Label-free quantitative proteomic analysis of the YAP/TAZ interactome

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THE COMPREHENSIVE KNOWLEDGE of the composition of protein complexes is a fundamental prerequisite for understanding protein functions and for unraveling physiological and pathophysiological mechanisms (11, 18). Recent substantial advancements in affinity purification techniques and in protein mass spectrometry analysis led to the development of powerful approaches to characterize protein complexes and discover unanticipated regulatory modules (29, 71). Accordingly, a bulk of exciting interaction and interactome studies dramatically increased the knowledge of protein functions and interaction networks (3, 14, 73, 86). However, the analysis of protein-protein complexes is often hampered by several aspects. Usually, proteins are exogenously expressed and fused with purification tags, either for single-step purification or a tandem-affinity purification (TAP) step. In conventional stable cell lines, transfected DNA may be integrated with more than one copy into the genome. In combination with highly active promoters, transgenes are likely to be markedly overexpressed compared with their physiological levels. This may lead to the discovery of false-positive interactors due to severely altered stoichiometry, posttranslational modifications, and other collateral effects. In addition, unspecific binding of proteins to beads or other proteins is a pitfall when analyzing pull-downs with mass spectrometry without appropriate controls and quantification. This has recently fostered the initiation of the “CRAPome” project, a contaminant repository for affinity purification (41).

To circumvent many of these technical limitations, we present an approach to statistically analyze pull-downs of affinity-tagged proteins expressed at near-physiological level. We applied this method to the oncogenic downstream effectors of the mammalian Hippo pathway, YAP (Gene symbol: Yap1) and TAZ (Gene symbol: Wwtr1) (49). The aim of this study was to resolve the well-characterized, biologically relevant YAP/TAZ interactome in a comprehensive manner.

The oncogenes YAP and TAZ were chosen due to their high relevance for cell proliferation and tumor biology (21, 25, 49, 74) and due to the fact that a reasonable number of interacting proteins have already been described in a number of studies (reviewed in Refs. 13, 24, 49). YAP and TAZ are mainly controlled by Lats1/2, the central kinase of the Hippo pathway. If the Hippo cascade is active, Lats phosphorylates its substrates YAP and TAZ among others at serine residues that are part of conserved 14-3-3 binding motifs. This enhances binding to 14-3-3 species proteins and results in cytoplasmic retention. In the inactive state of the Hippo pathway, YAP and TAZ are released from 14-3-3 binding, shuttle to the nucleus, bind to transcription factors such as TEAD, and promote pro-proliferative transcriptional programs. In addition, several Hippo-independent regulators of YAP and TAZ have been recently identified: Interaction of YAP with junctional proteins such as -catenin (60, 63) or angiomotin and angiomotin-like proteins (5, 75, 82) or with the tyrosine phosphatase PTPN14 (28, 36, 76) has been shown to inhibit its activity independently of Lats, while the interaction with ZO-2 activates YAP/TAZ.

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(46, 53). These findings further underline the importance of these transcriptional coactivators in many different biological processes. Thereby, TAZ and YAP have overlapping and but also distinct functions. In this study, we could implement a screening technique and we resolved the well-characterized, biologically relevant YAP/TAZ interactome in a near-comprehensive manner. In addition, our study uncovers differences in the YAP and TAZ interactome and identifies novel interacting proteins for YAP and TAZ thereby allowing new insights into potential regulatory modules acting in concert with or completely independent of the Hippo pathway.

MATERIALS AND METHODS

Cell culture. HEK293T and NIH-3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum.

Generation of stable cell lines. Murine TAZ and YAP cDNAs were provided by M. Yaffe. The respective GenBank accession numbers for the transcripts were NM_133784 and NM_009534. YAP and TAZ were subcloned into a modified pENTRA vector. The insert was transferred into the pG-LAP destination vector via LR clonase reaction (Gateway Technology; Life Technologies). NIH-3T3 Flp-in cells (Life Technologies) were cotransfected with the vector expressing the Flp recombinate (pOG44; Invitrogen) and the p-G-LAP plasmids (67) (plasmids obtained from Peter Jackson through www.addgene.org, #19702, #19706, #19704) encoding the gene of interest. Transfections were done in a six-well plate using Lipofectamine LTX (Invitrogen) according to the manufacturer’s recommendations. Transfection media was replaced with DMEM and 10% fetal bovine serum after 6 h. Medium containing hygromycin B (200 mg/ml) was added after 20 h to select for stable integrants. The selection was carried on for the entire experimental period. Experiments were carried out after the seventh passage after transfection.

Analysis of fluorescence of the transgene. Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min and afterwards washed with Dulbecco’s PBS. Coverslips were mounted onto glass slides using Prolong Gold antifade reagent with DAPI-containing mounting medium. Cells were visualized using an Axiovert 200 microscope (objectives: Plan Apochromat ×20/0.8, C-Apochromat ×63/1.22 W) equipped with a camera (AxioCam MRm; Carl Zeiss) and an imaging system (ApoTome; Carl Zeiss) using Axiosvision 4.8 software for acquisition and subsequent image processing (Carl Zeiss).

Immunoprecipitation for MS. For each replicate of the experiments, 15 × 15 cm dishes (80% confluency, containing ~6 × 10^7 cells) were used. 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 NaP2O7, 2 mM Na3VO4, and complete protease inhibitors (PIM; Roche)]. Cells were sonicated for 1 min 30 s at 10% power. Cell suspension was incubated on ice for 15 min. After centrifugation at 15,000 g for 15 min at 4°C and ultracentrifugation at 210,000 g for 30 min at 4°C, the supernatant was incubated at 4°C for 1 h 30 min with the anti-eGFP antibody covalently coupled to magnetic beads (10829018; Miltenyi Biotec). Before the addition of antibody, a small aliquot of each supernatant was preserved and diluted with 2× SDS-PAGE sample buffer for later immunoblot analysis. Columns were washed three times with wash buffer 1 (50 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol, and 0.05% IGEPal-CA-630) and five times with wash buffer 2 (50 mM Tris pH 7.5 and 150 mM NaCl). This procedure was modified from the protocol by Hubner and Mann (29). A simplified scheme of the entire workflow is depicted in Fig. 1A.

Purified protein complexes were then eluted nonspecifically by direct in-column digestion with trypsin. For this purpose, a buffer containing 2 M urea in 50 mM Tris (pH 7.5), 1 mM DTT, and 5 μg/ml trypsin was added to the column and incubated for 30 min at room temperature. Partially digested proteins were then eluted two times with 50 μl 2 M urea, 50 mM Tris pH 7.5, and 5 mM iodoacetamide and fully digested overnight at room temperature. DTT and iodoacetamide were present for the reduction and alkylation of disulfide bonds. Digestion was stopped the next day by adding 1% trifluoroacetic acid (29). Label-free pull-downs were performed as four biological replicates. Clean-up of peptides was performed using C18 StageTips (Sp301; Thermo Scientific) (52).

MS/MS methods. Purified peptides were analyzed using an LTQ Orbitrap Discovery (Thermo Scientific) mass spectrometer as previously described (56). Briefly, analysis was carried out using reversed phase liquid chromatography coupled to nano-flow electrospray tandem mass spectrometry [EASY nLC II nano-LC (Proxeon/Thermo Scientific) with a 150-mm C18 column (internal diameter: 75 μm, Dr. Maisch)]. Peptide separation was performed at a flow rate of 300 nl/min over 90 min (5 to 7% ACN in 5 min, 7 to 45% in 60 min, 45 to 50% in 5 min, 50 to 97% in 5 min, and wash at 100%; buffer A: 0.1% formic acid in H2O; buffer B: 0.1% formic acid in acetonitrile). Survey full scan MS spectra (m/z 300 to 2000) of peptides were acquired in the Orbitrap at a resolution of 30,000; m/z = 445.12,003 was used as a lock mass. Collision-induced dissociation (CID) was used as fragmentation mode. The mass spectrometer acquired spectra in “data dependent mode” and automatically switched between MS and MS/MS acquisition. Signals with unknown charge state were excluded from fragmentation. The dynamic exclusion option was enabled (1 min). The five most intense ions (charge state: z ≥ 2) were isolated and fragmented in the linear ion trap using CID fragmentation (“Top-5-Method”).

Bioinformatic analysis. Analysis of raw files was performed using the MaxQuant suite algorithms (v. 1.3.0.5) (9). Raw file spectra were searched against the mouse uniprot reference database using the target-decoy strategy (reversed database). Mass accuracy was 20 ppm in the first search and 6 ppm in the second search with enabled deisotoping option. For fragment ions, mass tolerance was 0.5 Da. Fixed modifications were carboxymethylation of cysteines (+57 Da). Variable modification was oxidation of methionine (+16 Da) with a number of four allowed modifications. Protein, peptide, and site false discovery rate (FDR) were adjusted to <0.01. All proteins identified with one or more peptides were taken for label-free quantification (LFQ) analysis (Fast LFQ option enabled). Intensity-based absolute quantification (iBAQ) values were calculated in the MaxQuant suite as previously described (62).

LFQ intensities for respective protein groups were uploaded in Perseus (10) and analyzed as previously described (29). Briefly, raw LFQ intensities were logarithmized and normalized by subtraction of the mean. At least two LFQ values per protein group needed to be present for the analysis. Contaminants and proteins resolved by PTM-sites only were removed from the dataset. To replace nonquantified values with low intensities, data imputation was performed based on normal distribution of LFQ intensities. Significant interactors were determined using a two-sample analysis (t test). Permutation-based FDR method in Perseus was used to perform multiple testing corrections. Gene Ontology (GO) terms, Corum terms, and PFAM domain terms were annotated using Perseus and analyzed using a Fisher’s exact test. As correction for multiple testing a Benjamin-Hochberg FDR of <0.05 was determined. Calculation of complex stoichiometry based on iBAQ values of significant interactors was performed in Microsoft Excel as previously described (64).

Immunoblot. 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 NaP2O7, 2 mM Na3VO4, and complete protease inhibitors (PIM; 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, 2× SDS sample buffer was added to the supernatants. Samples were resolved by SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and visualized with enhanced chemilumines-
ence after incubation of the blots with the respective antibody (anti-GFP; Santa-Cruz Biotechnology).

**Luciferase assays.** The Hippo reporter assay was performed using the GAL4-TEAD Luciferase Reporter System (H1001; Biomyx). 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, Rassf8, Tnpo, and C/EBPβ, and the control empty vector pcDNA6) into HEK293T cells in a 96-well format using Lipofectamine LTX (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) 24 h after transfection. Transfections and measurements

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**Fig. 1.** Validation and characterization of stably transfected NIH/3T3 flp-in cell lines. **A:** schematic overview of experimental procedure. NIH3T3 cells with single-copy integration of the transgenes [GFP.YAP or GFP.GFP fusion protein (termed here GFP protein)] were lysed and protein complexes were immunopurified using an anti-GFP matrix. After in-column tryptic digestion, single-shot mass spectrometry was performed. **B:** immunoblot analyses confirming the expressions of GFP.GFP (termed here: GFP), GFP.TAZ, and GFP.YAP fusion proteins in NIH/3T3 flp-in cells. Immunoblotting was performed using an anti-GFP antibody. Actin was used as a loading control. **C:** whole-cell lysates of Flp-in NIH/3T3 cells were stained with anti-Wwtr1 and anti-Yap, respectively showing that the fusion proteins are expressed at similar levels as the endogenous TAZ/YAP (represented by *). **D:** fluorescence microscopic analysis of the transgene localization of stably transfected NIH/3T3 flp-in cell lines expressing GFP, GFP.YAP, and GFP.TAZ. **E:** localization of GFP.YAP and GFP.TAZ stable cell lines in sparse and high-confluency cell conditions.
were performed in triplicates for each single experiment, and each experiment was repeated independently at least three times. Error bars shown in the figures represent SE. P values were calculated using an unpaired Student’s t-test.

**TaQMan assays.** HEK293T cells were transiently transfected using the calcium phosphate method with the indicated plasmids. The following day cells were harvested in Qiazol (Qiagen), and RNA was isolated using a RNA miniprep kit (Zymo Research). Reverse transcription was performed using an ABI HighCapacity cDNA Kit and quantitative (q)PCR analysis was performed with ABI Taqman assays to evaluate connective tissue growth factor (CTGF; Hs00170014_m1) expression levels. ActB (4326315E) served as endogenous control. All qPCR experiments were performed on the ABI 7900HT System and repeated independently at least three times. Error bars shown in the figures represent SE. P values were calculated using an unpaired Student’s t-test.

**Coimmunoprecipitation.** HEK293T cells were transiently transfected using the calcium phosphate method, and coimmunoprecipitation was performed as described previously (22). The following day, cells were harvested with ice-cold PBS and 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 Na4P2O7, 2 mM Na3VO4, and complete protease inhibitors (PIM; 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 2 h with the anti-FLAG (M2) antibody covalently coupled to agarose beads (Sigma-Aldrich). Before the addition of the beads, a small aliquot of each supernatant was preserved and diluted with 2× SDS-PAGE sample buffer for later immunoblot analysis (lysate). The beads were washed extensively with lysis buffer, and bound proteins were resolved by SDS-PAGE, blotted on to polyvinylidene fluoride membranes, and visualized with enhanced chemiluminescence after incubation of the blots with the respective antibodies.

**RESULTS**

We generated NIH-3T3 Flp-In cell lines with single-integration of GFP.YAP and GFP.TAZ as described in MATERIALS AND METHODS (Fig. 1A). Immunoblotting of whole cell lysates from the cell lines confirmed expression of the GFP.YAP fusion protein (termed here: “GFP”), GFP.YAP, and GFP.TAZ (Fig. 1B). The YAP isoform (NM_009534) used in the study is related to human isoform Yap1–alpha (16, 66). Immunoblotting revealed a comparable expression of endogenous YAP, TAZ, and the GFP-tagged protein (Fig. 1C). Fluorescence microscopy further revealed that GFP-tagged YAP and TAZ both localized to the nucleus and to the cytoplasm (Fig. 1D). To demonstrate that GFP.YAP and GFP.TAZ change their localization in response to physiological stimuli, we analyzed localization of the respective fusion proteins in sparse and high-confluence cell conditions. In fact, in high confluent cells, both proteins were predominantly localized extranuclear (Fig. 1E) as previously described for endogenous proteins (84).

After establishing the cell lines and optimizing the conditions for purification, we started analyzing the interactome of GFP.YAP and GFP.TAZ as described in MATERIALS AND METHODS and in Fig. 1A. Another Flp-in cell line with GFP.GFP as a single-copy gene served as negative control. We acquired samples for GFP.YAP vs. GFP as well as GFP.TAZ vs. GFP with four biological replicates (all prepared on different days). The entire measurement was accomplished within a 24-h measurement (gradient) time. In our samples, a total number of 780 proteins could be identified with high confidence. Of these, 685 proteins passed the criteria for LFQ. Principal component analysis (PCA) (55) revealed that all of the GFP samples clustered together and were markedly different from GFP.YAP and GFP.TAZ pull-downs (Fig. 2A). This clear separation of samples was evident although the pull-downs were performed as biologically independent replicates on different days. We performed hierarchical clustering of protein intensities to visualize protein abundance across all samples. Figure 2B highlights the intensities for all proteins discovered from the samples based on correlational clustering (yellow represents high intensity and blue represents low intensity; the respective proteins are depicted in the rows). The raw data of the quantification are added as Data Supplement S1 (Supplemental Material for this article is available online at the Am J Physiol Cell Physiol website). As expected from the PCA, biological experimental replicates strongly clustered together, with YAP and TAZ being more interrelated than YAP or TAZ and GFP. The majority of proteins was equally represented in the different sample groups. A more detailed view on the heat map revealed that several proteins had similarly high intensities in both YAP and TAZ pull-downs compared with GFP (Fig. 2C). This heat map further revealed YAP-specific and TAZ-specific enriched proteins.

To cut down the list and discriminate between background binders and significantly enriched proteins, we performed a statistical analysis of LFQ intensities of the GFP.YAP dataset compared with the GFP controls (Fig. 3). To reveal the significantly enriched proteins and also show their significance, we plotted the log2 ratio of GFP.YAP/GFP (LFQ intensities) against the negative log of the P value (so-called volcano plot, Fig. 3A). To correct for multiple testing, a permutation-based false discovery rate approach was applied (FDR = 0.05; s0 = 1) (9). This analysis separated significant interactors from background binders (Fig. 3A). As expected, YAP was the most enriched protein among the proteins in the pull-down. Taken together, our dataset revealed 37 interactors significantly enriched in the YAP-sample.

In contrast to relative quantification of protein abundances, parameters have been recently developed to confidently estimate absolute copy numbers of proteins (62). Protein copy numbers can be estimated using iBAQ (62), which is based on MS1 intensities and possible peptide numbers. This approach can be applied to analyze protein stoichiometry within immunopurified pull-downs (64). Since the iBAQ values obtained in the control samples indicate background binding, these iBAQ intensities can be subtracted from the iBAQ intensity obtained in the GFP.YAP pull-down (64). For this analysis, proteins sharing common peptides and high similarity, such as different 14-3-3 or TEAD species, have to be collapsed into one entry (64). We performed this analysis (Fig. 3B). The remaining values were scaled according to the abundance of the bait protein, resulting in the stoichiometry of the interactors relative to the bait. We arbitrarily set the abundance of all 14-3-3 proteins as 1 in the complex, since the binding of 14-3-3 with TAZ/YAP is very well understood and each TAZ/YAP protein has just one 14-3-3 binding site. The analysis of stoichiometry revealed that AMOTL2 and MPP5 were the most abundant proteins within the complex. The vast majority of proteins had a substoichiometric abundance within the complex compared with 14-3-3 proteins, while TEADs had an absolute abundance within the complex which was comparable to that of the 14-3-3 proteins.
The GFP.YAP pull-down dataset and its control was analyzed the same way (FDR = 0.05; s0 = 1; Fig. 4). Again, TAZ was the most abundant protein within the complex and in total 31 proteins were found to be significantly enriched within this pull-down (Fig. 4A). We also performed the identical analysis of stoichiometry for significant interactors in the GFP.TAZ pull-down. The result was similar, with AMOTL2 and MPP5 being the most abundant proteins, with 14-3-3 and TEAD sharing a similar complex stoichiometry (Fig. 4B) and the majority of proteins including Lats1, Pard3, or Runx2 showing a substoichiometric abundance.

We analyzed the combined interactor dataset and performed a literature search to find known or previously undescribed
Table 1) interactors of YAP and TAZ. The analysis revealed that the majority of interacting proteins were previously described to directly interact with YAP or TAZ or were known parts of the Hippo pathway. We also performed a statistical analysis to characterize the interactors more globally. To this end, we performed a Fisher's exact test to test for significant enrichment of Corum protein complexes (58) (Table 2), GO terms (Biological Processes, Cellular Compartment) (Table 3), as well as the enrichment of PFAM domains (Table 4) compared with the nonchanged proteins within the samples. Consistent with the nature of the pull-down, the Corum term analysis revealed a significant overrepresentation of the YAP-TAZ-TEAD complex but also for the apical polarity complex containing Par3, Patj, and Pals (Table 2). GO-term analysis for

Fig. 3. Quantitative proteomic analysis of the YAP interactome. A: logarithmized ratios are plotted against the negative logarithmic P value of a Student’s t-test. Background binding proteins are located close to the vertical 0-line. The proteins on the right side of the curved line were determined as interactors. This line was drawn to correct for multiple testing [false discovery rate (FDR) = 0.05; s0 = 1]. The data points of significant interactors are labeled with the gene symbols of the respective proteins as indicated in the Table 1. B: stoichiometry determination for GFP.YAP interactors. The abundance of the interactors in the GFP.YAP pull-down [intensity-based absolute quantification (iBAQ) value] was corrected for the obtained abundance in the control pull-downs. The remaining values were scaled according to the abundance of the bait protein.

Fig. 4. Quantitative proteomic analysis of the TAZ interactome. A: logarithmized ratios are plotted against the negative logarithmic P value of the Student’s t-test. Background binding proteins have a ratio close to 1:1 and are located close to the vertical 0-line. The proteins on the right side of the curved line were determined as interactors. This line was drawn to correct for multiple testing (FDR = 0.05; s0 = 1). The data points of significant interactors are labeled with the gene symbols of the respective proteins as indicated in Table 1. *Whp2 is not significantly changed. B: stoichiometry determination for GFP.TAZ interactors. The abundance of the interactors in the GFP.TAZ pull-down (iBAQ value) was corrected for the obtained abundance in the control pull-downs. The remaining values were scaled according to the abundance of the bait protein.
Acting proteins were used as background dataset. All noninteracting proteins were used as background dataset.

Table 2. Enrichment of Corum terms within the interacting proteins

<table>
<thead>
<tr>
<th>Corum Term</th>
<th>–log(P Value)</th>
<th>Benj. Hoch. FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEAD4-YAP DNA-protein complex</td>
<td>2.3</td>
<td>0.049</td>
</tr>
<tr>
<td>TEAD2-multiprotein complex</td>
<td>4.7</td>
<td>0.001</td>
</tr>
<tr>
<td>PAR3-PATJ-PALS1 complex</td>
<td>3.5</td>
<td>0.008</td>
</tr>
<tr>
<td>PALS1-PATJ-CRB3-PAR3-PAR6-αPKC-14-3-3-ζ complex</td>
<td>4.7</td>
<td>0.003</td>
</tr>
</tbody>
</table>

A Fisher’s exact test was performed with correction for multiple testing (Benjamin-Hochberg false discovery rate (Benj. Hoch. FDR)). All noninteracting proteins were used as background dataset.

References for known interactors or members of the Hippo pathway are given.

YAP and TAZ are homologs and share many functional similarities and common interactors (81). The activity of both is regulated through Lats1-induced phosphorylation and phospho-dependent binding to 14-3-3. Similar to YAP, TAZ also functions as a transcriptional coactivator and interacts with TEAD (38). Amino acid sequence comparison between YAP and TAZ shows that YAP contains a proline-rich NH2-terminal that is absent in TAZ. In addition, YAP has two WW-domains and TAZ only one. These differences are consistent with the binding affinities of 14-3-3 (30) reported for YAP and TAZ.

Table 3. Enrichment of Gene Ontology terms within the interacting proteins

<table>
<thead>
<tr>
<th>GO Category/Term</th>
<th>Count</th>
<th>–log(P Value)</th>
<th>Benj. Hoch. FDR</th>
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</thead>
<tbody>
<tr>
<td>Biological processes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive regulation of transcription from RNA polymerase II promoter</td>
<td>9</td>
<td>4.74</td>
<td>0.044852</td>
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<tr>
<td>Cell differentiation</td>
<td>10</td>
<td>2.79</td>
<td>0.049516</td>
</tr>
<tr>
<td>Cellular compartment</td>
<td></td>
<td></td>
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<tr>
<td>Synaptosome</td>
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<td>3.52</td>
<td>0.014525</td>
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<tr>
<td>Tight junction</td>
<td>9</td>
<td>9.83</td>
<td>3.19E-08</td>
</tr>
<tr>
<td>Cell-cell junction</td>
<td>9</td>
<td>6.62</td>
<td>3.43E-05</td>
</tr>
</tbody>
</table>

A Fisher’s exact test was performed with correction for multiple testing (Benj. Hoch. FDR). All noninteracting proteins were used as background dataset. GO, Gene Ontology.
compared with only one in TAZ (7) (Fig. 5A). Both interactomic analyses revealed that the majority of protein interactors (24 interactors) were identified in both samples (Fig. 5B). To specifically elaborate on differences in the YAP and TAZ interactome, we performed a direct comparison between the GFP.YAP and the GFP.TAZ samples (Fig. 5C). As expected, YAP and TAZ were the most enriched proteins in their respective pull-downs; YAP specifically interacts with Ptpn14 and Col16a1 while TAZ specifically interacts with FBxw11 and Skp1 confirming previous studies (34, 35).

Next, we selected three candidate proteins which were not previously known to interact with YAP and TAZ: Tmpo (thymopoetin), Rassf8, and CCAAT/enhancer-binding protein (C/EBP). We hypothesized that these interactors may be involved in regulating the YAP/TAZ-TEAD dependent transcriptional activity. In luciferase-based reporter assays, cotransfection of Rassf8 significantly increased YAP/TAZ-TEAD-dependent transcriptional activity (Fig. 6, A and B) while cotransfection of Tmpo did not alter the YAP/TAZ-TEAD dependent transcriptional activity (Fig. 6, C and D). The transcriptional activity of YAP/TAZ-TEAD was significantly reduced by cotransfection of C/EBPβ (Fig. 6, E and F). Cotransfection of neither Tmpo, Rassf8, or C/EBPβ altered the basal activity of the reporter compared with the vector control (data not shown). In addition to the reporter assays, we measured mRNA expression of CTGF, a bona fide target gene of YAP and TAZ (85) by quantitative PCR. Consistent with the findings from the reporter assays, YAP-dependent CTGF gene expression was further increased by the additional expression of Rassf8 (Fig. 6G) and decreased by cotransfection of C/EBPβ (Fig. 6H).

To confirm the interaction of proteins, we performed coimmunoprecipitation experiments of both Rassf8 and C/EBPβ in HEK293T cells. As expected, immunoblotting could confirm the coprecipitation of the respective V5.tagged proteins with either F.YAP and F.TAZ (Fig. 7, A and B). Since C/EBP is a transcription factor, we also tested whether presence of C/EBPβ may influence the biochemical interaction between YAP/TAZ and TEAD. To this end, we cotransfected V5.CEBPβ, GFP.TEAD as well as F.YAP. We performed coimmunoprecipitation of FLAG-tagged YAP and TAZ. Immunoblot analysis revealed strong interaction of GFP-tagged TEAD4, which was not diminished by additional cotransfection of V5.CEBPβ (Fig. 7C). Thus the mechanism of decreased YAP/TAZ-TEAD dependent transcription upon cotransfection with C/EBPβ remains to be elucidated.

**DISCUSSION**

Analysis of affinity-purified protein complexes by mass spectrometry typically results in extensive lists of identified possible interactors while the biological relevance often remains ambiguous. In contrast, we present a stringent approach widely applicable to the identification of significant protein interactions. While the use of the Flp-in system guarantees single-copy integration of transgenes of interest (59, 67), the use of GFP fusion proteins allows single-step immunopurification in parallel to visualization of the protein within living or fixed cells (Fig. 1). In addition, the method uses a “single-shot”
mass spectrometry run. This dramatically reduces measurement time compared with gel-based fractionation methods and facilitates a comprehensive analysis of the interactome within one sample, thereby enabling accurate bioinformatics analysis (29). Thereby, metabolic labeling of proteins, i.e., with stable isotope labeling by amino acids in cell culture (SILAC), is not mandatory for this approach (29). Our data provide an accurate and clear separation of pull-downed complexes obtained from different “bait” proteins, which even distinguishes between the two highly similar proteins YAP and TAZ (Fig. 2). In addition, this method uses a quantification algorithm, which quantifies the area under the curve of the precursor ions. This quantification mode has been shown to be more accurate and reliable than spectral counting (44). Our results demonstrate that the majority of proteins remain unchanged between YAP/TAZ and GFP-control samples, indicating the danger of determining “sticky” proteins as true interactors when not using accurate quantification and controls (Fig. 2) (41).

Recently, it has become feasible to use absolute MS1 intensities to gain insights into protein copy numbers within samples. The parameter iBAQ can be used as a direct measure of absolute protein copy numbers within a complex sample (62). This again can be used to reveal the stoichiometry of purified protein-protein complexes (64). Strikingly, analysis of complex stoichiometry revealed that TEAD and 14-3-3 were similarly abundant in the complex. Binding of phosphorylated YAP and TAZ to 14-3-3 proteins preferentially occurs in the cytoplasm, whereas binding of nonphosphorylated YAP and TAZ to TEADs preferentially occurs in the nucleus (21, 25, 49, 54, 74). To get a comprehensive picture, we chose conditions where YAP and TAZ showed both a cytoplasmic and a nuclear localization (Fig. 1D). GFP.YAP and GFP.TAZ, however, both
were physiologically regulated by confluency as expected from a protein expressed on near-endogenous levels (Fig. 1E).

Amotl2 and Mpp5 are the most-enriched proteins within the complex. However, for the majority of proteins, stoichiometric values were in fact lower than for 14-3-3 (for some candidates even by an order of magnitude). Among them were the kinase Lats1, the classic regulatory kinase of the pathway, and other established regulatory proteins such as Wbp2 (6), Skp1 (35), and the phosphatases Ppp1ca and Ptpn14 (34, 36). This finding emphasizes that regulation of protein function is often mediated through transient, highly dynamic interactions. These interactions are indeed expected to be represented in a stoichiometric abundance.

Strikingly, the majority of previously published interactors of YAP and TAZ could be confirmed in our screen; among them are TEADs (Tead1–4), AMOT, Wbp2, and almost all 14-3-3 isoforms (Table 1). Interestingly, the interaction partners found in this analysis have been mapped to all regions of the YAP/TAZ protein, among these are the NH2 terminus (TEAD1–4; Ref. 72), the COOH terminus (all PDZ domain containing proteins; Refs. 46, 53, 70), as well as the WW-domain (14-3-3 proteins; Ref. 24). This highlights the power of this stringent screening approach. A global view of these interactors revealed a significant overrepresentation of polarity proteins within the interaction dataset (Tables 2–4), which supports the hypothesis of a strong link between organization of polarity and Hippo signaling in a more quantitative manner (13, 70). This is indicated by the significant overrepresentation of the PFAM domains “Angiomotin_C” and “PDZ,” the significant overrepresentation of the Corum term “PALS/PATJ/CRUMBS” complex, as well as the GO terms “cell-cell contacts” and “differentiation” (Tables 2–4). All of these analyses underline the power of our screening approach; however, one constraint of our study is the use of only one fibroblast cell line. Although the interactome data contain the vast majority of previously known interactors, the data should not be regarded as representative of the entire YAP/TAZ interactome relevant to all tissues and cancers. Our screen, for example, failed to detect 

Fig. 7. Confirmation of functional YAP/TAZ interactors using coimmunoprecipitation. A: FLAG-tagged YAP and TAZ were immunoprecipitated (IP) using an anti-FLAG antibody on Sepharose beads. Elutes were analyzed for contents of coeluted V5-tagged Rassf8. WB, Western blot. B: FLAG-tagged YAP and TAZ were immunoprecipitated using an anti-FLAG antibody. Elutes were analyzed for contents of coimmunoprecipitated V5-tagged C/EBPβ. C: FLAG-tagged YAP was immunoprecipitated using an anti-FLAG antibody. Elutes were analyzed for content of GFP-tagged TEAD4 as well as V5-tagged C/EBP protein.
In addition, there is a significant bias of Lats1 interaction towards YAP, which is consistent with previous mass spectrometry studies (77). The degradative Skp1-complex, however, is specifically associated with TAZ as previously described (35).

The Hippo pathway interactome has been very recently addressed by two studies: the first one provided a comprehensive insight into the Drosophila pathway using S2R+ cell cultures and identified >200 protein-protein interactions (32) while the second study focused on the mammalian pathway by using lentivirally transduced HEK293T cells (77). Here, TAP purifications of the YAP/TAZ-associated complexes were performed without biological or technical repeats while spectral counting was used as a quantification method. A comparison of these data with our results reveals a large overlap of the interactomes of both respective studies (Fig. 8). However, several of the possible interaction partners from the TAP screen were found in our pull-down data set as well, but were lacking significance and therefore have been excluded in our study (marked with a “§” in Fig. 8). Finally, we suggest a core mammalian YAP and TAZ interactome deviated from the intersection of both studies (Fig. 8). Given the huge differences in pull-down analyses due to different species, cell culture systems, purification methods, (Fig. 8). Given the huge differences in pull-down analyses due to different species, cell culture systems, purification methods, mass spectrometry analysis, and statistical criteria, the definition of common standards to dynamically assess interactomes of individual proteins will be of critical importance for future studies. Development of next-generation mass spectrometers and more targeted quantification methods might help to guide this process (17, 40, 43).

Strikingly, our study identified a number of novel, significant interactors for YAP and TAZ (Table 1). Most of these candidates are associated with cancer. Given the high stringency of the analysis, these proteins can be considered to be important regulators of YAP and TAZ function and might play crucial roles downstream of the mammalian Hippo cascade. Among these novel interactors are the CCAAT/enhancer-binding proteins (C/EBPβ and C/EBPδ; gene symbol Cebp and Cebpd). C/EBPβ and δ belong to the C/EBP family of transcription factors that consist of C/EBPα to C/EBPγ, which have multiple functions in development of several tissues, in liver regeneration, and different types of cancer (reviewed in Rev. 61). Very recently, both C/EBPβ and C/EBPδ have been found to be crucial factors for epicardial activation in cardiac development and repair (27). Further cooperate functions of C/EBPβ and δ have been described before. Future, more detailed studies will have to address the question, whether each individual C/EBP or a C/EBP-β/δ heterodimer regulates YAP/TAZ activation and whether the interaction is mediated by the WW domains of YAP/TAZ and a classical PPXY motif that is present in C/EBPβ and C/EBPδ (4, 8, 65).

![Fig. 8. Comparison of the identified YAP (A) and TAZ (B) interactome with a recently published TAP screen in 293T cells. Proteins discovered in both studies were defined as member of the “core interactome.” §Interaction partners in the latter study not being found significant in our study.]
Another novel interactor identified in our study is the core-binding factor subunit β (Cbfb), a transcription factor known to be involved in different forms of leukemia (19). Cbfb is known to interact with Runx1 and Runx2, the latter is a YAP/TAZ interactor with relevance for development of the skeletomuscular system (31, 78, 80). The YAP/Runx2 interaction was confirmed within our experiments (Figs. 3–4). In addition, we could identify Rassf8, which was also found in the recent mammalian Hippo TAP screen (77). Rassf8 is a member of the Ras domain family (51). In contrast to Rassf 1–6 that interact with different components of the Hippo cascade via a COOH-terminal SARAH (Salvador-Ras-Hippo) domain and thus modulate YAP/TAZ function (20), Rassf8 is lacking the SARAH domain (68). Recently, Rassf8 has been suggested to be a candidate tumor suppressor protein in mammals and has been shown to modulate both Wnt and NF-κB signaling (37). Flies lacking the Drosophila homolog display overgrowth phenotypes with enlarged wings, rough and enlarged eyes, and increased weight (33). Rassf8 may represent a specific relay node for interconnection of Wnt and Hippo signaling transduction, an exciting field of future research (69). Surprisingly, our analyses of YAP/TAZ-TEAD activation with reporter assays and qPCR of target genes revealed that Rassf8 activates YAP/TAZ-TEAD-dependent transcriptional activity. This implicates that the tumor suppressor functions of Rassf8 might be independent on the regulation of YAP/TAZ-TEAD activity and that Rassf8 also has pro-proliferative effects by activating YAP/TAZ.

Taken together, our experimental setting identified novel TAZ/YAP interactors that both activate and reduce TEAD-activation as shown in luciferase assays and target gene expression. Since some of the novel interactors function as transcription factors or transcriptional coactivators themselves, further studies must address additional transcriptional networks downstream of YAP and TAZ rather than focusing on the TEAD family of transcription factors. While in our study we have chosen conditions under which YAP and TAZ show both cytoplasmic and nuclear localization indicating a moderate activation state, it would be striking to analyze the dynamic changes of the respective interactomes in response to changes in YAP or TAZ activity. These changes could be induced by either varying cell density (Fig. 1E), by GPCR activation (79), or as recently described through changes in substrate rigidity (1, 12). Analyzing the complexity of these dynamic interaction networks will help to gain a better understanding of common functions and the distinction of the two main downstream effectors of the mammalian Hippo tumour suppressor pathway.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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PTPN14 interacts with and negatively regulates the oncogenic TrCP E3 ligase.


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