Abstract

In recent years, proximity labelling has established itself as an unbiased and powerful approach to map the interactome of specific proteins. While physiological expression of the labelling enzyme is beneficial for the mapping of interactors, generation of the desired cell lines remains time-consuming and challenging. Using our established pipeline for the rapid generation of C- and N-terminal CRISPR-Cas9 knock-ins (KIs) based on antibiotic selection, we were able to compare the performance of commonly used labelling enzymes when endogenously expressed. Endogenous tagging of the µ subunit of the AP-1 complex with TurboID allowed identification of known interactors and cargo proteins that simple overexpression of a labelling enzyme fusion protein could not reveal. We used the KI-strategy to compare the interactome of the different adaptor protein (AP) complexes and clathrin and were able to assemble lists of potential interactors and cargo proteins that are specific for each sorting pathway. Our approach greatly simplifies the execution of proximity labelling experiments for proteins in their native cellular environment and allows going from CRISPR transfection to mass spectrometry analysis and interactome data in just over a month.

Introduction

The biological role of a protein is shaped by its subcellular localization and its interaction with other biomolecules. Therefore, mapping the interactors of a given protein can be crucial for understanding its biological function. In the past several years, proximity labelling with biotin in living cells has emerged as a complementary approach to classic affinity purification/mass spectrometry (AP/MS)-based methods for mapping protein-protein interactions in living cells and organisms. The proximity labelling is carried out by enzymes genetically fused to the protein of interest (POI) that catalyze the formation of a highly reactive biotin intermediate labeling proteins within a small radius (1-10 nm) in a promiscuous manner. A key advantage of proximity labelling-based interactome mapping compared to traditional approaches is that very weak and transient interactions can also be captured. The biotinylation itself provides a unique chemical moiety that can be used for subsequent enrichment and identification.

The enzymes used for proximity labelling are either engineered peroxidases (APEX, APEX2) or engineered biotin ligases (BioID, BioID2, TurboID and miniTurboID). APEX and APEX2 use H2O2 as a co-substrate to rapidly generate a highly reactive phenoxyl radical from biotin-phenol that reacts specifically with electron-rich side chains (primarily tyrosine). An attractive feature of APEX peroxidases is the fast labelling kinetics (labelling time: <1 min)
that enable probing with a high temporal resolution. However, on the downside, they require
H₂O₂, which causes oxidative stress in living cells and thus cannot be used for proximity
labelling in living organisms. In contrast, biotin ligases simply need non-toxic, highly soluble
biotin as a substrate which in an ATP-dependent reaction, is converted into a reactive
biotinoyl-5′-AMP intermediate that covalently tags proximal lysine residues. Although the
labelling time could be reduced from >18h for BioID to less than an hour with TurboID, labelling with biotin ligases is significantly slower than with APEX peroxidases.

To avoid artifact as a result of overexpression, such as mislocalization and protein
aggregation among others, physiological expression levels of the POI fusion protein are
preferred for interactome mapping experiments. Moreover, less abundant interactors that
would be hidden in the unspecific labelling background that is caused by non-physiological
expression levels might be identified this way. To control expression levels of the fusion
protein overall, various different approaches have been implemented. These include the use
of small-molecule induced expression systems in a safe DNA locus (such as the Flp-In™ T-
Rex™ system), CRISPR-Cas9 based genome editing or knock out and replacement
approaches in which the gene is first knocked out and then the fusion protein is integrated in
a safe locus. However, these methods either require the time-consuming selection and
testing of single cell clones (CRISPR-based approaches) or are limited to standardized
commercially available cell lines and not endogenous protein expression (inducible
expression systems).

Here, we combine a rapid KI strategy for C- or N-terminal tagging based on antibiotic
selection of positively edited cells with proximity-based proteomics to detect interactors at
physiological expression levels. This versatile approach enabled us to endogenously tag
proteins with the four most commonly used labelling enzymes (APEX2, BioID2, miniTurboID
and TurboID) and compare their performance when expressed endogenously. In the past,
proximity biotinylation approaches have been instrumental to map the interactome of
membrane-bound organelles and membrane associated proteins. Therefore, we tagged
the µ1A subunit of the adaptor protein complex AP-1 (AP1µA) at the C-terminus and clathrin
light chain (CLC) at the N-terminus. AP-1, together with CLC, mediates specific transport
between the trans-Golgi network (TGN) and endosomes and transiently associates to
membranes, leaving excess, non-membrane bound AP-1 subunits and CLC in the cytosol.
Our data reveals that endogenous tagging allows proximity labelling with higher specificity
compared to simple overexpression of the labelling enzyme. Known interactors of AP-1 as
well as specific cargo were more highly enriched or even exclusively found in the
experiments performed with a KI cell line. We identified TurboID to be best suited for knock-
in proximity labelling and propose a pipeline for rapid endogenous tagging to improve the
workflow and quality of proximity-based mass spectrometry experiments. The use of this
pipeline allowed us to compare the interactome of different AP complexes, including the low-
abundant AP-4 and assemble a list of potential interactors and cargo proteins for each
adaptor protein complex. Lastly, we compared the interactome of CLCa to AP-1 and AP-2,
which are both clathrin adaptors, to show that the here presented KI-strategy with TurboID
allows identification of pathway specific interactors.
Results

Endogenous C-terminal tagging of AP1µA with labelling enzymes

To evaluate the performance of commonly used labelling enzymes, we used the CRISPR-Cas9 system to genetically fuse APEX2, BioID2, miniTurboID and TurboID to the C-terminus of our chosen target AP1µA. The insertion of a geneticin (G418) resistance cassette downstream of a polyadenylation signal into the targeted gene locus (AP1M1) allowed us to rapidly select for cells that were successfully modified. In addition, a V5-tag was inserted for KI validation via western blot and immunofluorescence. The CRISPR strategy is illustrated in Fig. 1A. Cells were transfected with plasmids encoding for a gRNA targeting the genomic locus and homology repair plasmids containing sequences of the various labelling enzymes. Three days post transfection, G418 was added, and cells were allowed to grow back to confluency to perform downstream analysis. Expression of the fusion proteins in the four KIs was validated with western blot and immunofluorescence (Fig. 1B, C). Homology-directed repair is a rare event, mainly yielding a mixed population of heterozygous cells. Therefore, we manually evaluated the overall efficacy of each KI using immunofluorescence microscopy to detect positively edited cells and found KI rates of 43% (APEX2), 63% (BioID2), 45% (MiniTurboID), 59% (TurboID). After comparison to the expression levels of edited AP1µA on the western blot, we assume that most of the cells in our population are heterozygous for AP1µA fusion protein expression.

Next, we wanted to compare the performance of the various labelling enzymes when expressed at endogenous levels. For biotin ligases, labelling times between less than 1h (miniTurboID and TurboID) and at least 16 h – 24 h (BioID2) are reported. To ensure the amount of biotinylation is sufficient for visualization and comparable between the different biotin ligases, we treated all cell lines with 50 µM biotin for 24 h to initiate the labelling. For the APEX2 peroxidase, we used the established effective labelling time of 1 min to avoid prolonged exposure to toxic H2O2. To visualize biotinylated proteins, we used fluorescently labelled streptavidin. The cargo adaptor complex AP-1 orchestrates transport between the TGN and endosomes and is reported to predominantly localize to the TGN. Thus, we expected both the fusion protein and the pool of biotinylated proteins to localize in the TGN area. The different biotin ligases BioID2, miniTurboID and TurboID and the peroxidase APEX2 localize correctly when fused to endogenous AP1µA (Fig. 1D). Importantly, biotinylated proteins and AP1µA fusions are strongly concentrated to the TGN region, marked by the TGN resident protein p230 (Fig. 1D). However, we could not find any specific biotinylation for the APEX2 peroxidase when expressed at physiological levels (Fig. 1D). To exclude general handling errors with the APEX2 sample, we transiently overexpressed Vimentin-APEX2 as well as AP1µA-APEX2 fusions and found for both constructs a specific biotinylation pattern (Supplementary Fig. 1A, B).

Quantitative analysis of the biotinylation rate of the BioID2, miniTurboID and TurboID KIs in immunofluorescence (Supplementary Fig. 1C, D) and western blot (Supplementary Fig. 1E, F) showed that shorter (2 h) labelling with MiniTurboID and TurboID leads to higher levels of biotinylated proteins than longer BioID2 labelling (24 h). This correlates well to what was originally reported for those labelling enzymes. The low biotinylation rate of BioID2 makes it, in our opinion, unfavourable compared to the TurboID variants, as a high amount of biotinylation is crucial to enrich enough protein for the subsequent identification and quantification by mass spectrometry (MS). We decided to use the AP1µAEND-TurboID-V5 (EN=endogenous) cell line for our further experiments as the TurboID fusion yielded a higher biotinylation rate compared to the other fusions (Supplementary Fig. 1D).
Fig. 1: Rapid KI strategy allows for endogenous tagging of AP1μA with different labelling enzyme for proximity biotinylation.

A: Scheme of KI strategy. AP1μA was C-terminally tagged with the labelling enzyme, a V5 tag and a resistance cassette that allows for rapid selection of positive cells.

B: Blots of whole cell lysates from generated cell lines to verify the KI of the labelling enzymes by anti-AP1μA and anti-V5 blotting.

C: Cell lines expressing the labelling enzymes endogenously were fixed and stained for the V5 tag to
detect the labelling enzyme expression and localization. D: Cells endogenously expressing the different labelling enzymes were fixed and stained with anti-V5 antibody to detect the labelling enzymes, anti-p230 antibody to mark the TGN area and streptavidin-AF488 to detect biotinylated proteins. Cells were treated with 50 μM biotin for 24 h and AP1µAEN-APEx2-V5-expressing cells were incubated for 30 min with 500 μM biotin-phenol and labelling was induced for 1 min with H2O2.

EN = endogenous. Scale bars are 10 µm.

Physiological expression of TurboID fusions permits highly specific interactome mapping

Transient overexpression of a protein can lead to artefacts such as mislocalization or aggregation10-12, increasing the chances of detecting non-specific, artificial interactors such as abundant cytosolic proteins. Physiological expression of the labelling enzyme should allow for highly specific, locally confined biotinylation of natural interactors. By applying two-color stimulated emission depletion (STED) super-resolution microscopy, we were able to visualize the biotinylation pattern after 2 h of biotin addition in AP1µAEN-TurboID-V5 knock-in cells (Fig. 2A) and compare it with AP1µA-TurboID-V5 overexpression (Fig. 2B). The high resolution achieved on the STED microscope enabled visualization of distinct nanodomains occupied by AP-1. Notably, the biotinylated proteins are primarily localized to the very same nanodomains as the endogenous AP1µAEN-TurboID-V5 fusion (indicated by the white arrows in Fig. 2A), indicating a high local specificity of the proximity labelling of the TurboID. Overall, localization of the overexpressed AP1µA-TurboID-V5 was more diffused (Fig. 2B) and we observed areas where biotinylated proteins and fusion protein did not overlap (white and yellow arrows in Fig 2B). Many cells overexpressing AP1µA-TurboID-V5 exhibit a very high background biotinylation in the cytoplasm and in the nucleus (Supplement Fig. 2A), while this was not noticed in the KI cells. Quantification of the background biotinylation revealed significantly higher cytosolic biotinylation in the transiently overexpressed cells compared to the KI cells, even after short biotin incubation (Supplement Fig. 2B).

To probe whether endogenous tagging with TurboID allows biotin labelling of AP1µA-specific interactors with increased sensitivity compared to transient overexpression, we analyzed streptavidin-purified proteins by MS using label-free quantification. Biotin was added to the culture medium for 24 h to secure detectable protein labelling in the KI cells. We analyzed KI AP1µAEN-TurboID-V5 cells and cells transiently overexpressing an AP1µA-TurboID-V5 fusion protein. As controls we used cells transiently overexpressing a cytosolic TurboID-V5 as well as WT HeLa cells that were treated with biotin for 24 h. In total, we identified and quantified 4548 proteins (Supplementary Table 1). Proteins with at least a 2-fold increase in relative intensity compared to both controls (log2 fold change >1) and a p-value <0.05 were considered significantly enriched. We found 227 proteins significantly enriched in the overexpression sample and 189 proteins significantly enriched in the KI sample (Fig 2C,D). Overall, a larger number of proteins were enriched when AP1µA-TurboID was overexpressed compared to the KI condition, which is likely to be the result of mislocalization of AP1µA-TurboID-V5, possibly leading to the biotinylation of a larger set of proteins that naturally would not interact with AP-1. We found the two large subunits of the AP1 complex (AP-1β and AP-1γ)25 significantly enriched in overexpression condition (Fig. 2C) and even more enriched in the KI cells (Fig. 2D). Notably, the other small subunit of the AP-1 complex (AP-1σ)25 was only identified when AP1µAEN-TurboID-V5 was endogenously expressed. We next looked at known interactors of the AP-1 complex to test whether endogenous tagging improves enrichment of specific interactors. The AP-1 complex is recruited to the Golgi membranes where it directly binds to the clathrin adaptors EpsinR and GGAs (Golgi-localized, y-ear-containing, Arf-binding proteins)33,34. Cell motility of membrane-bound AP-1 is conferred through the kinesin-like protein KIF13A, a microtubule-dependent motor protein that directly interacts with AP-135. Most interactors were identified in the overexpression condition, but only GGA2 and KIF13A were found to be significantly enriched. Physiological
expression of AP1µA<sup>EN</sup>-TurboID-V5, on the other hand, allowed significant enrichment of KIF13A, EpsinR (CLINT1) and all GGA proteins (GGA1-3). The difference between physiological expression levels and overexpression was even more striking when we looked at the AP-1-specific cargo proteins ATP7A and Sodium-bicarbonate co-transporter NBCn1 (SLC4A7)<sup>36,37</sup>. These AP-1 cargo proteins were all not or only slightly enriched in the overexpression experiment but significantly enriched in KI cells.

The overall impression that physiological expression of the TurboID fusion enhances the specificity of the hits we derive from our interactome data compared to overexpression can be confirmed when looking at gene ontology (GO) term analysis. While both data sets show enrichment of general GO terms such as intracellular transport or vesicle-mediated transport, AP-1-specific GO terms including post-Golgi vesicle-mediated transport or endosomal transport, are more enriched in the KI sample (Fig. 2E). Likewise, direct comparison between the two data sets shows significantly higher enrichment of all known interactors, subunits and cargo proteins in proximity labelling MS experiments performed in KI cells when compared to simple overexpression of the labelling enzyme (Fig 2F, G). The exception here is AP1µA itself, as the overexpression of the fusion proteins leads to higher enrichment in the overexpression (OE) condition (Fig. 2G).

To finally evaluate the overall quality of the MS data from the CRISPR-KI AP1µA<sup>EN</sup>-TurboID experiment, we identified possible interactors of AP1µA from the MS data and compared the two data sets (KI vs OE). We defined potential interactors as proteins that were significantly enriched and are either known to localize to the Golgi/TGN, are involved in cellular trafficking, are transmembrane proteins that might be trafficked by AP1µA or might be involved in the regulation of membrane homeostasis (e.g. regulatory kinases). For the KI cell line, we found a total of 107 potential interactors (Supplementary Table 2), which corresponds to 56% of all significant enriched hits in the MS. Evaluation of the MS data from the overexpressed AP1µA-TurboID resulted in a list of only 77 potential interactors (34% of total number of significant hits) (Supplementary Table 3). The results of our analysis are illustrated in Fig. 2H. Taken together, our findings highlight that tagging the protein with the labelling enzyme endogenously increases the sensitivity of the proximity biotinylation and the MS measurement so that very transient but specific interactors, such as specific cargo proteins, can be identified.
Fig. 2: Endogenous tagging allows for more specific proximity labelling and interactome mapping than overexpression of the labelling enzyme.

A: STED micrographs of a fixed AP1µA-STUB1 cell stained with anti-V5 antibody and streptavidin-STARORANGE to detect biotinylated proteins. Cells were treated with 50 µM biotin for 2 h before fixation. Crops show distinct overlap of biotinylated proteins and AP1µA-STUB1 (marked by white arrows).

B: STED micrographs of a fixed cell transiently overexpressing AP1µA-STUB1 that was treated as described in A. Crops show that biotinylated proteins and AP1µA-STUB1 are co-localized.

C: Graph showing enrichment of proteins with cyt TurboID vs OE.

D: Graph showing enrichment of proteins with cyt TurboID vs KI.

E: GO term enrichment for OE and KI.

F: Table showing enrichment of protein complexes with OE and KI.

G: Graph showing enrichment of proteins with OE vs KI.

H: Venn diagram showing potential interactors and enriched proteins.
TurboID-V5 accumulate in distinct zones (white arrows indicate areas of biotinylation without AP1µA-TurboID-V5, yellow arrows indicate areas of accumulated biotin ligase without biotinylated proteins). Scale bars are 5 µm for STED images and 500 nm for crops in A, B. C: Volcano plot showing the changes in relative protein intensity between the overexpression (OE) experiment and control (cytosolic overexpressed TurboID). Significant hits are shown in the top right corner (p-value < 0.05 and log2 fold change > 1) separated by the orange lines. The volcano plot only includes proteins that were significantly enriched compared to WT cells. Subunits of the AP-1 complex (red), known interactors (blue) and known cargos (magenta) are marked. Entire protein list is shown in Supplementary Table 1. D: Volcano plot showing the changes in relative protein intensity between the KI experiment (AP1µA EN-TurboID-V5) and control. Same parameters as in C. E: GO term enrichment analysis showing enrichment of selected GO terms in overexpression (OE) and KI condition. F: Table of the analysed subunits, interactors and cargo proteins. Differences (Diff.) in log2 fold enrichment are indicated. G: Volcano plot showing the changes in relative protein intensity between KI experiment and OE experiment. Same parameters as in C. H: Venn diagram showing the number of potential interactors (defined by protein localization and function (see methods) and significant enrichment). Lists of potential interactors are shown in Supplementary Tables 2 and 3.

Interactome mapping of different AP-complexes with endogenous TurboID fusions

The comprehensive interactome data resulting from endogenous tagging of AP1µA with TurboID encouraged us to apply the rapid knock-in TurboID approach to different proteins to probe its versatility and, in particular, to reveal the native interactome of AP complexes. Five different AP complexes (AP-1, AP-2, AP-3 and AP-4 and the more recently discovered AP-5) are responsible for sorting cargo throughout the endo-lysosomal system of human cells. Our overall understanding of the intracellular role of the different AP complexes would greatly benefit from a better knowledge of their interactome. Aside from a few cargo proteins that are often used as model cargos, little is known about which proteins are sorted by which adaptor protein in mammalian cells. Recent proteomic studies have shed light on cargo sorting pathways in yeast cells, however such a comparative and comprehensive study is still lacking for mammalian cells. We also do not fully understand the mechanisms of recruitment of AP complexes to different membranes. Although AP-1, AP-3 and AP-4 are all known to be recruited by ARF1 to TGN/endosomal membranes, they localize to distinct endosomal buds, suggesting the presence of different unknown interactors. Due to their role as key regulators of intracellular trafficking, dysfunction of AP complexes is linked to a variety of diseases and a better understanding of the interaction partners could help reveal the underlying mechanistic basis of pathology. While AP-1 is known to locate to the TGN and endosomal membranes, where it coordinates clathrin-dependent trafficking between the two organelles, AP-2 is found at the plasma membrane, where it recruits clathrin for clathrin-mediated endocytosis. AP-3 is thought to localize to the same endosomes as AP-1 but has different cargo clients, which points towards a role in trafficking to the lysosome. AP-4 binds to TGN membranes, where it mediates transport of autophagosomal factor ATG9A. The low abundance of AP-4 (about 40 fold lower than AP-1 or AP-2 in HeLa cells) makes it an interesting target to test the endogenous TurboID tagging on a very low abundant protein. AP-5 is thought to be involved in the transport from the late endosome to the Golgi or the lysosome and to also contribute to lysosome maintenance. It is different from other AP complexes as it associates with two additional proteins and is not recruited to intracellular membranes by ARF1 as are AP-1, AP-3 and AP-4. However, as it is as comparably low abundant as AP-4 and was not precisely localized so far, we decided not to include it in this study as it would be difficult to confirm correct integration of the endogenously tagged subunit.

We C-terminally tagged the µ-subunit of the different AP complexes with TurboID and tested the expression and localization of the fusion protein with immunofluorescence imaging and western blot (Fig. 3A,B). After 24 h of biotin treatment, we were able to detect extensive biotinylation of proteins via western blot and in immunofluorescence imaging experiments for
all AP complexes, even for the low abundant AP4µEN-TurboID-V5 fusion. To map and
compare the interactome of the four AP complexes we analysed streptavidin-purified
biotinylated proteins by MS using label-free quantification for all the KIs. As a control we
used HeLa WT cells that were treated with biotin. In total, we identified and quantified 2574
proteins (Supplementary Table 4). In order to identify specific interactors and cargo proteins
for each AP complex, we compared the relative intensity of a given protein between data
sets for various AP complexes. Proteins with at least a 2-fold increase in relative intensity
(log2 fold change >1 or <-1) and a p-value <0.05 were considered significantly enriched. By
doing so, we were able to identify AP-2 complex specific interactors such as Epsin-1 (EPS1)
and Epsin-2 (EPS2), Synaptojain-1 (SYNJ1) and the protein numb like (NUMB) that are all
known for their role in clathrin mediated endocytosis (Fig. 3C). Importantly, the data set for
the low abundant AP4µEN-TurboID-V5, highlighted several known AP-4 specific interactors
such as Tepsin (ENTDH2), Hook1 (HOOK1) and a FHF complex subunit (FAM160A1)55 (Fig.
3D). Comparison of the interactome of various AP complexes allowed not only the
identification of interactors that are specific for one single AP complex (e.g. GGA proteins
and the PI4-kinase PI4K2B for AP-156,57), but also revealed potential common interactors
such as the SNARE protein Vamp7 that is enriched for AP-1 as well as AP-3 (Fig. 3D). Aside
from potential uncharacterized effectors, endogenous TurboID-tagging enabled the
identification of potential cargo specific for each AP complex. We identified known
cargo proteins (various integrins (ITGA5, ITGAV or ITGA1) for AP-2 or ATG9A for AP-4) and
also novel potential cargo proteins such as the ring finger protein 121 (RNF121), a Golgi
localized protein with anti-apoptotic effects in cancer cells58, the plasma-membrane-localized
cation channel TRPM7, the TGN-localized calcium transporter ATP2C2 for AP-1 and the
lysosomal Cl-/H+ Antipporter CIC-7 (CLCN7) for AP-3 (Fig. 3C,D). Those potential cargo
proteins all have at least one tyrosine-based YXXφ motif in their cytoplasmic domains that is
one of the motifs necessary for sorting via AP-1 or AP-3. We compared the significantly
enriched proteins from the data sets of each AP complex and assembled a list of potential
interactors and potential cargo proteins for each AP complex (Supplementary Table 5).
Potential interactors and cargo proteins were defined proteins that are known to be involved
in membrane trafficking, might be involved in regulation of membrane homeostasis (e.g.
regulatory phosphatases and kinases), have a transmembrane domain (potential cargo
proteins) or are known to play a role in clathrin mediated endocytosis (for AP-2). In total, we
identified more than 300 potential interactors and more than 200 potential cargo proteins.

We then further tested some of the unexpected hits we found in the AP1µA data. We
selected Integrin beta 1 (ITGB1), the SNARE protein VAMP7 and SCY1-like 2 (SCYL2).
ITGB1 could be a potential cargo protein of AP-159. VAMP7 is a component of a SNARE
complex composed of syntaxin-8, syntaxin-7, VAMP7 and VTI1B that is involved in
endosomal recycling of endocytosed material60, and interestingly, we found all four members
as potential AP-1 interactors. So far, VAMP7 was only reported to interact with AP-3, and no
direct interaction with the other adaptor complex, AP-1, was observed61,62. SCYL2 was
originally identified as a protein kinase for AP-264 but has also been connected to AP-1- and
AP-3-mediated trafficking64 as well as clathrin-dependent TGN export in plants65, but its
overall role remains poorly understood. We transiently expressed GFP-fusions of the three
proteins in an AP1µA EN-SNAP-V5 KI cell line, where AP1µA has been tagged with a SNAP
tag (Supplementary Fig. 3), enabling visualization of endogenous AP1µA in living cells. Live-
cell confocal microscopy showed that vesicular AP-1 structures can be found on tubular
compartments positive for either ITGB1 or VAMP7 (Fig. 3E). Similarly, we find punctuated
SCYL2 structures perfectly colocalizing with vesicular AP1µA (Fig. 3E). All three proteins are
thus likely to interact with the adaptor complex AP-1, as suggested by their close proximity in
living cells. Interestingly, ITGB1 is found together with AP-1 in the peripheral areas of the
cell, pointing towards a possible role for AP-1 in the endocytic recycling of the integrin, a role
that has been described for AP-1 in the recycling of the transferrin receptor\textsuperscript{66}. The close proximity of SCYL2 and VAMP7 to vesicular AP-1-positive structures suggests some regulatory functions of those proteins in AP-1 mediated trafficking.

**Fig. 3:** Interactome comparison of AP complexes with endogenous TurboID-tagging. 

A: HeLa KI cells expressing the endogenous AP \(\mu\) subunits 1-4 fused to TurboID-V5 were fixed and stained with anti-V5 antibody to detect the labelling enzyme and streptavidin-AF488 to detect
visualization of TurboID activity in all four KI cell lines on a western blot. Cells were treated with 50 µM biotin for 24 h before fixation. **B:** Volcano plot showing the changes in relative protein intensity between AP1µAEN-TurboID-V5 and AP2µ EN-TurboID-V5. Proteins that show significant changes in their relative intensity are shown in the top left (AP-1) and top right (AP-2) corner (p-value <0.05 and log2 fold change >1 or <-1) separated by the orange lines. Subunits of the AP complexes (red), potential interactors (blue) and potential cargos (magenta) are marked. **D:** Volcano plot showing the changes in relative protein intensity between AP3µAEN-TurboID-V5 and AP4µEN-TurboID-V5. Same parameters as in **C.**

**E:** HeLa AP1µAEN-SNAP-V5 KI cells labelled with JFX650-BG that were transiently transfected with plasmids encoding for ITGB1-eGFP, eGFP-VAMP7 and eGFP-SCYL2 (left to right). Crops show where AP1µA domains are observed in close proximity to structures defined by the various proteins tested (marked by yellow arrows). Scale bars are 10 µm and 1 µm for crops in **A, E.**

**N-terminal endogenous tagging to map the interactome of CLCa**

To test whether we can apply our rapid KI strategy also for N-terminally tagged proteins, we fused the different labelling enzymes to the N-terminus of endogenous clathrin light chain A (CLCa). To create N-terminal fusions, the resistance cassette is inserted between LoxP sites upstream of the start codon of the V5-tag and the TurboID18. In a second step, the resistance cassette is then excised via transfection of Cre recombinase. This step becomes necessary as the presence of the resistance cassette in the genome could possibly isolate the gene from its promoter region and cause it not to be transcribed. The strategy used for N-terminal tagging is illustrated in Fig. 4A. Successful expression of the fusion protein was confirmed on western blot and with immunofluorescence microscopy (Fig. 4B, C). Moreover, immunofluorescence microscopy of the CLCa-fusion proteins together with clathrin heavy chain (CHC) shows that both proteins colocalize, suggesting correct localization and function of the fusion proteins (Fig. 4D). The overall KI rates were comparable to C-terminal tagging (55% (APEX2), 25% (BioID2), 51% (MiniTurboID), 71% (TurboID)). Alike for C-terminally tagged AP1µA, we observed locally confined biotinylation for the biotin ligases but no specific biotinylation for the APEX2 fusion protein (Fig. 4D).

We then used the V5-TurboID-CLCaEN KI cell line to map the CLCa interactome by analyzing streptavidin-purified biotinylated proteins with MS using label-free quantification (Supplementary Table 6). CLCa fulfils multiple roles in intracellular trafficking such as coating membranes during endocytic events or trafficking intermediates shuttling between the Golgi/TGN and endosomes67. Clathrin does not directly bind membranes but uses adaptor proteins such as adaptor protein complexes AP-1 for Golgi-endosome trafficking and AP-2 for clathrin-mediated endocytosis25. As we already have performed interactome mapping experiments for those two adaptors, a direct comparison between the data sets generated from CLCa and AP-1 or AP-2 TurboID KIs allows the sequestering of CLCa interactors that are involved in endocytosis from those involved in intracellular post-Golgi transport. A comparison of CLCa against AP-1 shows strong enrichment of proteins such as Epsin-1 (EPS1), Synaptotagmin-1 (SYNJ1) and the protein numb like (NUMB) that are known for their role in clathrin-mediated endocytosis (Fig. 5A). Furthermore, GO terms such as endocytosis, clathrin-mediated endocytosis and import into cell are strongly enriched (Fig. 5B). On the contrary, comparison of the interactome of CLCa with AP-2 shows proteins enriched that are involved in AP-1 dependent post-Golgi transport such as the GGA proteins and the HEAT repeat containing 5B (HEATR5B)86 (Fig. 5C). Here, among the most enriched GO terms we find intracellular transport and Golgi-vesicle transport (Fig. 5D). In conclusion, endogenous N-terminal tagging of CLCa with TurboID granted a highly specific interactome data set that, when combined with data sets from AP-1 or AP-2, allowed mapping of pathway-specific interactors.
Fig. 4: Endogenous N-terminal tagging of CLCa with labelling enzymes.

A: Scheme of KI strategy. CLCa was N-terminally tagged with the labelling enzyme and a V5 tag. The integrated resistance cassette can be excised with the loxP-Cre-system after transfection with the Cre recombinase.

B: Blots of whole cell lysates from generated cell lines to verify the KI of the labelling enzymes by anti-V5 blotting.

C: Cell lines expressing the labelling enzymes endogenously were fixed and stained for the V5 tag to detect the labelling enzyme expression.

D: Cells endogenously expressing the different labelling enzymes were fixed and stained with anti-V5 antibody to detect the labelling enzymes, anti-CHC antibody to detect the endogenous clathrin and streptavidin-AF488 to detect biotinylated proteins. Cells were treated with 50 μM biotin for 24 h and
V5-APEX2-CLCa<sup>EN</sup> expressing cells were incubated for 30 min with 500 μM biotin-phenol and labelling was induced for 1 min with H2O2. Scale bars are 10 μm.

**Fig. 5:** Comparison of interactome data set allows mapping of pathway specific clathrin interactors.

A: Volcano plot showing the changes in relative protein intensity between AP1µA<sup>EN</sup>-TurboID-V5 and V5-TurboID-CLCa<sup>EN</sup>. Significant hits (p-value <0.05 and log<sub>2</sub> fold change >1) are separated by the orange lines in the top right. Clathrin chains (red) and exemplary proteins involved in clathrin-mediated endocytosis (blue) are marked.

B: GO term enrichment analysis showing the most enriched GO terms comparing enrichment for CLCa against AP1µA.

C: Volcano plot showing the changes in relative protein intensity between AP2µ<sup>EN</sup>-TurboID-V5 and V5-TurboID-CLCa<sup>EN</sup>. Same parameters as in A. Clathrin chains (red) and exemplary proteins involved in post-Golgi transport (blue) are marked.

D: GO term enrichment analysis showing the most enriched GO terms comparing enrichment for CLCa against AP2µ.

**Discussion**

Employing an antibiotic-based CRISPR KI strategy for C- and N-terminal tagging, we endogenously fused the commonly used labelling enzymes APEX2, BioID2, miniTurboID and TurboID to AP1µA and CLCa (Fig. 1, 4). We identified the biotin ligase TurboID as best suited for the KI approach in combination with proximity labelling experiments, as it combines high KI rates with favourable labelling kinetics (Supplementary Fig. 1). The lower KI rates of miniTurboID compared to TurboID might be result of lower stability of miniTurboID fusion proteins, which has already been reported in other studies<sup>9,68</sup>. Faster labelling kinetics are
not only beneficial to reliably generate sufficient amounts of biotinylation to enrich enough
protein for the subsequent identification and quantification by MS but are also advantageous
when designing experiments that require short labelling times. Generally, the incubation time
with the biotin might be adapted according to the abundance of the POI, when working with
highly abundant proteins, incubation times shorter than 24 h with biotin could be used.
Previous work has already demonstrated the TurboID variants outperform BioID or
BioID29,27,28,68, however BioID2 is still a commonly used enzyme. Recently, new variants of
the BioID2 were introduced, microID and ultraID69. With a molecular weight below 20 kDa,
they are significantly smaller than the here presented labelling enzymes. Especially the
ultraID is reported to have labelling kinetics similar to TurboID with less background activity.
In addition, an ancestral BioID variant called AirID was developed with faster labelling
kinetics than BioID and more specific labelling compared to TurboID70. Another possibility to
reduce labelling background of TurboID is the use of a light-controlled variant of the TurboID
(LOV-Turbo)71. It would be interesting to see how these variants perform when used for
tagging at the endogenous locus. Practically, to prevent background biotinylation of TurboID,
cells can be grown in biotin-free media 72 h prior to the experiment. This becomes crucial in
interactome mapping experiments that, for example, require induced changes of the cell
state, as labelling should only occur after the change was induced. To our surprise, we could
not achieve any biotinylation with the APEX2 peroxidase expressed as a low abundance
endogenous fusion (Fig. 1, 4). The short labelling time in combination with the low
physiological expression does not yield detectable proximity labelling in our hands.
Generally, CRISPR KI approaches to tag endogenously with APEX2 were reported to be
functional15,72; however, for us, multiple attempts to induce labelling failed. We can only
speculate that highly concentrated APEX2 is needed to get sufficient biotinylation when it is
endogenously expressed. Specific confined cellular environments, such as nucleus or stress
granules, would favour such a high concentration of an APEX2 fusion protein. On the other
hand, soluble cytosolic proteins may not be optimal for an APEX2 approach. Yet, labelling
with TurboID and biotin worked reliably and was easy to induce for peripheral cytoplasmic
machinery like adaptors and clathrin.

Using the AP1μAEN-TurboID-V5 cell line created with the KI strategy, we were able to
compare the enrichment of known interactors and cargo proteins of the AP-1 complex in
quantitative MS measurements against data sets generated with cells overexpressing
AP1μA-TurboID-V5. Strikingly, we found known interactors to be significantly more enriched
when AP1μAEN-TurboID-V5 was expressed at endogenous levels (Fig. 2). Especially relevant
for our research is the strong enrichment of AP-1 cargo proteins that can be only observed
when TurboID is endogenously fused to AP1μA, highlighting the importance of matching the
endogenous expression levels. The increased sensitivity for real interactors is likely a result
of less unspecific labelling due to mislocalization- or aggregation artefacts induced by
overexpression of the labelling enzyme fused to the POI. A larger background of peptides
from unspecifically labelled proteins increases the sample complexity and therefore lowers
the overall sensitivity of the MS measurements, as all peptides compete for ionization and
detection. Endogenous protein tagging with a biotin ligase that allows for highly confined
biotinylation (Fig. 2A), therefore increases the chances for detection of specific interactors,
especially if they are of low abundance. Importantly, endogenous expression of the
AP1μAEN-TurboID fusion resulted in a more comprehensive list of potential interaction
partners compared to overexpression of AP1μA-TurboID (Supplementary Tables 2, 3 + Fig.
2E,H). Using the described KI-pipeline, we tagged the μ-subunit of different AP complexes
with TurboID and comparably analyzed their interactome in quantitative MS measurements
(Fig. 3). The provided lists of potential interactors and cargo proteins for each individual AP
complex (Supplementary Table 5) not only demonstrate the versatility of the approach but
also present a database that can contribute to the better understanding of AP-driven
intracellular sorting. Our data sets can be used as a starting point for studies aimed at unravelling the mechanisms of spatially confined recruitment of different TGN- and endosome-associated AP complexes. Furthermore, linking the AP complexes to the different potential cargo proteins will shine light on the intracellular sorting and trafficking routes of those proteins. By picking three proteins from our list of potential AP-1 interactors (ITGB1, VAMP7 and SCYL2) and studying their localization in respect to AP-1 in living cells (Fig. 3E), we could show that they all localize in the vicinity of AP-1. Lastly, we mapped the interactome of the N-terminally TurboID-tagged CLCa and compared the data to the data sets of AP1µA and AP2µ (Fig 5, Supplementary Table 6). By doing so, we were able to identify pathway-specific interactors of CLCa.

In summary, endogenous tagging with biotin ligases enables highly specific proximity labelling and increases the sensitivity for real interactors that might be transient or of low abundance. Our pipeline presents an alternative to classical CRISPR-based approaches that would require single-cell selection of positive TurboID clones and can be applied to any cell line as long as it can be reliably transrected and selected. The pipeline for the generation of CRISPR KIs, based on the integration of a resistance cassette, allows rapid generation of endogenously tagged cells and can be applied to low abundant target proteins like AP-4.

Aside from obvious time and work reduction, it also avoids artefacts that arise from single clone behaviour. In particular, for naturally low abundant proteins, inducible systems may still trigger non-physiological protein expression.

Rapid endogenous tagging with TurboID enabled us to map and compare the interactome of 4 different AP complexes and revealed known but also novel AP specific interactors. The ease and speed of the KI generation makes it an attractive alternative to transient overexpression of the labelling enzyme or classical KI approaches using single cell selection, as the MS experiments can be done in about 4-5 weeks after the CRISPR transfection. Additionally, the tools provided here can be used in a variety of different cell lines for the identification of cell type-specific and physiological cargoes.

**Acknowledgments**

We acknowledge the whole lab for the fruitful discussion during the planning and writing phase of this manuscript. This project was supported by Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) grants SFB958 (Project A25), SFB/TRR186 (Project A05 (CF) and Project A20 (FB)) and a major research instrumentation grant for the acquisition of the STED microscope. For mass spectrometry, we acknowledge the assistance of the Core Facility BioSupraMol supported by the Deutsche Forschungsgemeinschaft (DFG).

**Methods**

*Antibodies and Streptavidin conjugates*

All dyes and antibodies used in this study are provided in Supplementary Table 7 in the supplementary information.

*Mammalian cell culture*

All experiments were carried out in HeLa cells ATCC CCL-2 (ECACC General Collection) grown in a humidified incubator at 37°C with 5% CO₂ in DMEM (Gibco-ThermoFisher) supplemented with 10% fetal bovine serum (Corning) and penicillin/streptomycin (Lonza Bioscience) to prevent contamination. For transient transfection, HeLa cells at 70-80% confluency were transfected with FuGENE HD Transfection Reagent (Promega) according to the supplier’s protocol.
Generation of CRISPR-Cas9 knock-in cell lines

See supplementary information.

Plasmid design of overexpression plasmids

See supplementary information.

Immunofluorescence

For all immunofluorescence samples, 40,000 cells were seeded on fibronectin-coated coverslips. Biotinylation was induced by replacing the growth media with medium containing 50 µM biotin and samples were incubated for 24 h or 2 h as indicated in the figures. Biotinylation for AP1µA\textsuperscript{EN}-APEX2-V5 cells was induced as described by Hung et al.\textsuperscript{29}. All cells were washed twice with PBS and then fixed in 4% PFA for 10 min at RT. Subsequently, they were rinsed three times with PBS and incubated for 3 min in permeabilization buffer (0.3% NP40 (Roth), 0.05% Triton-X 100 (Sigma Aldrich) and 0.1% BSA (IgG free) (Roth) in PBS). Cells were blocked for 1 h in blocking buffer (0.05% NP40, 0.05% Triton-X 100 and 5% goat serum (Jackson ImmunoResearch) in PBS) at RT and then incubated with primary antibodies in blocking buffer overnight rocking at 4°C. On the next day, the samples were washed three times 5 min in washing buffer (0.05% NP40, 0.05% Triton-X 100 and 0.2% BSA (IgG free) in PBS) before incubation with the respective secondary antibodies in blocking buffer for 1 h rocking at RT. For visualization of biotinylated proteins either Streptavidin-Alexa488 (for confocal microscopy) or Streptavidin-STARORANGE (for STED microscopy) was added to the secondary antibody mix. The cells were then washed three times 5 minutes with wash buffer and then dipped in dH\textsubscript{2}O before mounting with ProLong Gold Antifade Reagent (Thermo Fisher Scientific). Mounted samples were let to harden overnight at RT and then stored at 4°C until imaging.

Live-cell imaging

For live-cell imaging experiments, 100,000 cells were seeded on glass-bottom dishes (3.5 cm diameter, No. 1.5 glass; Cellvis), coated with fibronectin (Sigma) beforehand. One day after seeding, KI cells expressing the AP1µA-SNAP fusion were labelled using an O\textsuperscript{6}-benzylguanine (BG) substrate (JFX650-BG) (1µM) in culture medium for 1 h.\textsuperscript{73} After the labelling, cells were washed for at least 1 h in culture medium. Live-cell imaging was carried out in FluoroBrite DMEM (Gibco) supplemented with 10% FBS, 20 mM HEPES (Gibco) and GlutaMAX (Gibco). For live-cell imaging experiments a microscope incubator (Okolab) was used to keep the stage and sample at 37°C.

Imaging and image processing

Confocal and STED imaging was carried out on a commercial expert line Abberior STED microscope equipped with 485 nm, 561 nm and 645 nm excitation lasers. For two-color confocal live-cell imaging both signals were detected simultaneously, detection windows were set to 498 to 551 nm and 650 to 756 nm. For two-color STED experiments both dyes were depleted with a 775 nm depletion laser. The detection windows for the dyes were set to 498 to 551 nm, 571 to 630 nm and 650 to 756 nm. Excitation power was kept constant between samples in the same experiment to be able to quantify differences in expression levels and biotinylation. The pixel size was set to 60 nm for confocal and 20 nm for STED.

All images shown were smoothed using a Gaussian filter with 1-pixel SD using ImageJ.\textsuperscript{74} For better representation of the AP4µ\textsuperscript{EN}-TurboD-V5 and live-cell images, the background was subtracted using a rolling ball radius of 50.0 pixels.

Image analysis and statistical analysis
All image analysis was carried out with ImageJ. To determine the ratio of biotinylated proteins and V5-tagged AP1µA-labelling enzyme fusions in Supplementary Fig. 1D, a small region in the Golgi area was selected in the raw image and the average grey values were measured for both channels. The ratio between the mean intensity fluorescence of the biotinylated proteins in the Golgi area versus the mean fluorescence intensities from the V5 channel was then calculated. For each condition at least 40 cells were analysed from three independent experiments.

To analyse background biotinylation in Supplement Fig. 2B, a small region in a cytoplasm was selected and the mean intensity fluorescence was measured. At least 20 cells from each condition were analysed.

Statistical analysis (t-tests) was carried out with Prism. P-values are indicated in figure legends.

**Quantification of KI rate**

To estimate the number of gene-edited cells, V5-immunostained cells for each KI cell line were screened for the presence of V5 signal using confocal light microscopy. 100 cells were screened for each cell line.

**Knock-in verification via western blot**

For each knock-in cell line, 180 000 cells were seeded on a 6-well plate. 24 h later cells were washed twice with PBS and harvested in 350 μl of Laemmli sample buffer. The samples were boiled for 10 min at 98°C before loading 30 μl of each sample on two separate 4-12% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham) via wet blotting. Both membranes were washed once with phosphate buffered saline with 0.05% Tween (PBST) and then blocked for 1 h with 5% (wt/vol) milk powder and 1% BSA in PBST rocking at RT. After that, membranes were washed once 5 min with PBST and two times 5 min with PBS before incubation with the respective antibodies overnight rocking at 4°C. On the next day, the membranes were washed three times 5 min with PBST and incubated with a secondary antibody coupled to HRP in 5% (wt/vol) milk powder and 1% BSA in PBST for 30 min rocking at RT. The membrane was washed twice with PBST and twice with PBS for 5 min each. To develop the membrane the ECL western blot substrate was added for 2 min and then the membrane was imaged.

**Western blot analysis of biotinylated proteins**

For detection of biotinylated proteins on a western blot, 800 000 HeLa WT and all KI cell lines were seeded on a 10 cm cell culture dish. Starting on the next day, the media was replaced with biotin-containing medium (50 μM biotin) and samples were incubated for 24 h or 2 h at 37°C. The biotin addition was timed in a way that all samples could be harvested at the same time. Biotinylation for AP1µA<sup>EN</sup>-APEX2-V5 cells was induced as described Hung et al. For harvesting, the cells were washed twice with ice-cold PBS and then extracted in 400 μL of ice-cold lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 0.5 mM DTT and protease inhibitors). Cells were then centrifuged for 10 min at 4°C at 14,000g to clarify the cell lysates. 20 μL of the whole-cell lysates were mixed with Laemmli buffer and boiled at 95°C for 10 min before loading on a 4-12% SDS-polyacrylamide gel. Two separate gels were used, one for detection of biotinylated proteins and the other for detection of the fusion protein. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham) via wet blotting. To visualize biotinylated proteins on the membrane, after blocking (5% (wt/vol) milk powder in PBST), the blot was incubated with 0.3 μg/mL
streptavidin-HRP in 3% BSA in PBST for 30 min rocking at RT. The western blot to detect
the fusion protein with the V5 tag was carried out as described above.

For quantification of the biotinylated proteins in relation to the available labelling enzyme
(Supplementary Fig. 1F) the intensity of the line on the biotinylation blot was measured in
ImageJ and set in relation to the intensity of the corresponding V5 line. To measure the
intensity of a line of biotinylated proteins in ImageJ, a box was drawn in the middle of the
measured signal and the average intensity value of this area was used for analysis. The
respective position and size of the box was kept constant for each line of a blot. The V5 tag
intensity was measured accordingly except that the box was always placed around the area
of the strongest signal. For each experiment the values were normalized to the AP1μA\textsuperscript{EN-}
TurboID-V5 sample that was incubated for 24 h with biotin. The experiment was repeated
two times.

Preparation of MS samples, LC-MS and data analysis
See supplementary information.

Identification of potential interactors of AP1μA comparing KI and overexpression
For identification of potential interactors only proteins that were significantly enriched (p-
value <0.05 and \( \log_2 \) fold change > 1) against both WT cells and cytosolic TurboID control
were considered. These proteins were then screened with Uniprot to identify proteins that are
either known to localize to either the Golgi or TGN, are thought to be involved in cellular
trafficking, or are transmembrane proteins that might be trafficked by AP1μA or could control
trafficking events (e.g. kinases, phosphatases). Together with all uncharacterized proteins
these proteins were then considered potential interactors and summarized in Supplementary
Tables 2, 3.

Identification of potential interactors and cargo proteins comparing different AP complexes
For identification of potential interactors and cargo proteins only proteins that were
significantly enriched (p-value <0.05 and \( \log_2 \) fold change > 1) compared to at least one other
AP-complex and also higher enriched than in the WT control (\( \log_2 \) fold change > 0) were
taken into consideration. These proteins were then screened with Uniprot to identify proteins
that are either known to localize to either the Golgi and endosomal system (or to CCP in the
case of AP-2), are thought to be involved in cellular trafficking, or are transmembrane
proteins that are potential cargo proteins or could control trafficking events (e.g. kinases,
phosphatases). All of those proteins that have at least one transmembrane domain were
considered potential cargo proteins, all others were termed potential interactors. The results
were summarized in Supplementary Table 5 (switch between sheets for different AP-
complexes) and it was stated in which AP comparisons these proteins were identified.

GO term analysis
For GO Term analysis only proteins were used that were significantly enriched (p-value
<0.05 and \( \log_2 \) fold change > 1) against both WT cells and cytosolic TurboID control (in case
of KI vs overexpression, Fig. 2E) or against WT cell control and AP1μA/ AP2μ sample (for
V5-TurboID-CLCa, Fig. 5). GO Term analysis was performed using the GO::TermFinder
open source software\textsuperscript{75}.

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