaves similar to the TAZ S314A mutant, which could also lead to inhibition SCFβ-TrCP mediated TAZ ubiquitylation and degradation leading to nuclear translocation and enhanced target gene expression (33). Stability and cytoplasmic retention of TAZ depends on physical interaction with 14-3-3 (32), and angiomiotonin (34). We speculate that the conformational change caused by C315A mutation could prevent interaction between TAZ and 14-3-3 or angiomiotonin leading to enhanced transactivation. However, further mechanistic studies are warranted to validate the importance of C315 residue in redox activation of TAZ.

It has been previously demonstrated that phosphorylation of Mst1/2 as well as MOB and Lats1 can be induced by H2O2 exposure (35). H2O2 induced phosphorylation of MST1 leads to neuronal cell death (36), however the role of the classical hippo cascade and its downstream effectors YAP and TAZ remained unclear in this study. H2O2 induced activation of the canonical hippo cascade could promote phosphorylation and nuclear localization of the downstream effectors which has been recently demonstrated for YAP (37). This inactivation of YAP would promote cell death, and consistently it has been shown, that both decreased Lats1 activity (31) as well as increased YAP activity could protect cardiomyocytes against H2O2-induced cell death (38). However, the role of TAZ is not as clear. TAZ could also undergo similar regulation due to its conserved phosphorylation sites with YAP at the 14-3-3 binding motif, however our data indicate that cysteine s-glutathionylated TAZ fails to undergo Lats1/2 induced phosphorylation at S89 residue despite a potentially high Mst1/2 activity (Fig. 2D). Therefore, we assume that TAZ s-glutathionylation could counteract hippo-dependent phosphorylation and deactivation, and thereby partially circumvent Mst1/2-dependent H2O2-induced apoptosis.

GPCR agonists play an important role in cell signaling to mediate cell growth, migration and gene expression via elevation of physiological ROS (38-40). These different signaling pathways converge at downstream targets such as PKC that upon activation triggers PI3K and subsequent activation of small GTPase Rac, which is required for the assembly of a functional NADPH oxidase (NOX) complex (14). Our data show that LPA induced ROS promotes TAZ s-glutathionylation and target gene expression in HEK 293T cells. These observations highlight an exclusive regulatory mechanism of TAZ in a physiological setting.

TAZ is highly expressed in the renal cortex and TAZ knockout mice develop polycystic kidneys implying a fundamental role in renal development (9,10). Furthermore, our study indicates lower YAP levels in murine kidneys suggestive of a critical role of TAZ in renal homeostasis. NAD(P)H oxidase is a multimeric enzyme complex composed of rac1, gp91phox, p67phcox, p47phox and p22phox subunits. Several NOX isoforms have been documented with tissue specific expression and activation (41). Our data indicate renal I/R injury lead to TAZ s-glutathionylation and nuclear localization in vivo. This validates our hypothesis that TAZ
and YAP could undergo differential regulation in response to I/R induced oxidative stress.

We demonstrate that NOX4 derived H$_2$O$_2$ modulates TAZ activity in vitro suggestive for a novel redox regulation of the hippo pathway. Furthermore, it has to be noted that NOX4 is the major isoform expressed in the kidney and constitutively generates H$_2$O$_2$ (23). LPA receptors (LPA$_1$, LPA$_2$ and LPA$_3$) are expressed in both renal cortical and medullary regions (42). At lower concentrations LPA initiates proliferative pathways, however excess LPA precursors have been observed upon I/R injury leading to pro inflammatory signaling and tissue damage (39). Basal TAZ s-glutathionylation observed in the kidney could be modulated by NOX4 derived ROS at physiological conditions. Increased TAZ s-glutathionylation following I/R injury could indicate oxidative stress stabilizes TAZ from degradation leading to enhanced CTGF expression and may contribute to tissue repair or renal fibrosis.

In summary, we have demonstrated a unique regulatory mechanism by which TAZ undergoes s-glutathionylation leading to enhanced stability and function. Since ROS is an important signal transducer in both physiological and pathological mechanisms, TAZ could function as a redox sensor of hippo pathway (Fig. 5H). However, further studies are needed to determine the redox activation of TAZ in various pathological conditions of renal fibrosis, inflammation and cancer for effective therapeutic strategies.

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**Author Contributions:** RKG, BS and TB conceived the idea for the project, analyzed the results, and wrote most of the paper. RKG, BW, MJ, MB, MHA and MR conducted most of the experiments. MAJ conducted in vivo I/R experiments. All authors reviewed the results and approved the final version of the manuscript.
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FIGURE LEGENDS

Figure 1. H2O2 elevates total protein levels of TAZ and increases TAZ/TEAD activity. A. HEK 293T cells were challenged with 50 µM, 100 µM, and 200 µM of H2O2 for 1 h. After treatment cells were loaded with cellular ROS indicator (H2DCF-DA) and changes in fluorescence were quantified using a microplate reader. B-C. HEK 293T cells expressing FLAG.TAZ or FLAG.YAP were challenged with 50 µM, 100 µM, 200 µM of H2O2 for 1 h. Western blot and densitometry analysis reveals elevated protein levels of TAZ but not YAP. D. HEK 293T cells expressing FLAG.TAZ or FLAG.YAP were pretreated with cycloheximide (40µg/ml) for 1 h. Cells were then challenged with 50 µM, 100 µM, and 200 µM of H2O2 for additional 1 h. Western blot and densitometry analysis reveals stable protein levels of TAZ but not YAP. E-F. HEK293T cells treated with 50 µM, 100 µM, 200 µM of H2O2 for 6 h. H2O2 enhances activity of the transcriptional coactivators TAZ (E) but not YAP (F) in TEAD reporter assays as described in methods. G. HEK 293T cells expressing FLAG.TAZ or FLAG.YAP were co-transfected
with NOX4. NOX4 enhances activity of the transcriptional coactivators TAZ but not YAP in TEAD reporter assays. **H.** Immunoblotting of whole cell lysates reveals that increasing amounts of NOX4 degrades YAP in a dose-dependent manner but not TAZ. **I-J.** HEK293T cells expressing FLAG.TAZ and V5.TEAD4 or FLAG.YAP and V5.TEAD4 were treated with increasing concentration of H$_2$O$_2$ for 1 hour. Co-immunoprecipitation reveals H$_2$O$_2$ does not significantly alter TAZ/TEAD4 interaction but YAP/TEAD4 interaction is perturbed. **K-L.** CTGF expression levels analyzed by qPCR reveal that TAZ induces CTGF expression following 6 h H$_2$O$_2$ which is reversed by antioxidants pretreatment. In contrast, YAP induced CTGF expression is not significantly altered by H$_2$O$_2$. **M.** NIH3T3 TAZ or YAP GFP flip-in stable cells were exposed to 250 µM H$_2$O$_2$ for 1 hour. Representative confocal images and quantification of GFP fluorescence. Scale bar: 50 µm. Data are mean ±SEM from three independent experiments (*P<0.05, **P<0.01 ***P<0.001)

**Figure 2.** H$_2$O$_2$ induces s-glutathionylation of TAZ at three cysteine residues. **A.** Sequence alignment of mouse TAZ (NP_598545.2) reveals conserved cysteine residues at position C261, C315 and C358. **B.** Coomassie staining of recombinant His.TAZ protein following 100 µM H$_2$O$_2$ exposure for 30 mins in vitro. **C.** HEK 293T cells transiently co-transfected with FLAG.TAZ and FLAG.YAP were treated with 100 µM H$_2$O$_2$ for 1 h. 1 mM DTT was added to cells 30 min post H$_2$O$_2$ exposure. After immunoprecipitation (IP) with anti-GSH antibody, western blot analysis revealed that wild type FLAG.TAZ (55 kDa) undergoes s-glutathionylation, but not YAP (72 kDa). **D.** HEK 293T cells transiently transfected with FLAG.TAZ were treated 50 µM, 100 µM, 200 µM H$_2$O$_2$ for 1 h. After IP with anti-GSH antibody, western blot analysis revealed that wild type FLAG.TAZ (WB) undergoes s-glutathionylation in a dose dependent manner which was inhibited by antioxidants GSH and NAC. **E-I.** HEK 293T cells transiently transfected with FLAG.TAZ cysteine mutants as indicated were treated with 50 µM, 100 µM, 200 µM H$_2$O$_2$ for 1 h. After IP with anti-GSH antibody western blot analysis revealed that TAZ undergoes s-glutathionylation in all mutants analyzed (E) C261A, (F) C315A, (G) C358A, (H) C261/C315A and (I) C261/C315/C358A indicating the presence of multiple s-glutathionylation sites within TAZ. Mouse IgG (isotype) is shown as control. Representative data from three independent experiments.

**Figure 3.** H$_2$O$_2$ mediated activation of TAZ depends on s-glutathionylation at three cysteine residues. **A.** TEAD reporter assay shows increased activity of TAZ wild type whereas TAZ triple mutant fails to elicit the H$_2$O$_2$ induced response. **B.** Basal TEAD reporter activity of TAZ cysteine mutants. Immunoblotting of whole cell extracts from luciferase assay showing basal TAZ protein levels. **C-D.** qPCR analysis reveals increased basal CTGF and CYR61 expression in TAZ C315A mutant when compared to wild-type in HEK 293 cells. **E.** HEK293T cells expressing FLAG.TAZ and V5.TEAD4 were immunoprecipitated using M2 beads. Co-immunoprecipitation reveals TAZ cysteine mutants do not alter TAZ/TEAD4 interaction. **F.** Basal total and phosphorylated (S89) levels of TAZ cysteine
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mutants in HEK 293 cells. Data is presented as mean ±SEM from three independent experiments (*P<0.05, **P<0.01 ***P<0.001)

Figure 4. Lysophosphatidic acid (LPA) induces TAZ s-glutathionylation. A. HEK 293T cells were treated with 5 µM of LPA for 1 h. After treatment cells were loaded with dihydroethidium (DHE) and superoxide elevation was quantified in a fluorescence plate reader. B. HEK 293T transiently expressing FLAG.TAZ were challenged with 1 µM and 5 µM LPA for 1 h. After IP with anti-GSH antibody, western blot analyses revealed that wild-type TAZ undergoes s-glutathionylation in a dose dependent manner which was inhibited with pretreatment of antioxidants and pan NOX inhibitor apocynin (APO) or diphenyliodonium (DPI). Mouse IgG IP (isotype) is shown as control. C. TEAD reporter activity of TAZ wild-type and cysteine triple mutant 6 hour following LPA treatment. D. TEAD reporter activity of YAP wild-type 6 hour following LPA exposure. E-F. HEK 293T cells transiently expressing FLAG.TAZ were challenged with 1 µM or 5 µM LPA for 6 h. TAZ target genes CTGF and CYR61 levels were quantified by qPCR. LPA induces TAZ mediated CTGF and CYR61 expression which is attenuated with pretreatment with 2 mM n-acetylcysteine (NAC) and 10 µM apocynin (APO). Data are mean ±SEM from three independent experiments (*P<0.05, **P<0.01, ***p<0.001)

Figure 5. TAZ s-glutathionylation is induced by ischemia-reperfusion in the kidney.

A. Immunohistochemical staining of wild-type mouse kidneys with total TAZ and YAP antibodies. B. Positive pixel count analysis of sections in A shows expression levels of TAZ and YAP (n=3). C. TAZ/YAP was immunoprecipitated using total anti-YAP/TAZ antibody from whole kidney lysates of wild-type mice. S-glutathionylated TAZ (TAZ-SSG) was detected using anti-GSH antibody (WB) under non-reducing conditions. D. In vivo elevation of superoxide (nuclear DHE) in tubular epithelial cells upon renal I/R injury by 2P microscopy (n=3). E. Whole kidney lysates from healthy and I/R kidney reveal increased TAZ glutathionylation following I/R injury (n=3). Rabbit IgG IP (isotype) is shown as control. F. Immunohistochemical staining of wild-type mouse kidneys following I/R injury showing increased cytoplasmic and nuclear TAZ and CTGF levels in I/R kidneys as compared to the control. (n=3). G. Proposed model in which ROS derived from physiological or oxidative stress pathways induces TAZ glutathionylation leading to activation and stability. Data are mean ±SEM from three independent experiments (*P<0.05, ***P<0.001). Scale bar: 200 µm
Figure 1

A

B

C

D

E

F

G

H

I

J

K

L

M
|                  | M.musculus | C315 | C358 |
|------------------|------------|------|------|
| H.sapiens       | RQEAALCRQLPME...SGLGLCNSVPTT...FPDFLPDPNTNV |      |      |
| X.tropicalis    | RQEAALCRQLPME...SGLGLCNSVPTT...FPDFLPDPNTNV |      |      |

Figure 2

A

|                  | C261 | C315 | C358 |
|------------------|------|------|------|
| Hist.TAZ         |      |      |      |

B

|                  |        |
|------------------|--------|
| $H_2O_2$ (uM)    | 0      |

C

- Isotype 0
- Isotype 100
- Isotype 100 nM DTT
- $H_2O_2$ (uM)
- pH (mM)
- precipitates anti-GSH
- lysates anti-Flag

D

|                  |        |
|------------------|--------|
| $H_2C_2$ (uM)    | 0      |

E

- TAZ WT
- TAZ C261A
- TAZ C358A
- TAZ C315A

F

- TAZ C261A
- TAZ C358A

G

- TAZ C315A

H

- TAZ C315A

I

- TAZ C315A

by guest on March 23, 2020
Figure 3

A

Firefly/renilla ratio

Vector  0  100  200 nM
H₂O₂

TAZ WT  TAZ C261/315/358A

B

Firefly/renilla ratio

Vector  TAZ WT  TAZ C261A  TAZ C315A  TAZ C358A  TAZ C261/315/358A

C

Relative fold change (CTGF)

Vector  TAZ WT  TAZ C261A  TAZ C315A  TAZ C358A  TAZ C261/315/358A

D

Relative fold change (Cyr61)

Vector  TAZ WT  TAZ C261A  TAZ C315A  TAZ C358A  TAZ C261/315/358A

E

55 kD
55 kD
55 kD
55 kD

F.TAZ
F.TAZ
F.TAZ
F.TAZ

β-actin
β-actin
β-actin
β-actin

lysates
lysates
lysates
lysates
Figure 4

(A) Graph showing DHE (f.a.u.) levels in different conditions: Control, 5µM LPA, 5µM LPA+GSH, 5µM LPA+APO.

(B) Western blot analysis with labels: Isotype, 0, 1, 5, 5+GSH, 5+APO, 5+DPI.

(C) Bar graph comparing Firefly/renilla ratio for TAZ WT and TAZ C261/315/358A.

(D) Bar graph showing Firefly/renilla ratio for Vector, Vehicle, LPA (1µM), LPA (5µM).

(E) Bar graph showing Relative fold change (CTGF) for Vehicle, 1µM LPA, 5µM LPA, 5µM LPA+NAC, 5µM LPA+APO.

(F) Bar graph showing Relative fold change (CYR61) for Vehicle, 1µM LPA, 5µM LPA, 5µM LPA+NAC, 5µM LPA+APO.
Figure 5

A
Neg.control  wild-type kidney  wild-type kidney
TAZ  YAP  TAZ  YAP

B
Neg.control  wild-type kidney
Neg.control  wild-type kidney
TAZ  TAZ  TAZ  TAZ

C
55 kD  35 kD
IP: YAP/TAZ
WB: anti GSH
TAZ-SSG

D
HOECHST  DHE  MERGE
Control
Ischemia/reperfusion

E
Isotype  Control  I/R
TAZ-SSG

F
TAZ  YAP  CTGF
Control
I/R

G
Decreased degradation
Enhanced transcription
Nuclear
TAZ
TAZ

Physiological stimuli
RCS elevation
Oxidative stress
O2/•H2O2
Antioxidants, NAC, DTT
SSG
TAZ-glutathiolation
TAZ

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