

Improved Proximity Labeling for Quantitative Proteomic Profiling of Primary Cilia in Previously Inaccessible Cell Types

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"The fox rejoices in the whirlwind."

– Greek proverb

I dedicate this work with deep gratitude
to my family and friends who have
accompanied me on this journey.

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I. Summary

Gaining insight into the molecular framework of primary cilia biology is undoubtedly important to better our understanding of molecular processes involved in cell fate determination and homeostasis. This niche domain of the cell is a highly dynamic, specialized compartment that is minute compared to the total cell volume (100-fold less surface area and 2500-fold less volume than a cell body). As a membrane protrusion open to the cell side, methods for proteomic analysis are lacking, as methods such as deciliation and a large number of protein-protein interaction studies cannot reflect the full picture of the proteome. Therefore, a proteomic tool with particularly high sensitivity and specificity is required.

Deciphering new ciliary candidates expands our knowledge of well-established signaling pathways and potentially enables surprising new connections to other cellular domains. Therefore, it is vital to explore primary cilia in various cellular contexts and compare the intricate proteomic differences and similarities between vastly different tissues. Here, engineered ascorbate peroxidase (APEX2)-based proximity labeling (PL) offers remarkable mapping capabilities with unmatched spatial and temporal resolution. Unfortunately, the system requires the addition of exogenous hydrogen peroxide (H_2O_2), which has so far limited the use of the system in *in vivo* studies, as the compound causes apoptotic oxidative cell damage. This has led to a lack of alternatives in a field dominated by biotin ligases (e.g., BioID or TurboID) that can be used in different cell contexts, as biotin supplementation is sufficient to perform proximity labeling.

In the first part of this work, I tested the specificity and temporal resolution of the APEX2 system against the biotin ligase-based PL of TurboID in primary cilia of murine inner medullary collecting duct cells (IMCD3). This cell line is a model system for kidney primary cilia research and was subject of well-established proteomic studies using APEX2 and BioID. However, a direct comparison of the two proteomic methods has never been carried out. The resulting comparison demonstrated some proteomic overlap between APEX2 and TurboID in primary cilia with enzyme-specific target differences potentially due to the enzymes' different covalent biotin-attachment mechanisms. TurboID exhibited higher cilia non-specific labeling background with lower temporal resolution compared to the APEX2 equivalent. The observed superiority of the ascorbate peroxidase in proteome coverage in a much shorter time prompted me to explore ways of using the proteomics tool in all cell types.

Integration of the APEX2 system in other well-established murine cell types (i.e., fibroblasts NIH/3T3, myoblasts C2C12 and preadipocytes 3T3-L1) was a challenge as non-targeted excess of H₂O₂ and tyramide substrate led to unspecific labeling at other sites of the cell by endogenous carboxylases and peroxidases.

To consolidate an improved APEX (hereafter termed “iAPEX”) method, I proposed a system in which both the flavoenzyme D-amino acid oxidase (DAAO) from yeast *Rhodotorula gracilis* and APEX2 are genetically targeted to primary cilia to allow concatenation of *in situ* H₂O₂ production and compartment-specific proximity labeling. DAAO catalyzes the multi-step stereoselective oxidative deamination of natural D-amino acids into their respective α -keto acids. The enzymatic reaction is dependent on noncovalently bound flavin-adenine dinucleotide (FAD) that becomes reduced to FADH₂ in the first half reaction. The resulting imino acid dissociates from the enzyme and spontaneously hydrolyzes thereby producing ammonium. FADH₂ is reoxidized by molecular oxygen in the second half reaction thereby releasing H₂O₂. A scavenging APEX2 in the vicinity should then be able to biotinylate surrounding proteins, given substrate is available. This cascade-like two-component system bypasses the need for exogenous H₂O₂.

The proposed iAPEX two-component system was established and then thoroughly tested in IMCD3 cells. The iAPEX proteomic workflow enabled the identification of new ciliary candidate proteins, which were confirmed in the further course of the project. The method was then expanded onto the previously inaccessible cell types for APEX2 proximity labeling to verify the efficacy and specificity of the proposed method. This objective demanded the development of a system for rapid cell line generation using lentiviruses by means of elaborate multicistronic vector systems. Finally, the proteomic findings of iAPEX proximity labeling in IMCD3 and NIH/3T3 cells were compared to reveal similarities and differences in their primary cilia contents.

This work contributes to advanced molecular studies, by providing an exploratory proteomics tool that allows specific, spatially restricted labeling of even narrow hair-like cellular domains. iAPEX now alleviates exploration of a potential cross-tissue cilia protein atlas and their cell type specific differences. By improving proteomic approaches such as APEX2 proximity labeling in terms of their availability and specificity, this will in the future enable the identification of novel drug targets for the development of potential treatments to alleviate the conditions of patients previously limited to symptom-orientated options.

II. Zusammenfassung

Ein Einblick in die molekularen Grundlagen der Biologie der primären Zilien ist zweifellos wichtig für ein besseres Verständnis der molekularen Prozesse, die an der Bestimmung des Zellschicksals und der Homöostase beteiligt sind. Diese Nischendomäne der Zelle ist ein hochdynamisches, spezialisiertes Kompartiment, das im Vergleich zum gesamten Zellvolumen winzig ist (100-mal weniger Oberfläche und 2500-mal weniger Volumen als der Zellkörper). Da es sich um eine zur Zellseite hin offene Membranausstülpung handelt, fehlt es an Methoden für die Proteomanalyse, da Methoden wie die Dezilierung und eine große Anzahl von Protein-Protein-Interaktionsstudien nicht das vollständige Bild des Proteoms widerspiegeln können. Daher wird ein proteomisches Werkzeug mit besonders hoher Empfindlichkeit und Spezifität benötigt.

Die Entschlüsselung neuer Kandidatenproteine in Primärzilien erweitert unser Wissen über gut etablierte Signalwege und ermöglicht es, überraschende neue Verbindungen zu anderen zellulären Bereichen herzustellen. Daher ist es von entscheidender Bedeutung, primäre Zilien in verschiedenen zellulären Kontexten zu untersuchen und die proteomischen Unterschiede und Gemeinsamkeiten zwischen sehr unterschiedlichen Geweben zu vergleichen. Hier bietet die verbesserte Ascorbatperoxidase (APEX2)-basierte Näherungsmarkierung (eng.: „proximity labeling“) bemerkenswerte Proteom-Kartierungsmöglichkeiten mit unübertroffener räumlicher und zeitlicher Auflösung. Leider erfordert das System die Zugabe von exogenem Wasserstoffperoxid (H_2O_2), was die Verwendung in *in vivo*-Studien bisher eingeschränkt hat, da diese Verbindung apoptotische oxidative Zellschäden verursacht. Dies hat zu einem Mangel an Alternativen in einem Bereich geführt, der von Biotin-Ligasen (z. B. BioID oder TurboID) beherrscht wird, die in verschiedenen Zellkontexten verwendet werden können, da eine Biotin-Ergänzung ausreicht, um eine Näherungsmarkierung durchzuführen.

Im ersten Teil dieser Arbeit testete ich die Spezifität und zeitliche Auflösung des APEX2-Systems gegenüber der Biotin-Ligase-basierten Näherungsmarkierung von TurboID in primären Zilien von murinen inneren medullären Sammelgangzellen (IMCD3). Diese Zelllinie ist ein Modellsystem für die Erforschung der primären Zilien der Niere und war bereits Gegenstand etablierter proteomischer Studien mit APEX2 und BioID. Ein direkter Vergleich der beiden proteomischen Methoden wurde jedoch noch nie durchgeführt. Der in dieser Arbeit durchgeführte Vergleich zeigte eine proteomische Überschneidung zwischen APEX2

und TurboID in primären Zilien mit enzyspezifischen Unterschieden, die möglicherweise auf die verschiedenen kovalenten Biotin-Bindungsmechanismen der Enzyme zurückzuführen sind. TurboID wies im Vergleich zum APEX2-Pendant einen höheren unspezifischen Markierungshintergrund in den Zilien bei gleichzeitig geringerer zeitlicher Auflösung auf. Die beobachtete Überlegenheit der Ascorbatperoxidase in der Proteomabdeckung in viel kürzerer Zeit veranlasste mich, nach Möglichkeiten zu suchen, das Proteomik-Werkzeug in allen Zelltypen einsetzen zu können.

Die Integration des APEX2-Systems in andere etablierte murine Zelltypen (z. B. Fibroblasten NIH/3T3, Myoblasten C2C12 und Präadipozyten 3T3-L1) war eine Herausforderung, da ein nicht zielgerichteter Überschuss an H_2O_2 und Tyramid-Substrat zu einer unspezifischen Markierung an anderen Stellen der Zelle durch endogene Carboxylasen und Peroxidasen führte.

Zur Konsolidierung einer verbesserten APEX-Methode (im Folgenden als "iAPEX" bezeichnet) schlug ich ein System vor, bei dem sowohl das Flavoenzym D-Aminosäure-Oxidase (DAAO) aus der Hefe *Rhodotorula gracilis* als auch APEX2 genetisch auf primäre Zilien ausgerichtet sind, um die Verknüpfung von *in situ*- H_2O_2 -Produktion und Kompartiment-spezifischer Näherungsmarkierung zu ermöglichen. DAAO katalysiert die mehrstufige stereoselektive oxidative Desaminierung natürlicher D-Aminosäuren zu ihren jeweiligen α -Ketosäuren. Die enzymatische Reaktion ist abhängig von nicht kovalent gebundenem Flavin-Adenin-Dinukleotid (FAD), das in der ersten Reaktionshälfte zu $FADH_2$ reduziert wird. Die entstehende Iminosäure dissoziiert vom Enzym und wird spontan hydrolysiert, wobei Ammonium entsteht. $FADH_2$ wird in der zweiten Halbreaktion durch molekularen Sauerstoff reoxidiert, wodurch H_2O_2 freigesetzt wird. Ein H_2O_2 -abfängendes APEX2 in der Nähe sollte dann in der Lage sein, die umgebenden Proteine zu biotinylieren, vorausgesetzt, ein entsprechendes Substrat ist verfügbar. Dieses kaskadenartige Zwei-Komponenten-System umgeht die Notwendigkeit von exogen zugesetztem H_2O_2 .

Das vorgeschlagene iAPEX-Zweikomponentensystem wurde etabliert und anschließend in IMCD3-Zellen gründlich getestet. Der iAPEX Proteomik-Arbeitsablauf ermöglichte die Identifizierung neuer ziliärer Kandidatenproteine, die im weiteren Verlauf des Projekts bestätigt wurden. Die Methode wurde dann auf die bisher unzugänglichen Zelltypen für APEX2 Näherungsmarkierung ausgeweitet, um die Wirksamkeit und Spezifität der vorge-

schlagenen Methode zu überprüfen. Diese Zielsetzung erforderte die Entwicklung eines Systems zur schnellen Zelllinien-Generierung unter Verwendung von Lentiviren mittels aufwändiger multicistronischer Vektorsysteme. Schließlich wurden die proteomischen Ergebnisse der iAPEX-Näherungsmarkierung in IMCD3- und NIH/3T3-Zellen verglichen, um Gemeinsamkeiten und Unterschiede in ihren primären Zilieninhalten aufzuzeigen.

Diese Arbeit trägt zu fortgeschrittenen molekularen Studien bei, indem sie ein exploratives Proteomik-Werkzeug bereitstellt, das eine spezifische, räumlich begrenzte Kartierung selbst schmaler, haarähnlicher zellulärer Domänen ermöglicht. iAPEX erleichtert nun die Erforschung eines gewebeübergreifenden Zilien-Protein-Atlases und ihrer zelltypspezifischen Unterschiede. Durch die Verbesserung von proteomischen Ansätzen, wie die der APEX2 Näherungsmarkierung in Bezug auf ihre Verfügbarkeit und Spezifität, wird dies in Zukunft die Identifizierung neuartiger medikamentöser Zielproteine für die Entwicklung potenzieller Behandlungen zur Linderung der Beschwerden von Patienten ermöglichen, die bisher auf symptomorientierte Optionen beschränkt waren.

III. Abbreviations

%	percent
°C	degree Celsius
$\times g$	times gravity, relative centrifugal force
μg	microgram
μm	micrometer
μM	micromolar
3T3	3-day transfer, <i>inoculum</i> 3×10^5 cells (3T3 Swiss albino fibroblast cell
3T3-L1	3T3 Swiss albino fibroblast subclone (murine preadipocytes)
ACN	acetonitrile
acTub	acetylated tubulin
ADPKD	autosomal dominant polycystic kidney disease
AF350	Alexa Fluor 50 conjugate
AF488	Alexa Fluor 88 conjugate
AF555	Alexa Fluor 55 conjugate
AF647	Alexa Fluor 47 conjugate
AKAP	A-kinase anchoring protein
ANOVA	single factor analysis of variance
APEX	ascorbate peroxidase
APEX2	engineered ascorbate peroxidase
APS	ammonium persulfate
ARL13B	ADP ribosylation factor-like protein 13
<i>att</i>	bacteriophage recombination sites
<i>attB</i>	bacterial attachment site
<i>attL</i>	“left” attachment site
<i>attP</i>	phage attachment site
<i>attR</i>	“right” attachment site
BBS	Bardet Biedl syndrome
BBSome	Bardet Biedl syndrome-associated protein complex
Blast	blasticidin S hydrochloride
BN	Blue native
BSA	bovine serum albumin

BT	biotin tyramide
C2C12	C2C12 mouse myoblast cell line
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cilia-APEX	engineered ascorbate peroxidase 2 localized to primary cilia
CMV	human cytomegalovirus promoter
CMV	truncated CMV
CatN	catalog number
CO ₂	carbon dioxide
DAAO	D-amino acid oxidase
D-Ala	D-alanine
dH ₂ O	distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMEM F-12	Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 1:1 mix
D-Ala	D-alanine
D-Met	D-methionine
dNTPs	deoxynucleoside triphosphates
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTBT	desthiobiotin tyramide
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
e.g.	<i>exempli gratia</i>
EDTA	ethylenediaminetetraacetic acid
EGTA	triethylene glycol diamine tetraacetic acid
eng.	english
EtOH	ethanol
FA	formic acid
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
f.c.	final concentration

fCTS	fibrocystin cilia targeting sequence
Fig.	figure
fwd	forward (5'-3' strand)
GFP	green fluorescent protein
GLI1	glioma-associated oncogene homolog 1 (zinc finger protein)
GLI2	glioma-associated oncogene homolog 1 (zinc finger protein)
GLI3	glioma-associated oncogene homolog 1 (zinc finger protein)
GPCR	G protein-coupled receptor
GPR161	G protein-coupled receptor 161
h	hour(s)
HCl	hydrogen chloride
HEK	human embryonic kidney cell line 293T
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IAA	2-iodoacetamide
i.e.	<i>id est</i>
IF	immunofluorescence
IFT	intraflagellar transport
IMCD3	inner medullary collecting duct clone 3
IRES	internal ribosomal entry site
kDa	kilodalton
KO	knock-out
L	Liter
L-Ala	L-alanine
L-Met	L-methionine
Lys-C	endoproteinase/serine protease Lys-C isolated from <i>Lysobacter sp.</i>
M	molar
MeOH	methanol
MIN6 _β	mouse pancreatic islet cell line 6 _β
min	minute(s)
mM	millimolar

MKS	Meckel Gruber Syndrome
ml	milliliters
mol	mole
MOPS	3-(N-morpholino)propanesulfonic acid
MS	mass spectrometry
n.a.	not applicable
n.s.	not significant
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
NL	NorthernLights™ antibody conjugate
norm.	normalized
NPHP3	nephrocystin 3
PAGE	polyacrylamide gel electrophoresis
PALD1	phosphatase domain containing paladin 1
PBS	Dulbecco's phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	<i>potentia hydrogenii</i>
PIC	protease inhibitor cocktail
PIP	phosphatidylinositolphosphate
PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKA	cyclic adenosine monophosphate-dependent protein kinase
PKA-RI _α	protein kinase A regulatory subunit RI _α
PKA-C	protein kinase A catalytic subunit C
PL	proximity labeling
PMSF	phenylmethylsulfonyl fluoride
PTCH1	Patched I
Puro	puromycin
rev	reverse (3'-5' strand)
RFU	relative fluorescence units

<i>R.gracilis</i>	<i>Rhodotorula gracilis</i>
RNA	ribonucleic acid
RPE	retinal pigment epithelium
RPM	revolutions per minute, centrifuge rotor speed
RT	room temperature
s	second(s)
SAG	Smoothened antagonist
SD	standard deviation
sdAb	single-domain antibody
SDS	sodium dodecyl sulfate
SHH	Sonic hedgehog protein
Shh-N	Sonic Hedgehog N terminus
SMO	Smoothened
<i>sp.</i>	species
Tab.	table
TBS	tris buffered saline
TBS-T	tris buffered saline with Tween20
TEMED	Tetramethylethylenediamine
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid, vitamin E analog
TZ	transition zone
U-ExM	ultrastructure expansion microscopy
v/v	volume per volume
WB	Western blot
WT	wild type
w/v	weight per volume

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1. INTRODUCTION

1.1 Preface to Primary Cilia

When examining vertebrate cells, it becomes apparent that most of them have a single, hair-like structure on their surface, the so-called primary cilium. Cilia themselves encompass a group of cellular membrane protrusions which are structurally similar and are found on most eukaryotic cell types, but exhibit specialized adaptations to their respective environments (Satir & Christensen, 2008; Mill *et al*, 2023). On the one hand, motile cilia, for instance found in large numbers in the respiratory tract, beat in synchronized patterns to ensure that particles and fluids can be moved in a directed manner. In addition, they are capable of sensing mechanical and chemical stimuli (Verdugo, 1982; Petit *et al*, 2023). A primary cilium on the other hand is an isolated structure that resembles a cellular antenna. In fact, the primary cilium of higher eukaryotes is believed to originate from ancestral cilia that combined locomotion with compartmentalized signaling to actively sense the passing environment (Mitchell, 2017; Derderian *et al*, 2023). Vertebrate primary cilia have since lost their elaborate motility and developed into highly specialized structures that perform signaling functions tailored to the specific needs of tissues and the organism as a whole. They are found in almost all cell types of the human body, from neuronal cells in the brain to cells of the renal epithelium (Spasic & Jacobs, 2017).

For a long time, the seemingly featureless primary cilium was regarded as an evolutionary remnant of the cell compared to the dynamic motile cilia. The decisive turning point arrived with the pioneering work on intraflagellar transport (IFT) proteins, which were found to be conserved from algae to vertebrates and could be linked to primary cilia through observed developmental disease progression (Pazour *et al*, 2002; Pazour & Rosenbaum, 2002; Bloodgood, 2009). Since then, two decades of research have established this minute compartment to coordinate crucial signaling events to control cellular processes that influence both the development and homeostasis of various tissues and organs (Mill *et al*, 2023). The Hedgehog signaling pathway plays a central role in primary cilia, and is responsible for proper embryonic development and tissue regeneration (Bangs & Anderson, 2017; Gigante & Caspary, 2020). Notably, Hedgehog receptors and sensing molecules involved in signaling transmission occupy and shape the primary cilium (Ho & Stearns, 2021).

When a primary cilium is dysfunctional or its structure is altered due to genetic mutations, a spectrum of diseases termed ciliopathies can develop (Badano *et al*, 2006; Braun & Hildebrandt, 2017). To date, more than 30 different ciliopathies and at least 247 genes associated with these ciliopathies have been identified (Mill *et al*, 2023). The most prevalent ciliopathies include polycystic kidney disease (PKD), Bardet-Biedl syndrome and Joubert syndrome (Oud *et al*, 2017). These diseases present a broad array of partially overlapping symptoms that can affect single or multiple organs—in isolation or on a syndromic level—and often affect the kidneys, eyes, liver and brain (Waters & Beales, 2011). This observed heterogeneity of ciliopathies cannot be explained by a cross-tissue ciliary blueprint, but rather indicates tissue-specific functions of cilia proteins (Gopalakrishnan *et al*, 2023). In order to better understand the syndromic landscape of primary cilia, "omics" approaches have gained momentum in the field. These methods capture the molecular profiles of cilia across tissues and analyze emerging patterns under varying conditions (Heydeck *et al*, 2018; Nishimura *et al*, 2019). Proximity labeling enzymes are remarkable proteomics tools that allow the study of protein populations in selected cellular domains (Kang & Rhee, 2022). In this context, proximity labeling of proteins using a genetically engineered ascorbate peroxidase (APEX or APEX2) (Martell *et al*, 2012; Rhee *et al*, 2013; Lam *et al*, 2015) or biotin ligase (BioID or TurboID) (Roux *et al*, 2012; Branon *et al*, 2018) has enabled high spatial and temporal resolution of the dynamic ciliary proteome. However, proximity labeling enzymes have limitations that have so far prevented the comprehensive analysis of complex animal systems. For instance, the need for exogenous addition of highly cell toxic hydrogen peroxide (H_2O_2) has limited the application of APEX2 in *in vivo* mouse studies, prioritizing biotin ligase-based proximity labeling in this area, which is controlled through supplementation with vitamin B7 (biotin) (Guo *et al*, 2023). The disadvantage of a biotin ligase-based approach is the search for a feasible route of administration for efficient uptake of biotin in complex organisms as these enzymes have been shown to sequester endogenous biotin, leading to maldevelopment (Watanabe *et al*, 2009; Báez-Saldaña *et al*, 2009; Branon *et al*, 2018; Suzuki *et al*, 2023). Therefore, improvements in these technologies are essential to provide better mapping of the interfaces and consequent differences between different cilia populations in diverse tissues.

1.2 Life Cycle of the Primary Cilium

In a quiescent or differentiated cell, a primary cilium is templated from the cell's modified mother centriole, the so-called basal body, which is locked in place at the plasma membrane through nine-fold symmetric centriolar distal appendages (transition fibers) (Marshall, 2008; Schmidt *et al*, 2012). The centriolar distal ends give rise to the ciliary axoneme, a microtubule doublet scaffold that follows the same symmetry and acts as a protein highway for structural and signaling molecules (**Fig. 1A**, right inset) (Satir *et al*, 2010; Linck *et al*, 2016). The microtubule cytoskeleton arches the continuous plasma membrane outward, thereby creating a hair-like cell-surface structure that is open to the cellular side. Here at the interface between basal body and ciliary space, forked protein fibers, termed Y-links, compartmentalize the primary cilium (Wang *et al*, 2022). These electron dense fibers connect the axoneme with the adjacent ciliary membrane thereby forming a gating structure called the transition zone (TZ) (Williams *et al*, 2011; Gonçalves & Pelletier, 2017). Ciliary cargoes must pass through the TZ as they enter and exit the primary cilium, which is facilitated by IFT protein complexes (commonly referred to as "trains") that move bidirectionally along the microtubule tracks with the help of specific molecular motors (**Fig. 1A**, left inset) (Lechtreck, 2015). The heterotrimeric motor protein kinesin 2 propels the IFT-B train together with the associated IFT-A complex towards the cilia tip, along with any cargo that is directly bound to specific IFT proteins (Pazour *et al*, 2000; Shao *et al*, 2024). These cargoes include cytoskeletal building blocks (tubulin), signaling molecules or even integral membrane proteins, such as G protein-coupled receptors (GPCRs) (Cole *et al*, 1998; Qin *et al*, 2004; Hao *et al*, 2011; Jiang *et al*, 2023). The return path from the cilia tip to the base is controlled by the rearrangement of the IFT trains, which are moved by dynein 2 (Garcia-Gonzalo & Reiter, 2017; Kobayashi *et al*, 2020). Membrane proteins destined to leave the primary cilium are chaperoned by another protein complex genotypically associated with Bardet-Biedl syndrome, termed BBSome (Nachury *et al*, 2007; Nachury, 2018). This complex acts as a cargo adaptor for GPCRs and the IFT train to support the exit from the cilium (Liew *et al*, 2014; Liu *et al*, 2021b). Subdistal appendages of the mother centriole allow the structure to function as a microtubule organizing center (Uzbekov & Alieva, 2018). Both the mother centriole and its proximally attached and perpendicularly positioned daughter centriole form the centrosome. When established, the cilia-centrosome complex acts as a sensory organelle that plays crucial roles in signal transduction pathways that govern cell growth and differentiation (**Fig. 1A**) (Pedersen *et al*, 2012).

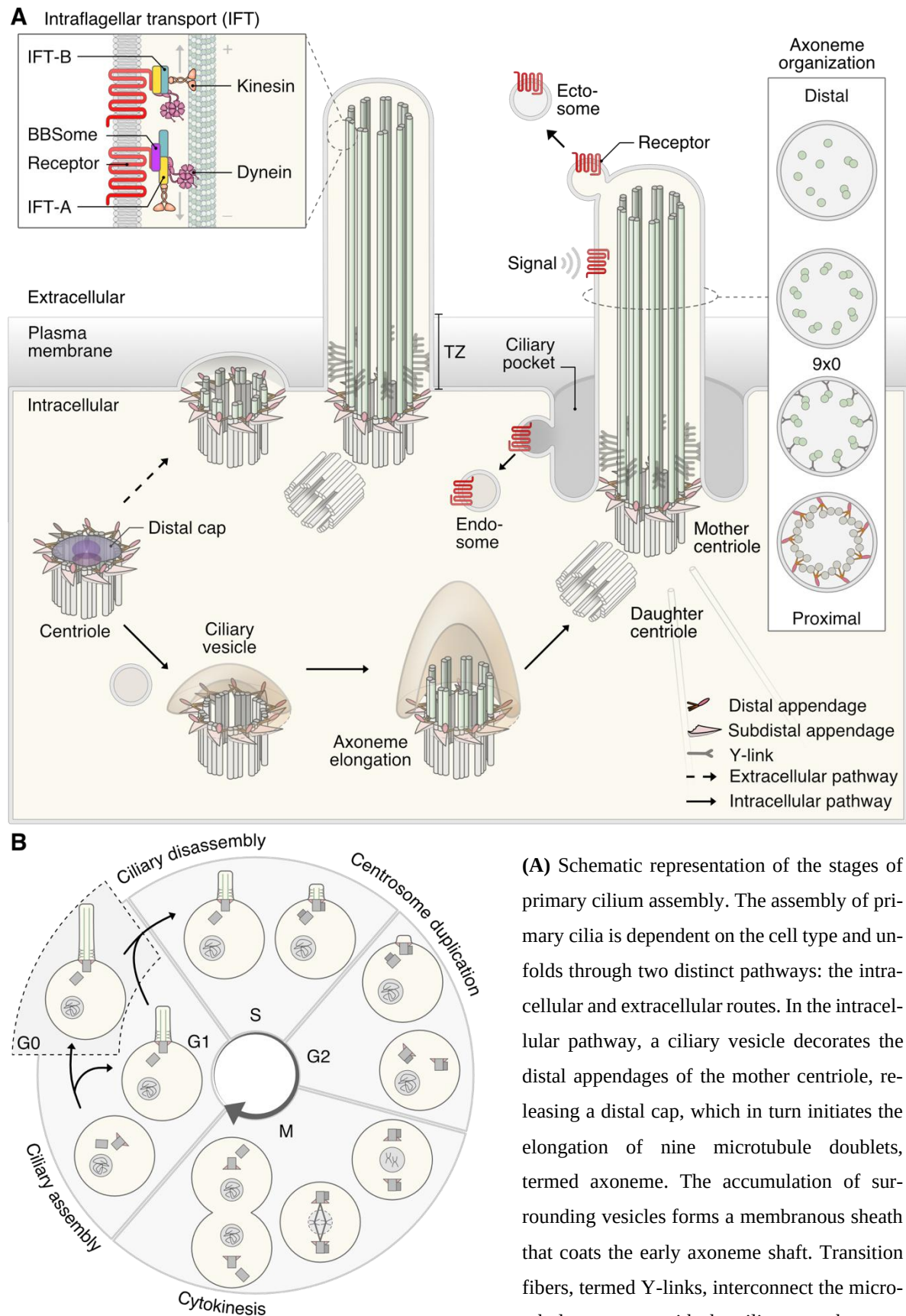


Fig. 1: The intricate assembly, dynamics and organization of the primary cilium.

The sheath eventually fuses with the plasma membrane, exposing the nascent intracellular cilium to the extracellular space with its proximal part embedded within a membranous depression, termed the ciliary pocket. The final axonemal organization is shown ordered from distal to proximal. In the extracellular pathway, however, the mother centriole anchors itself directly to the plasma membrane via its distal appendages, which causes the cilium to protrude outwards into the extracellular milieu. During and after assembly, the microtubule scaffold enables the directed transport of proteins destined for or to be removed from the cilium through so-called intraflagellar transport (IFT) to maintain structure and signaling function. Receptors lining the ciliary membrane produce signaling events and are regulated by the release of ectosomes at the distal part or the uptake of endosomes at the periciliary membrane. **(B)** Simplified depiction of primary cilium assembly and disassembly within the cell cycle. Primary cilia formation follows a cyclic pattern. Following cytokinesis, assembly occurs in each cell cycle during G0/G1 phase. At this stage, the centrioles are locked in place at the plasma membrane. S phase initiates DNA replication, centriole duplication and ciliary resorption. During the transition from the G2 phase to mitosis (M phase), the centrioles detach from the plasma membrane. Mother and daughter centrioles are separated in order to position themselves in the direction of the cell nucleus and form the spindle poles. In the next cycle, each mother centriole forms a new daughter centriole.

However, when a cell enters a proliferative state at the end of the G1 phase of the cell cycle, the primary cilium begins to disassemble and centrosome duplication initiates; processes that can last until the G2 phase (**Fig. 1B**) (Quarmby & Parker, 2005; Pugacheva *et al*, 2007; Ford *et al*, 2018). Here, ciliary disassembly is a prerequisite for centrosome release (Sánchez & Dynlacht, 2016). Each centriole sprouts another centriole, thus producing engaged pairs which then separate into single centrosomes. The duplication event produces centrioles of different age, the matured template (mother) and the younger copy (daughter) (Loncarek *et al*, 2008). These, in turn, enable the assembly of the bipolar spindle and ultimately chromosomal segregation (Tsou & Stearns, 2006). After cytokinesis in the newly formed daughter cells, the centrosomes can form new primary cilia in response to mitogen deprivation or differentiation cues that terminate the cell cycle (Tucker *et al*, 1979a, 1979b).

Dependent on the cell type, ciliogenesis progresses along two different paths (**Fig. 1A**) (Pedersen *et al*, 2012; Bernabé-Rubio & Alonso, 2017; Shakya & Westlake, 2021). The extracellular pathway describes the route of the mother centriole observed, for instance, in polarized, epithelial lung and kidney cells (Sorokin, 1968). The centrosome migrates toward the plasma membrane and becomes anchored to it through centriolar distal appendages on the mother centriole. Once anchored, a proteinaceous distal cap that inhibits microtubule growth is removed from the distal end, allowing the ciliary axoneme to extend (Spektor *et*

al, 2007; Williams *et al*, 2011; Tsang & Dynlacht, 2013). This leads to an immediate protrusion from the plasma membrane. Mesenchymal cells like fibroblasts, as well as smooth muscle cells and retinal epithelial cells follow the intracellular pathway (Sorokin, 1962). As the name implies, ciliogenesis in the intracellular pathway begins in the cytoplasm, where Golgi-derived vesicles attach to the distal appendages of the mother centriole to form a precursor of the ciliary membrane (Westlake *et al*, 2011). An early axoneme elongates into a growing space ensheathed by a ciliary vesicle until the established basal body docks to the plasma membrane, causing a characteristic depression from which the primary cilium is exposed to the extracellular space (Molla-Herman *et al*, 2010; Schmidt *et al*, 2012; Lu *et al*, 2015).

In order to analyze primary cilia in lab-grown cell culture systems, cell cycles are synchronized by serum starvation which induces cilia assembly through deprivation of mitotic growth factors (Tucker *et al*, 1979a; Vorobjev & Chentsov, 1982). Many progenitor cell types such as hepatoblasts (precursors of liver hepatocytes and cholangiocytes in the bile duct) or myoblasts (precursors of skeletal muscle) display primary cilia on their surfaces and lose them once they differentiate into their destined cell lineage (Bodle & Lobo, 2016; Patel & Tsiokas, 2021). In an undifferentiated state, Sonic Hedgehog (SHH) signaling plays a critical role for progenitor cell expansion, thereby allowing tissue repair and development (Le *et al*, 2008; Bangs & Anderson, 2017). In the context of liver development, mutations in cilia-related genes disrupt SHH signaling, leading to congenital anomalies such as biliary atresia and fibrocystic liver diseases (Whitley *et al*, 1987; Davey *et al*, 2014; Grzelak *et al*, 2014). An imbalance in SHH signaling in skeletal myogenesis can lead to rhabdomyosarcoma, a rare soft tissue cancer, arising from defective myoblast differentiation, which is a type of neoplasm that is predisposed to develop in patients with ciliopathy (Fu *et al*, 2014).

1.3 Sonic Hedgehog Signaling

The primary cilium has the ability to dynamically fine-tune its lipid and protein content in response to external cues (Nachury & Mick, 2019). In SHH signaling, ligand sensing dictates rearrangements in the ciliary membrane composition, orchestrating changes at the second messenger level that alter downstream processing of transcription factors ultimately governing transcriptional responses (Kong *et al*, 2019).

In the off state without ligand (**Fig. 2**, left side), the transmembrane protein Patched (PTCH1) is localized to the ciliary membrane and suppresses the G-protein coupled receptor Smoothed (SMO) from local accumulation (Rohatgi *et al*, 2007). SMO is in turn ubiquitinated (Ub) and removed from cilia as a cargo of the BBSome protein complex (Shinde *et al*, 2020; Desai *et al*, 2020). Concurrently, the constitutively active G-protein coupled receptor GPR161 retains ciliary localization which stimulates nearby adenylate cyclases (AC) via $G_{\alpha s}$ which synthesize elevated levels of cAMP (Mukhopadhyay *et al*, 2013). Excess cAMP is sensed by the regulatory PKA subunit $I\alpha$ (RI α), which results in the phosphorylation of target proteins, such as GLI transcription factors, through released PKA catalytic subunit (PKA-C) (Kim *et al*, 2007). The phosphorylation of full-length GLI yields ubiquitination and proteolytic cleavage into the GLI repressor form (GLI-R). GLI-R translocates to the nucleus to suppress target gene expression (Haycraft *et al*, 2005; Huangfu & Anderson, 2005; Wang *et al*, 2014). In the on state (**Fig. 2**, right side), exposure to the ligand is sensed by PTCH1, which then leaves the primary cilium in a presumably Ub-dependent manner, allowing SMO to take its place (Milenkovic *et al*, 2009, 2015; Tukachinsky *et al*, 2016). SMO accumulation and subsequent activation effect GPR161 phosphorylation by GRK2 and PKA, which is recognized by β -arrestin2, driving BBSome-mediated removal of GPR161 from the primary cilium (Pal *et al*, 2016; Nager *et al*, 2017; Ye *et al*, 2018). The signaling circuit cumulates in inhibited AC activity and a drop in ciliary cAMP levels, leaving GLI unaltered as a transcriptional activator (GLI-A), which translocates to the nucleus finally activating the expression of target genes (Kim *et al*, 2009; Humke *et al*, 2010).

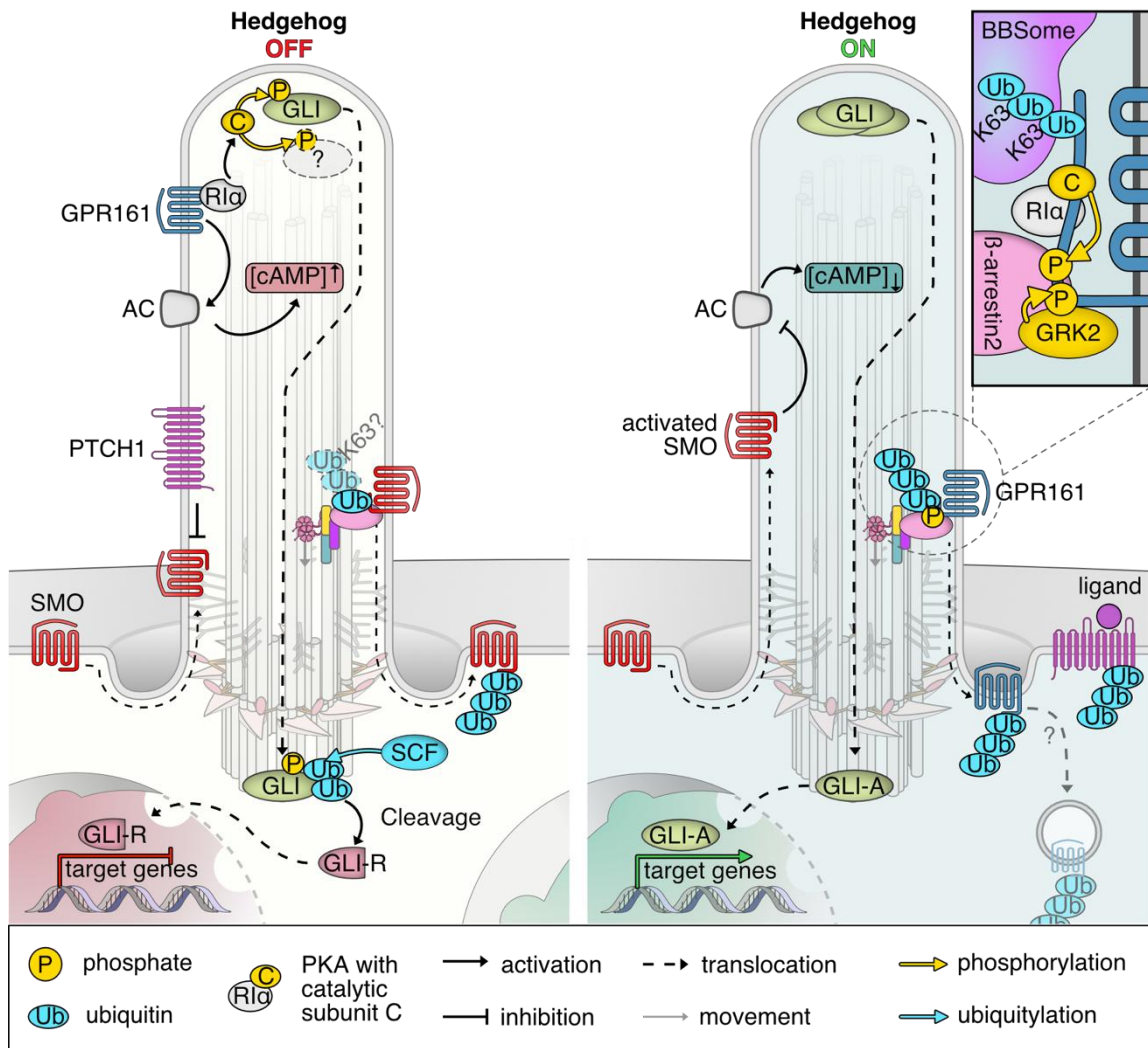


Fig. 2: The Sonic Hedgehog signaling pathway is specific to the primary cilium in mammalian cells. Visualization of the off and on state of the sonic hedgehog signaling pathway in mammalian cells (modified from May *et al*, 2021). Detailed description of the depicted processes can be found in the aforementioned text.

1.4 Ciliopathy Landscape

The pathophysiological landscape of primary cilia is diverse encompassing overlapping clinical phenotypes among syndromes that range in their severity from embryonal lethality to late onset of retinal degeneration in adulthood (Braun & Hildebrandt, 2017). Depending on the degree of primary cilia dysfunction caused by the patient's specific genotype, these disorders can affect single or multiple organs (Oud *et al*, 2017; Grochowsky & Gunay-Aygun, 2019; Cruz *et al*, 2022). This phenotypical heterogeneity is further complicated by mutations in the identical ciliary gene that lead to different ciliopathies, e.g., CEP290 or CC2D2A, which can cause Meckel-Gruber or Joubert syndrome, respectively (**Fig. 3A**) (Frank *et al*, 2008; Mougou-Zerelli *et al*, 2009; Shaheen *et al*, 2016). The involvement of individual structural cilia elements, which can be affected in a single syndrome (**Fig. 3B**), reveals another level of complexity that cannot be represented by genomic studies alone (Elliott *et al*, 2023). In this context, proteomic changes can compensate for functional disorders in less pronounced phenotypes or, in more severe cases, lead to failure of the overall structure (Mill *et al*, 2023). Besides direct ciliary involvement, the so-called "second-order ciliopathies" include diseases that overlap with the ciliopathy phenotype spectrum. These include genetic alterations in non-ciliary proteins that affect the expression of products destined for cilia, which blurs the line in disease aetiology (Reiter & Leroux, 2017). Therefore, multi-omics approaches need to be improved and integrated into ciliopathy studies to ensure a better understanding of the underlying cellular processes in primary cilia and ultimately enable the development of potential treatments.

1.5 Proteomics

The field of proteomics research is based on liquid chromatography-mass spectrometry (LC-MS), which enables the detailed analysis of peptides from complex protein samples (Köcher & Superti-Furga, 2007; Karpievitch *et al*, 2010). The general proteomic bottom-up workflow involves digesting proteins into peptides using specific proteases, such as trypsin and Lys-C. Trypsin digestion generates peptides with defined termini, specifically cleaving at the C-terminus of lysine and arginine residues, which helps in MS by producing peptides with predictable masses and charges, facilitating accurate identification and analysis. The Lys-C protease efficiently cleaves lysine sites, reducing the number of failed lysine cleavages by trypsin in tightly folded proteins (Saveliev *et al*, 2013). After digestion, peptides are separated through liquid chromatography (LC), where reverse-phase chromatography is often used, which separates peptides based on their hydrophobicity (Shen *et al*, 2001, 2005). In this process, peptides are passed through a column filled with hydrophobic material, and those with higher hydrophobicity elute later than more hydrophilic peptides allowing for better resolution and identification of complex protein mixtures (Köcher *et al*, 2011). After separation, the peptides are ionized, either by matrix-assisted laser desorption/ionization (MALDI) or, more commonly, electrospray ionization (ESI), before entering the mass spectrometer. ESI involves applying a high voltage to the liquid exiting the chromatography column, converting peptides from an aqueous phase into charged ions in a fine aerosol, which is then introduced into the mass spectrometer (Nadler *et al*, 2017). In the instrument, mass-to-charge ratios (m/z) of peptides are measured, generating an MS1 spectrum that provides a profile of intact peptide ions. When tandem mass spectrometry (LC-MS/MS) is employed, precursor ions from the MS1 spectrum are selected and further fragmented by colliding with inert gases, producing MS2 spectra. These spectra provide detailed information on the peptide sequence and post-translational modifications by analyzing the resulting fragment ions (Hunt *et al*, 1986; Eng *et al*, 1994; Perkins *et al*, 1999). Mass spectrometry is not inherently quantitative because the ionization efficiency can vary significantly between different peptides, leading to inconsistent signal intensities that do not directly correlate with peptide abundance (Pappireddi *et al*, 2019). Additionally, factors such as sample preparation and instrument variability can further affect the accuracy of the MS signal, making additional techniques necessary to achieve reliable quantification (Piehowski *et al*, 2013).

Quantification in proteomics can be achieved through several methods, including label-free quantification, heavy isotope labeling, and isobaric tagging (Pappireddi *et al*, 2019). Label-free quantification compares peptide ion intensities directly between samples, while heavy isotope labeling incorporates stable isotopes into peptides, allowing for accurate quantification by comparing the relative intensities of isotopically distinct peptides (Cox *et al*, 2014). Stable isotope labeling by amino acids in cell culture (SILAC) employs incorporation of heavy isotopes (such as arginine containing carbon-13 instead of carbon-12) into proteins during translation within living cells, even before sample preparation (Ong *et al*, 2003; Wang & Huang, 2014). Peptides generated from samples grown in media containing either heavy isotope-labeled amino acids or normal amino acids are combined and analyzed together in the mass spectrometer, allowing comparison of peptide abundance based on their mass-to-charge ratios (m/z) (Mann, 2006). Isobaric tagging of digested peptides allows for simultaneous, multiplexed quantification of proteins across multiple samples in one mass spectrometry run. Peptides which are reacted with Tandem Mass Tags (TMT), i.e., isobaric chemical labels with identical overall mass, produce distinct reporter ions of different masses upon fragmentation, allowing for the multiplexed quantification (Dayon *et al*, 2008; McAlister *et al*, 2012; Thompson *et al*, 2019; Li *et al*, 2020a). To enhance quantification precision and address ion interference and ratio distortion in MS2, TMT-based LC-MS3 introduces an additional fragmentation step. In this process, MS2 ions are further fragmented to produce an MS3 spectrum, increasing confidence in peptide identification and enabling accurate quantification of low-abundance proteins with greater precision (Ting *et al*, 2011; Aebersold & Mann, 2016).

1.6 Cilia Proteomics History

The study of primary cilia proteomes has its roots in the investigation of simple eukaryotic model organisms, such as *Chlamydomonas reinhardtii* and *Trypanosoma brucei*, which have significantly contributed to our understanding of ciliary structure and function due to the evolutionary conservation of ciliary components (Dean *et al*, 2016; Sigg *et al*, 2017; Marshall, 2024).

C. reinhardtii, a unicellular green alga, has two flagella which are structurally and functionally analogous to mammalian cilia, making it an ideal system for ciliary proteomic studies. A pivotal study by Pazour *et al* (2005) provided one of the first comprehensive proteomic analyses of the flagella, identifying numerous proteins that were later found to be conserved in mammalian cilia; including motor proteins like dyneins and kinesins responsible for intraflagellar transport (IFT), as well as signaling proteins, receptors, and ion channels essential for ciliary signaling cascades. Research on the single flagellum of the protozoa *T. brucei* provided further insight into the conserved nature of ciliary proteins by identifying essential components, such as radial spokes and central pair complexes, together with ATP-dependent molecular motors, for flagellar motility (Broadhead *et al*, 2006).

In parallel, advancements in human ciliary proteomics have underscored the critical connections between ciliary structure, function, and pathophysiology (Pazour & Rosenbaum, 2002). Early studies faced limitations in isolating motile and immotile cilia, often relying on chemical or mechanical deciliation of tissues combined with subcellular fractionation techniques to enrich ciliary components, followed by protein/peptide separation methods to allow analysis by high resolution liquid-chromatography mass spectrometry (LC-MS) (Chen *et al*, 2022b; Quarmby & Mahjoub, 2023). By analyzing isolated bronchial epithelial cilia axonemes for example, researchers established a foundational framework for understanding the human ciliary microtubule proteome (Ostrowski *et al*, 2002). Additionally, a comprehensive study of the human centrosome led to the discovery of new candidates within the pericentriolar matrix and centrosomal proteins, linking these components to developmental defects (Andersen *et al*, 2003). A comparative genomics study further revealed the evolutionary conservation of *C. reinhardtii* flagellar and basal body proteins to their human orthologs, connecting these components to human ciliopathy genes such as BBS5 (Bardet-Biedl syndrome 5 protein) (Li *et al*, 2004). Research on highly specialized primary cilia,

which were easier to obtain, has illuminated the signaling capabilities of these compartments and expanded our understanding of the complex adaptations within ciliary tissues. For instance, the proteomic analysis of the photoreceptor sensory cilium complex (PSC) in mouse retina has identified specific proteins critical to phototransduction processes, among these were many IFT particles with their molecular motors and proteins directly linked to ciliary syndromic diseases (e.g., BBS1 and BBS7) (Liu *et al*, 2007). Furthermore, a comprehensive proteomic dataset from rat olfactory sensory neurons (OSN) advanced our knowledge of the ciliary membrane protein composition and the underlying second messenger physiology of the sensory organelle (Mayer *et al*, 2009), which has been extended by work of Kuhlmann *et al* (2014) in mouse OSN. Proteomic analysis of prototypical mammalian primary cilia from inner medullary collecting duct cells led to the discovery of novel ciliary proteins and pathways involved in ciliary assembly, maintenance, and function. This provided significant insights into renal ciliopathies and related disorders (Ishikawa *et al*, 2012).

The initial studies were primarily focused on generating exploratory proteomic data from isolated ciliary fractions, aiming to offer a comprehensive overview of potential proteins within this compartment. However, these isolation methods have their limitations. They are prone to contamination by co-fractionated cellular components, and lack essential structural elements required for comprehensive mapping (Gopalakrishnan *et al*, 2023). To unravel molecular details with greater precision, ciliary protein-protein interaction (PPI) networks have been used analyzed through genetically encoded affinity tags in the past; which, when incorporated in the DNA sequence of a protein of interest, allow for the expression of a tagged „bait“-protein (Beeckmans, 1999; Kimple *et al*, 2013). Subsequently, this bait can be utilized for affinity-based isolations of potential interaction partners (“preys”), enabling more accurate purification and in-depth characterization of protein complexes, such as those derived from primary cilia, through subsequent mass spectrometry analysis (Morris *et al*, 2014).

Research on PPIs within primary cilia of various cell types has revealed significant insights into the molecular mechanisms governing ciliogenesis, ciliary membrane composition, trafficking and signaling function. Using BBS4 as bait, a stable core complex of evolutionary conserved Bardet-Biedl Syndrome (BBS) proteins was discovered and found to collaborate with the GTPase Rab8 to facilitate vesicle docking at the ciliary base essential for ciliary membrane biogenesis (Nachury *et al*, 2007). Further dissection of the BBS4 interactome strengthened the link of the BBSome complex to cilia assembly through interaction with

BBIP10 (Loktev *et al*, 2008). TULP3, used as a bait, was shown to directly interact with core proteins of the IFT-A complex (specifically IFT122, IFT140 and WDR19) to facilitate ciliary entry and trafficking of a subset of GPCRs establishing it as a ciliary shuttle (or gating) protein (Mukhopadhyay *et al*, 2010). An interactome study of B9D1 identified a complex of nine proteins, including TMEM231 and CC2D2A, which was found to be essential for selective localization of ciliary membrane proteins and exclusion of periciliary plasma membrane proteins, therefore acting as a diffusion barrier at the transition zone to compartmentalize the primary ciliary space (Chih *et al*, 2012). Similarly, another transition zone complex (including Tctn1, Tctn3 and seven Meckel-Gruber syndrome proteins) that regulates mammalian ciliogenesis and ciliary membrane composition in a tissue-specific manner was identified using affinity-tagged Tctn1 (Garcia-Gonzalo *et al*, 2011). Pulldown-based proteomic analysis of phosphoinositide phosphatase INPP5E interactors revealed a functional ciliary targeting network involving ARL13B, PDE6D, and CEP164, distinct from previously described transition zone localized complexes (Humbert *et al*, 2012).

The purification of protein complexes from primary cilia using affinity capture techniques has made it possible to propose molecular connections and show the direct interaction of complex partners. However, biochemical isolation is compromised by the potential loss of candidates due to the insolubility of proteins and transient or weak interactions that may be disrupted during the isolation process (Roque & Lowe, 2008; Hayes *et al*, 2016; Gentzel *et al*, 2019).

It is worth mentioning that the yeast two-hybrid (Y2H) system, a high-throughput genetic technique, has also been used to establish ciliary protein-network connections, such as exploring the interactomes of BBS proteins (Oeffner *et al*, 2008) and the Alström syndrome-causing protein ALMS1 (Collin *et al*, 2012). This system evaluates potential protein-protein interactions (PPI) between a bait protein and a library of prey proteins, relying on transcriptional readout of auxotrophic and colorimetric reporter genes that manifest as yeast phenotypes. The interaction restores a split transcription factor, regulating transcription based on interaction strength (Brückner *et al*, 2009). The ability to screen vast libraries for potential interactions efficiently makes Y2H a valuable tool for mapping protein networks and discovering novel interactions. However, Y2H has limitations, including a high number of false-positive and false-negative results, artifacts from indirect transcriptional activation,

and the artificial yeast environment (Koegl & Uetz, 2007). This environment can cause structural alterations in fusion proteins due to forced overexpression and mandatory nuclear localization, while lacking the machinery for post-translational modifications (Van Crielinge & Beyaert, 1999; Stynen *et al*, 2012). Additionally, proteins that naturally localize to the extracellular space or plasma membrane may be underrepresented as they compete with nuclear localization signals, thus capturing only a subset of the interactome (Parrish *et al*, 2006; Koegl & Uetz, 2007).

In 2012, the emergence of two independent proximity labeling (PL) enzymes significantly enhanced future proteomic studies. PL involves enzymes that create reactive species to generate chemical handles that covalently react with nearby proteins in living cells allowing subsequent purification from complex lysates bypassing the need for cell fractionation (Qin *et al*, 2021). Using for instance covalent attachment of biotin to proximal proteins in their native environment in living cells allows capture by strong affinity to streptavidin ($K_d = 10^{-14}$ to 10^{-15} M) even under denaturing conditions while preserving the spatial information about potential protein relationships (Bayer & Wilchek, 1990; Hirsch *et al*, 2002; Niinae *et al*, 2021). Weak, dynamic or transient interactors that are otherwise too volatile to be detected can be effectively mapped if they are within the PL enzyme labeling radius regardless of the duration of the interaction, although no information on direct interactions between proteins can be extrapolated from the spatiotemporal proteomic data (Guo *et al*, 2023).

The first PL enzyme to be developed was the 35-kDa monomeric BioID (proximity-dependent biotin identification) (**Fig. 4AB**) (Roux *et al*, 2012). Previously employed in highly specific biotinylation of a minimal recognition sequence termed biotin acceptor tag (BAT), *Escherichia coli* biotin protein ligase (BirA) was mutated (R118G; termed BirA*) to allow release of an electrophilic activated biotin ester (biotinoyl-5'-AMP) (Beckett *et al*, 1999; Kulman *et al*, 2007). With the new enzyme variant, the selective biotinylation of a single acceptor peptide was altered to untargeted or “promiscuous” tagging of surface-exposed lysine of proximal proteins (Roux *et al*, 2012). The labeling radius of the activated biotin molecules has been estimated to be 10 nm based on proximity labeling experiments on the nuclear pore complex (Kim *et al*, 2014; Mair & Bergmann, 2022).

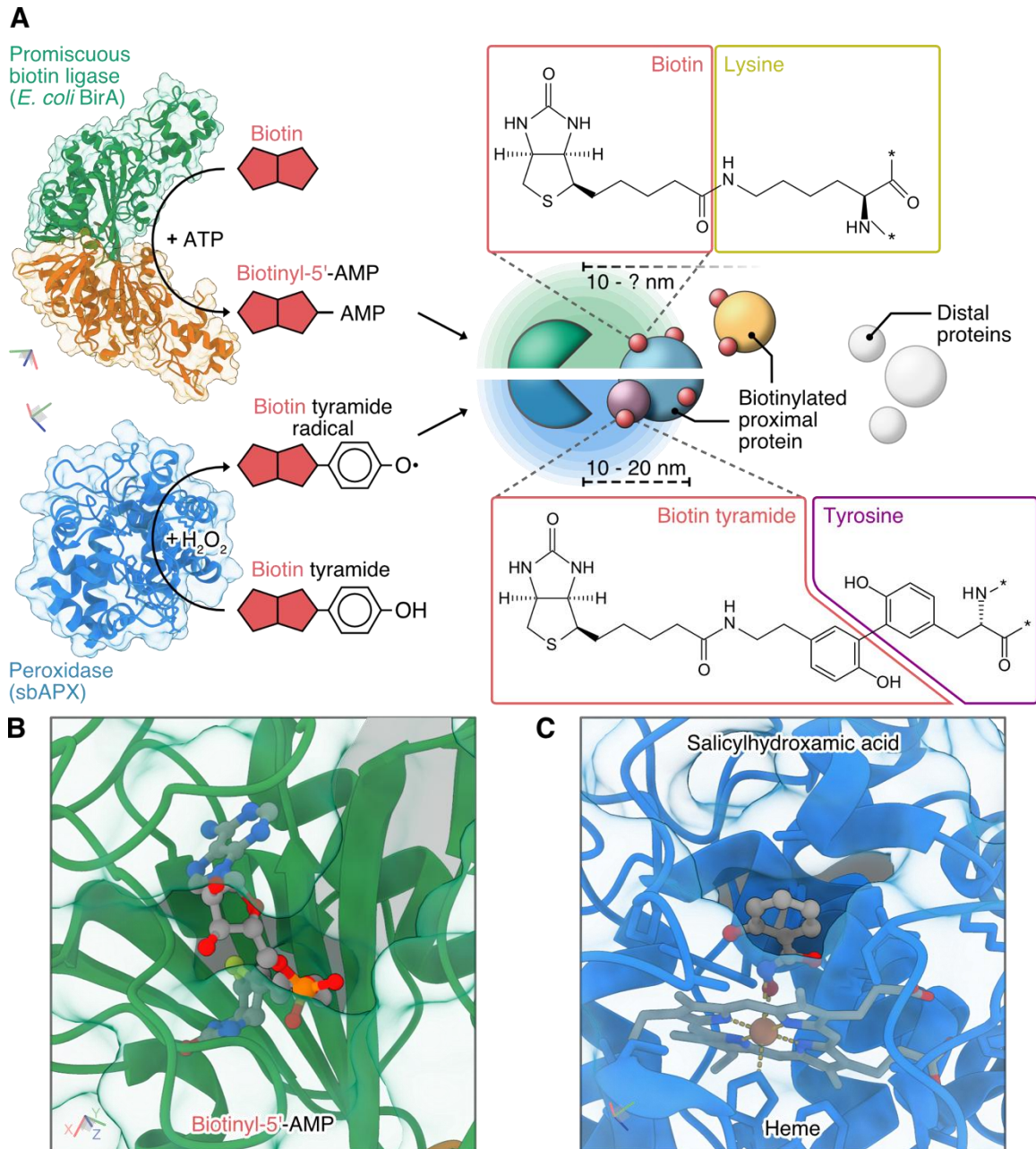


Fig. 4: Enzyme-catalyzed proximity labeling of nearby proteins through covalent biotinylation.

(A) Schematic representation of the proximity labeling reactions catalyzed by the promiscuous biotin ligase BirA from *E. coli* and the soybean ascorbate peroxidase APEX (sbAPX). Both enzyme crystal structures are shown in ribbon representation with surface-filling (*E. coli* BirA PDB: [2EWN](#); sbAPEX PDB: [1V0H](#)). BirA generates ATP-activated reactive biotin molecules that diffuse to potential target proteins, while APEX catalyzes the oxidation of biotin tyramide to a highly reactive short-lived radical in the presence of hydrogen peroxide. The covalent biotinylation of nearby proteins occurs at lysine for BirA and preferentially at tyrosine in the case of APEX. **(B)** Magnification of the BirA active site for biotin binding and activation. The binding site is occupied by an intermediate biotinyl-5'-AMP. **(C)** Magnification of the APEX catalytic site with incorporated heme and bound substrate salicylhydroxamic acid.

Concurrently, Rhee *et al* (2013) introduced a second proximity labeling enzyme: APEX, a genetically encodable 28-kDa monomeric heme-containing ascorbate peroxidase, originally derived from a constitutive homodimer from pea or soybean (**Fig. 4AC**) (Mandelman *et al*, 1998). First used for high-resolution electron microscopy (EM) imaging, the enzyme's useful proteomic potential was quickly discovered in its H₂O₂-dependent ability to oxidize phenolic compounds to short-lived phenoxyl radicals which can covalently react with tyrosine residues (and is proposed to react with other electron-rich amino acids such as cysteine, histidine and tryptophan) on surrounding proteins (Takahama, 2004; Martell *et al*, 2012; Rhee *et al*, 2013). Phenoxyl radical half-life time in aqueous solution has been shown to be below 1 ms, thereby estimating the proximal protein tagging range to less than 20 nm (Martell *et al*, 2012; Sato & Nakamura, 2019; Seath *et al*, 2021). APEX differs from biotin ligases like BioID in its structural positioning of the heme-containing substrate binding pocket (Fig. 4, insets). In APEX the aromatic substrate binding site is partially exposed to solvents, which results in weak substrate specificity. Therefore, diverse probes with different chemical enrichment handles, linker sizes, and aromatic moieties could be developed for PL (Kang & Rhee, 2022).

Prior to the discovery of APEX, heme-containing horseradish peroxidase (HRP) from plants was widely used in immunocytochemistry and immunohistochemistry, from light to electron microscopy, as well as for the HRP-coupled antibody detection of proteins in Western blotting using luminol-based chemiluminescence (Grigorenko *et al*, 2015). Similar to APEX, HRP catalyzes the solvent-exposed oxidation of aromatic phenols in the presence of H₂O₂ (Danner *et al*, 1973). However, HRP functionality and heme incorporation depend on structural requirements, including four disulfide bonds and presence of two Ca²⁺ prosthetic groups. In the reducing environment of the cell, the loss of Ca²⁺ and weakened disulfide bonds result in the misfolding of HRP, which leads to reduced catalytic activity and thermostability and limits the use of HRP for proximity labeling (Krainer & Glieder, 2015; Chen & Perrimon, 2017).

Mick *et al* (2015) used proximity labeling with a cilia-targeted APEX enzyme to analyze the proteome of wild-type mouse inner medullary collecting duct cell line (IMCD3) primary cilia *via* biotin-streptavidin affinity capture. The spatial and temporal specificity of APEX was coupled with the sensitivity of tandem-mass-tag LC/MS2, which enabled the discovery of novel ciliary components and subsequent molecular dissection of their ciliary functions.

Among the newly identified ciliary proteins were the kinases PKA, AMPK, and LKB1. The study proposed a ciliary localization of PKA regulating Hedgehog signaling and a signaling axis encompassing a cilia-localized STRAD β -LKB1-AMPK holoenzyme. Comparative proteomic analysis of IFT27-deficient cilia revealed accumulation of the BBSome complex and identified β -arrestin 2 and the viral receptor CAR as potential BBSome cargoes.

Bioengineering approaches have further ameliorated limitations of predecessor PL enzymes by enhancing their labeling abilities by, for example, reducing enzyme size, required substrate concentrations, labeling times and increasing stability as well as activity (Kang & Rhee, 2022; Mair & Bergmann, 2022). BioID2, the successor to BioID, was developed from the biotin ligase of *Aquifex aeolicus* to reduce the size of the PL tag (from 35 kDa to 27 kDa) and thus improve the correct localization of proteins in their intended environment. The enzyme was shown to require less biotin compared to BioID while maintaining a comparable time window for the labeling reaction (between 18-24 hours) (Kim *et al*, 2016). This major limitation barred biotin ligase-based approaches of mapping dynamic changes and transient interactions within cellular structures and prompted the development of TurboID and miniTurbo (Branon *et al*, 2018). Using yeast display, these enzymes were evolved from BioID to efficiently biotinylate vicinal proteins in a remarkably short time span. This approach resulted in TurboID, which had 15 mutations compared to the wild-type enzyme reducing the labeling time to about 10 min in different challenging cellular environments. miniTurbo exhibited smaller size (28 kDa) comparable to BioID2 due to deletion of its N-terminal region and encompassed 13 mutations relative to wild-type. The enzyme had a similar labeling period to TurboID, but had a two-fold reduction in activity and exhibited lower expression and stability. Directed evolution using yeast-display was also used to engineer soybean APEX for improved activity and sensitivity suitable for various cellular compartments, resulting in a single point mutant (A134P), termed APEX2 (Lam *et al*, 2015).

1.7 Quantitative Primary Cilia Proteomics

May *et al* (2021) then combined the evolved APEX2 PL with quantitative TMT-based proteomics using LC-MS3 to map the dynamic changes in the primary ciliary proteome upon Sonic Hedgehog (SHH) morphogen-dependent pathway activation. The proteomic profiling revealed distinct changes in the ciliary protein composition over time. Upon SHH signaling activation, PKA and GPR161 were quickly expelled from cilia. This rapid exit is a critical step in the signal transduction cascade, suggesting that the removal of these proteins from the ciliary compartment is necessary for the propagation of the sensed signal. PKA exit is particularly interesting as it indicates a shift in the cAMP signaling pathway, which is known to regulate ciliary function and SHH signaling (Tschaikner *et al*, 2020). In contrast to PKA and GPR161, the study identified that Paladin, a putative phosphatase, translocates into cilia upon SHH signaling activation (May *et al*, 2021a). This entry suggests that Paladin plays a significant role in modulating the ciliary environment, potentially by dephosphorylating ciliary proteins and thereby regulating their activity and stability.

Recently, the individual strengths of the advanced PL enzymes were further extended by a combinatorial approach termed TransitID (trafficking analysis by sequential incorporation of tags for identification) of sequential proximity labeling for mapping of protein trafficking. TurboID and APEX2 were used at distant sites in the cell to label their subcellular proteomes in succession to reveal the transported components (Xu *et al*, 2023).

The list of PL enzymes was further expanded by a recently developed 35 kDa bacterial tyrosinase from *Bacillus megaterium*, called BmTyr, which uses a type-3 copper enzyme which uses molecular oxygen to oxidize phenols to reactive o-quinones that react with nearby protein nucleophiles (cysteine, lysine and histidine) (Shuster & Fishman, 2009; Zhu *et al*, 2024). Though the enzyme does not require exogenous H₂O₂ compared to APEX2, the BmTyr-based labeling reaction in cells was shown to be dependent on supplementation of copper (Shuster & Fishman, 2009; Kanteev *et al*, 2013; Zhu *et al*, 2024). Further, endogenous tyrosinase may cause background labeling in melanocytes and retinal pigment epithelial cells, which permits the use of this enzyme for these cell types (Bentley *et al*, 1994).

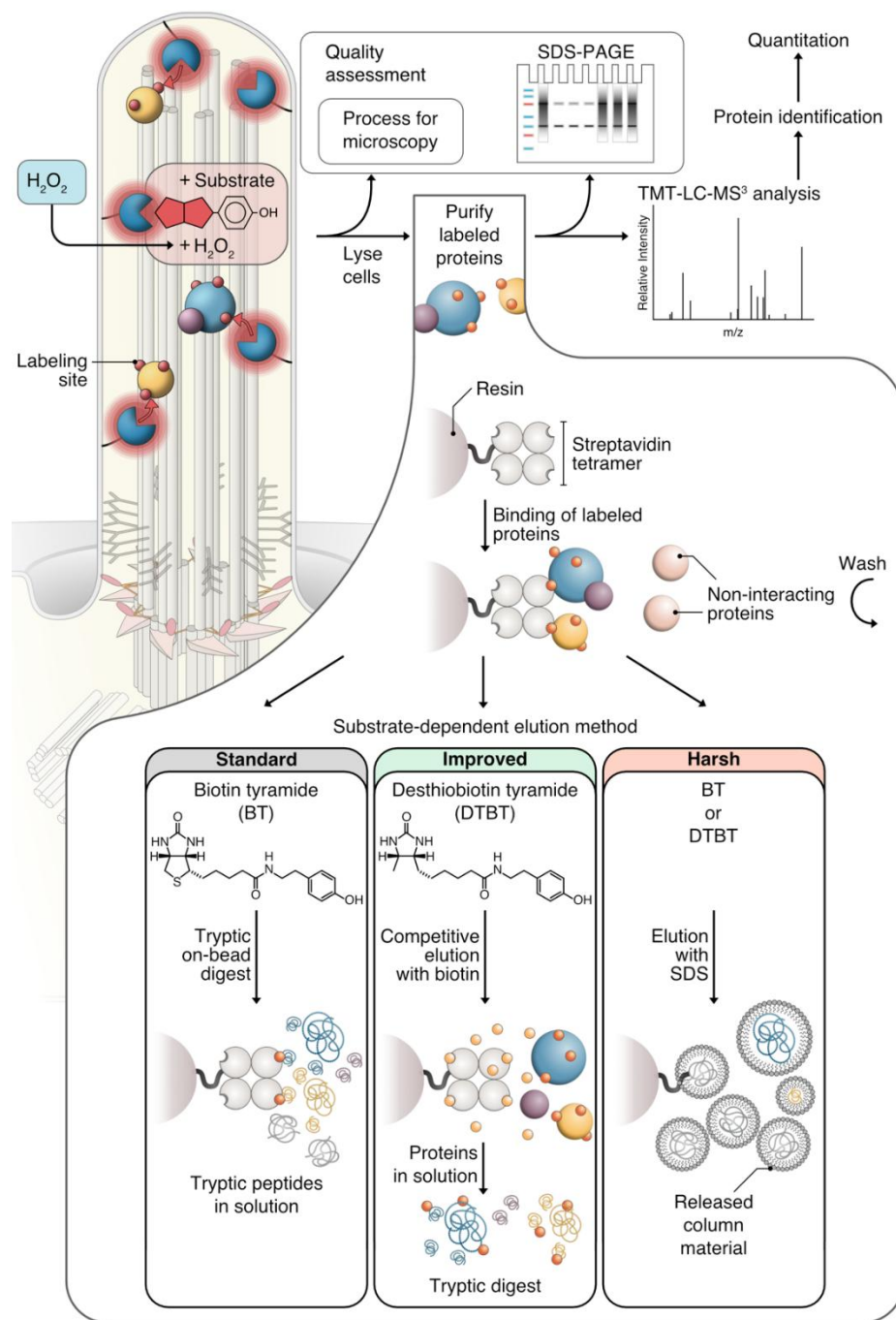


Fig. 5: Quantitative primary cilia proximity labeling proteomics.

Illustration of the primary cilia proteomics workflow. APEX is genetically targeted to primary cilia to locally catalyze a radical reaction which covalently links biotin to nearby proteins. Labeling capacity of handled specimen is assessed by microscopy and SDS-PAGE. Cell lysates are processed by affinity chromatography using streptavidin for biotin capture. Depicted are substrate-dependent elution methods which result in tryptic peptides that can be quantified by tandem-mass-tag liquid chromatography mass spectrometry with multiple rounds of fragmentation (TMT-LC-MS3). Elution with SDS is not recommended for MS analysis and is rather used to evaluate protein isolation efficiency.

1.8 Objectives

Primary cilia are crucial cellular organelles involved in signal transduction and cellular communication, playing key roles in various physiological processes. Despite their importance, the proteomic composition of primary cilia remains incompletely understood, particularly across different cell types and tissues (Mill *et al*, 2023). This work aims to address this gap by employing advanced proximity labeling techniques to achieve high-resolution and specific ciliary proteomic data. By elucidating the protein composition of diverse primary cilia, this study seeks to advance our fundamental understanding of these cellular compartments, which might aid in identifying novel therapeutic targets for ciliopathies and related conditions in the future. The specific objectives of this work include:

1. **Implement a quantitative labeling technique which can be used across cell types**

Apply a quantitative proximity labeling technique to a diverse range of murine cell types. This involves establishing a robust labeling protocol that can be consistently applied across different cellular contexts.

2. **Develop efficient methods for generating stable cell lines for quantitative proximity labeling in primary cilia**

Create a workflow for fast stable cell line generation in different cell types to allow comprehensive proteomic profiles of their primary cilia. This comparison will help identify tissue-specific variations and contribute to a broader understanding of primary cilia across different biological contexts.

3. **Conduct proteomic profiling and identify candidates**

Perform detailed proteomic profiling of primary cilia to identify new candidate proteins associated with primary cilia. This objective aims to expand the understanding of the protein composition and functional mechanisms of primary cilia. Validate the identified candidate proteins through subsequent experiments and comparisons with existing data, ensuring that the new findings are accurate and reliable.

By achieving these objectives, this research will provide significant insights into the molecular composition and function of primary cilia.

2. MATERIAL AND METHODS

2.1 Material

2.1.1 Antibiotics

Antibiotic	Stock Concentration (mg/ml)	Working Concentration (µg/ml)	Supplier/ CatN	Purpose
Ampicillin	100	100	Carl Roth K029.2	Bacterial Culture
Blasticidin S hydrochloride	10	4	Thermo Fisher A1113903	Cell Culture
Chloram- phenicol	25	25	Carl Roth 3886.2	Bacterial Culture
Kanamycin sulfate	50	50	Fisher Scientific 11578676	Bacterial Culture
Penicillin- Streptomycin (100x)	10	100	Fisher Scientific 15140122	Cell Culture
Puromycin Dihydrochlo- ride	10	1	Fisher Scientific 12122530	Cell Culture

2.1.2 Antibodies

Primary Antibodies

Antibody (clone)	Supplier/ CatN	Species	IgG	Clonality	Dilution IF	Dilution WB
acTub (6-11B-1)	Sigma-Aldrich (Merck) T7451	mouse	IgG2b	monoclo- nal	1:2000	1:2000
ALFA sdAb rab- bit-fc	NanoTag Biotechnologies N1583	camelid	IgG	polyclonal	1:500	1:500
ALFA sdAb mouse-fc	NanoTag Biotechnologies N1582	camelid	IgG1	monoclo- nal	1:500	1:500
Annexin V	Proteintech 66245-1-Ig	mouse	IgG1	monoclo- nal	n.a.	1:10000
ARL13B	Proteintech 17711-1-AP	rabbit	IgG	polyclonal	1:1000	n.a.
FLAG (M2)	Sigma-Aldrich (Merck) F1804	mouse	IgG1	monoclo- nal	1:1000	1:1000
GAPDH (1E6D9)	Proteintech 60004-1-Ig	mouse	IgG2b	monoclo- nal	n.a.	1:2000
GFP (3H9)	Proteintech (Chromotek) 3h9	rat	IgG2a	monoclo- nal	n.a.	1:1000
"GFP34"	self-made	rabbit	IgG	polyclonal	n.a.	1:500
IFT20	Proteintech 13615-1-AP	rabbit	IgG	poly-clonal	1:2500	n.a.

Continued on the next page.

Primary Antibodies (Continued)

Antibody (clone)	Supplier/ CatN	Species	IgG	Clonality	Dilution IF	Dilution WB
IFT57	Proteintech 11083-1-AP	rabbit	IgG	poly-clonal	n.a.	1:500
IFT88	Proteintech 13967-1-AP	rabbit	IgG	poly-clonal	1:200	1:1000
mCherry (16D7)	Thermo Fisher Scientific M11217	rat	IgG2a	monoclo- nal	1:1000	1:1000
PALD1	Sigma-Aldrich (Merck) HPA017343	rabbit	IgG	poly-clonal	1:200	1:1000
SMO (E-5)	SantaCruz sc-166685	rabbit	IgG	poly-clonal	1:250	n.a.
V5	Fisher Scien- tific 10347642	mouse	IgG2a	monoclo- nal	1:200	1:5000

Fluorescent Secondary Antibodies and Reagents

Antibody	Supplier/ CatN	Host	Reac- tivity	Target Isotype	Dilution IF	Dilu- tion WB
FluoTag®-X2 α-ALFA Alexa Fluor™ 647	NanoTag Biotechnolo- gies N1502-AF647	camelid sdAb	ALFA	n.a.	1:500	n.a.
FluoTag®-X2 α-ALFA IRDye® 800CW	NanoTag Biotechnolo- gies N1502-Li800	camelid sdAb	ALFA	n.a.	n.a.	1:1000
Revert™ 700 Total Protein Stain	LI-COR 926-11011	n.a.	n.a.	n.a.	n.a.	n.a.
Streptavidin Alexa Fluor™ 647	Fisher Scien- tific S21374	n.a.	biotin	n.a.	1:1000	n.a.
Streptavidin IRDye® 680RD	LI-COR 926-68079	n.a.	biotin	n.a.	n.a.	1:1000
Streptavidin IRDye® 800CW	LI-COR 926-32230	n.a.	biotin	n.a.	n.a.	1:1000
Streptavidin Northern- Lights™ 557	RD Systems NL999	n.a.	biotin	n.a.	n.a.	1:2000
α-mouse IgG HRP conju- gate	Merck AP127P	goat	mouse	IgG	n.a.	1:2000
α-mouse IgG1 Alexa Fluor™ 488	Thermo Fisher Scientific A-21121	goat	mouse	IgG1	1:500	n.a.

Continued on next page.

Fluorescent Secondary Antibodies and Reagents (continued)

Antibody	Supplier/ CatN	Host	Reac- tivity	Target Isotype	Dilution IF	Dilu- tion WB
α -mouse IgG1 Cyanine3	Thermo Fisher Scientific M30010	goat	mouse	IgG1	1:500	n.a.
α -mouse IgG1 Cyanine5	Thermo Fisher Scientific A10524	donkey	mouse	IgG	1:500	n.a.
α -mouse IgG2a Alexa Fluor™ 350	Thermo Fisher Scientific A-21130	goat	mouse	IgG2a	1:500	n.a.
α -mouse IgG2a Alexa Fluor™ 488	Thermo Fisher Scientific A-21131	goat	mouse	IgG2a	1:500	n.a.
α -mouse IgG2a Alexa Fluor™ 555	Thermo Fisher Scientific A-21137	goat	mouse	IgG2a	1:500	n.a.
α -mouse IgG2b Alexa Fluor™ 488	Thermo Fisher Scientific A-21141	goat	mouse	IgG2b	1:500	n.a.
α -mouse IRDye® 680RD	LI-COR 926-68070	donkey	mouse	IgG	1:500	n.a.
α -rabbit IgG Alexa Fluor™ 350	Thermo Fisher Scientific A-11046	goat	rabbit	IgG	1:500	n.a.
α -rabbit IgG Alexa Fluor™ 488	Thermo Fisher Scientific A-11008	donkey	rabbit	IgG	1:500	n.a.

Continued on next page.

Fluorescent Secondary Antibodies and Reagents (continued)

Antibody	Supplier/ CatN	Host	Reac- tivity	Target Isotype	Dilution IF	Dilu- tion WB
α -rabbit IgG Alexa Fluor™ 555	Thermo Fisher Scientific A-21428	donkey	rabbit	IgG	1:500	n.a.
α -rabbit IgG Alexa Fluor™ 647	Thermo Fisher Scientific A-21245	donkey	rabbit	IgG	1:500	n.a.
α -rabbit IgG Alexa Fluor™ plus555	Thermo Fisher Scientific A-31572	donkey	rabbit	IgG	1:500	n.a.
α -rabbit IgG HRP-conju- gate	Sigma-Aldrich AP187P	goat	rabbit	IgG	n.a.	1:2000
α -rabbit IRDye® 800CW	LI-COR 926-32211	donkey	rabbit	IgG	n.a.	1:10000
α -rat Cyanine5	Thermo Fisher Scientific A10525	donkey	rat	IgG	1:500	n.a.
α -rat IgG IRDye® 680RD	LI-CO 926-68076	goat	rat	IgG	n.a.	1:10000
α -rat IgG IRDye® 800CW	LI-COR 926-32219	goat	rat	IgG	n.a.	1:10000

2.1.3 Bacterial Strains

Bacterial Strain	Genotype	Supplier
One Shot™ <i>ccdB</i> Survival™ 2 T1R Competent Cells	F*- <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 ara</i> Δ139 Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str) <i>endA1</i> <i>nupG fhuA::IS2</i>	Thermo Fisher Scientific A10460
One Shot™ Mach1™ T1 Phage-Resistant Chemically Competent <i>E. coli</i>	F*- φ80(<i>lacZ</i>)ΔM15 Δ <i>lacX74 hsdR</i> (r*K*-, m*K*+) Δ <i>recA1398 endA1 tonA</i>	Thermo Fisher Scientific C862003
One Shot™ Stbl3™ Chemically Competent <i>E. coli</i>	F*- <i>mcrB mrrhsdS20</i> (r*B*-, m*B*-) <i>recA13 supE44 ara-14 galK2 lacY1 proA2</i> <i>rpsL20</i> (Str) <i>xyl-5 λ-leumtl-1</i>	Thermo Fisher Scientific C737303
Subcloning Efficiency™ DH5α Competent Cells	F*- φ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (r*K*-, m*K*+) <i>phoA</i> <i>supE44 thi-1 gyrA96 relA1 λ-</i>	Thermo Fisher Scientific 18265017

2.1.4 Buffers and Solutions

Agarose Gel Electrophoresis

TAE buffer (50×)	500 mM Tris 5.7% (v/v) Glacial acetic acid (100%) 50 mM EDTA (500 mM, pH 8.0) in water
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APEX/iAPEX Labeling

Biotin tyramide	50 mM in DMSO
Desthiobiotin tyramide	50 mM in DMSO

Continued on next page.

Buffers and Solutions (continued)

D-Methionine	250 mM D-Methionine in water
L-Methionine	250 mM L-Methionine in water
Quenching buffer (Q)	10 mM Sodium L-ascorbic acid 10 mM Sodium azide in 1x PBS (pH 7.2)
Quenching buffer + Trolox (QT)	10 mM Sodium L-ascorbic acid 10 mM Sodium azide 5 mM Trolox in 1x PBS (pH 7.2)
RIPY+Q buffer (1×)	0.5% Triton X-100 (10%) 0.1% SDS (10%) 50% (v/v) RIPY+Q buffer (2x) 0.5 mM PMSF 2% (v/v) PIC (50x) in water
RIPY+Q buffer (2×)	600 mM NaCl 200 mM Tris/HCl (pH 7.5) 20 mM Sodium L-ascorbate 20 mM Sodium azide 10 mM Trolox 20% (v/v) Glycerol in water
Tris/HCl elution buffer + biotin	100 mM Tris/HCl (pH 7.5) 5 mM Biotin in water
Urea elution buffer + biotin	5 mM Biotin in urea wash buffer
Urea wash buffer	2 M Urea 10 mM Tris/HCl (pH 7.5) in water

Continued on next page.

Buffers and Solutions (continued)

Bacterial Culture

Lysogeny Broth	1% (w/v) Tryptone 0.5% (w/v) yeast extract 0.5% (w/v) NaCl 1.6% (w/v) Agar-agar in water
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Blue Native PAGE

Acrylamide	49.5% Acrylamide 3% N,N'-Methylenebisacrylamide in water
Anode buffer (40×)	2 M Bis-Tris in water (pH 7.0 adjusted with HCl)
BN PAGE gel buffer (3×)	200 mM ϵ -Amino n-caproic acid 150 mM Bis-Tris/HCl in water (pH 7.0)
Cathode buffer blue	500 mM Tricine 150 mM Bis-Tris/HCl 0.2% Coomassie G in water (pH 7.0)
Cathode buffer white	500 mM Tricine 150 mM Bis-Tris/HCl in water (pH 7.0)
Coomassie loading dye (10×)	5% Coomassie G 500 mM ϵ -amino n-caproic acid 100 mM Bis-Tris in water (pH 7.0 adjusted with HCl)
Coomassie staining solution	40% (v/v) EtOH (100%) 10% (v/v) Glacial acetic acid 0.5% (w/v) Coomassie R in water

Continued on next page.

Buffers and Solutions (continued)

Destaining solution	40% (v/v) EtOH (100%) 10% (v/v) Glacial acetic acid in water
SDS running buffer (10×)	250 mM Tris 1.91 M Glycine 10% (w/v) SDS in water
Solubilization buffer (4×)	80 mM Tris/HCl (pH 7.4) 200 mM NaCl 40% (v/v) Glycerol 0.4 mM EDTA in water
Solubilization elution buffer (1×)	2% (v/v) ALFA peptide (100x) in solubilization wash buffer (1x)
Solubilization lysis buffer (1×)	50% (v/v) Solubilization buffer (2x) 0.2-0.4% (v/v) NP-40 (10%) 1 mM PMSF 2% (v/v) PIC (50x) in water
Solubilization wash buffer (1×)	50% (v/v) Solubilization buffer (2x) 0.02% (v/v) NP-40 (10%) in water

Copper-Catalyzed Azide-Alkyne Cycloaddition

Alkyne-phenol	50 mM Alkyne-phenol in DMSO
Azide-fluor545	10 mM Azide-fluor545 in DMSO
Biotin-azide	10 mM Biotin-azide in DMSO
BTAA	50 mM BTAA in water

Continued on next page.

Buffers and Solutions (continued)

CuSO ₄	50 mM CuSO ₄ in water
Cu-BTTAA stock (25×)	12.5 mM : 25 mM Cu-BTTAA stock 1:2:1 CuSO ₄ :BTTAA:water
Sodium ascorbate	25 mM in 1× PBS

General

DTT	1 M DTT in water
PMSF	200 mM PMSF in 100% EtOH
SAG	100 mM SAG in DMSO
SDS (10%)	10% (w/v) SDS in water
Tris/HCl	0.1 or 1 M Tris in water (pH 6.8, 7.5 or 8.5; all adjusted with HCl)
Triton X-100 (0.2%)	0.2% (v/v) Triton X-100 in water
Triton X-100 (10%)	10% (v/v) Triton X-100 in water

Immunofluorescence

Blocking buffer	5% (v/v) Fetal bovine serum 3% (w/v) BSA 10% (v/v) PBS (10x) in water
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Buffers and Solutions (continued)

PBS (10×)	27 mM KCl 18 mM KH_2PO_4 1369 mM NaCl 81 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in water (pH 7.4 adjusted with HCl)
PFA (4%)	4% (v/v) PFA (16%) 10% (v/v) PBS (10x) in water

Mass Spectrometry Preparation

DTT buffer	50 mM DTT in 2 M Urea wash buffer
HEPES (pH 8.0)	1 M HEPES (pH 8.0 adjusted with NaOH) in water
IAA	50 mM IAA in 8 M Urea/Tris (pH 8.5)
IAA buffer	10 mM IAA in 2 M Urea wash buffer
Urea/HEPES (pH 8.0)	8 M Urea in 1 M HEPES (pH 8.0)
Urea/Tris (pH 8.5)	8 M Urea in 0.1 M Tris/HCl (pH 8.5) (0.75 g in 2 ml reaction tube, dissolved in 1 ml Tris results in about 1.5 ml 8 M urea)

SDS PAGE

Bis-Tris buffer (3×)	1 M Bis-Tris/HCl in water (pH 6.4)
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Continued on next page.

Buffers and Solutions (continued)

Laemmli sample buffer (5×)	10% (w/v) SDS 50% (v/v) Glycerol 300 mM Tris/HCl (pH 6.8) 0.05% (w/v) Bromophenol blue in water
MOPS (10×)	500 mM MOPS 500 mM Tris 1% (w/v) SDS 10.25 mM Na ₂ EDTA*2H ₂ O in water (pH approx. 7.6)

Sucrose Gradient Density Ultracentrifugation

EDTA	500 mM EDTA in water (pH 8.0)
EGTA	500 mM EGTA in water (pH 8.0)
HEPES (pH 7.4)	1 M HEPES (pH 7.4 adjusted with NaOH) in water
HMDEK buffer (4×)	40 mM HEPES (1 M, pH 7.4) 20 mM MgSO ₄ *7H ₂ O 2 mM EDTA (500 mM) 100 mM KCl (2 M) in water
HMDEK buffer + sucrose	25% (v/v) HMDEK buffer(4x) 1 mM DTT X% (v/v) Sucrose (80%) in water
KCl	2 M KCl in water
LAP _{200ng} buffer (4×)	200 mM HEPES (pH 7.4) 800 mM KCl (2 M) 2 mM EGTA (500 mM) 2 mM MgCl ₂ (1 M) in water

Continued on next page.

Buffers and Solutions (continued)

LAP _{200ng} buffer + sucrose	25% (v/v) LAP _{200ng} buffer (4x) 1 mM DTT X% (v/v) Sucrose (80%) in water
MgCl ₂ *6H ₂ O	1 M MgCl ₂ *6H ₂ O in water
MgSO ₄ *7H ₂ O	1 M MgSO ₄ *7H ₂ O in water
Sucrose (80%)	80% (w/v) Sucrose in water (dissolved by slow addition while stirring under heating (60 °C))

Western blot

LICOR Revert Destaining solution	100 mM Sodium hydroxide 30% (v/v) MeOH (100%) in water
LICOR Revert Wash solution	6.7% (v/v) Glacial acetic acid 30% (v/v) MeOH (100%) in water
Semi-dry buffer (10×)	200 mM Tris 1500 mM Glycine 0.2% (v/v) SDS (10%) in water (add 10% (v/v) EtOH (100%) when making 1x)
TBS (25x)	500 mM Tris/HCl 3.125 M NaCl in water (pH 7.5 adjusted with HCl)
Transfer buffer (10×)	250 mM Tris 1920 mM Glycine in water (add 10% (v/v) EtOH (100%) when making 1x)

2.1.5 Cell Lines

Parental	Species	Cell Line/ Plasmid	Expression of	Reference
293 [HEK-293]	human	293 [HEK-293]	n.a.	ATCC CRL-1573
3T3-L1	mouse	3T3-L1	n.a.	ATCC CRL-173
		3T3-L1 pTS100	Nphp3(1-200)-eGFP-APEX2-IRES- Nphp3(1-200; opt.)-FLAG-DAAO- ALFA	this work (Lea Sanwald)
C2C12	mouse	C2C12	n.a.	ATCC CRL-1772
		C2C12 pTS100	Nphp3(1-200)-eGFP-APEX2-IRES- Nphp3(1-200; opt.)-FLAG-DAAO- ALFA	this work (Lea Sanwald)
IMCD3 Flp-In™	mouse	IMCD3 Flp-In™	n.a.	Mukhopadhyay 2010
		IMCD3 pDaM181 (clone 6)	Nphp3(1-200)-eGFP-APEX2	this work
		IMCD3 pDaM209 (clone 9)	Nphp3(1-200; G2A)-eGFP-APEX2	
		IMCD3 <i>Cep164</i> ^{-/-} pDaM181 (clone 8)	Nphp3(1-200)-eGFP-APEX2	

Continued on next page.

Cell Lines (continued)

Parental	Species	Cell Line/ Plasmid	Expression of	Reference
IMCD3 Flp-In™	mouse	IMCD3 <i>Ift88</i> ^{-/-} (clone 1)	IMCD3 Flp-In™	Lea Sanwald
		IMCD3 <i>Ift88</i> ^{-/-} pTS106 (clone 4)	Nphp3(1-200)-eGFP-APEX2 + ALFA-IFT88	this work
		IMCD3 <i>Palld1</i> ^{-/-}	IMCD3 Flp-In™	Patrick Schuster
		IMCD3 <i>Palld1</i> ^{-/-} PALD1-ALFA (clone 6)	PALD1-ALFA	
		IMCD3 pTS010 (clone 1)	Nphp3(1-200)-GFP- TurboID-V5	this work
		IMCD3 pTS012 (clone 6)	Nphp3(1-200; G2A)-eGFP-Tur- boID-V5	
		IMCD3 pTS069 (clone 8)	Nphp3(1-200)-eGFP-APEX2 + Nphp3(1-200)-FLAG-DAAO-NES	
		IMCD3 pTS086 (clone 3)	Nphp3(1-200)-eGFP-APEX2 + Nphp3(1-200; G2A)-FLAG- DAAO-NES	
		IMCD3 pTS093 (clone 4)	mNphp3(1-200)-FLAG-DAAO- ALFA-APEX2	

Continued on next page.

Cell Lines (continued)

Parent- al	Species	Cell Line/ Plasmid	Expression of	Reference
IMCD3 Flp-In™	mouse	IMCD3 pTS094 (clone 3)	mNphp3(1-200; G2A)-FLAG- DAAO-ALFA-APEX2	this work
		IMCD3 pTS100	Nphp3(1-200)-eGFP-APEX2-IRES- Nphp3(1-200; opt.)-FLAG-DAAO- ALFA	this work (Lea Sanwald)
		IMCD3 pTS115	Nphp3(1-200)-eGFP-APEX2-T2A- fCTS-FLAG-DAAO-ALFA	
		IMCD3 pTS124	Nphp3(1-200; G2A)-eGFP-APEX2- T2A- fCTS-FLAG-DAAO-ALFA	
NIH/3T3 Flp-In™	mouse	NIH/3T3 Flp-In™	n.a.	Thermo Fisher Scientific R76107
		NIH/3T3 pDaM181 (clone 5)	Nphp3(1-200)-eGFP-APEX2	this work
		NIH/3T3 pTS069 (clone 7)	Nphp3(1-200)-eGFP-APEX2 + Nphp3(1-200)-FLAG-DAAO-NES	
		NIH/3T3 pTS086 (clone 3)	Nphp3(1-200)-eGFP-APEX2 + Nphp3(1-200; G2A)-FLAG- DAAO-NES	

Continued on next page.

Cell Lines (continued)

Parent- al	Species	Cell Line/ Plasmid	Expression of	Reference
NIH/3T3 Flp-In™	mouse	NIH/3T3 pTS093 (clone 3)	mNphp3(1-200)-FLAG-DAAO- ALFA-APEX2	this work
		NIH/3T3 pTS094 (clone 5)	mNphp3(1-200; G2A)-FLAG- DAAO-ALFA-APEX2	
		NIH/3T3 pTS100	Nphp3(1-200)-eGFP-APEX2-IRES- Nphp3(1-200; opt.)-FLAG-DAAO- ALFA	this work (Lea Sanwald)
		NIH/3T3 pTS115	Nphp3(1-200)-eGFP-APEX2-T2A- fCTS-FLAG-DAAO-ALFA	
		NIH/3T3 pTS124	Nphp3(1-200; G2A)-eGFP-APEX2- T2A- fCTS-FLAG-DAAO-ALFA	
RPE-1 Flp-In™ T- REx™	human	RPE-1 Flp-In™ T-REx™	n.a.	(Čajánek & Nigg, 2014) ATCC CRL-4000
		RPE-1 pTS100	Nphp3(1-200)-eGFP-APEX2-IRES- Nphp3(1-200; opt.)-FLAG-DAAO- ALFA	this work (Lea Sanwald)

2.1.6 Cell Culture Media and Supplements

Media/Supplements	Supplier/CN
Bovine Calf Serum	Sigma-Aldrich 12133C-500ML
BAMBANKER [®] serum-free cell freezing medium	Wako Chemicals 302-14681
Gibco [™] Bacto [™] tryptone	Fisher Scientific 16279751
Gibco [™] DMEM, high glucose, pyruvate	Fisher Scientific 11594486
Gibco [™] DMEM/F-12, HEPES	Fisher Scientific 11594426
Gibco [™] PBS, pH 7.2	Fisher Scientific 11530546
Gibco [™] Trypsin-EDTA (0.05%), phenol red	Fisher Scientific 11580626

2.1.7 Chemicals

Chemical	Supplier/CN
2-Propanol	VWR International 20842.323
Acetonitrile	Sigma-Aldrich 34851
Acrylamide	Carl Roth 7906.2

Continued on next page.

Chemicals (continued)

Chemical	Supplier/CN
Acrylamide solution, 40%	Sigma-Aldrich A4058
Agarose, low melt	Carl Roth 6351.5
Alfa Aesar™ D-Alanine	Fisher Scientific 11483923
ALFA peptide (#1787)	Martin Jung n.a.
ALFA Selector PE incl. ALFA elution peptide	Nano-Tag N1510
Alkyne-phenol	Hölzel Biotech HY-131442
Amplex™ UltraRed Reagent	Fisher Scientific 10737474
APS Ammonium peroxydisulphate	Grüssing 10316250U
Azide-fluor545	Sigma-Aldrich 760757-1MG
Bacto™ Agar solidifying agent	BD Diagnostics 214010
Bacto™ Yeast extract	BD Diagnostics 212720
Biotin-azide	Hölzel Biotech HY-129832-10MG

Continued on next page.

Chemicals (continued)

Chemical	Supplier/CN
Biotin tyramide	Iris Biotech LS-3500.1000
BIS-TRIS Bis-(2-hydroxyethyl)-imino-tris-(hydroxymethyl)-methane	Carl Roth 9140.8
Bromophenol Blue sodium salt	SERVA 15375
BSA Bovine Serum Albumin Fraction V, IgG free	Carl Roth 3737.3
BTAA 2-(4-((bis((1-(tert-butyl)-1H-1,2,3-triazol-4-yl) methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid	Jena Bioscience CLK-067-100
Carl Zeiss™ Immersol™ Immersion oil	Fisher Scientific 10539438
Complete™ EDTA-free Protease Inhibitor Cocktail tablets	Sigma-Aldrich 5056489001
Coomassie Brilliant blue G 250	SERVA 35050
CuSO ₄ Copper(II) sulphate	Roth CP86.2
Cytiva Streptavidin Sepharose™ High Performance Medium	Fisher Scientific 11565015
CytochalasinD, from <i>Zygosporium mansonii</i>	Sigma-Aldrich C8273-10MG
D-Methionine	Carl Roth 7885.2

Continued on next page.

Chemicals (continued)

Chemical	Supplier/CN
DDM n-dodecyl--D-maltoside	Avanti Polar Lipids 850520P-5g
Desthiobiotin	Fisher Scientific 12753064
Desthiobiotin tyramide	Iris Biotech LS-1660.0250
Disodium hydrogen phosphate dihydrate	Carl Roth 4984.2
DMSO Dimethylsulfoxid	Carl Roth 4720.1
dNTP Deoxynucleotide solution mix	New England Biolabs N0447S
DTT 1,4-Dithiothreitol	Fisher Scientific 10578170
EDTA Ethylenediaminetetraacetic acid disodium salt dihydrate	Carl Roth 8043.2
EGTA Ethylene Glycol Bis(-aminoethylether) tetraacetic acid	Carl Roth 3054.1
Ethanol,99% denatured with 1% methyl ethyl ketone	BCD Chemie 10012343
Ethanol, absolute	Geyer 2273.1000
Formaldehyde solution, 36.5-38%	Sigma-Aldrich F8775

Continued on next page.

Chemicals (continued)

Chemical	Supplier/CN
Formaldehyde, 16%	Polysciences 18814-20
Formic acid	Thermo Fisher Scientific 85178
Glacial acetic acid	Carl Roth 6755.1
Glycine Pufferan	Carl Roth 3908.3
Glycerol	Sigma-Aldrich G5516
Hydrogen peroxide solution 30% (w/w) in H ₂ O	Sigma-Aldrich H1009
Hydroxylaminesolution 50% aqueous solution	abcr AB 131585
IAA Iodoacetamide	Sigma-Aldrich I6125
IBMX, 3-Isobutyl-1-methylxanthine	Fisher Scientific 10617572
Invitrogen™ Fluoromount-G™	Fisher Scientific 15586276
Invitrogen™ Hoechst 33342 trihydrochloride, trihydrate	Thermo Fisher Scientific H3570
Jetprime® DNA and siRNA transfection reagent	VWR International 101000046.

Continued on next page.

Chemicals (continued)

Chemical	Supplier/CN
L-Methionine	Sigma-Aldrich M9625-5G
L(+)-Ascorbic acid sodium salt	Carl Roth 3149.1
LICOR Intercept (TBS) protein-free blocking buffer	LI-COR Biosciences 927-80003
Magnesium chloride hexahydrate	Carl Roth 2189.2
Magnesiumchloride hexahydrate	Carl Roth 2189.2
Magnesium Sulfate Heptahydrate	Sigma-Aldrich M2773
Mangan(II)-chlorid tetrahydrate	Carl Roth T881.3
Methanol	VWR International 20864.320
Molecular Probes™ D-Biotin (50 mM)	Fisher Scientific 11554167
MOPS 3-(N-Morpholino)-propane sulphonic acid	Carl Roth 6979.3
N,N'-Methylenebis(acrylamide)	Sigma-Aldrich 146072
N,N'-Methylenebis(acrylamide) solution, 2%	Sigma-Aldrich 1-6095

Continued on next page.

Chemicals (continued)

Chemical	Supplier/CN
neoLabLine Bath Stabil [®] , water bath stabilizer	Neolab 1-6095
NP-40 Surfact-Amps [™] Detergent Solution, 10%	Thermo Fisher Scientific 85124
Pierce [™] Premium grade TCEP-HCl Tris(2-carboxyethyl)phosphin-hydrochlorid	Fisher Scientific 15295763
PMSF Phenylmethylsulfonylfluorid	Carl Roth 6367.1
Poly-D-Lysine	Fisher Scientific A3890401
Potassium Chloride	Sigma-Aldrich P-9541
Potassium dihydrogen phosphate	Grüssing 120171000
Peqlab peqGREEN DNA/RNA dye	VWR International 732-3196
ROTI [®] Quant Bradford solution	Carl Roth K015.1
ROTIPHORESE [®] Gel 30 (37.5:1)	Carl Roth 3029.1
SAG dihydrochloride Smoothened Agonist	Sigma-Aldrich SML1314-1MG
SDS Sodium dodecyl sulfate	AppliChem A2572,1000

Continued on next page.

Chemicals (continued)

Chemical	Supplier/CN
Sequencing grade modified trypsin	Promega V5113
Skimmed milk powder	Sucofin n.a.
Sodium acrylate	Sigma-Aldrich 408220
Sodium azide	Carl Roth K305.1
Sodium chloride	Fisher Scientific 10428420
Sodium hydroxide	Merck 6498
Strep-Tactin [®] Sepharose [®] 50% suspension	IBA lifesciences 2-1201-002
Surface disinfectant Kohrsolin [®] FF	Carl Roth L301.2
TCA Trichloroacetic acid	Carl Roth 8789.4
TFA Trifluoroacetic acid, Optima [™] LC/MS grade	Fisher Scientific 10723857
Tricine	Sigma-Aldrich T-5816-25g
TRIS Pufferan	Carl Roth AE15.1

Continued on next page.

Chemicals (continued)

Chemical	Supplier/CN
Triton-X100	Thermo Fisher Scientific A16046.AP
Trolox(R), 97%	Fisher Scientific 10782831
Tween [®] 20	Carl Roth 9127.2
Urea, ultra-pure	MP Biomedicals 821527
V5 synthetic peptide	Sino Biological PP100378-5
Water, Optima™ LC/MS grade	Fisher Scientific 10728098
ε-Amino n-caproic acid	Sigma-Aldrich A7824-100G

2.1.8 Consumables

Item	Supplier/CN
Bemis™ Parafilm™ Laboratory Wrapping Film	Fisher Scientific 11772644
Bio-One™ CELLSTAR Test Tubes, 15 ml	Fisher Scientific 10384601
Bio-One™ CELLSTAR Test Tubes, 50 ml	Greiner Bio-One 227261

Continued on next page.

Consumables (continued)

Item	Supplier/CN
Bio-One™ Cryo.s™ Cryogenic Tubes	Greiner Bio-One 122263
Bio-One™ Filter Pipet Tips 1000 µl	Greiner Bio-One 740288
Bio-One™ Filter Pipet Tips 200 µl	Greiner Bio-One 739288
Bio-One™ Filter Pipet Tips, 20 µl	Greiner Bio-One 774288
Blotting Paper	Macherey-Nagel 742111
Bolt™ 4-12% Bis-Tris Plus Gels, 15-well	Thermo Fisher Scientific NW04125BOX
Brand™ Soda Lime Glass Pasteur Pipets	Fisher Scientific 15202699
Cell Culture Flask, T-25	Sarstedt 83.3910.002
Cell Culture Flask, T-75	Sarstedt 83.3911.002
Cell Culture Multiwell Plate, 24-well	Greiner Bio-One 662160
Cell Culture Multiwell Plate, 48-well	Greiner Bio-One 677180
Cell Culture Multiwell plate, 6-well	Greiner Bio-One 657160

Continued on next page.

Consumables (continued)

Item	Supplier/CN
CELLSTAR Tissue Culture Dish, 10 cm	Greiner Bio-One 664160
CELLSTAR Tissue Culture Dish, 15 cm	Greiner Bio-One 639160
Corning™ TC-Treated Culture Dishes, Square 500 cm ²	Fisher Scientific 10350602
Cytiva Amersham™ Protran™ Premium NC Nitrocellulose Membrane	Fisher Scientific 15269794
Disposal Bags	Sarstedt 86.1200
Eppendorf Tubes™ Conical Tubes, 5 ml	Fisher Scientific 15128344
Filter Pipet Tips, 10 µl	Brand 732724
Fisherbrand™ Cell Lifters	Fisher Scientific 11577692
Fisherbrand™ Grade 913 Lens Cleaning Tissue	Fisher Scientific 11517362
Fisherbrand™ Premium Microscope Slide Box	Fisher Scientific 11334145
Gel Loading Dye Purple 6x	New England Biolabs B7024A
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific SM0311

Continued on next page.

Consumables (continued)

Item	Supplier/CN
Gibco™ Bottle Weight	Fisher Scientific 10025683
Graduated Pipet Tip, 10 µl	STARLAB S1111-3700
Graduated Pipet Tip, 1000 µl	STARLAB S1111-6701
Graduated Pipet Tip, 200 µl	STARLAB S1113-1706
Graduated Polypropylene Culture Tubes	Carl Roth AEY1.1
Inoculation Loops	Sarstedt 86.1562.050
Invitrogen™ NuPAGE™ -12% Bis-Tris Midi Protein Gels, 26-well	Thermo Fisher Scientific WG1403BX10
Microtubes, 2 ml	Sarstedt 72,694,007
Mobicol „F“, fixed outlet plug, inserted 35 µm filter small	MoBiTec M105135F
PCR strips	Carl Roth XT90.1
pipetus® PTFE Membrane Filters	Sigma-Aldrich Z315028-1EA
PlusOne® HMW Native Marker Kit	VWR International 17-0445-01

Continued on next page.

Consumables (continued)

Item	Supplier/CN
PYREX 8x8mm Cloning Cylinders	Sigma-Aldrich CLS31668-125EA
Reaction Tubes 1,5ml	Sarstedt 72,690,001
Rogo Sampaic™ Glass Microscope Slides	Fisher Scientific 11874782
Round Coverslip, 12 mm, Type 1.5	Fisher Scientific 11846933
Round Coverslip, 25mm Type 1.5	Fisher Scientific 10593054
Sartorius™ Midisart™ 2000 PTFE Air Filter	Fisher Scientific 10109660
Serological Pipette, 10 ml	Sarstedt 703234
Serological Pipette, 2 ml	Sarstedt 86.1252.025
Serological Pipette, 25 ml	Sarstedt 86.1685.001
Serological Pipette, 5 ml	Sarstedt 86.1253.001
Serological Pipette, 50 ml	Sarstedt 86.1256.001
StarGuard® Sensitive, Nitrile Gloves, M	Starlab SG-N-M

Continued on next page.

Consumables (continued)

Item	Supplier/CN
Syringe Filters ROTILABO® PVDF, 0,22 µm	Carl Roth P666.1
Syringe Filters ROTILABO® PVDF, 0,45 µm	Carl Roth P667.1
Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa	Fisher Scientific 11832124
Thermo Scientific™ Pierce™ 18 Spin Columns	Fisher Scientific 11814141
Transfer Membrane Immobilon®-P PVDF	Carl Roth T831.1

2.1.9 Enzymes

Enzyme	Supplier/CN
BamHI	New England Biolabs R0136S
BbsI	New England Biolabs R3539S
Bsp119I	Thermo Fisher Scientific FD0124
DpnI	New England Biolabs R0176S

Continued on next page.

Enzymes (continued)

Enzyme	Supplier/CN
EcoRI	Thermo Fisher Scientific FD0274
Gateway™ LR Clonase™ Enzyme mix	Thermo Fisher Scientific 11791100
HindIII	New England Biolabs R0104S
MluI	Thermo Fisher Scientific ER0561
NcoI	New England Biolabs R3193S
NdeI	New England Biolabs R0111S
NotI	New England Biolabs R3189S
NsiI	New England Biolabs R0127S
Phusion® High-Fidelity DNA Polymerase	New England Biolabs M0530S
Q5® High-Fidelity DNA Polymerase	New England Biolabs M0494S
Shrimp Alkaline Phosphatase (rSAP)	New England Biolabs M0371S
SpeI	New England Biolabs R3133S

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Enzymes (continued)

Enzyme	Supplier/CN
SphI	New England Biolabs R3182S
XbaI	New England Biolabs R0145S
XhoI	New England Biolabs RO146S

2.1.10 Equipment

Equipment	Manufacturer
Amersham™ ImageQuant 800 GxP biomolecular imager	Cytiva
Attofluor™ Cell Chamber	Thermo Fisher Scientific
B6 Function Line Microbiological Incubator	Heraeus
Beckman Optima™ MAX Ultracentrifuge Series	Beckman
CellXpert® C170 cell incubator	Eppendorf
Centrifuge 5804 R	Eppendorf
Centrifuge Heraeus Megafuge 16R	Thermo Fisher Scientific
Combi Racks	Starlab
Counting chamber, Neubauer	Hirschmann

Continued on next page.

Equipment (continued)

Equipment	Manufacturer
Criterion™ Blotter with Wire Electrodes	Bio-Rad
Eppendorf™ Cooled Centrifuge 5424 R	Fisher Scientific
Fisherbrand™ Hand Tally Counter	Fisher Scientific
Fisherbrand™ Isotemp™ General Purpose Deluxe Water Baths	Fisher Scientific
Fisherbrand™ Isotemp™ Stirring Hotplate	Fisher Scientific
Fisherbrand™ SafeVIEW MINI2 LED Transilluminator	Fisher Scientific
Hoefer™ SG Series Gradient Makers	Fisher Scientific
Ice pan without lid 9L	VWR International
Inverse LED Microscope Leica DMI1	Leica Microsystems
Inverted Microscope Leica DMI8	Leica Microsystems
Invitrogen™ Mini Gel Tank	Thermo Fisher Scientific
Invitrogen™ Cell4 SureLock™ Midi-Cell	Thermo Fisher Scientific
LaminAir® HB 2448 K Laminar Flow Hood	Heraeus
Mini Blot Module	Thermo Fisher Scientific
Mini Gel Tank	Thermo Fisher Scientific

Continued on next page.

Equipment (continued)

Equipment	Manufacturer
Mini Trans-Blot Module	Bio-Rad
Mini-PROTEAN [®] Tetra Vertical Electrophoresis Cell	Bio-Rad
neoLabLine neoVAQ Maxi Suction system	Neolab Migge
Odyssey [®] DLx	LI-COR Biosciences
PCR rack	Carl Roth
Shaking cabinets with incubation or refrigeration, Ecotron	Infors HAT
StarStore 81	Starlab
StarRack 80	Starlab
Tecan Reader SPARK 10M	Tecan Group
Thermo Scientific [™] Owl [™] HEP Series Semidry Electroblotting Systems	Fisher Scientific
Thermomixer-Mixer HC	Starlab
Tilt/roller mixer RS-TR series model RS-TR 05	Phoenix Instruments
TLS-55 Swinging-Bucket Rotor	Beckman
Totally Tubular [™]	Starlab
Vortex-Genie [®] 2	Scientific Industries
Zeiss LSM880	Zeiss
Zeiss LSM900 with AiryScan	Zeiss

2.1.11 Kits

Kit	Supplier/CN
Gibson Assembly [®] Master Mix	New England Biolabs E2611S
Macherey-Nagel [™] NucleoBond [™] Xtra Midi	Fisher Scientific 1279840
Macherey-Nagel [™] NucleoSpin [™] Plasmid Columns	Fisher Scientific 12353358
Macherey-Nagel [™] NucleoSpin [™] Gel- and PCR Clean-up	Fisher Scientific 12303368
Quick Ligation	New England Biolabs M2200S

2.1.12 Oligonucleotides and Plasmids

Several oligonucleotides (i.e., primers) were specifically designed to interact with a diverse array of plasmids, facilitating either the amplification of existing DNA sequences or the insertion of new genetic material. In this work, elaborate dual-expression plasmids for mammalian cells were developed to allow for the simultaneous expression of two target genes from a single plasmid:

1. **pTS069:** A Flp-In-compatible dual-expression vector with two individual promoters (Δ CMV and EF1 α -TATA-box-mutant) (**Fig. 6**). The first locus is occupied by the cilia-APEX2 transgene, whose expression is controlled by the Δ CMV promoter. The second locus is controlled by the EF1 α -TATA-box-mutant promoter and expresses the cilia-DAAO transgene. The plasmid backbone pVC08, which is accessible through Gateway-compatible entry clones for fast expression clone generation, was originally generated by Valerie Chaumet.
2. **pTS100/pTS115:** A modular lentivirus-compatible dual-expression vector system controlled by a single promoter was constructed (**Fig. 7**). The expression of the transgene cassette is controlled by the Δ CMV promoter, a truncated version of the full length promoter. Duality is given through the following characteristics:
 - (a) Two genes of interest can be nested between an internal ribosomal entry site (IRES) sequence. This allows for a polycistronic mRNA which will result in co-expression of two separate gene products through ribosomal internal initiation of translation (Al-Allaf *et al*, 2019; Marques *et al*, 2022).
 - (b) Two genes of interest, where each starts with a preceding start codon and the second gene is followed by a stop codon, can be nested between a self-cleaving peptide sequence T2A (*Thosea asigna virus 2A*). In contrast to IRES, no initiation of translation within the polycistronic mRNA molecule occurs. Once translation of the first sequence reaches the T2A motif, steric hindrance and successful ribosome skipping lead to a failure of glycyl-prolyl peptide bond and continued translation, resulting in two "cleaved" proteins (Kim *et al*, 2011; Daniels *et al*, 2014).

Synthesis of pTS100 was performed by BioCat (<https://www.biocat.com/>). For a comprehensive list of the primers and associated plasmids generated and used in this work, please refer to the supplementary material in **Tab. 6**, **Tab. 7** and **Tab. 8**.

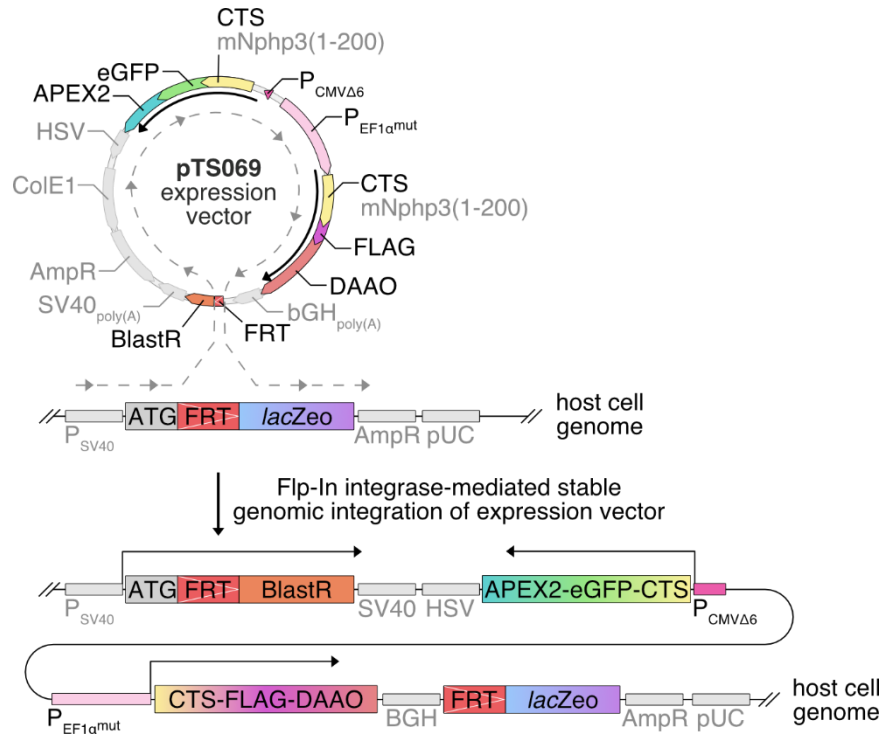


Fig. 6: Optimized dual-expression vector for targeted Flp-In integrase-mediated stable genomic integration of the iAPEX two-component system.

Schematic of the “pTS069” expression vector used in this work for rapid Flp-In integrase-based genomic manipulation of pre-existing cell lines with a Flp recombination target (FRT) site. P_{CMVΔ6}: truncated low-expressing promoter version of the cytomegalovirus “delta 6”; P_{EF1αmut}: modified low-expressing promoter EF1α; CTS: cilia-targeting sequence; FLAG: protein tag; DAAO: D-amino acid oxidase; BGH_{poly(A)}: bGH_{poly(A)}: bovine growth hormone polyadenylation terminator; BlastR: Blastocidin resistance gene; eGFP: enhanced green fluorescence protein; APEX2: engineered ascorbate peroxidase; HSV: herpes simplex virus terminator; ColE1: origin of replication; AmpR: ampicillin resistance gene; SV40_{poly(A)}: simian virus 40 polyadenylation terminator; ATG: start codon; lacZeo: lacZ-Zeocin fusion gene – coding for β-galactosidase and Zeocin resistance gene product; pUC: origin of replication.

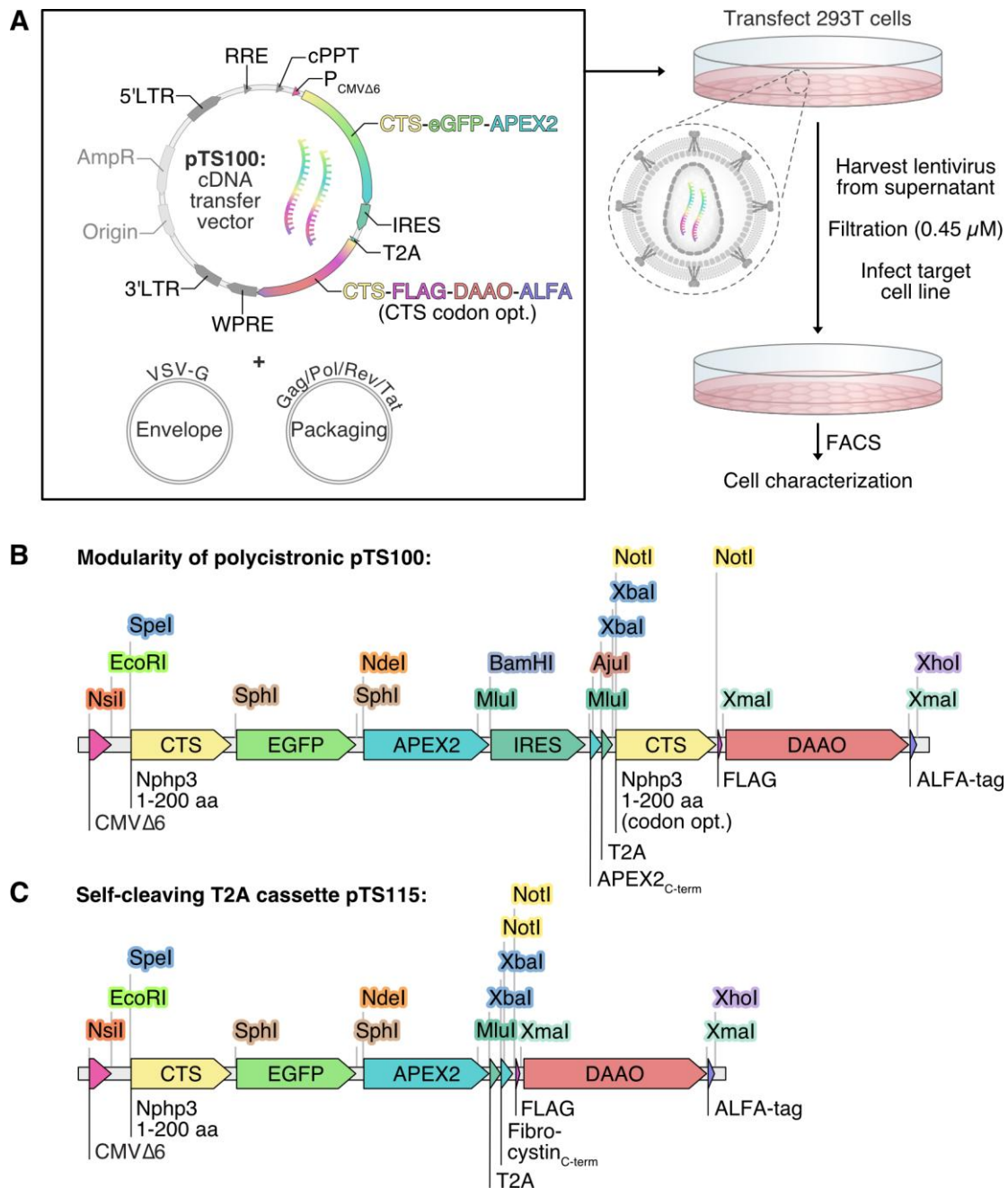


Fig. 7: Engineered lentiviral cDNA transfer vector harboring a polycistronic cassette for the iAPEX two-component system expression.

(A) Schematic of the “pTS100” cDNA transfer vector for lentivirus production in 293T cells by transfection of envelope, packaging and pTS100 vector. Abbreviations match Fig. 5 with addition of: RRE: Rev response element; cPPT: central polypurine tract; IRES: internal ribosome entry site; T2A: *Thosea asigna* virus 2A self-cleaving peptide; WPRE: Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element; 5' LTR: constitutive promoter; 3' LTR: polyadenylation terminator; VSV-G: Vesicular stomatitis virus G glycoprotein; Gag/Pol/Rev/Tat: HIV genes for lentivirus packaging-related proteins; FACS: fluorescence-activated cell sorting. **(B)** The modularity of the exchangeable polycistronic pTS100 plasmid elements is made clear by the highlighting of the restriction enzymes surrounding them. **(C)** Modularity is maintained for pTS115, excluding IRES and introducing the self-cleaving peptide strategy for improved polycistronic expression.

2.1.13 Software

Name	Publisher	Version	Purpose
Affinity Designer	Serif Ltd.	1.10.6	Data representation and vector graphic design
Benchling	Benchling	2019-2024	Digital molecular biology tool: • Design and management of plasmids and oligonucleotides • Multiple sequence alignments
ChimeraX	UCSF RBVI	1.7.1	Molecular graphics and analyses
Fiji (ImageJ)	Open Source	2.14.0/1.54f	Immunofluorescence data analysis: • ciliaQ (Hansen <i>et al</i>, 2021): primary cilia intensity and length measurements • Specially generated macros for image analysis and image preparation (this work)
Graphpad Prism	GraphPad Software	10.1.1	Statistical analysis of data
Image Studio™ Lite	LI-COR	5.2.5	Fluorescence-based Western blot analysis
LAS X Life Science Microscope Software Platform	Leica Microsystems	3.7.0.20979	Acquisition and management of immunofluorescence microscope data

Continued on next page.

Software (continued)

Microsoft Office 365	Microsoft	16.78.3	Excel: • Data analysis and management • Generation of calculation templates PowerPoint: • Presentation design Outlook and MS Teams: • Outreach Word: • Conceptualization of texts
SparkControl	TECAN	1.1	• Nucleic acid quantitation • Protein concentration determination
Zotero	Open Source Digital Scholar	6.0.30	Management of citations

2.2 Methods

2.2.1 Molecular Cloning

Agarose Gel Electrophoresis

In this work 1 % agarose gels were used to separate or visualize DNA (fragments) of varying sizes. For this, agarose was added to TAE buffer in a flask, then heated in a microwave until fully solved. The hot flask was placed in a room-temperature water bath to cool down the mixture. Once cooled down to 55 °C, 4 µl of the intercalating DNA dye peqGREEN was added per 100 ml of mixture, mixed well by swirling the flask and poured into a pre-assembled gel chamber. A comb was placed into the still setting gel. After polymerization, the gel chamber was placed into an electrophoresis cell and filled with 1× TAE buffer until fully submerged. Finally, the samples were added to adjacent wells in the gel together with a molecular weight marker. The electrophoretic separation run time was set to 45 min at constant 80 V. After the run, the DNA gel was lifted out of the gel chamber and imaged with a DNA imaging system.

Cloning Strategies

Multiple cloning strategies have been employed for DNA manipulation in this work which will be subject of the following chapters. The cloning strategy used for a specific plasmid is indicated in the plasmid list in the appendix (see supplementary **Tab. 6**).

Classical Cloning

Classical cloning enabled the manipulation of double-stranded DNA sequences by restriction enzymes that cut precisely at palindromic sequences specific to each enzyme. DNA of interest from one plasmid (insert) was inserted into another plasmid (backbone) by cutting with the identical enzyme(s). This procedure generated 5' or 3' single-strand extensions (known as sticky-ends) which were ligated by T4 DNA ligase to produce a new plasmid. The insert originated from a plasmid or from a PCR reaction (see page **69**), restriction digest (see page **71**) or annealed oligonucleotides with sticky-ends (see page **68**), which needed an additional phosphorylation step. In any case, the generated DNA fragment was purified before the ligation reaction (see page **68**).

For the ligation mixture, the following ingredients were added together in a PCR tube:

Ingredient	Volume (µl)
DNA (e.g., annealed oligos, PCR product)	3
Pre-digested plasmid (10 ng/µl)	1
2x Quick Ligase Buffer	5
NEB Quick Ligase	1
Water filled up to	10

The mixture was incubated at RT for 30 minutes and immediately transformed into competent bacteria (see page 67).

Gateway Cloning

The LR reaction was catalyzed by the LR Clonase™ II Enzyme Mix which facilitated the recombination of an *attL* (entry clone) with an *attR* substrate (destination vector) to generate an *attB*-containing expression clone (Katzen, 2007). LR reactions were carried out by mixing:

Ingredient	Volume (µl)
Destination vector	1
Entry Vector	1
Water	1
LR Clonase™ II Enzyme Mix	1

The mixture was incubated at 25 °C for 1 h in a thermomixer. The reaction was stopped by the addition of proteinase K solution and incubating for 10 min at 37 °C. The reaction mix was

transformed into chemically-competent bacterial cells (see page 29, not DB3.1) to propagate the desired expression clone.

Gibson Assembly

Gibson cloning was used to seamlessly join DNA fragments with overlapping sequences together by relying on an enzyme mix consisting of 5' exonuclease, a DNA polymerase, and a DNA ligase (Avilan, 2023). These fragments were designed to have overlapping regions (around 20-40 base pairs) at their ends, were obtained or amplified using PCR (see page 69) and then purified (see page 68) to remove excess primers and contaminants.

Next, the purified DNA fragments were mixed with the Gibson Assembly[®] Master Mix:

Ingredient	Volume (μl)
Gibson mix	5
Restriction enzyme digest DNA	1
PCR product	1

This reaction mixture was incubated at 50 °C for 1 h to allow for assembly. Competent bacterial cells were then transformed (see below) with the assembled DNA. Following transformation, the cells were plated onto selective media to select cells containing the desired construct.

Bacterial Transformation

Heat-shock-induced DNA uptake of chemically-prepared competent *E. coli* bacterial cells was used to replicate plasmid DNA for molecular cloning or cell culture purposes (Hasegawa *et al*, 2018; Green & Sambrook, 2021). Typically, 50-100 μl of DH5α[™] competent cells were slowly thawed on ice. Once thawed, 1-10 ng of plasmid DNA was added to the cells, mixed by tapping and incubated on ice for 20 min. A heat shock at 42 °C was administered in a thermomixer for 45 s, followed by a 2 min incubation on ice. The thermomixer was cooled down to 37 °C. Once cool, 350-500 μl of SOC medium was added to the bacterial suspension and the mixture was incubated for 60 min at 37 °C and 350 rpm. The resulting suspension

was evenly spread on an LB agar plate—supplemented with the appropriate antibiotic dictated by the plasmid resistance gene—using a Drigalski spatula. To allow improved bacterial colony formation for re-transformations, only 10% of the suspension was spread. The plates were incubated upside down overnight at 37 °C. Upon successful transformation, bacterial colonies emerged. Single colonies were carefully selected using sterile pipette tips or inoculation loops and inoculated in liquid LB medium cultures supplemented with the appropriate antibiotic. Positive clones were confirmed through sequencing analysis.

DNA Purification

Any DNA fragment of interest (e.g., PCR amplicons) for downstream processing was separated from reactants and contaminants by agarose gel electrophoresis (see page 65). Once separated, the DNA was shortly visualized on a blue light transilluminator and excised from the gel using a scalpel. The small gel piece was weighed and then purified using the silica membrane-based NucleoSpin® Gel and PCR Clean-up Kit according to the manufacturer's instructions (Macherey-Nagel, March 2023).

Nucleic Acid Quantitation

Nucleic acid (e.g., plasmid DNA, amplicon) quantity and quality was assessed using the NanoQuant Plate and SparkControl® Software of the TECAN Spark® 10M according to the manufacturer's instructions (Tecan, 2022).

Oligonucleotide phosphorylation and annealing

In order to add new DNA fragments to digested plasmids without the use of PCR amplification from other vectors, overlapping oligonucleotides with plasmid-complementary sticky ends were designed and ordered. To phosphorylate and anneal the primers, the following mix was prepared in a PCR tube:

Ingredient	Final conc.	Volume (μl)
5' to 3' oligo (100 μM)	5 μM	1
3' to 5' oligo (100 μM)	5 μM	1
5x PNK buffer A	1x	4
10 mM ATP (pH 7.2)	1 mM	2
T4 PNK		1
Water to		20

In the thermocycler, the mixture was incubated at 37 °C for 30 min (phosphorylation reaction), before heating to 95 °C for 5 min (T4 PNK inactivation). The temperature was ramped down to 25 °C at 1 °C/5 s intervals (annealing step) before holding forever at 4 °C. Added 40 μl dH₂O to the annealed oligos before ligation (see page 65).

Plasmid Isolation

Prior to plasmid isolation, 3-5 ml of LB medium, supplemented with antibiotic dependent on the plasmid to be purified, was inoculated with a single bacterial colony and incubated at 37 °C at 180 rpm overnight. The extraction and purification of plasmid was performed with the NucleoSpin® Plasmid Kit according to the manufacturer's instructions (Macherey-Nagel, 2023).

Polymerase Chain Reaction

In order to amplify DNA sequences *in vitro*, all reagents were assembled on ice and quickly transferred to a thermocycler. Both, Phusion® High-Fidelity DNA Polymerase and Q5® High-Fidelity DNA Polymerase were used for amplification reactions.

Reaction mix when using Phusion[®] High-Fidelity DNA Polymerase:

Component	Volume (µl)	Final Concentration
5x Phusion [®] HF Buffer	5.0	1x
10 mM dNTPs	0.5	200 µM
10 µM reverse primer	1.25	0.5 µM
10 µM forward primer	1.25	0.5 µM
Template DNA	variable	≤ 1 ng
Phusion [®] High-Fidelity DNA Polymerase	0.25	1.0 units/50 µl PCR
DMSO	0.75	3%
Nuclease-free water	to 25	

Reaction mix when using Q5[®] High-Fidelity 2× Master Mix:

Component	Volume (µl)	Final Concentration
10 µM reverse primer	1.25	0.5 µM
10 µM forward primer	1.25	0.5 µM
Template DNA	variable	≤ 1 ng
Q5 [®] High-Fidelity 2× Master Mix	12.5	1×
DMSO	0.75	3%
Nuclease-free water	to 25	

To prevent exonuclease-driven primer degradation prior to thermocycling, the DNA polymerases were added last in the reaction.

Mutagenesis

Point mutations were introduced by PCR using oligonucleotides designed with specific changes to the template sequences in their partially complementary 3'-overhangs. Following the PCR amplification, the reaction mixture contained both the original methylated plasmid and the newly synthesized mutated plasmid. To ensure propagation of only the mutated plasmid, the mixture was treated with DpnI, an enzyme that digests methylated (parental) DNA. This digestion step removed the original template, allowing only the newly synthesized, mutated plasmid to be introduced into bacterial cells for transformation and subsequent propagation (see page 67).

Restriction Enzyme Digest

The following reaction components were pipetted together in a 1.5 ml reaction tube for a restriction digest:

Component	Volume (µl)
DNA (1 µg)	1
10 × recommended buffer for restriction enzyme	2
Restriction enzyme(s)	each 0.5-1
Water	filled up to 20

The mix was incubated at 37 °C for 60 min in a thermomixer, then heat-inactivated at 65 °C or 80 °C, depending on the used enzyme, for 20 min. After incubation, a fraction of the digest was mixed with 6 × Gel Loading Dye and analyzed by agarose gel electrophoresis (see page 65).

Sequencing

Every generated construct was verified by sanger sequencing at MicroSynth Seqlab (<https://www.microsynth.com/home-de.html>) using either standard or designed primers. Plasmid DNA was mixed with water to a total volume of 15-20 µl with a final concentration of 100 ng/µl. Resulting sequence chromatograms were analyzed by alignment in Benchling using the MAFFT v7 algorithm.

2.2.2 Cell Culture

Cell culture work was handled under a laminar flow hood with EtOH- and UV-sterilized surfaces. Cultivated cell lines were kept in a cell incubator at 37 °C in a 5% CO₂ atmosphere. All cultivation steps were performed either in DMEM or DMEM F-12 culture medium supplemented with 7.5% FBS depending on the cell line. For propagation, 10 cm cell culture dishes, T-25 or T-75 culture flasks were used. Antibiotics were not used in regular cell culture, except for prolonged cultivation of FACS-sorted cell clones, transfection selection and virus-associated cell culture.

Experiments were performed with a fixed cell number. Seeded cells grown for one or two days were synchronized to induce formation of primary cilia by removal of serum. For this, culture medium was removed, cells were washed with 1× PBS and culture medium supplemented with 0.2% FBS was added for further 24 h.

Given the high variety of cell culture dishes and flasks, the following table summarizes the numbers used for cultivation, propagation, APEX labeling and cell lysis:

Tab. 1: Cell culture seeding cell numbers and condition volumes

Dish or plate	Growth area (cm²)	Seeding cell number	Media volume (ml)	Wash volume (ml)	Trypsin volume (ml)	Lysis buffer volume (ml)	APEX label volume (ml)
μ-Slide 8	1	$5-11 \times 10^4$	0.3	0.3	0.1	/	0.3
well							
6-well	10	0.25×10^6	2.0-2.5	1	0.25	0.1	2.5
24-well	2	5×10^4	0.5	0.5	0.1	/	0.5
96-well	0.3	1.25×10^4	0.1	0.1	0.1	/	/
10 cm	78	1×10^6	10	4	1	0.3	4
15 cm	175	2.5×10^6	25	10	2.5	0.8	8
500 cm ₂	500	$12-17 \times 10^6$	100	50	/	3	25
T-25	25	0.7×10^6	5	5	0.5	/	/
T-75	75	2.1×10^6	10	5	1	/	/

In order to propagate cells or seed a fixed cell number for an experiment, growing cells were detached from the culture vessel by washing with 1× PBS and trypsinizing. Trypsin was incubated for 5 min at 37 °C in the cell incubator, then the cells were resuspended in culture medium. A fraction of the cells was transferred to a fresh vessel (1:10 on weekdays, 1:20 over the weekend), remaining cell suspension was collected in a conical tube for cell counting or freezing. Cell number of any culture was determined manually using a Neubauer counting chamber and 10 μl of cell suspension.

For long term-storage in an ultra-low temperature freezer (-80 °C), cells were pelleted at 200 × g, medium was aspirated and the cells were resuspended in Bambanker freezing medium to be aliquoted.

Ascorbate Peroxidase-Based Proximity Labeling

Ascorbate peroxidase-based proximity labeling (APEX) was performed on cells *in vivo*. Biotin derivatives (i.e., biotin tyramide (BT) or desthiobiotin tyramide (DTBT)) were used as substrates for APEX to achieve covalent labeling of surface-exposed electron-rich amino acids of nearby proteins in the presence of hydrogen peroxide (H_2O_2) (Rhee *et al*, 2013; Mick *et al*, 2015; May *et al*, 2021a). In this work, APEX labeling was carried out under two conditions:

- “Standard APEX” by incubating with a biotin derivative and exogenous H_2O_2 (only making use of the APEX enzyme in dual expression systems)
- “Improved APEX” (hereafter referred to as iAPEX) by incubating with a biotin derivative and a D-amino acid (i.e., D-Alanine (D-Ala) or D-Methionine (D-Met)) for endogenous H_2O_2 production of D-amino acid oxidase (DAAO)

Labeling in culture dishes was controlled by placing coverslips inside. “No labeling” coverslips were taken out from each plate before starting the experiment. Coverslips were then transferred to a 24-well plate with pre-laid 4% PFA in $1\times$ PBS and fixed for 15 min at RT. To ensure accurate labeling times when handling multiple plates, these were processed in succession in a nested sequence. Before substrate treatment, medium volume was adjusted according to table 2.14. For standard APEX labeling, a 50 mM BT stock solution was added to f.c. 50 μM on a plate under laminar flow and then incubated in a cell incubator for 30 min. On bench, 100 mM H_2O_2 was added to f.c. 1 mM to start the labeling reaction. Plate was placed on a rocker shaker; reaction was stopped after 2-3 min by placing the plate on ice and quickly rinsing with cold (4 °C) quenching buffer and subsequently adding cold quenching buffer with trolox to incubate cells for 5 min on ice. During the incubation time, coverslips were taken out of the plate, fixed and processed (see page 77). Lysates were prepared by adding $1\times$ RPY+Q lysis buffer to the cells and scraping them in solution. The resulting cell suspension was transferred to a suitable reaction tube to be placed on ice until further processed (see page 77).

For DAAO-dependent iAPEX labeling, exogenous H_2O_2 was not added. Instead, 250 mM D-Met stock solution was added directly at the beginning to f.c. 10 mM and incubated together with BT for 15-30 min. Handling was identical to standard APEX labeling plates.

Once all cells were lysed, cell suspensions were kept on ice for another 20 min, then spun at max. speed for 30 min in a cold centrifuge (4 °C). Supernatants were transferred to fresh reaction tubes and small fractions were taken for protein content determination and Western blot analysis. The samples were shock-frozen in liquid nitrogen to be stored at -80 °C or used immediately.

Proximity labeling on coverslips in a 24-well plate was performed identically as described above, except that the labeling reaction was stopped using trolox-containing quenching buffer directly in the plate without taking out the coverslips. The cells were then immediately fixed with 4% PFA in 1× PBS followed by steps further described in (see page 77).

Biotin Ligase-Based Proximity Labeling

Biotin ligase-based proximity labeling using TurboID was performed on cells *in vivo*. Biotin was used as a substrate for TurboID to catalyze ATP-dependent activation of biotin molecules for covalent labeling of surface-exposed electron-rich lysines of nearby proteins (Branon *et al*, 2018). The preparation and handling of cells was the same as described in the chapter before (see page 74).

For biotin ligase proximity labeling, a 50 mM biotin stock solution was used to add f.c. 500 μM to cells to incubate at 37 °C in a cell incubator for 30 min. The reaction was stopped by placing the culture dish on ice and washing the cells twice with cold PBS. After 5 min of incubation, the cells were lysed by scraping in 1 × RIPPY+Q lysis buffer.

Copper-catalyzed Azide-Alkyne Cycloaddition Post Labeling

By using alkyne-phenol (alkyne tyramide) as the APEX2 substrate for proximity labeling in living cells, copper-catalyzed azide-alkyne cycloaddition (CuAAC), also known as “click chemistry”, could be applied post-fixation to chemically react azide compounds with functional moieties for downstream visualization or potential enrichment.

The alkyne-tyramide substrate was prepared as a 100× stock solution at 50 mM in DMSO and substituted BT in both standard and improved APEX labeling (see page 74). The azide compound with functional moiety (e.g., biotin-azide or azide-fluor545) was prepared as a 100× stock solution, which was prepared at 10 mM in DMSO and stored at -80 °C in single-use aliquots. Cu-BTTAA stock solution was prepared at 25× concentration as a

12.5 mM:25 mM (1:2) solution in water. The stock was stored at -20 °C in single-use aliquots. A 10× stock solution of sodium ascorbate was prepared at 25 mM in 1x PBS and stored at -20 °C.

Cells were seeded in a 24-well format according to **Tab. 1** two days prior to the experiment. On the day of the experiment, substrate A (alkyne-phenol; Alk-ph) was dissolved in DMSO to create a 50 mM stock solution. If already prepared, the stock was thawed at room temperature. Substrate B (D-methionine; D-Met) was reconstituted with water to create a 250 mM stock solution or thawed as necessary.

Directly before adding to cells, Substrate A and Substrate B stocks were mixed in a 1:4 ratio (e.g., 10 µl Alk-ph + 40 µl D-Met) to create the "iAPEX labeling stock," which was a 20× concentration. The "iAPEX labeling stock" was then added to the medium to a final concentration of 1×. The medium was mixed well, and cells were incubated for 30 min under standard growth conditions (37 °C with 5% CO₂ for regular tissue culture cells). If standard labeling was performed, Alk-ph was incubated alone for 30 min and f.c. 1 mM H₂O₂ was added to the cells for another 3 min. After incubation, the medium containing labeling reagents was removed and substituted with quenching buffer, which was incubated for 5 min on the cells on ice. Cells were fixed with 4% PFA for 10 minutes at RT.

Cells were quickly rinsed with 1× PBS and permeabilized in ice-cold methanol (MeOH) at -20 °C for 10 minutes. After permeabilization, cells were rehydrated in 1× PBS for at least 10 minutes. A CuAAC reaction solution was prepared (300 µl per reaction) fresh by mixing:

- 255 µl 1× PBS
- 30 µl 25 mM sodium ascorbate (final concentration: 2.5 mM)
- 12 µl of 12.5 mM Cu-BTTAA (1:2) stock (final concentration: 0.5 mM)
- 3 µl of azide (e.g., biotin-azide, final concentration: 0.1 mM)

Cells were washed once or twice with 1× PBS, and all PBS was removed by suction. The CuAAC solution was added to the cells (300 µl per sample), and were incubated for 1 h at 37 °C in an incubator. After incubation, the solution was removed, and cells were washed with 1× PBS. From here, steps in the immunofluorescence protocol were followed (see page 77).

Cell Lysis

All cells were lysed in cold $1\times$ RIPPY+Q + 0.5% Triton X-100 using a cell scraper, unless used for native complex analysis. For the latter, cold $1\times$ solubilization buffer with varying detergent concentrations was used. Cell lysates were transferred to suitable reaction tubes and incubated for 20 min on ice. After shortly vortexing the cell lysates, these were spun at $20.000\times g$ for 30 min at 4 °C. The resulting supernatant was transferred to a fresh reaction tube, then kept on ice for subsequent processing or shock-frozen using liquid nitrogen and kept at -80 °C for long-term storage.

Fluorescence Activated Cell Sorting

Cells that underwent virus-mediated transduction (see page **81**) analyzed by fluorescence activated cell sorting (FACS) to isolate unique cell clones and complex cell pools (Galbraith *et al*, 1998). Transduction efficiency was calculated from cells effectively expressing the fluorescent protein GFP compared to fluorescence background generated from wild type cells. The FACS analyses and clonal selections were performed by Lea Sanwald who was kindly assisted by Prof. Dr. Daniela Yildiz at the Pharmacology and Toxicology Institute in Homburg. For FACS, cells were first trypsinized and transferred to a 15 ml conical tube, then centrifuged for 10 min at $200\times g$ to remove medium. Cells were washed with 5 ml $1\times$ PBS and centrifuged again to remove PBS. Added 1 ml $1\times$ PBS to count cells (expected cell number approx. $1-2\times 10^6$) and put cells through a cell strainer. Kept cells on ice until sorted into a 96-well plate with warm medium supplemented with 10% FBS, 20 mM HEPES and antibiotics (1 µg Kanamycin and 1% Pen/Strep). Clones and pools were then grown for multiple days to verify successful genomic integration of polycistronic gene cassette by IF (see text below) or SDS-PAGE and WB (see pages **88** and **89** respectively).

Immunofluorescence Microscopy

Immunofluorescence (IF) assays in this work were performed on 12 mm glass coverslips typically laid in a 24-well plate prior to cell seeding, unless otherwise stated. Cells were seeded and cultured as described in **Tab. 1**. To begin, coverslips from cell culture dishes other than a 24-well plate were carefully transferred to a 24-well plate. Cells were fixed with 300 µl 4% PFA in $1\times$ PBS for 15 min at RT. Fixed cells were washed with $1\times$ PBS and stored overnight at 4 °C. The next day, cells were semi-permeabilized with 300 µl 0.2% Triton X-100 in $1\times$ PBS/well for 20 min at RT, then washed three times with $1\times$ PBS. Coverslips

were transferred from the 24-well plate to 1× PBS droplets on Parafilm in a culture dish wrapped in aluminum foil to protect the samples from light. PBS was aspirated, 100 µl blocking buffer was added per coverslip and incubated for 30 min at RT. In the meantime, a primary antibody mix was prepared in blocking buffer. Subsequently, the solution was aspirated and 40 µl of mix was applied to each coverslip incubating for 1 h at RT or 4 °C overnight. Cells were washed three times with 1× PBS, then 40 µl of a secondary antibody mix in blocking buffer was added to each coverslip and incubated for 30 min to 1 h at RT. Lastly, coverslips were again washed with 1× PBS and embedded in Fluoromount G™. Cells were imaged at the Leica DMI8 inverted microscope using a 63× oil objective (NA 1.4, Plan Achromat).

Live-Cell Imaging of Peroxidase-Catalyzed Amplex™ UltraRed Oxidation

Cells were seeded onto 25 mm coverslips in a 6-well plate or in a µ-Slide 8 well chambered coverslip according to **Tab. 1**. Cells were imaged using a Zeiss LSM900 confocal microscope with a 63× oil objective (NA 1.4, Plan Achromat). When working with cells grown on a 25 mm coverslip, it was placed in a round chamber and covered with cold (4 °C) 400 µl physiological imaging buffer containing HEPES (total volume was 800 µl). Recording was started before substrate addition to the cells. The missing 400 µl imaging medium was supplemented with 100 µM Amplex™ UltraRed and 20 mM D-Met or 2 mM H₂O₂, then directly and carefully pipetted into the center of the solution dome to yield halved final concentrations. Using µ-Slide 8 well coverslips, culture medium in wells was replaced with 300 µl imaging medium containing HEPES. The recording was started before substrate addition. Using two pipettes, 50 µM Amplex™ UltraRed and 10 mM D-Met or 1 mM H₂O₂ were applied directly from stock solutions into the imaging medium. Recordings were stopped at various time points.

Transfection

The introduction of exogenous DNA in the form of plasmids into various mammalian cell systems was essential to study their respective primary cilia proteomes. A defined set of plasmids for mammalian cell transfection were constructed, many containing coding information for engineered APEX2 and DAAO, which were each genetically targeted to or distant from primary cilia (see page **60** and supplementary **Tab. 6**).

jetPRIME[®] DNA/siRNA transfection reagent (hereafter abbreviated as jetPRIME), a positively charged polymer reagent, was used for gene transfer in transient and stable transfections, allowing exogenous DNA expression for a limited (transient) or persistent (stable) period of time. Alternatively, lenti- and retroviral transduction of suitable plasmids was used to generate stable cell lines.

Stable Transfection

Cell lines possessing a pre-integrated, characterized genomic locus for targeted gene expression, namely the Flp-In locus, were used for rapid cell line generation (Szczesny *et al*, 2018). By co-transfecting a Flp-In integrase-containing plasmid with a Flp-In-compatible plasmid, both plasmids needed to be taken up by the cell to allow targeted exchange of the pre-integrated genomic segment with the entire co-transfected plasmid. This process facilitated persistent and controlled expression of desired protein(s) under the regulation of the plasmid-given promoter. The process, from transfection to clone verification, lasted two to three weeks for each cell line. For stable transfection, 0.25×10^6 cells were seeded per well in a 6-well plate before transfection (termed forward transfection).

The next day, for each well a transfection mix was prepared by pipetting 200 μ l jetPRIME buffer to a respective reaction tube. To each solution, a plasmid mix containing

- I. 1 μ g plasmid of interest
and
- II. 3 μ g pOG44 (Flp-In integrase)

was added. For mock transfections, plasmid of interest was omitted. jetPRIME reagent was added in ratio 3:1 (reagent : DNA(μ g)), then vortexed for 1 s and incubated for 10 min at RT on bench. The mix was added drop-wise to the respective well and the cells were placed back in the cell incubator. After 4 h incubation, the medium was aspirated and replaced with fresh medium.

The next day, cells were split from a 6-well plate into 15 cm dishes in antibiotic-containing medium, dictated by the transfected plasmid of interest. Small fraction of cell suspension was retained in the 6-well plate and filled up to 2 ml (“backup plate”). Cells were kept in selective medium for 2 weeks.

Single cell clones were isolated from the 15 cm culture dishes with cloning rings. For that, clones were marked on the underside of the dish. Cloning rings were pre-coated with Vaseline on one side and placed in another culture dish for fast access. The medium on the clone culture dish was aspirated, cells were washed with 1× PBS and the plate was aspirated dry. Cloning rings were placed on top of the marked clones, then 50 µl trypsin was added to each ring. After a short incubation time in the cell incubator, 150 µl medium was used to resuspend each clone and add the suspension to a fresh 6-well plate. Any remaining cell clones on the plate were pooled by rinsing the plate with more trypsin and medium.

Cells were split from confluent 6-well plate in Blasticidin-containing medium in another 6-well plate and a drop of cells was added to a 24-well plate with a pre-laid coverslip to test for correct expression of protein in these cells. Remaining cell fractions were collected in individual 1.5 ml reaction tubes and centrifuged at $200 \times g$ for 5 min. Supernatants were discarded and cell pellets were resuspended in BamBanker freezing medium to be stored at -80 °C.

Cell clones were verified by immunofluorescence (see page 77), SDS-PAGE (see page 88) and Western blot (see page 89).

Transient Transfection

For rapid analysis of protein (co-)localization and (co-)expression, cells were transiently transfected using jetPRIME. For experiments studying protein expression, the protocol for stable transfection (see text before) was followed, using 4 µg plasmid of interest without pOG44 vector. For localization studies, cells were prepared in a 24-well plate as described in **Tab. 1** one day before transfection. For each well, a mix containing 50 µl jetPRIME buffer, 2 µg DNA and jetPRIME reagent (ratio 3:1, jetPRIME : DNA) was prepared, following the steps as described before.

The next day, cells were deprived of serum by changing to starvation medium for 24 h. At this stage, the cells were ready for further processing by APEX proximity labeling (see page 74) or immunofluorescence (see page 77).

Virus-Mediated Transduction

In cases where Flp-In cell lines were unattainable, polycistronic gene cassettes were encapsulated within lentivirus (Elegheert *et al*, 2018). Through transduction, these lentiviral vectors facilitated the generation of cell lines by random integration into the host genome. The protocol for virus production follows multiple days over the course of two weeks. On the starting day (Friday), HEK293 cells were propagated in DMEM + 7.5% FBS into five T-75 cell culture flasks to ensure high enough number of cells for seeding on Monday. Up to three 6-well plates were prepared by seeding 1×10^6 cells per well. The next day, cells were transfected with 2nd generation lentivirus plasmids (packaging psPAX2 & envelope vector pMD2g-VSV-G) and a transfer plasmid (cilia-iAPEX^{viral} pTS100). The plasmids were mixed in 200 μ l 150 mM NaCl water in the ratio 1:1:2 (packaging : envelope : transfer) using 500 ng lentivirus plasmid per well. The mixture was vortexed briefly, then incubated for 20 min at RT. During the incubation, cells were brought to the S2-safety level laboratory, where the transfection mix was added drop-wise to each well (200 μ l per well). Cells were placed in the incubator to allow virus formation at 37 °C at 5% CO₂. The next day, medium was replaced in each 6-well plate, increasing the volume to 4 ml per well. In the S1-safety level lab, cell types to be infected were prepared by seeding at least 1×10^5 into a 6-well plate. On the fourth day, HEK293 supernatant was collected and filtered using a 0.45 μ m PES filter with a syringe. 2.5 ml polybrene was added to 4 ml supernatant before infection of cells in the prepared 6-well plates. Additionally, a 24-well plate was prepared to test infection of cells on coverslips for the expression of desired construct. Infected cells in the 6-well plate were passaged the next day and up to two more times in the following days before FACS sorting (see page 77). Cells were sorted into different pools dependent on their respective GFP expression profile (low and high expressers) against a WT control.

Ultrastructure Expansion Microscopy

The modified ultrastructure expansion microscopy (U-ExM) protocol by Gambarotto *et al* (2021) was used to allow imaging of physically magnified cellular structures in high resolution detail using conventional confocal microscopy. Cells were seeded in a 24-well plate with pre-laid coverslips as described before in **Tab. 1** and APEX proximity labeling was performed (see page 74). Quenching buffer was aspirated and 300 μ l cross-linking solution was added to the each well. Cells were incubated for 5 h at 37 °C. For gelation, an ice-cold humid chamber laid with parafilm was placed on ice, coverslips were transferred to a tissue

paper and 90 μ l monomer solution was mixed with 5 μ l APS and 5 μ l TEMED. Two drops of 35 μ l mixed solution was pipetted onto parafilm and rapidly covered by coverslips with the cells facing into the solution. For improved gel penetration, cells were incubated for 5 min on ice, then incubated at 37 °C for 1 h. To detach the gels from the coverslips, they were transferred to a 6-well plate, with each well filled with 1 ml of denaturation buffer. The plate was incubated for 15 min at RT gently shaking on an orbital shaker. Once detached, the gels were carefully transferred into a 1.5 ml reaction tube filled with fresh denaturation buffer, where they were further incubated for 1 h and 30 min at 95 °C in a thermal shaker. Afterwards, the gels were expanded for the first time in individual 250 ml beakers filled with dH₂O, where the water was replaced three times every 10 min, agitating at 100 rpm in an incubator at RT. To calculate the expansion factor, the gels were measured after the first round of size increase. Subsequently, gels were shrunk for antibody incubation by draining the water and replacing it with 1× PBS for 30 min while gently shaking. The gels were cut into quarters with a spatula and transferred into a 24-well plate with 200 μ l of primary antibody diluted in PBS-BSA (2%) to be incubated overnight in a cold room with agitation. The next day, gels were washed three times 10 min with 1× PBS + Tween (0.1%) at RT while gently shaking. Buffer was removed and each gel piece was incubated in 200 μ l of secondary antibody diluted in PBS-BSA (2%) for 2 h 30 min at 37 °C at 130 rpm, protected from light using aluminum foil. After another triple wash, gels were expanded again, this time in a 6-well plate with dH₂O supplemented with 0.02% sodium azide to incubate over the weekend at 4 °C. In the meantime, 25 mm coverslips were coated with 100 μ g/ml Poly-D-Lysine overnight at 4 °C, then washed three times with dH₂O, dried and stored in the fridge. Imaging was performed at the Zeiss LSM900 confocal microscope with AiryScan using a 63× oil objective (NA 1.4, Plan Apochromat), the convex gel side placed facing the glass surface of a coated 24 mm coverslip placed in a round chamber.

2.2.3 Protein Analysis

Affinity Chromatography

Affinity chromatography separates molecules using their unique interactions with immobilized ligands on a solid support, enabling the purification of target molecules from complex mixtures through their specific affinity to these binding partners. To enable targeted isolation, the proteins of interest were genetically modified such that a known epitope (affinity tag) was attached to the N- or C-terminus (Götzke *et al*, 2019; Pina *et al*, 2021). These recombinant proteins were then stably expressed in cells. Using epitope-specific antibodies cross-linked with agarose beads, the desired proteins could be precipitated from lysates, under optimal conditions together with their protein interaction partners, a process known as co-immunoprecipitation (Co-IP). Proteins covalently modified with a biotin derivative after APEX proximity labeling (see page 74) *in vivo* were captured using agarose sepharose cross-linked to the homo-tetrameric bacterial protein streptavidin, which has exceptionally high affinity ($K_D \sim 10^{-15}$ M) to the small molecule modification and allows a non-covalent interaction (**Fig. 8**) (Hirsch *et al*, 2002). At the beginning of each chromatography, cells were lysed (see page 77) the protein content was assessed (see page 86) and the lysates were adjusted with lysis buffer to contain equal protein amounts (ranging from 10-40 mg) in the same volume. Each centrifugation step was performed at $200 \times g$ for 30 s, unless otherwise indicated, where one wash equaled a centrifugation step. Buffers used in chromatographies destined for subsequent mass spectrometry analysis (see page 91) were filter-sterilized beforehand. Prior to protein loading, Mobicol spin column was filled with 50% Sepharose slurry where

- 50-150 μ l ALFA Selector^{PE} (≈ 3.5 μ l packed beads per 1 mg protein)
- 50-200 μ l streptavidin (≈ 2.5 μ l packed beads per 1 mg protein)

was used. Beads were cleared by centrifugation, washed with $1 \times$ PBS, then equilibrated twice with $1 \times$ lysis buffer. After spin-out, beads were incubated with lysate for 1 h at RT or 4 °C while rolling on tube roller or overhead rotor. To settle the beads, mixture was centrifuged and the cleared buffer was saved in new microcentrifuge tube (unbound material). Settled beads were resuspended in Mobicols and washed 10 times with 500 μ l lysis buffer. From here, different elution methods were used depending on the experimental conditions, which are explained in the following two sections.

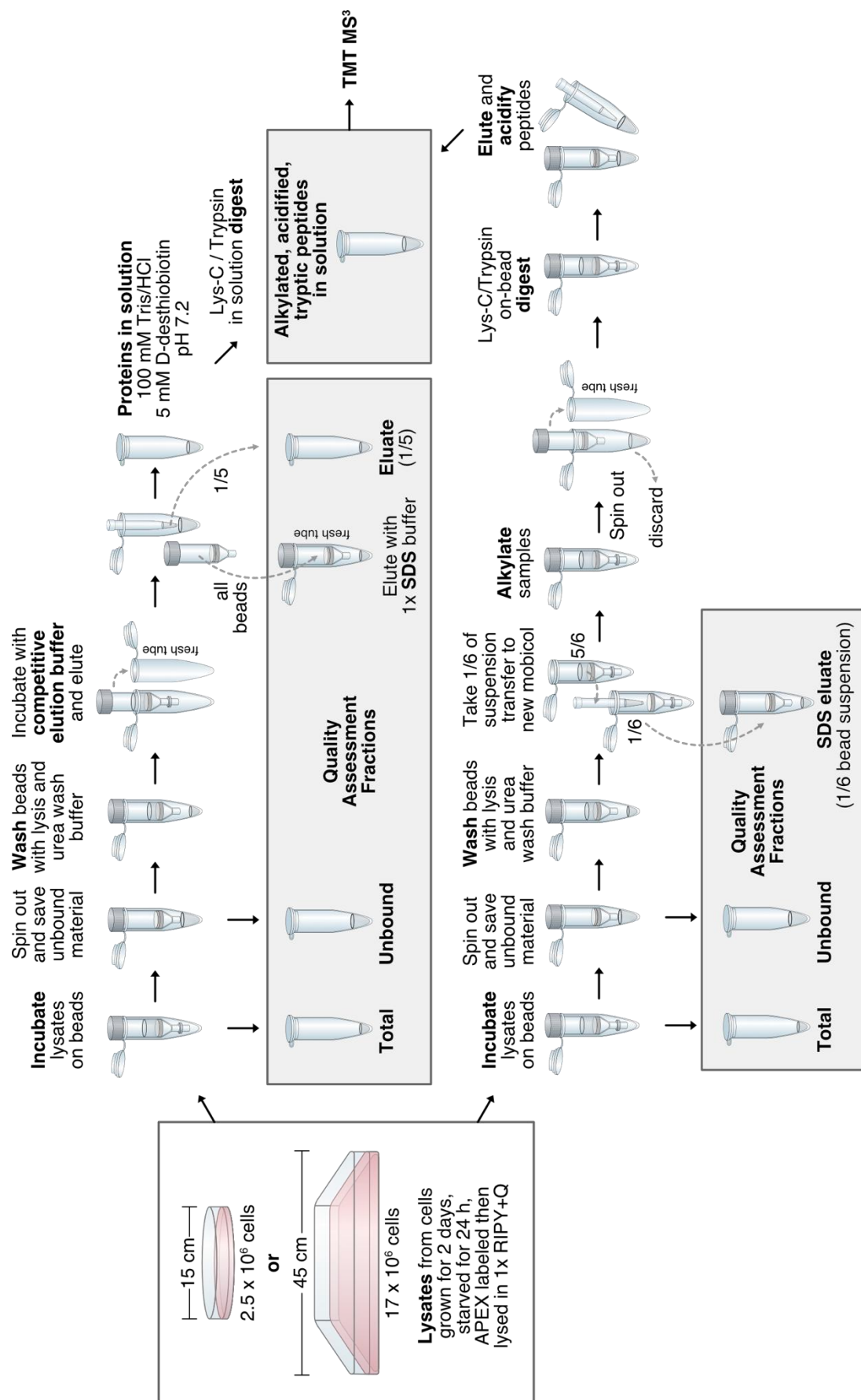


Fig. 8: Streptavidin affinity chromatography workflow following APEX proximity labeling.

Competitive Elution

In competitive elution (**Fig. 8**, top route), abundance of a ligand-specific additive with higher affinity to the stationary phase allows the release of bound target proteins in dependence of additive concentration, reaction temperature and volume. Protein release from solid into mobile phase was achieved by soaking beads in Mobicols two times consecutively in 500 µl competitive elution buffer containing

- 200 µg ALFA peptide for ALFA Selector^{PE} captured ALFA-tagged proteins and
- 5 mM biotin for streptavidin sepharose bound to desthiobiotin tyramide labeled proteins.

Each elution step was shaken at 950 rpm for 30 min at RT or 4 °C. Both elution fractions were collected in a new tube by centrifugation at $200 \times g$ for 2 min and pooled. Dry stationary phase was cleared by cooking packed beads in SDS elution buffer (1:1 ratio to bead bed) at 95 °C, 650 rpm for 5 min and spun out hot into a new reaction tube at $200 \times g$ for 2 min. Before further analysis the dilute eluate had to be concentrated either through trichloroacetic acid precipitation (see page **88**) or the use of an Amicon[®] centrifugal filter. The latter was used for mass spectrometry sample preparation (see page **91**), where 500 µl volume was typically reduced to ~67 µl in 5 min at $14.000 \times g$. The retentate was recovered and 10% was taken for analysis by SDS PAGE and Western blotting (see pages **88** and **89** respectively). All samples were shock-frozen with liquid nitrogen and stored at -80 °C.

On-Bead Peptide Generation

Following the standard conditions of APEX or TurboID proximity labeling (see pages 74 and 75) proteins were labeled by biotin tyramide, which is inapplicable for competitive elution (**Fig. 8**, bottom route). That is due to the exceptionally high affinity and low dissociation rate ($k_{off} \sim 10^{-6} \text{ s}^{-1}$) of the biotinylated targets relative to the stationary streptavidin (Hirsch *et al*, 2002). Therefore, the proteins bound to beads were washed 5 times in 2 M urea wash buffer, with the last two washes containing 2 mM biotin to saturate the remaining free binding sites of the column material, allowing the streptavidin to fold more compactly and ensuring stability for subsequent peptide elution. At this point, a fraction (1/6th) of the beads was saved in another Mobicol for SDS elution and subsequent enrichment analysis by SDS PAGE and Western blotting (see pages **88** and **89** respectively). From here, incubation

steps were carried out at 37 °C 900 rpm in a thermoshaker. The remaining bead bed was processed further by incubating the beads in 200 µl dithiothreitol (DTT) buffer for 20 min. After two washes with 2 M urea wash buffer, proteins were alkylated in 200 µl iodoacetamide (IAA) buffer in the dark for another 20 min. Alkylation was quenched by washing twice with 2 M Urea wash buffer containing DTT. Primed for digestion, bead-protein mixture was incubated for 4 h in 80 µl 8 M urea/HEPES (pH 8.0) buffer to which 2.5 µg Lys-C was freshly added. Subsequently, 500 µl MS-grade dH₂O was added together with 4 µg trypsin to incubate for another 16 h. Peptides were retrieved by centrifugation and beads were soaked in 200 µl acetonitrile for 5 min. Spin-out was combined with peptide eluate and recovered peptides were acidified by addition of 30 µl formic acid (FA). Resulting eluate was snap-frozen in liquid nitrogen and stored at -80 °C prior to mass spectrometry analysis (see page 91).

Bradford Assay

Following cell lysis (see page 77) protein concentration of lysates was determined using ROTI[®] Quant. Binding of Coomassie Brilliant Blue-G250 to proteins, particularly to basic amino acid residues, resulted in a protein amount-proportional shift of absorption maximum from 465 to 595 nm, which could be measured spectrophotometrically using the TECAN Spark[®] 10M. The protein concentration was determined against a defined IgG standard curve.

This assay was performed in a 48-well plate. Six wells were pre-filled with 50 µl water to allow a serial dilution of IgG standard (1.49 µg/µl). 50 µl standard solution was added to the first well, mixed thoroughly and 50 µl was transferred to the next well onward. Reaching the penultimate well, content was mixed well and 50 µl was discarded, keeping the last well with only water to measure the blank dye value in absence of protein. Each sample was tested in triplicate, filling three wells with 48 µl water and adding 2 µl sample to each well. The 5× concentrated Bradford reagent was diluted to 1× and 700 µl was added to each filled well of the 48-well plate. The samples were immediately measured using the micro plate reader.

Polyacrylamide Gel Electrophoresis (PAGE)

Blue Native(BN)-PAGE

Blue Native (BN) PAGE was performed to analyze native protein complexes from compound lysates and affinity chromatography-purified samples by Coomassie-derived charge separation in an acrylamide mesh. 4 to 16.5% gradient or stacked continuous gels were prepared using a gradient maker linked to a peristaltic pump using the following composition:

%	4	6	10	16.5	Stack
BN PAGE gel buffer (3×) (ml)	3	3	3	3	2.5
Acrylamide (ml)	0.73	1.07	1.82	3.05	0.6
Glycerol (ml)	-	-	1.8	1.8	-
10% APS (μl)	38	20	30	20	60
TEMED (μl)	3.8	2	3	2	6
dH ₂ O (ml)	5.228	4.888	2.347	1.117	4.367

The gels were topped with a stacking gel for equal protein entry into the acrylamide mesh. Gels were cooled on a cold room for a few hours and shortly before running the gel pockets were filled with Coomassie G-250 loaded samples. Samples were carefully overlaid to the brim with cold 1× cathode buffer. Once gel was assembled with a buffer reservoir, 1× cathode buffer blue was added carefully at the top and the entire setup was transferred into a BN PAGE tank, where 1× anode buffer has been cooled to 4 °C with a water-cooling system. Proteins were separated at a constant 25 mA per gel, where small gels took about 2 h and big gels took up to 5 h or were run overnight at 80 V. 1× cathode buffer blue is replaced with 1× cathode buffer white after 15 min of run time. Samples ran alongside an unstained marker (50 μl per lane, 4 °C) and empty lane buffer containing the respective detergent used during cell lysis or chromatography. The run came to a hold once the blue Coomassie running front

emerged from the bottom of the gel. The gel was stripped from the cast and placed in SDS running buffer to soak until further processed by Western blotting (see page **89**).

Sodium Dodecyl Sulfate (SDS)-PAGE

Sodium dodecyl sulfate (SDS)-PAGE was performed to analyze individual proteins from a complex lysate or a chromatographically prepared sample according to their respective mass. Proteins were separated in this work using pH-neutral Bis-Tris gel chemistry, using either a 4-12% Bis-Tris midi gel (Invitrogen) or a small 10% Bis-Tris gel prepared in-house.

The following table shows the composition of a self-made gel:

Ingredients	Running Gel (10%)	Stacking Gel (4.50%)
Acrylamide (30%)	30	4.5
Bis-Tris buffer (3×)	30	10
dH ₂ O	30	15.5
APS	0.45	0.3
TEMED	0.045	0.03

The gel tank was assembled and filled with 1× MOPS buffer. Protein mixtures adjusted with Laemmli sample buffer were pipetted into the gel wells together with a pre-stained protein ladder. The proteins were separated at a constant 130 V until the blue running front emerged from the bottom of the gel. Once stripped from the gel cast and soaked in transfer buffer, separated proteins were transferred electrically to a suitable membrane through Western blotting (see page **89**).

Trichloroacetic acid (TCA) Precipitation

In order to analyze diluted protein solutions from various experimental procedures in their full extent, samples were precipitated using trichloroacetic (TCA) acid and reconstituted in smaller volumes. For this, a solution was adjusted to ~0.5% Triton-X100 or NP-40 and chilled on ice. TCA was added to ~12-15%, where the final concentration was calculated

for 15% relative to the sample volume. Complete precipitation was ensured by mixing well and a 30 min incubation on ice. Protein precipitate was collected by centrifugation at 13.000 rpm for 5 min in a cold centrifuge. The supernatant was discarded and the oily pellet was washed by addition of 1 ml ice-cold acetone. After a further round of centrifugation, the supernatant was removed and the pellet was air-dried until it no longer smelled of acetone. The pellet was resuspended in SDS buffer. Aiding re-solubilization, sample was sonicated in six cycles of 10 s, each consisting of 5 s sonication pulses followed by 5 s breaks to prevent overheating and foaming.

Western Blot

Proteins separated by charge and mass in SDS-PAGE (see page 88) or by charge and shape in BN-PAGE (see page 87) were further processed by Western blotting. Proteins were transferred to different membranes depending on the detection method:

- Polyvinylidene fluoride (PVDF) for horseradish-peroxidase (HRP)-based chemiluminescence detection
- Nitrocellulose for fluorescence-based detection (LI-COR)

Prior to use, PVDF membrane needed to be activated by soaking in EtOH for 10 s, followed by equilibration in transfer buffer for 2 min. Nitrocellulose, on the other hand, was only soaked in transfer buffer. BN PAGE gels were blotted exclusively on PVDF since methanol-based Coomassie Brilliant Blue R-250 staining (see page 90) was used to visualize the unstained protein marker. Therefore, the membranes were not suitable for the fluorescence-based detection due to background generation caused by Coomassie.

For **semi-dry blotting**, gels were directly placed on wet blotting paper in a semi-dry electroblotting system. The gel was overlaid with a membrane, followed by another wet blotting paper. The transfer was performed in semi-dry buffer containing 10% EtOH for 135 min at a constant 350 mA.

For **wet blotting**, gels were assembled in a wet transfer sandwich with blotting paper and a membrane of choice, then placed in a blotter chamber or Trans-Blot module. Protein membrane immobilization was conducted using transfer buffer containing 10% EtOH over the course of an hour at a constant 100 V.

This process allowed the separated proteins to maintain their previous spatial gel arrangement as they moved onto the membrane. At this point, membranes were dried, stained (see page 90) or immediately blocked using 5% milk in 1× TBS or LI-COR TBS blocking solution for 1 h. Dried membranes had to be re-hydrated in 1× TBS for 5 min before blocking. Subsequently, the membrane underwent cold overnight incubation with a specific antibody in 5% milk in 1× TBS targeted against a protein of interest, followed by multiple washing steps with 1× TBS containing 0.05% (HRP) or 0.1% Tween-20 (LI-COR). For the detection of multiple proteins, the membrane was either cut horizontally into pieces of different protein mass before antibody incubation, or another primary antibody mixture was added after the first incubation. Finally, secondary antibody, coupled to HRP or a fluorescent dye, directed against the primary antibody host was used to detect the protein. This mix was incubated for 1 h, then washed thoroughly as mentioned before. For LI-COR, the secondary antibody mix was prepared in 1× TBS. The membrane was then imaged at the Amersham™ ImageQuant or at the LI-COR Odyssey® imager.

Coomassie Brilliant Blue R-250

In order to visualize protein complexes and unstained protein marker used in BN PAGE, proteins transferred onto PVDF were stained using Coomassie staining solution for 5 s. The stain was decanted and the membrane was washed two times with destaining solution. The visible marker bands were drawn onto the membrane, then MeOH was used to completely destain. Once all blue color had vanished, membrane was soaked in 1× TBS, then processed further as described in the chapter before.

LI-COR Total Protein Stain

To visualize total protein content of individual samples, membranes were stained for total protein content using 5 ml LI-COR Revert Total Protein stain incubating for 5 min while gently shaking. Stain was decanted and membranes were washed two times with LI-COR wash solution for 30 s each. Membranes were rinsed with dH₂O and kept in water until imaged at the LI-COR Odyssey® imager. Once imaged, membranes were destained for 10 min in LICOR destaining solution and then washed with 1× TBS. Membranes were then handled as described before (see page 89).

Mass Spectrometry

The following protocol has been adapted from the original text by Per Haberkant, who performed the mass spectrometric analysis of all samples in this work at the Proteomics Core Facility at EMBL in Heidelberg. The materials and methods are mentioned in the text for the sake of completeness, rather than in the previous chapters, as they were not part of my work. Frank Stein performed the initial bioinformatic data analysis. Prior to conducting mass spectrometry analysis, eluates concentrated using Amicon filters were thawed and underwent an in-solution tryptic digestion. The tryptic peptides were produced using a modified version of the Single-Pot Solid-Phase-enhanced Sample Preparation (SP3) technology (Hughes *et al*, 2014; Moggridge *et al*, 2018). A slurry of hydrophilic and hydrophobic Sera-Mag Beads (Thermo Scientific, #4515-2105-050250, 6515-2105-050250) was prepared by mixing 20 μ l of the beads, washing them with water, and then reconstituting them in 100 μ l of water. From this slurry, 5 μ l were added to 50 μ l of the eluate along with 55 μ l of acetonitrile. The subsequent steps were automated using the King Fisher Apex System (Thermo Scientific). The beads, once bound to the peptides, were washed three times with 100 μ l of 80% ethanol before being transferred to 100 μ l of a digestion buffer (50 mM Hepes/NaOH pH 8.4 with 5 mM TCEP, 20 mM chloroacetamide (Sigma-Aldrich, #C0267), and 0.25 μ g trypsin (Promega, #V5111)). The samples were digested overnight at 37 °C. Post-digestion, the beads were removed, and the peptides were dried and reconstituted in 10 μ l of water. Subsequently, 80 μ g of TMT10plex (Thermo Scientific, #90111) (Werner *et al*, 2014) labeling reagent dissolved in 4 μ l of acetonitrile were added to the mixture, which was then incubated for 1 hour at room temperature. Any excess TMT reagent was neutralized by adding 4 μ l of a 5% hydroxylamine solution (Sigma, 438227). The mixed peptides were then cleaned using a reverse phase step (OASIS HLB 96-well μ Elution Plate, Waters #186001828BA). These peptides were fractionated under high pH conditions (Hughes *et al*, 2014) into 12 fractions, which were then analyzed by multi-stage mass spectrometry (MS3) using a Lumos system (Thermo Scientific).

Peptide separation was achieved using an Ultimate 3000 nano RSLC system (Dionex), equipped with a trapping cartridge (Precolumn C18 PepMap100, 5 mm, 300 μ m i.d., 5 μ m, 100 Å) and an analytical column (Acclaim PepMap 100, 75 $\times$ 50 cm C18, 3 mm, 100 Å) connected to a nanospray-Flex ion source. Peptides were loaded onto the trap column at a flow rate of 30 μ l/min using solvent A (0.1% formic acid) and eluted with a gradient from

2% to 80% solvent B (0.1% formic acid in acetonitrile) over 2 hours at 0.3 μ l/min, using LC-MS grade solvents.

The Orbitrap Fusion Lumos was operated in positive ion mode with a spray voltage of 2.5 kV and a capillary temperature of 275 °C. Full scan MS spectra were acquired in profile mode over a mass range of 375–1,500 m/z at a resolution of 120,000, with a maximum fill time of 50 ms and an automatic gain control (AGC) Target set to Standard, and a radio frequency (RF) lens setting of 30%. Ion selection in the quadrupole used a 1.5 m/z isolation window. Fragmentation was performed by higher-energy collisional dissociation (HCD) with a collision energy of 36%, and ions were analyzed in the ion trap with a maximum fill time of 50 ms and an AGC target set to Standard. The top 5 precursors were chosen using synchronous precursor selection (SPS) between 400 to 2,000 m/z, with a precursor ion exclusion width of -18 and +5 m/z. Further fragmentation by HCD was conducted at 70% collision energy, and ions were analyzed in the Orbitrap at a resolution of 50,000, with a maximum injection time of 105 ms and an AGC Target set to Custom and 200%.

Data acquired were analyzed with FragPipe (Kong *et al*, 2017) using the Uniprot *Mus musculus* FASTA database (UP000000589, ID10090 with 21,968 entries, downloaded on January 11, 2023). Modifications considered included Carbamidomethyl (C, fixed), TMT10plex (K, fixed), Acetyl (N-term, variable), Oxidation (M, variable), and TMT10plex (N-term, variable). The mass error tolerance was set at 10 ppm for full scan MS spectra and 0.02 Da for MS/MS spectra, allowing up to 2 missed cleavages. A minimum of two unique peptides with at least seven amino acids in length and a false discovery rate below 0.01 were required at both the peptide and protein levels (Savitski *et al*, 2015).

2.2.4 Statistics

Line and violin plots were generated using tabular data in Excel and GraphPad Prism. The TMT mass spectrometric data was processed by Frank Stein at the Proteomic Core Facility in Heidelberg using IsobarQuant or FragPipe, followed by R Studio. After annotating the experimental conditions, contaminants and duplicates were removed. The data was logarithmically transformed and filtered to include only proteins quantified by more than two unique peptides in at least two of the three replicates. Subsequently, the data was fitted to a linear model to mitigate batch effects using Linear Models for Microarray and RNA-Seq

Data (limma) analysis. A variance stabilization normalization method was applied to the log2-transformed raw data (Smyth *et al*, 2003).

CiliaQ Analysis

Acquired images were analyzed using CiliaQ, a specialized ImageJ plugin for ciliary structure quantification (Hansen *et al*, 2021). The plugin was employed to measure fluorescence intensities across selected channels within defined primary cilia regions. Initially, images were imported into CiliaQ, and regions of interest (ROIs) for individual cilia segmentation were manually defined by selecting an appropriate thresholding method for a ciliary marker. The software then automatically detected ciliary structures, allowing for precise quantification of fluorescence intensity along each cilium. Finally, data were exported from CiliaQ for subsequent statistical analysis.

Cilia Gold Standard List

Protein IDs were compared to a manually curated "Cilia Gold Standard" (CGS) list that I compiled for conditional filtering. I created the CGS list by integrating data from the Syscilia consortium van Dam *et al* (2013), Dam *et al* (2019) and studies by Mick *et al* (2015) and May *et al* (2021). The list comprises not fully characterized ciliary proteins obtained by multiple methods, such as phenotype validation, subcellular localization studies, and proteomics (interaction data and cilia-specific proteome). These methods have been applied to these organisms and combined in the publicly accessible Syscilia Gold Standard List:

- *Caenorhabditis elegans*
- *Danio rerio* morpholinos
- *Homo sapiens* retinal pigment (hTert-RPE1) and lung epithelial cells
- *Mus musculus* retina cross-sections, chondrogenic teratocarcinoma cells (ATDC5) and inner medullary collecting duct cells (IMCD3)

The applied criteria ensured a careful balance between avoiding false positives while keeping false negatives low, focusing on proteins significantly enriched in cilia while excluding those with similar abundance inside and outside of cilia.

DeepVenn

Area-proportional Venn diagrams were generated to highlight overlaps between acquired mass spectrometry datasets using the web application (<https://www.deepvenn.com/>) (Hulsen, 2022). Protein identifiers (IDs) were added as individual sets to yield circles showing intersecting areas. Intersecting and unique area IDs were exported and compared using gene set enrichment analysis.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was performed on the individual proteomic sets in R Studio to determine statistically significant differences between proximity labeled samples and their respective controls (Subramanian *et al*, 2005). The datasets were ranked by fold change in a descending order. The GSEA results were plotted as a ridge plot together with a dot plot.

For analysis of DeepVenn extracted protein ID sets, lists were analyzed using the gene set enrichment web application ShinyGO 0.80 (<http://bioinformatics.sdstate.edu/go/>) (Ge *et al*, 2020).

Volcano Plot Analysis

The normalized data was then further analyzed by me using Perseus and R Studio. A two-sample unequal variances (Welch's) t-test was performed to compare experimental groups. Volcano plots were generated to visualize proteomic differences, plotting $\log_2$ -transformed fold changes against the negative $\log_{10}$ -transformed Welch's t-test p-values for each sample condition.

3. RESULTS

3.1 Differential Labeling Dynamics of APEX2 and TurboID in Primary Cilia

Proximity labeling (PL) technologies have significantly enhanced our comprehension of cellular micro-environments, extending beyond protein-protein interaction networks by enabling precise spatiotemporal sampling. The initial challenge in conducting quantitative analyses lies in the selection of the appropriate enzyme for PL, a decision shaped by various factors. These considerations involve choosing between *in vivo*, *ex vivo* or *in vitro* studies, assessing the compatibility of cell types with the selected PL enzyme, evaluating the efficiency of substrate uptake by these cells, and determining the required time resolution for the experiment.

Prior to this study in 2015, Mick *et al* pioneered the establishment of an IMCD3 cell line with stable expression of ascorbate peroxidase (APEX) genetically targeted to primary cilia (termed cilia-APEX) to study the compartment's proteomic content using PL. In the same year, APEX2 was developed by Lam *et al* through protein engineering following yeast surface display mutagenesis, exhibiting superior performance compared to its predecessor. In 2021, May *et al* combined the technological enzymatic advances with TMT labeling for peptide quantitation and investigated proteomic remodeling of primary cilia involved in Sonic Hedgehog signaling using an improved version of the enzyme, termed cilia-APEX2.

While numerous studies have aimed to unravel the proteome composition of primary cilia using proximity labeling techniques based on either biotin ligase (Gupta *et al*, 2015; Bozal-Basterra *et al*, 2018, 2020; Aslanyan *et al*, 2023) or ascorbate peroxidase (Mick *et al*, 2015; Kohli *et al*, 2017; May *et al*, 2021a) none of these studies conducted a comparative analysis to assess proteome coverage and overlap.

3.1.1 Generation and Characterization of Primary Cilia-Targeted TurboID Cell Line

Therefore, the first aim of this work was to generate an IMCD3 cell line comparable to May *et al* that expresses a cilia-localized promiscuous biotin ligase. For this purpose, the more advanced biotin ligase TurboID was selected, as it enables faster labeling compared to its predecessor BioID (Branon *et al*, 2018). The shorter reaction time made it possible to perform experiments in tandem with the APEX PL workflow.

In the above mentioned studies, both cilia-APEX and -APEX2 were N-terminally fused to a primary cilia targeting sequence (“cilia-“) consisting of the first 200 amino acids of NPHP3, followed by the enhanced green fluorescent protein (eGFP) for detection. To mitigate non-physiological alterations resulting from ectopic expression, a less potent promoter (pCMVΔ6) replaced the original pEF1α promoter in the genomic Flp-In expression cassette. An expression vector compatible with the Flp-In system was generated by replacing APEX2 with the TurboID transgene (cilia-eGFP-TurboID; short cilia-TurboID). In addition, a cytosolic counterpart was constructed by introducing a point mutation into the cilia-targeting sequence (CTS), resulting in a myristylation-deficient mutant (G2A) that does not target primary cilia (control-eGFP-TurboID; short control-TurboID).

After generation of the stable IMCD3-TurboID cell lines, the ectopic expression of the transgenes was assessed by Western blot (WB; see method page 89) analysis in comparison to wild-type (WT) cells after staining with GFP antibodies (**Fig. 9A**). All fusion proteins showed correct localization in cell imaging with no basal biotinylation activity (**Fig. 9C**). In order to test the labeling capacity of the TurboID cell lines in comparison to their APEX2 equivalents, PL was performed in these cells and stopped at various time points to be analyzed by WB (**Fig. 9B**) and immunofluorescence (IF; see method page 77) (**Fig. 9C**). The significantly different labeling times were chosen because they are adapted to the different modes of action of TurboID and APEX2. TurboID relies on the release of activated biotin molecules, which inherently leads to longer labeling times.

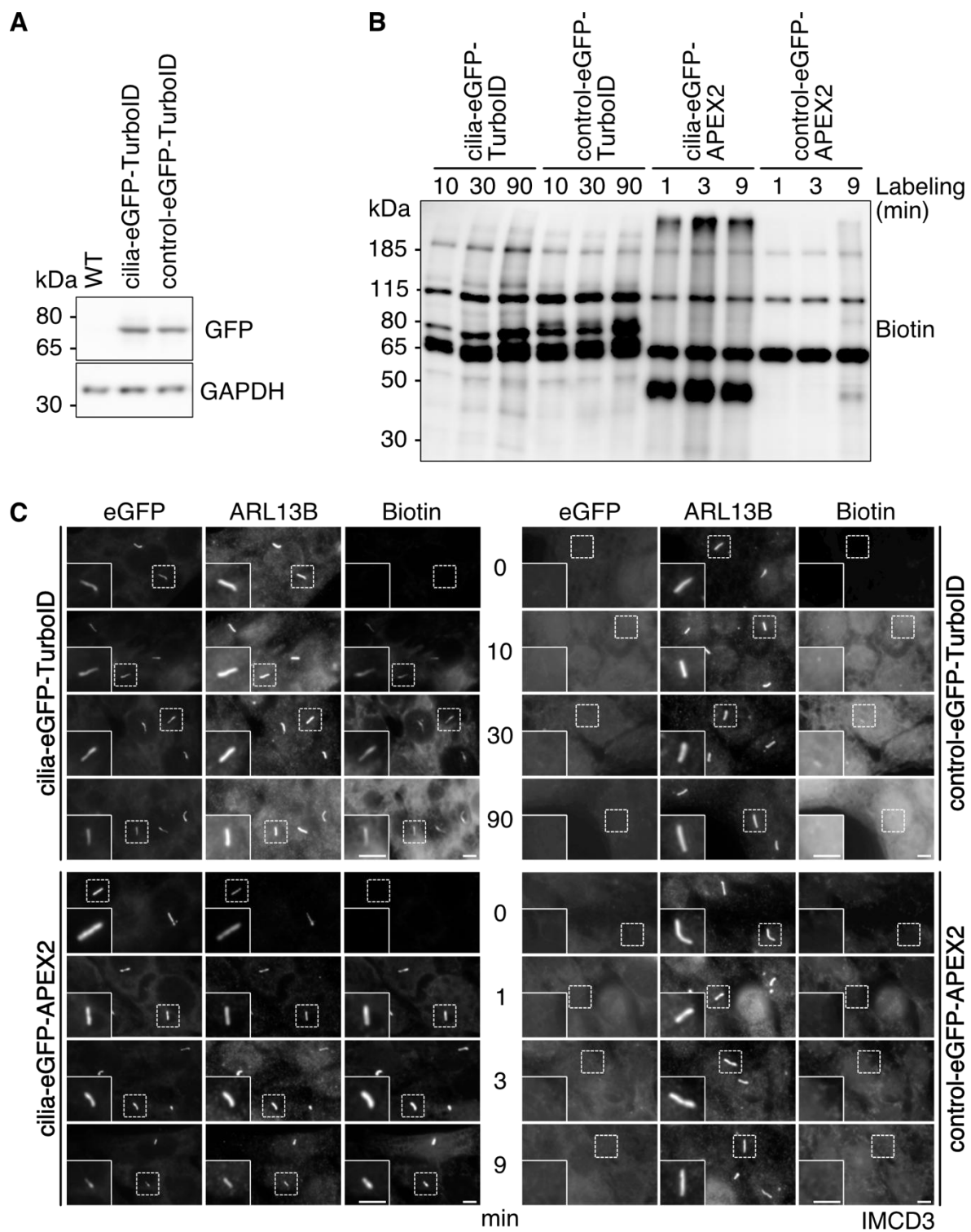


Fig. 9: Targeted labeling of primary cilia with APEX2 results in stronger ciliary signals while producing less cellular background compared to TurboID.

(A, B) A semi-quantitative Western blot analysis was conducted on IMCD3 cells expressing TurboID and APEX2 variants either localized to primary cilia or the cytosol post-proximity labeling at different time points. TurboID labeling was performed using 500 μ M biotin, whereas APEX2 samples were labeled using 500 μ M biotin tyramide (BT, 30 min) with varied 1 mM H_2O_2 incubation times. Each lane was loaded with 20 μ g protein. Signals were obtained through horseradish peroxidase (HRP)-conjugated antibodies and streptavidin

using luminol chemiluminescence. **(C)** Representative immunofluorescence (IF) images of experimental data shown in (A) and (B) depict gradually increasing proximity labeling. Primary cilia were stained by ARL13B antibody staining. GFP fluorescence marked TurboID and APEX2 constructs, with biotin detected by fluorescently labeled streptavidin. Scale bar = 5 μ m.

Conversely, APEX2 catalyzes a radical reaction that occurs significantly faster, resulting in shorter labeling times. This is reflected in the observed signal intensities detected by fluorescent streptavidin in the WB analysis. The PL biotinylation of cilia-APEX2, which was stopped after 1 min (**Fig. 9B**, lane 6), exceeded that of cilia-TurboID, which only reached a comparable level at 90 min (**Fig. 9B**, lane 3).

The pattern was different for the cytosolic controls, where control-TurboID displayed increased biotinylation at 10 min (**Fig. 9B**, lane 4) compared to control-APEX2 at 1 min (**Fig. 9B**, lane 10). This observation was consistent with the accompanying IF results from the same experiment, which showed increased cellular biotinylation for control-TurboID compared to the control-APEX2 cell line (**Fig. 9C**). Surprisingly, cellular accumulation of biotinylated proteins was also observed in the cilia-TurboID cell line, in contrast to the focused signal that was localized specifically to primary cilia when APEX2 PL was used.

3.1.2 Labeling Specificity and Temporal Resolution

Given the high disparity between labeling modes and times, the enzymes were further tested for their ability to specifically label primary ciliary proteins. For this, PL was performed in triplicate for APEX-labeled samples of cilia-APEX2 and control-APEX2 cells, along with duplicate samples of cilia-TurboID and control-TurboID cells (**Fig. 10A**) to be subsequently analyzed by mass spectrometry. After cell lysis, biotinylated proteins were isolated from the complex cell lysates by streptavidin affinity chromatography (described in detail in methods section, see page 83).

To assess isolation specificity of the mass spectrometry samples, total cell lysates (chromatography input) of cilia- as well as cytosolic-localized (control) PL enzymes were analyzed by semi-quantitative WB against their eluate fractions (**Fig. 10B**). The biotinylation rate was evaluated by probing with HRP-conjugated streptavidin, which exhibited efficient enrichment and isolation for all conditions and replicates visible as “smear” between prominent endogenous biotin-associated carboxylases (**Fig. 10B**, lanes 11-20). When streptavidin is used on total cell lysates (**Fig. 10B**, lanes 1-10), a four-band pattern is usually observed,

consisting of acetyl-CoA carboxylase (ACC; above 185 kDa), pyruvate carboxylase (PC; above 115 kDa), methylcrotonyl-CoA carboxylase (MCC; above 65 kDa) and propionyl-CoA carboxylase (PCC; slightly below MCC) (Bramwell, 1987; Wang *et al*, 2005; Tong, 2013).

Eluates from cilia-TurboID and -APEX2 both enriched IFT88, a component of the IFT-B protein complex that is important for ciliary cargo trafficking (**Fig. 10B**, lanes 11-12 and 15-17). Further, acetylated tubulin (acTub) was isolated in high amounts from cilia-APEX2 samples (**Fig. 10B**, lanes 15-17), but not from cilia-TurboID. A possible explanation for this difference could be the inaccessibility of activated biotin molecules to the mostly luminal and therefore protected lysines of α - and β -tubulin, some of which are masked by post-translational modifications, such as acetylation of K40 (Portran *et al*, 2017; Prokop, 2022). Re-probing the WB membrane with a GFP antibody revealed some biotinylation of all PL fusion enzymes except control-APEX2 in the isolated samples. It cannot be ruled out that the enzymes label themselves (in *cis*) or their neighbors (in *trans*) during PL. Faint IFT88 and acTub signals also appeared in the cytosolic controls (**Fig. 10B**, lanes 13-14 and 18-20). These could arise from cytoplasmic fractions of these ciliary proteins, specifically basal body associated IFT88 or cellular acTub. Altogether, the eluates of samples that possessed cilia-localized PL enzymes also showed a specific enrichment of cilia proteins in the WB analysis.

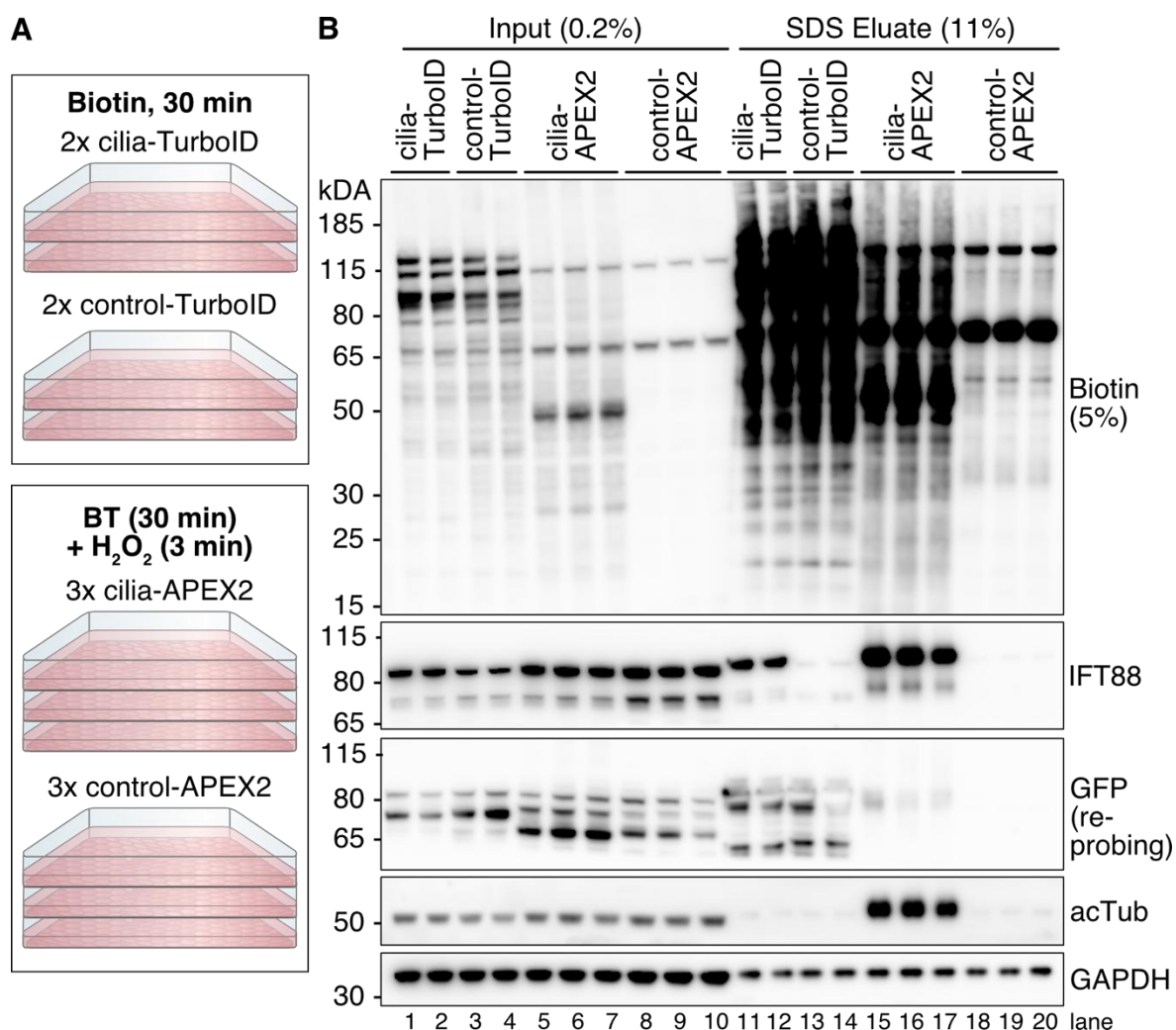


Fig. 10: Proximity labeling using biotin ligase yields enrichment patterns comparable yet different from those generated by APEX.

(A) Scheme of cell culture plates used in TurboID or APEX2 PL described in detail in (B). MM section XY describes the cell seeding numbers and affinity chromatography handling details. **(B)** A semi-quantitative Western blot analysis was performed on IMCD3 cells expressing TurboID and APEX2 variants, targeted either to primary cilia (cilia) or the cytosol (control) following proximity labeling. TurboID labeling employed 500 μ M biotin for 30 minutes, while APEX2 samples were labeled using 500 μ M BT for 30 minutes, supplemented with 1 mM H₂O₂ for 3 minutes. TurboID samples were carried out in duplicate, APEX2 samples in triplicate. Biotin signals were detected on one membrane, analyzing 5% of the eluate using streptavidin-HRP, while signals for IFT88, GFP, acTub, and GAPDH (all from 11% eluate) were detected on another membrane using HRP-conjugated antibodies. All signals were obtained through luminol chemiluminescence. Each lane was loaded with 0.2% of the chromatography input, and the percentage in brackets indicates the quantity analyzed from 16% of the eluate, derived from isolation using 10 mg total protein.

3.1.3 Quantitative Primary Cilia Proteome Comparison between APEX2 and TurboID

The remaining streptavidin chromatography isolates were subjected to trypsin/Lys-C proteolysis and the resulting peptides were labeled on their lysine residues with tandem-mass-tag (TMT) labels to be analyzed by LC-MS3 (see page 91). Hierarchical clustering of protein abundances obtained from the multiplexed experiment revealed high reproducibility among the triplicate and duplicate samples (**Fig. 11A**). Distinct clusters of candidate ciliary proteins emerged in the cilia-APEX2 and cilia-TurboID dataset when compared to their respective controls, with non-ciliary proteins forming separate clusters (**Fig. 11B**; **Sup.-Fig. 1**). When looking at the distribution of the mean values of relative protein abundances, tendencies for cilia clusters could be recognized (**Sup.-Fig. 1B**). Further, candidate ciliary proteins were identified through statistical analyses comparing the relative enrichment between cilia-APEX2 or cilia-TurboID samples and mislocalized cilia controls. For this, proteomic data was represented in a volcano plot (for method see page 94), where proteins were classified as ciliary, if they exhibited over twofold enrichment in cilia samples compared to control samples (fold change ≥ 2.0), along with a statistically significant enrichment in a two-sample t-test (p value ≤ 0.05) (**Fig. 11BC**, red circles). In order to benchmark potential primary ciliary hits, I compiled a curated cilia proteome gold standard (CGS) list by merging data from the Syscilia consortium van Dam *et al* (2013) and studies conducted by Mick *et al* (2015) and May *et al* (2021). Proteins that met the fold change and p value criteria and additionally appeared in the CGS were labeled as clearly ciliary (**Fig. 11BC**, red filled circles). The applied criteria ensured a careful balance between avoiding false positives while keeping false negatives low, focusing on proteins significantly enriched in cilia while excluding those with similar abundance inside and outside of cilia.

Out of a total of 4240 quantified proteins, this filtering approach yielded 12.26% (520 proteins) significantly enriched ciliary candidates for cilia-APEX2 over control-APEX2, of which about one-sixth (15.58%, 81) were identified as clearly ciliary. Among the 7.76% (329) significantly depleted proteins, only 1.52% (5) were ciliary (**Fig. 11B**). In the comparison between cilia-TurboID and control-TurboID, 7.08% (300) of the proteins were significantly enriched, with the proportion of enriched ciliary proteins (29) being about one-tenth (9.67%) and the proportion of depleted ciliary proteins being 1.65% (6 out of 363)

(**Fig. 11C**). From this analysis, APEX2 PL exhibited 1.6 times more significantly enriched ciliary proteins compared to the TurboID equivalent.

To understand the lower number of enriched proteins in cilia-TurboID, the condition was tested against control-APEX. The overall enrichment of labeled proteins increased significantly from 7% to almost 18% (17.83%). Despite this increase, the proportion of ciliary proteins remained relatively low at 7.01% (53 out of 756) (**Sup.-Fig. 2A**). This suggests that cilia-TurboID was more effective at labeling proteins compared to cilia-APEX2 while labeling more non-ciliary proteins non-specifically. However, a direct comparison is challenging, given the vastly different labeling times between TurboID and APEX2.

To further investigate the observed difference in enrichment, control-TurboID was compared to control-APEX2, showing a significant enrichment of 20.97% (889 proteins), with 4.72% (42) being ciliary proteins (**Sup.-Fig. 2B**). This indicates that control-TurboID labels a substantial number of ciliary proteins, implying increased nonspecific interactions. This non-specific labeling by control-TurboID likely contributes to the apparent loss in ciliary hits observed in the cilia-TurboID sample, thereby affecting its specificity (**Fig. 11C**).

Interestingly, a direct comparison of the cilia-localized PL enzyme samples indicated distinct ciliary protein target preferences, both exhibiting unique ciliary protein enrichment on either side (13.4% enrichment for APEX2, 10.32% for TurboID) (**Sup.-Fig. 2C**).

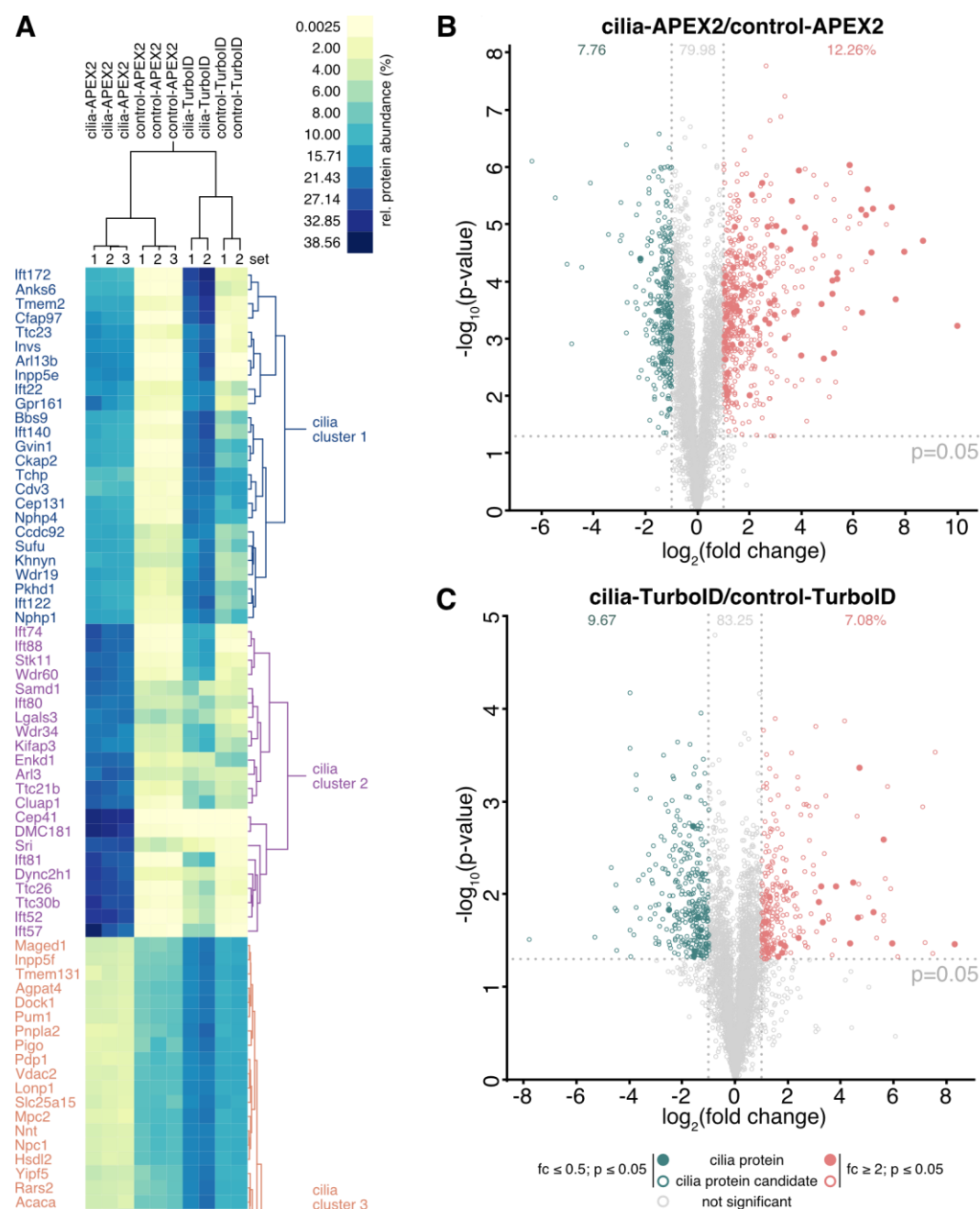


Fig. 11: Quantitative primary cilia proteomics reveals similarities and differences in mapping between TurboID and APEX proximity labeling.

(A) Hierarchical two-way cluster analysis using Ward's minimum variance method was conducted on proteomics data obtained from samples in **Fig. 10**. The heatmap columns represent individual samples within each condition set, clustered against the identified proteins listed in the rows. The relative abundance of each protein was determined by dividing its individual TMT signal by the sum of TMT signals across all samples. The color legend for relative abundance (expressed in percent) is displayed in the top right corner. The row dendrogram indicates the similarity of protein groups, with deeper splits indicating dissimilarity. In this analysis, clusters containing cilia proteins were highlighted. The complete data for the hierarchical clustering can be found in **Sup.-Fig. 1**. **(B, C)** Volcano plot representations of the cilia-APEX2 and cilia-TurboID TMT experiment data. Proteins are plotted by the negative logarithm (base 10) of the two-sample t-test p-values (y-axis) against the

logarithm (base 2) of the TMT fold change (fc; x-axis) between conditions cilia-APEX2 and control-APEX2 (A) or cilia-TurboID and control-TurboID (B). Colored circles in the top corners indicate proteins with high statistical significance ($p \leq 0.05$) and large fold changes (green $fc \leq 0.5$; red $fc \geq 2$). Filled circles mark cilia proteins matched against a self-compiled gold standard set. The horizontal dashed gray line represents $p = 0.05$, and the vertical lines denote fc indicated before. Proteins not meeting the threshold criteria are shown in gray. The colored numbers at the top of each quadrant indicate the percentage of proteins that meet the specified criteria, out of the total 4240 quantified proteins. Volcano plots illustrating differences between the other tested conditions are available in **Sup.-Fig. 2**.

Comparative Analysis of APEX2 and TurboID Proteomic Datasets

The comparative analysis of IMCD3 primary cilia proteomes using APEX2 and TurboID PL has elucidated critical differences in specificity, temporal resolution, and proteome depth. Notably, the distinct labeling patterns obtained in streptavidin WB staining (**Fig. 10**) hinted at potential variations in the proteomic outcomes between these two PL methods.

Despite the shorter labeling duration of 3 min for APEX2 compared to the 30 min for TurboID, APEX2 PL demonstrated a higher enrichment of ciliary proteins (**Fig. 11**). When comparing the significantly enriched proteins of each proteomic dataset against the manually curated Cilia Gold Standard (CGS) list of core ciliary components, APEX2 identified 163 shared ciliary proteins, surpassing the 49 identified by TurboID (**Fig. 12**). Of these, 40 proteins were shared among APEX2, TurboID, and CGS, underscoring the superior specificity and temporal resolution of APEX2. Nonetheless, both PL methods resulted in a shared proteomic overlap of 78 proteins, representing 30% of enriched hits in the TurboID set, with 38 being potential new ciliary candidates. Interestingly, the number of non-overlapping enriched proteins identified with APEX2 was approximately twice that of TurboID. This discrepancy could be attributed to either APEX2-independent labeling, potentially caused by exogenous H_2O_2 -induced activity of other cellular peroxidases (Mick, 2019), or the artificial inflation of ciliary protein coverage within the manually curated Ciliary Gene Set (CGS). The overrepresentation in the CGS could result from incomplete characterization of candidate genes in the referenced studies, raising the possibility that some entries may be false positives (Mick *et al*, 2015; Dam *et al*, 2019; May *et al*, 2021a).

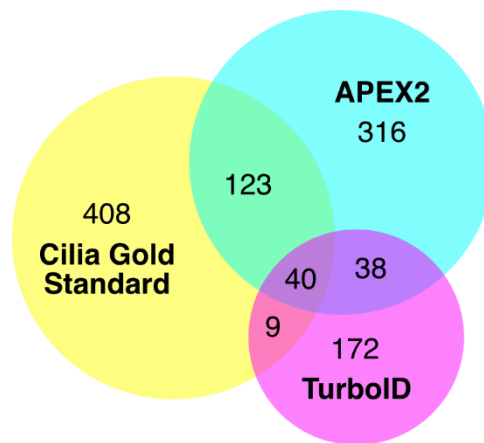


Fig. 12: APEX2 cilia proteome maps more cilia proteins than the TurboID equivalent.

Venn diagram depicting proteomic overlap of enriched ciliary hits between cilia-APEX2 and cilia-TurboID against a manually curated cilia gold standard list. Ciliary hits were determined by comparing cilia-APEX2 and cilia-TurboID to their respective controls. Extracted enriched proteins from Volcano plot (see **Fig. 11**; Welch's t-test $p \leq 0.05$ and fold change ≥ 2).

When the individual sections of the Venn diagram (**Fig. 12**) were analyzed for GO term enrichment (for method see page **94**), the datasets revealed distinct biological processes (**Sup.-Fig. 3**). APEX2 unique proteins (316) were enriched in biological processes such as cytoskeletal organization, centrosome cycle and microtubule-based processes, rather indicating a focus on cellular structural roles than cellular peroxidase background. The overlap between APEX2 and TurboID (38) highlighted proteins involved in intracellular transport and cytoskeleton organization, suggesting shared targets related to cellular localization mechanisms. TurboID unique proteins (172) showed enrichment in cellular component biogenesis, protein modification regulation, and catabolic processes, emphasizing a distinct focus on regulatory and metabolic activities.

A further comparative Gene Set Enrichment Analysis (GSEA) was performed on the proteomic data from cilia-APEX2 and cilia-TurboID samples against their respective controls (**Fig. 13**; for method see page **94**). The analysis was performed based on analysis of the overrepresented Gene Ontology (GO) terms in the dataset (Harris *et al*, 2004; Roncaglia *et al*, 2017). This was done to visualize enrichment distributions based on protein fold change (ridge plot) of one condition over the corresponding control and the respective gene ratios (dot plot) of enriched or depleted sets of proteins involved in distinct biological processes and cellular compartment categories.

Notably, APEX2 and TurboID comparisons against their respective controls revealed shared enrichment in cilia-related GO terms, affirming the relevance of APEX2 and TurboID in identifying cilia-specific proteins. Interestingly, TurboID showed overrepresentation of a set of proteins involved in heart development and morphogenesis (**Fig. 13B**). The common enrichment of cilia-associated terms suggests that both methodologies effectively captured specific proteins involved in ciliary function and structure, thus validating their use in cilia proteome studies.

However, the differences between the two datasets were also significant. The depleted GO terms varied between the cilia-APEX2 and cilia-TurboID datasets, suggesting that each method may have distinct biases or sensitivities. As mentioned before, these differences could arise from the unique biochemical properties and labeling mechanisms inherent to APEX2 and TurboID. Overrepresentation of depleted mitochondrial GO terms in the APEX2 dataset pointed to increased mitochondrial background labeling arising from the control cell line (**Fig. 13A**). In contrast, the labeling mechanism of TurboID is based on diffusive biotin-AMP, which might have led to a different subset of proteins being labeled and detected. This was reflected in the overrepresentation of nucleus- and DNA-associated terms seen in the analysis.

This divergence in depleted terms highlights that while both methods are robust for enriching cilia-related proteins, they might capture different spectra of the proteome, thus providing complementary views of the cilia's molecular landscape.

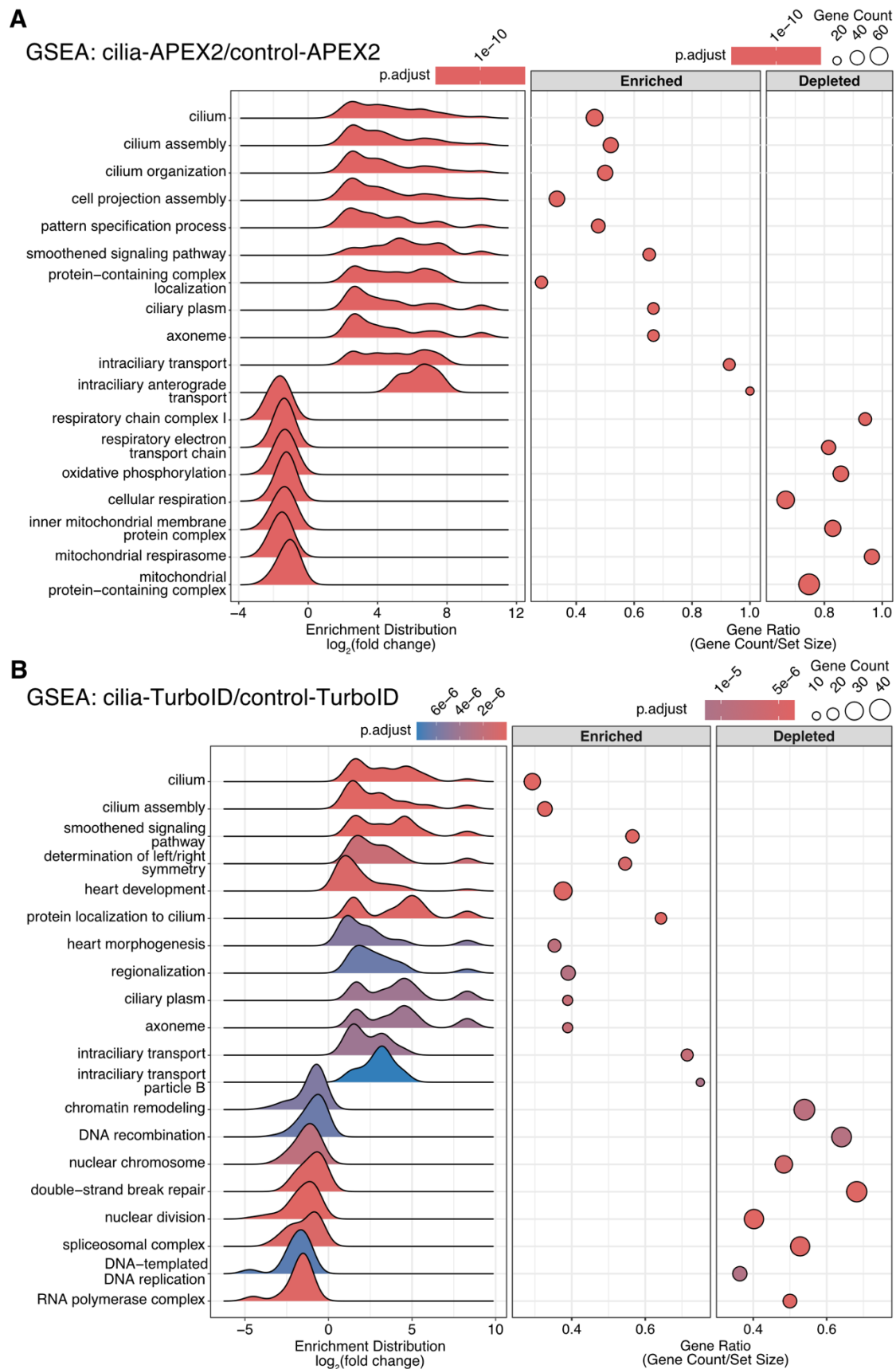


Fig. 13: Gene Set Enrichment Analysis shows shared enriched cilia representation between APEX2 and TurboID sets with differing depleted Gene Ontology terms.

(A) Ridge and dot plot representation of Gene Set Enrichment Analysis (GSEA) results from cilia-APEX2 proteomic data. Protein fold changes between cilia-APEX2 and control-APEX2 were calculated, sorted in descending order, and used to generate a ranked list. GSEA was performed, and the top scoring enriched and depleted Gene Ontology (GO) terms for biological processes and cellular compartments were plotted. The gene count represents the number of genes associated with each GO term, while the gene ratio indicates the percentage of significantly correlated genes relative to the total number of genes linked to that process or compartment. **(B)** GSEA results as in (A) for cilia-TurboID data compared to control-TurboID.

3.1.4 Restricted Substrate Flexibility of TurboID Limits Usability

APEX2 and TurboID displayed differential labeling dynamics in primary cilia with a partial overlap of accessible targets. Nevertheless, TurboID generated higher cell background in the biotinylation reaction and did not achieve the spatiotemporal resolution of APEX2.

Another disadvantage is the substrate limitation of TurboID. The enzyme's active site exclusively allows biotin and ATP to bind, initiating the formation of a biotinoyl-5'-AMP intermediate which diffuses away from the enzyme to react with nearby proteins (Branon *et al*, 2018). On the contrary, APEX2 can use a variety of different phenolic compounds that can selectively label proteins, RNA and DNA molecules (Kang & Rhee, 2022). To illustrate the aforementioned substrate limitation, cilia-TurboID IMCD3 cells were incubated with either biotin or desthiobiotin, a sulfur-free biotin analogue (**Fig. 14A**). Similarly, cilia-APEX2 IMCD3 cells were incubated with the phenolic equivalents and labeled for different time points. The fixed and stained micrographs showed that TurboID was selective for biotin and did not accept desthiobiotin as a substrate, which is in line with findings of previous studies. In contrast, APEX2 demonstrated substrate flexibility by accepting either phenolic substrate, which showed identical labeling capacity (**Fig. 14B**).

The use of desthiobiotin tyramide (DTBT) in APEX2 proximity labeling offers significant benefits for protein interaction and complex analysis. Importantly, the binding affinity (K_d) of streptavidin for biotin is extremely high ($K_d = 10^{-14}$ M), which is advantageous for stable protein capture. However, the slightly weaker affinity for desthiobiotin ($K_d = 10^{-11}$ M) allows for effective competitive elution using biotin without compromising the capture efficiency during the chromatography (Hirsch *et al*, 2002; Niinae *et al*, 2021).

Competitive elution worked under varying conditions, which allows for precise control over the release of biotinylated proteins from streptavidin beads enhancing the recovery and analysis of truly labeled proteins while retaining endogenous biotin-associated proteins (**Sup.-Fig. 4**). This approach provides flexibility in isolating proteins under different temperature and duration conditions, enabling detailed studies of protein interactions within specific cellular compartments such as cilia.

Further, proximity labeling using DTBT coupled with streptavidin affinity capture, competitive elution and subsequent sucrose density gradient ultracentrifugation (SGDU), allows for the isolation, separation and analysis of intact protein complexes under non-denaturing conditions, preserving their native state and interactions (**Sup.-Fig. 5**). The DTBT APEX2 proximity labeling workflow facilitates the identification and characterization of complex protein assemblies, enhancing our understanding of cellular processes and protein networks.

In summary, TurboID is a valuable tool in the proximity labeling toolbox and can be used independently or in combination with APEX2 to draw a more complete picture of the cilia proteome. The disadvantages of substrate limitation could be mitigated by further protein engineering. The reaction time and the generation of a biotin cell background then still depend on the substrate release dynamics and the labeling radius of the activated molecule in question (Guo *et al*, 2023). APEX2 seemed to have eliminated all these shortcomings. Though APEX2 has one major drawback that made the use of TurboID much more attractive in the past: its dependence on exogenously added H₂O₂ with unclear cytotoxicity, depending on cell type (Chidawanyika & Supattapone, 2021). It is currently recommended that *in vivo* animal studies with APEX2 be performed *post mortem*, as intravenously injected H₂O₂ rapidly breaks down into water and oxygen, resulting in air bubbles in the bloodstream, which can be fatal (Kang & Rhee, 2022; Rayaprolu *et al*, 2022). Additionally, H₂O₂ leads to oxidation of cysteine and other amino acids causing harm to the animal (García-Santamarina *et al*, 2014). If proteomic changes in an organism are to be investigated in connection with pharmacological agents or signaling pathways, TurboID was previously a gentler method for the animal, since biotin as a dietary vitamin supplement (vitamin B₇) exhibits negligible cell toxicity. Here, the already expressed TurboID in living tissue represents a possible limitation, as the enzyme could deplete the endogenous biotin pools leading to disrupted cell homeostasis or developmental defects (Branon *et al*, 2018).

3.2 iAPEX: Advancing APEX2 Proximity Labeling through *In Situ* Hydrogen Peroxide Production

Despite their universal presence and similar structural features, primary cilia exhibit distinct proteomic compositions and functional variations depending on the tissue type, which are essential to understand their specific roles in health and disease (Anvarian *et al*, 2019; Mill *et al*, 2023). Among the various cell types used in primary cilia studies, NIH/3T3 fibroblasts, 3T3-L1 preadipocytes, C2C12 myoblasts, and retinal pigment epithelial (RPE) cells stand out due to their distinct physiological contexts and relevance to specific ciliopathy phenotypes.

NIH/3T3 cells, fibroblasts derived from minced NIH Swiss mouse embryos (Todaro & Green, 1963), are a mesenchymal connective tissue model widely used in research. Their robust nature and ability to differentiate into various lineages make them valuable for investigating molecular mechanisms of cell differentiation (Dastagir *et al*, 2014; Rahimi *et al*, 2022). In these cells, primary cilia are crucial for the regulation of signaling pathways such as Hedgehog (Pusapati *et al*, 2018; Kong *et al*, 2020; Gómez *et al*, 2022), which influences cell differentiation, proliferation and wound healing (Ford *et al*, 2018).

3T3-L1 cells are a widely used model for studying adipogenesis, the process by which preadipocytes differentiate into adipocytes (Zebisch *et al*, 2012). Primary cilia in 3T3-L1 cells play a critical role in regulating several signaling pathways, including the IGF1 receptor pathway (Zhu *et al*, 2009), Hedgehog signaling (Suh *et al*, 2006; Fontaine *et al*, 2008) and Wnt signaling (Donati *et al*, 2014; Zhu *et al*, 2016), which are essential for adipocyte differentiation and function (Forcioli-Conti *et al*, 2015; Hilgendorf *et al*, 2019). Research of primary cilia in these cells is particularly relevant for understanding ciliopathy-linked obesity and other metabolic disorders (Wu *et al*, 2023).

C2C12 cells are myoblasts derived from mouse muscle satellite cells and are widely used as a model for studying myogenesis, the process of muscle cell differentiation. In these cells, primary cilia are essential for the regulation of key signaling pathways, such as the Sonic Hedgehog (Fu *et al*, 2014) and Wnt signaling pathways (Ozawa, 2015; Abraham, 2016), which are crucial for muscle differentiation and growth. Primary cilia in C2C12 myoblasts reduce excessive cell proliferation and control the cell cycle, ensuring that muscle precursor

cells remain in a state of quiescence for tissue regeneration (Venugopal *et al*, 2020). Further, genetic ablation of primary cilia in adult muscle satellite cells (MuSCs) has been shown to dramatically decrease their self-renewal and regenerative capacity *in vivo* (Palla *et al*, 2022). This highlights the importance of primary cilia in muscle formation and regeneration and makes C2C12 cells a valuable model for studying muscular dystrophies and other muscle-related ciliopathies.

Retinal pigment epithelial (RPE1) cells, derived from human retinal pigment epithelium, are used extensively to study retinal diseases and ciliopathies (Bharti *et al*, 2006, 2022). Photoreceptor outer segments, which function as highly specialized primary cilia, maintain a close relationship with retinal pigment epithelial (RPE) cells. These RPE cells are integral to the visual cycle, as they recycle retinol and phagocytose the old membrane discs shed from the photoreceptor outer segments (Wright *et al*, 2010). Primary cilia in RPE1 cells are vital for photoreceptor function and the maintenance of retinal health (May-Simera *et al*, 2018). RPE1 cilia are Hedgehog responsive and can be studied for primary cilia formation and disassembly (Spalluto *et al*, 2013; Gómez *et al*, 2022). Research on primary cilia in RPE1 cells is particularly relevant for understanding retinal degenerative diseases, such as retinitis pigmentosa and age-related macular degeneration, where ciliary dysfunction plays a significant role (May-Simera *et al*, 2018; Bachmann-Gagescu & Neuhauss, 2019).

Despite versatile cell studies, the proteomic differences between primary cilia in different tissues have not yet been thoroughly investigated. This is particularly significant as ciliopathies—disorders caused by ciliary dysfunction—exhibit drastically different symptoms across multiple systems despite the proposed uniform functions of ciliary proteins (Reiter & Leroux, 2017). Investigating these tissue-specific differences using proximity labeling techniques can reveal novel proteins and pathways unique to each tissue, thereby enhancing our understanding of ciliary functions and their varied implications in ciliopathies.

3.2.1 Development of the iAPEX System

Depending on the cell type to be analyzed, APEX2 PL exhibits dramatic labeling resulting from mitochondrial peroxidases that can scavenge the substrates and produce off-target biotinylation (**Fig. 15**). This rendered APEX2 unusable for the exploration of diverse tissues in animal models, for which a solution is proposed in the following chapter.

The addition of exogenous hydrogen peroxide is a clear obstacle to efficient labeling of APEX2 *in vivo*. Therefore, the search for an endogenous hydrogen peroxide vehicle would allow proteomic endeavors in previously inaccessible cell and animal systems.

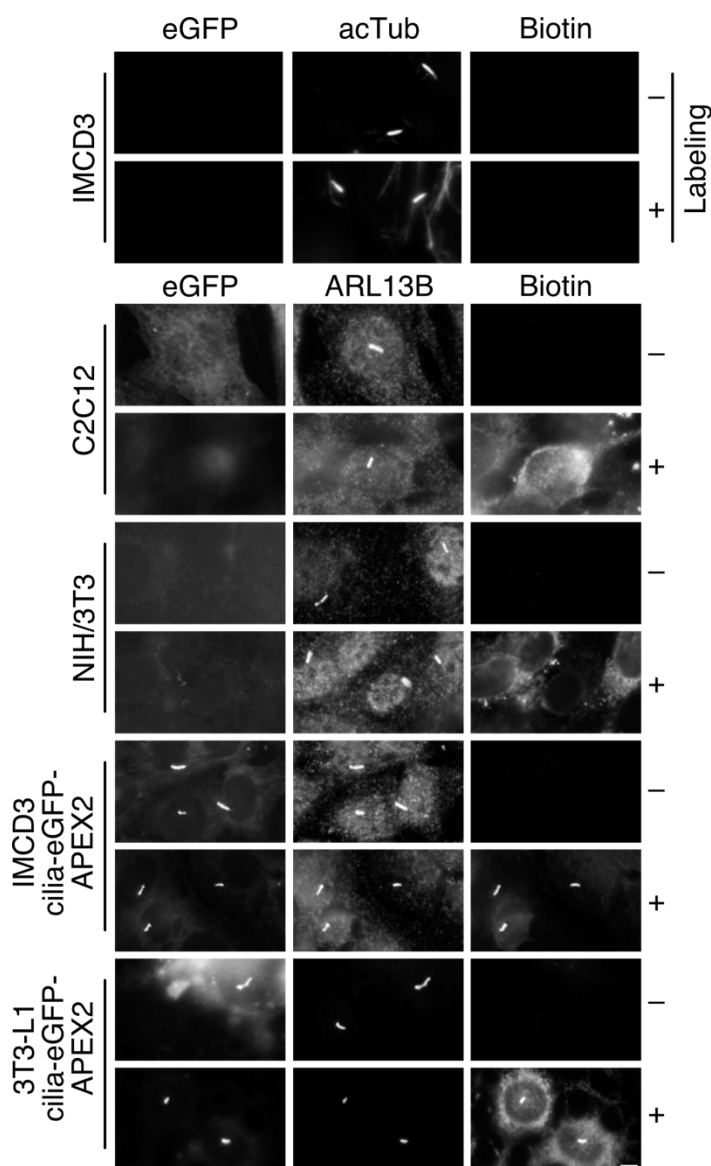


Fig. 15: Cell-dependent biotinylation background that arises from APEX substrates for proximity labeling limits the possible applications. Representative immunofluorescence (IF) micrographs of various cell types (IMCD3, C2C12, NIH/3T3), including cell lines stably expressing cilia-eGFP-APEX2 (IMCD3, 3T3-L1). Cells were left untreated (-) or were incubated for 30 min with 500 μ M biotin tyramide followed by 3 min incubation of 1 mM hydrogen peroxide (+). After fixation, primary cilia were visualized by antibodies targeted against acetylated tubulin (acTub) or ARL13B. Biotinylation was detected by fluorescently labeled streptavidin. Scale bar = 5 μ m.

Mechanism of *In Situ* Hydrogen Peroxide Production in Primary Cilia

To establish an improved APEX method, I hereby refer to as "iAPEX", I proposed a system where both the flavoenzyme D-amino acid oxidase (DAAO) from the yeast *Rhodotorula gracilis* (Pollegioni & Simonetta, 1991; Pollegioni *et al*, 1992; Boselli *et al*, 2004; Rosini *et al*, 2008) (**Fig. 16A**) and APEX2 are specifically targeted to primary cilia through genetic engineering. This approach combines *in situ* H₂O₂ production with proximity labeling that is specific to the ciliary compartment (**Fig. 16C**). DAAO, with the help of its coenzyme

flavin adenine dinucleotide (FAD), catalyzes the oxidative deamination of D-amino acids to their respective α -keto acids. In the multi-step reaction, FAD is first reduced to its hydroquinone form (FADH₂) which generates an imino acid intermediate that dissociates from the enzyme and spontaneously hydrolyzes thereby releasing ammonium (NH₄⁺).

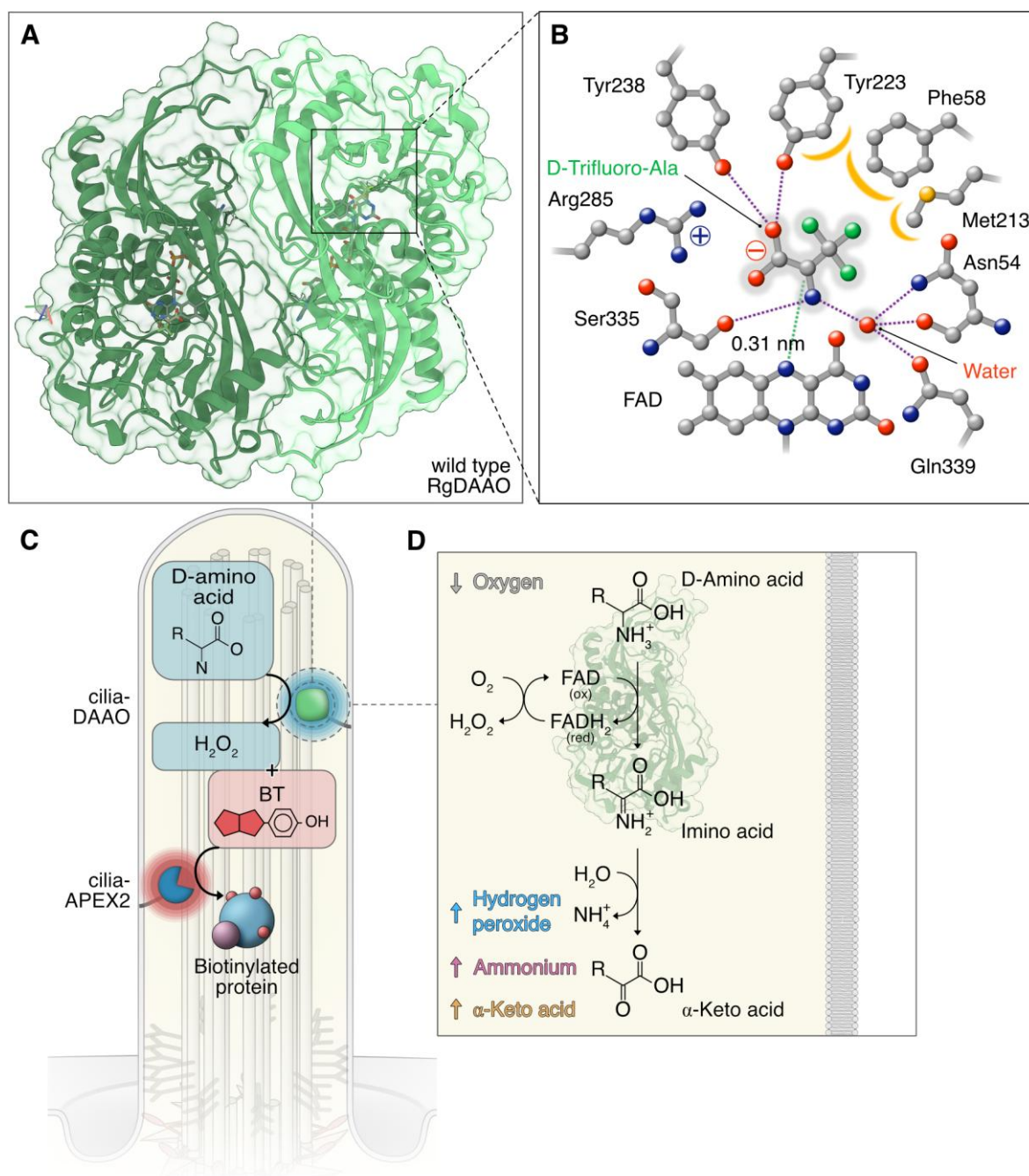


Fig. 16: *In situ* D-amino acid oxidase-mediated hydrogen peroxide production allows APEX2 proximity labeling.

(A) Quaternary structure of wild-type *Rhodotorula gracilis* D-amino acid oxidase (RgDAAO) dimer represented as ribbons with surface filling (enzyme monomers: dark and light green; PDB: 1C0L). DAAO is shown complexed with FAD and D-trifluoro-alanine. **(B)** Magnification of the RgDAAO active site with bound D-114

trifluoro-alanine. Residues at a distance <0.4 nm surrounding the substrate are shown in atomic model following the CPK color scheme (C atoms: gray; N atoms: blue; O atoms: red; S atoms: yellow). The distance between substrate C $_{\alpha}$ and flavin N(5) is shown in green with H-bonds shown in purple. Hydrophobic interactions are shown by yellow crescents (modified after Pollegioni & Molla, 2011). **(C)** Genetically cilia-targeted DAAO and APEX act in concert to biotinylate proteins in vicinity. The two-component system is termed “improved APEX” or short iAPEX. DAAO produces hydrogen peroxide (H₂O₂) that allows APEX to generate biotin tyramide (BT) radicals to permanently modify nearby proteins. **(D)** Inset depicts detailed DAAO-catalyzed D-amino acid oxidation to α -keto acid through an imino acid intermediate using flavin adenine dinucleotide (FAD) as a coenzyme. Reduction of FAD to FADH₂ is resolved by re-oxidation by molecular oxygen (O₂) yielding H₂O₂ in the process. Meanwhile, the imino acid spontaneously hydrolyzes thereby releasing ammonium (NH₄⁺).

Molecular oxygen (O₂) oxidizes FADH₂ back to its quinone form (FAD), which generates H₂O₂ (Pollegioni & Molla, 2011; Kiss & Ferenczy, 2019) (**Fig. 16BD**). By incubating serum deprived cells with a compound combination of D-amino acid and biotin tyramide, DAAO locally generates H₂O₂ in the primary cilium that nearby APEX2 can scavenge to covalently biotinylate proteins in the vicinity through oxidized phenoxyl radicals (**Fig. 16C**). Therefore, this cascade-like two-component system bypasses the need for exogenous H₂O₂.

3.2.2 Generation and Characterization of iAPEX Cell Lines

In order to test the proposed system, stable cell lines were generated (see page 60; supplementary **Tab. 6** and **Tab. 7** for plasmid details; for method see page 79) with different localizations and fusions of the enzymes (**Fig. 17**). Multiple vectors were constructed for Flp-In cell line generation (early part of the work) or lentiviral transduction (later stage) (see page 81). The constructs are as follows:

1. cilia-iAPEX: In this construct, individual DAAO and APEX2 enzymes were each targeted to primary cilia using a cilia-targeting sequence (CTS), allowing for separate localization.
2. cilia-iAPEX^{fused}: In this construct, DAAO and APEX2 were fused together with an N-terminal CTS, creating a single polypeptide directed to primary cilia.
3. control-iAPEX: Designed to test the need for proximity in the cascade reaction, this construct restricted DAAO's access to cilia by fusing it to a myristylation-deficient CTS while maintaining APEX2 localization to cilia.
4. control-iAPEX^{fused}: A cytosolically trapped fusion construct using the same myristoylation-deficient CTS.

A further control cell line, in which DAAO is localized in the cilium and APEX2 in the cytosol, was considered but not included in the initial test phase of the system.

Due to the considerable size of the fusion construct, a fluorescent protein was omitted, and a small, diverse, synthetic ALFA tag was added instead. The transgene structure of cilia-APEX2 was maintained in the separate constructs, with DAAO fused to a FLAG tag for detection. Both tags were detected in fixed cells using antibodies, thus confirming their correct localization (**Fig. 17**).

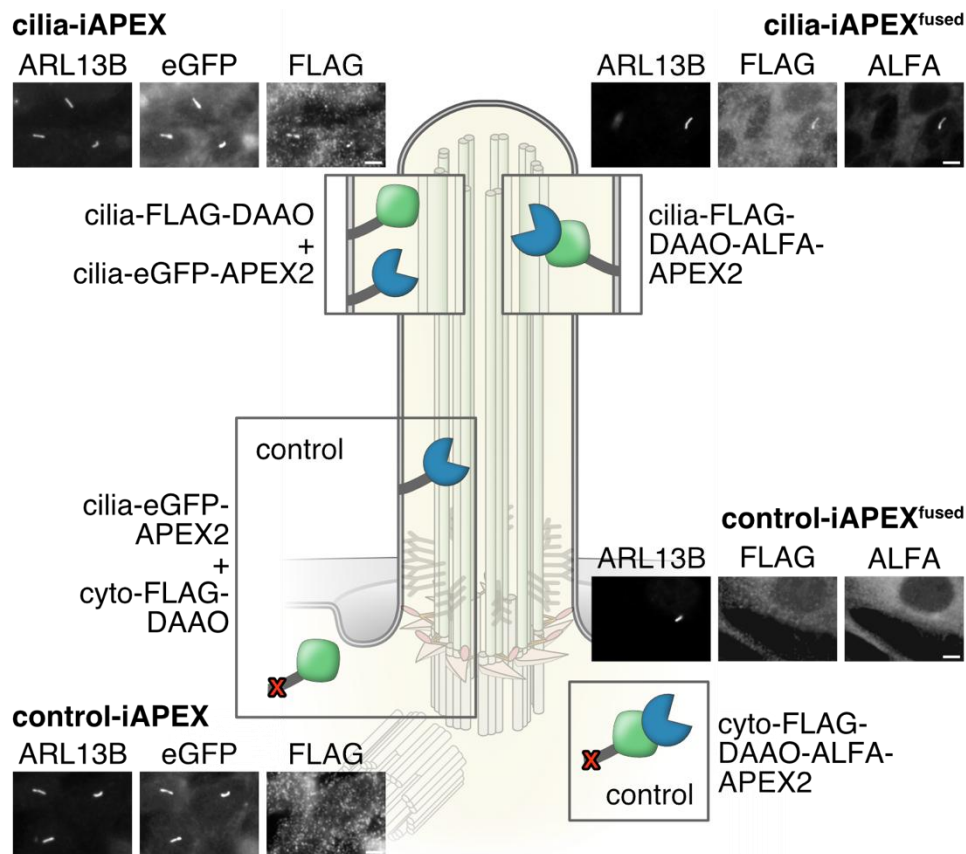


Fig. 17: The iAPEX two-component system allows flexible combinations for targeted and control cell lines. Schematic of a primary cilium with differently localized and tagged variations of APEX2 and DAAO. Red X represents myristoylation mutant of Nphp3 cilia targeting signal. IF micrographs of established IMCD3 cells depict stable expression of four different iAPEX combinations (cilia-iAPEX, cilia-iAPEX^{fused}, control-iAPEX, control-iAPEX^{fused}). GFP fluorescence shows cilia-APEX2 localization. Cells were fixed and subsequently stained with an antibody targeting ARL13B to visualize primary cilia. Additionally, FLAG and ALFA antibodies were employed to visualize DAAO alone or as a chimeric APEX fusion.

As the resolution of standard fluorescence microscopy methods is limited, the cilia-targeted iAPEX enzymes were inspected for their respective localization in the ciliary membrane by expansion microscopy.

For that, stable iAPEX RPE-1 and IMCD3 cell lines were embedded in an acrylamide gel matrix to be physically expanded (**Fig. 18**; method see page **81**). Proximity labeling was initially tested in IMCD3 cells, where cells were left untreated or were incubated with a subset of substrates (**Fig. 18B**). To test whether APEX2 is functional in the dual-expression system, cells were incubated with biotin tyramide and exogenous H_2O_2 . To evaluate DAAO-mediated biotinylation, exogenous H_2O_2 was omitted and instead biotin tyramide and D-methionine, a common DAAO substrate, were added together on the cells. Acetylated tubulin was used to stain the cilia-basal body complex, where APEX and DAAO exclusively decorated the ciliary membrane in RPE1 cells (**Fig. 18A**). A similar localization pattern was observed for IMCD3 cells (**Fig. 18B**), where PL biotinylation was additionally monitored by fluorescent streptavidin. In both test conditions, biotinylation occurred and was confined specifically to the cilioplasm. At this stage, the iAPEX system was working as proposed.

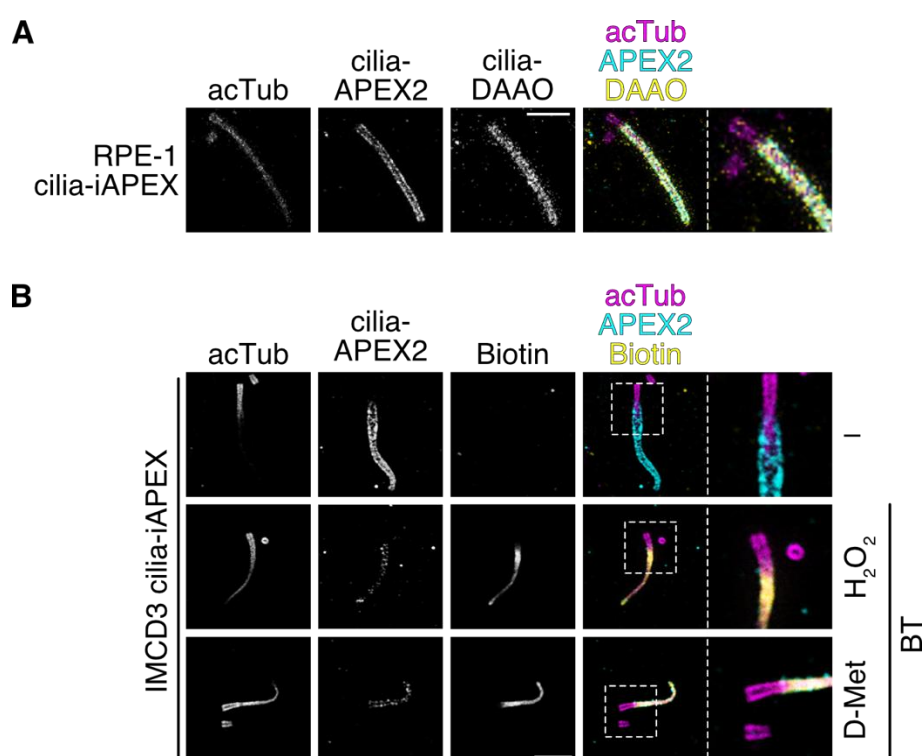
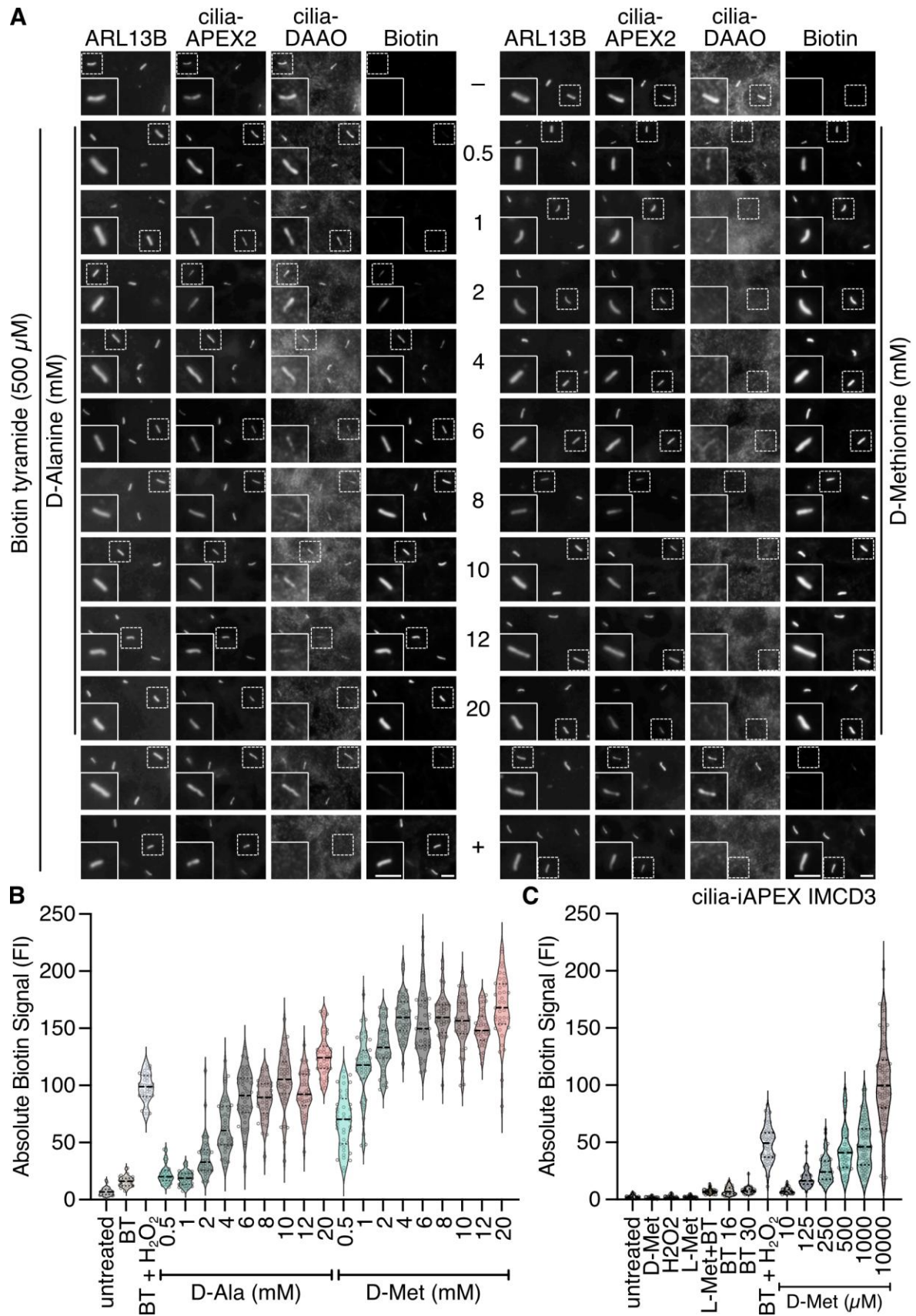


Fig. 18: Expansion microscopy of cilia-iAPEX expressing cells reveals distinct ciliary membrane localization of APEX2 and DAAO with biotinylation restricted to the cilioplasm.

(A) IF image of an expanded primary cilium from an RPE-1 cell line stably expressing cilia-iAPEX. Cells were first fixed, then cross-linked, and finally embedded in water-expandable gel. After denaturation, specimens were probed with antibodies targeting acetylated tubulin (for primary cilia), GFP (for cilia-APEX2), and ALFA (for cilia-DAAO). Scale bar = 5 μ m with an expansion factor of 4. **(B)** IF micrographs of IMCD3 cilia-iAPEX cell line. Cells were left untreated (-) or labeled with 500 μ M biotin tyramide (BT; 30 min) and hydrogen peroxide (H_2O_2 ; 3 min) or BT and 10 mM D-methionine (D-Met) (together for 30 min). Expansion protocol was performed as in (A). Cells were stained by antibodies directed against acTub (for primary cilia) and GFP (for cilia-APEX2). Biotin was visualized by fluorescently labeled streptavidin. Scale bar = 5 μ m with an expansion factor of 4.3.

Evaluating Substrate Specificity of D-Amino Acid Oxidase via Titration

To rule out that the ascorbate peroxidase was acting independent of DAAO—by using cellular H_2O_2 —stereoselectivity, substrate- and concentration-dependent H_2O_2 production of DAAO were tested in IMCD3 cells. For this, D-alanine (D-Ala), D-methionine (D-Met) (**Fig. 19A**) and L-methionine (L-Met) (**Sup.-Fig. 6**) were tested respectively together with biotin tyramide. The resulting biotinylation was used as a relative readout (**Fig. 19BC**). D-Ala and D-Met concentrations were compared by titration, as they are the predominant substrates for DAAO in the literature. Additionally, single substrate effects on PL were tested (**Sup.-Fig. 6**). After 30 minutes of simultaneous substrate incubation, a weak biotinylation for D-Ala was observed, which started at 2 mM and increased fivefold with 10-times substrate concentration (**Fig. 19AB**). D-Met led to ciliary biotinylation even at much lower concentrations of only 125 μ M. In contrast, incubation of L-Met with BT did not result in biotinylation, confirming DAAO stereoselectivity in the IMCD3 cell context. Incubation of individual substrates also resulted in no labeling, confirming that there is no tendency for DAAO to produce basal H_2O_2 and that APEX2 activity is now coupled to that of DAAO. Compared to 2 min exogenous H_2O_2 addition (standard), D-Ala at 6 mM achieved a similar ciliary biotin signal, and biotinylation did not increase significantly with more substrate (**Fig. 19B**). D-Met exceeded the highest signals obtained with D-Ala at 1 mM and reached up to 1.4-fold higher signals at 6 mM. The standard labeling values were already comparable at 500 μ M D-Met (**Fig. 19C** and **Sup.-Fig. 6**). Thus, D-Met was chosen as the DAAO substrate for all further experiments in this work. Noteworthy is the decrease in ciliary FLAG signal when proximity labeling was performed. The epitope recognition by antibody could be altered by potential tyrosine labeling within the tag.



(A) Representative IF micrographs of DAAO substrate titration in IMCD3 cells stably expressing cilia-iAPEX. APEX labeling was performed by incubating cells for 30 min with 500 μ M biotin tyramide and varying concentrations (range from 0.5-20 mM) of D-amino acids alanine or methionine, respectively. Micrographs marked with (-) show cells which were left untreated, () were incubated with BT only and (+) were incubated with BT (for 30 min) and subsequently with 1 mM H₂O₂ (for 2 min). Antibody staining against ARL13B labeled primary cilia, while staining against FLAG marked cilia-DAAO. GFP was employed to observe cilia-APEX2, while biotin was detected by fluorescent streptavidin. Scale bar = 5 μ m. **(B)** Quantification of absolute ciliary biotin fluorescence signals from (A) shown as violin plots. The quartiles are shown as dotted lines surrounding the median represented by a dashed line. Quantification using CiliaQ is described on page 93. n= 40 cilia per condition. **(C)** Violin plots as described before displaying data from **Sup.-Fig. 6**. n = 77 cilia per condition.

Comparative Analysis of Separate and Fused iAPEX Constructs in Proximity Labeling

Stable expression and biotinylation capacity of the iAPEX constructs in IMCD3 cells were further tested by comparative WB (**Fig. 20**) and IF analysis (**Fig. 21**). Here, iAPEX cell lines were compared with the preceding APEX2 system (cilia- and control-APEX2) and WT cells. For this purpose, the cells were either left untreated or subjected to PL with the respective substrate requirements, whereby APEX2 and iAPEX systems were tested in all cell lines. As expected, GFP staining showed expression of APEX2 in all cell lines (**Fig. 20**, lanes 4-15) except WT and fusion constructs, in which the eGFP-tag was omitted (**Fig. 20**, lanes 1-3 and 16-21). For the latter, anti-ALFA and anti-FLAG antibodies were used to detect the fusion constructs, demonstrating stable expression with no signs of degradation. FLAG probing also detected the separate DAAO enzymes in cilia and control iAPEX, with the latter showing lower expression, likely due to reduced stability in the cytosol through lack of N-myristylation. Similarly, GFP expression was also reduced in control-APEX2 (**Fig. 20**, lanes 7-9) compared to the cilia-localized counterpart (**Fig. 20**, lanes 4-6). Biotinylation capacity was probed by fluorescently labeled streptavidin. The highest amount of biotinylation in WB analysis was observed under exogenous H₂O₂ conditions for separate APEX2 localized in cilia, regardless of the context of dual expression (**Fig. 20**, lanes 5, 11 and 14). DAAO conditions only led to biotinylation in cell lines with DAAO in proximity to APEX2 within a confined space (**Fig. 20**, lanes 12 and 18; **Fig. 21**), showcased by absent biotinylation with mislocalized DAAO in control-iAPEX (**Fig. 20**, lane 15; **Fig. 21**). Cytosolic control-iAPEX^{fused} (**Fig. 20**, lane 21), together with WT (**Fig. 20**, lane 3) and APEX2 cell lines (**Fig. 20**, lanes 6 and 9) did not yield visible biotinylation above background using BT and D-Met as substrates.

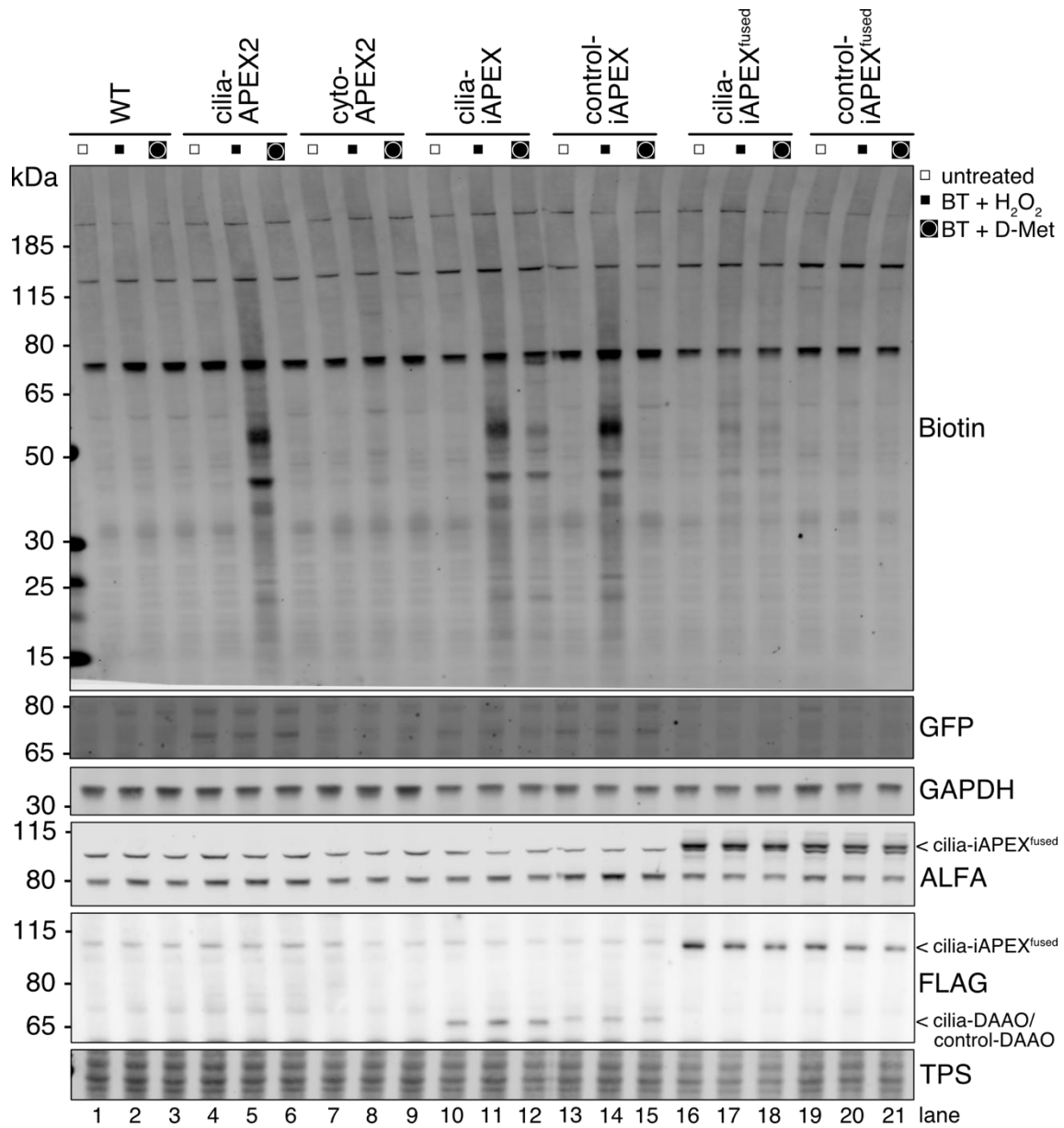


Fig. 20: The iAPEX-based system demonstrates robust but overall reduced biotinylation compared to the preceding system.

Western blot comparison was performed using cell lysates from various APEX- and iAPEX-expressing IMCD3 cells. Cell lines were lysed before (no labeling, □) or after APEX proximity labeling, with standard labeling using 500 μ M BT and 1 mM H_2O_2 (■, 3 min) or DAAO-dependent labeling using BT and 10 mM D-Met (●, 30 min). Lysates were subjected to SDS-PAGE and analyzed by quantitative Western blotting. Biotin was detected using fluorescently labeled streptavidin, while GFP, GAPDH, ALFA, and FLAG were detected using specific antibodies. GAPDH and total protein stain (TPS) were included to ensure equal protein loading (20 μ g/lane). Biotin, GFP, and GAPDH signals were detected on one membrane, while ALFA, FLAG, and TPS signals were detected on another membrane using the same lysate samples.

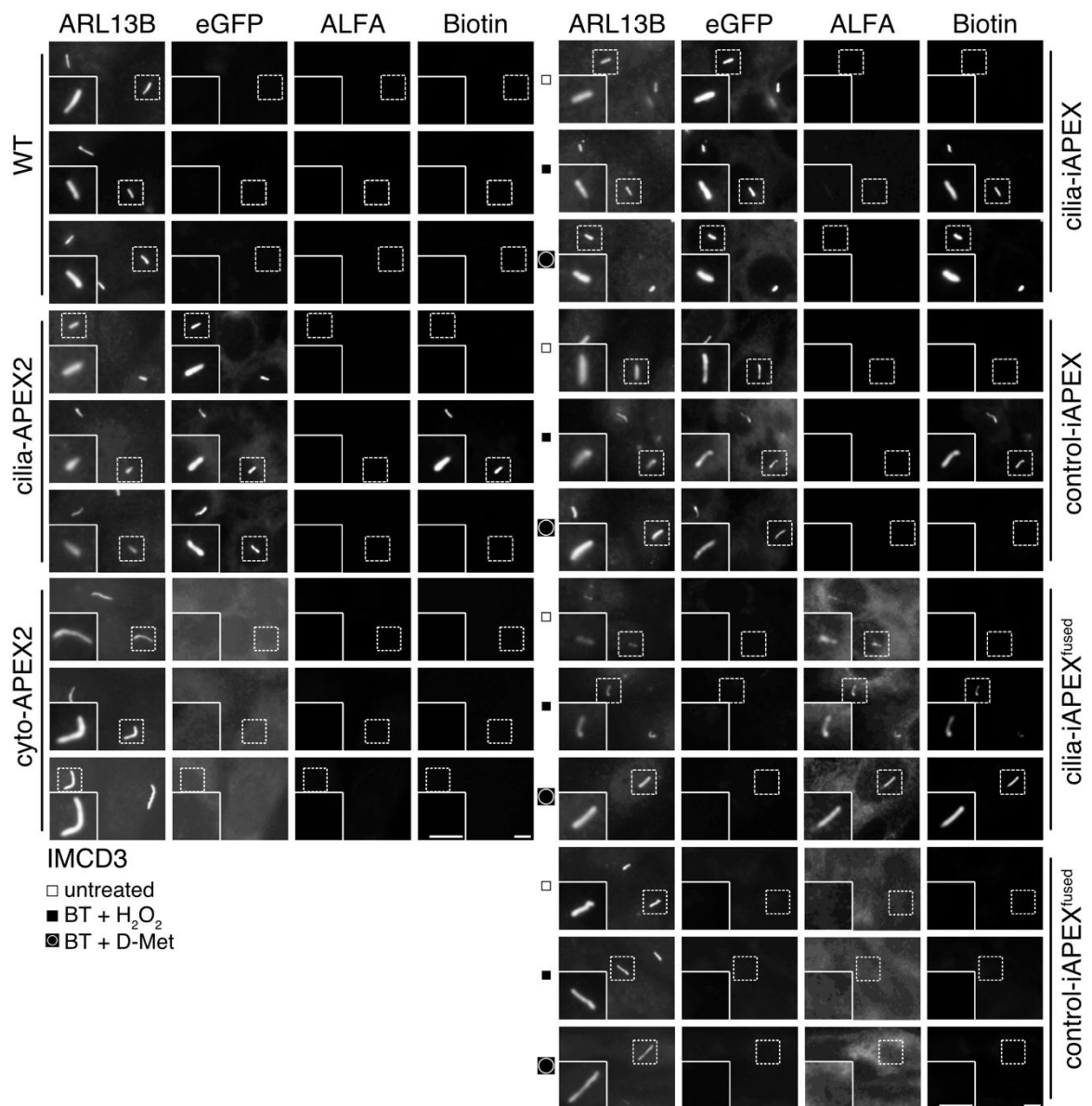


Fig. 21: The amount of ciliary labeling is not the cause of the overall lower biotinylation observed in cilia-iAPEX cells.

Exemplary IF images illustrating the biotinylation status of IMCD3 cells, from which lysates were generated for the Western blot analysis presented in **Fig. 20**. Antibodies were used to stain ARL13B for primary cilia and ALFA for fusion constructs. GFP fluorescence marked APEX2 in cilia-APEX2, cyto-APEX2, cilia-iAPEX and control-iAPEX. Biotin was detected by fluorescent streptavidin. Scale bar = 5 μm.

Live-Cell Proximity Labeling Monitoring

To determine the origin of the additional biotinylation signal in standard APEX labeling compared to iAPEX, live-cell imaging was conducted using 10-Acetyl-3,7-dihydroxyphenoxazine (ADHP), also known as Amplex Red (chemical structure in **Fig. 22A**; method see page **78**). ADHP, a non-fluorescent and colorless peroxidase substrate, is oxidized in the presence of H_2O_2 to produce highly fluorescent resorufin (**Fig. 22A**). Primary cilia were recognized by GFP from cilia-APEX2 and monitored for resorufin emergence in the course of 4.4 min upon substrate addition (**Fig. 22B**). When H_2O_2 production was catalyzed by DAAO, resorufin was detected at 24 s whereas exogenous H_2O_2 addition led to visible resorufin as early as 4.8 s. Along with an initial ciliary accumulation of the fluorescent substance through exogenous H_2O_2 , a pulse-like distribution throughout the entire cell could be observed, which started at about 48 s (**Fig. 22A**, bottom). This cell-wide signal could not be observed when using DAAO D-amino acid catalysis as H_2O_2 source (**Fig. 22B**, top). A possible explanation for the observed pulse could be that Amplex can be oxidized by mitochondrial carboxylesterases or peroxidases by excess H_2O_2 (Dębski *et al*, 2016; Miwa *et al*, 2016), which saturates or inactivates other cellular peroxidases, such as cytochromes (Valderrama *et al*, 2002). Oxidized resorufin accumulates in the cell body before it is released from the cell to the outside, which reduces the amount of fluorescent dye in the cell (Erikstein *et al*, 2010; Martell *et al*, 2017). Accordingly, DAAO seemed to generate a constant ciliary H_2O_2 pool that was directly scavenged by APEX2 before it reached the cell body.

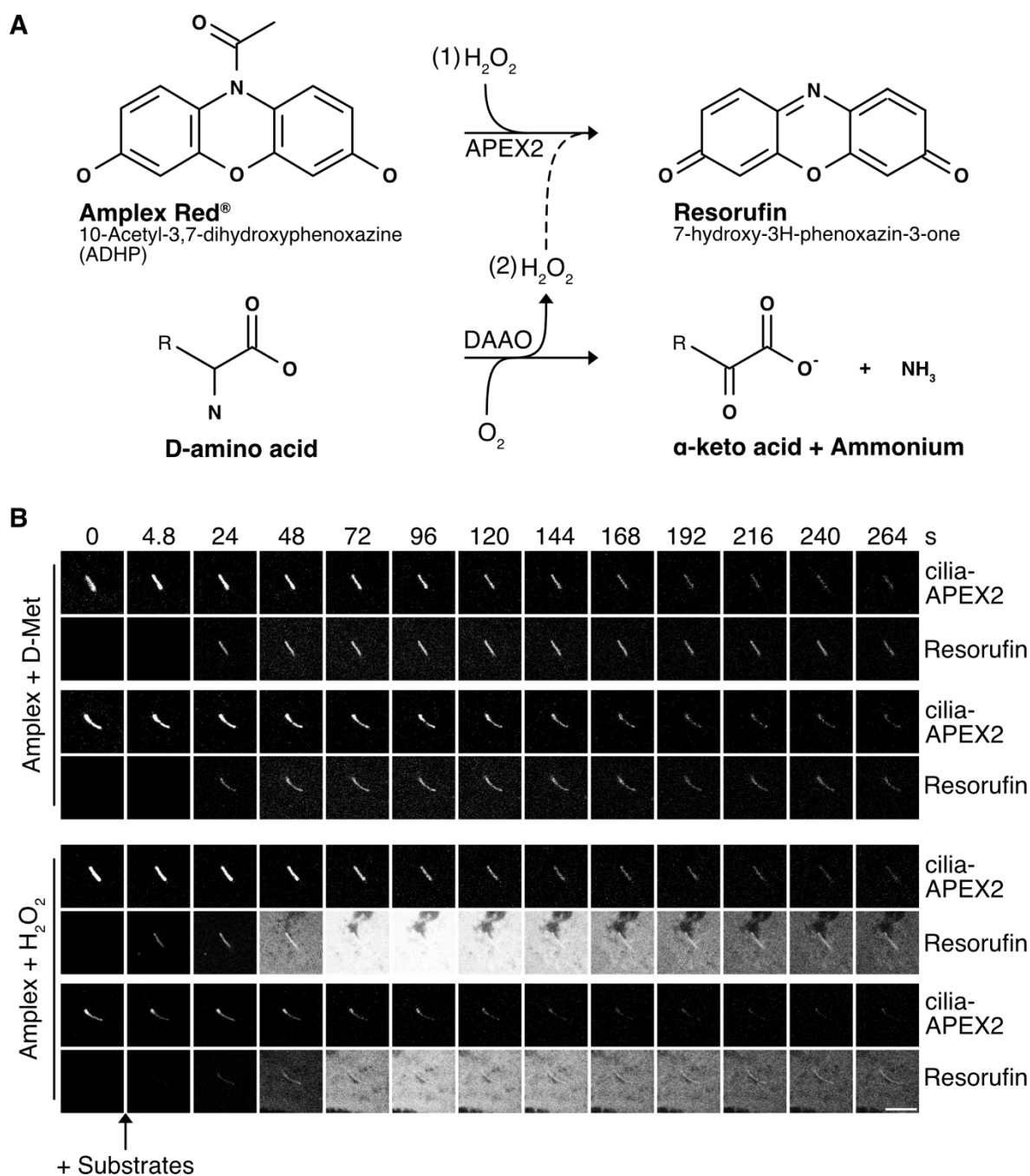


Fig. 22: APEX2 activity monitoring demonstrates exclusive ciliary resorufin emergence under iAPEX conditions in contrast to pulsed cellular emergence through exogenous H_2O_2 addition.

(A) Schematic illustrating the oxidation of Amplex UltraRed® by APEX2 in the presence of H_2O_2 , which can be introduced exogenously (1) or produced endogenously as a by-product of DAAO-mediated oxidative deamination (2). **(B)** Live-cell imaging micrographs were captured to observe Amplex UltraRed® oxidation in IMCD3 cells stably expressing cilia-iAPEX. Cells were treated with 50 μ M Amplex together with either 10 mM D-Met or 1 mM H_2O_2 . GFP and resorufin were monitored using diode lasers 488 and 561 nm at 4.8-second intervals over a total duration of 264 s.

3.2.3 Impact of Substrate Order and Labeling Duration on Biotinylation Efficiency

To explore the flexibility of the cilia-iAPEX labeling system, given its specific labeling in the cilium and lack of cellular H₂O₂ sequestration, several experiments were conducted to understand the influence of substrate incubation times on biotinylation efficiency. First, the effect of different incubation times of the two required substrates on biotinylation was investigated. This included both simultaneous incubation and sequential incubation with one substrate followed by the other. Untreated and single substrate controls were performed to exclude the effects of the individual substrates on potential biotinylation (**Fig. 23**).

Cells were incubated with biotin tyramide (BT) and D-methionine (D-Met) simultaneously, and the biotinylation outputs were assessed at various time points (**Fig. 23AC**). DTBT was tested in parallel to BT, as it enables competitive elution in subsequent streptavidin affinity chromatography, which facilitates further proteomic analyses of isolated biotinylated proteins (**Sup.-Fig. 7**; method on page 85).

The results showed that untreated controls or those treated only with D-Met or H₂O₂ lacked detectable signal above background (**Fig. 23D**). BT or DTBT incubated alone on cells displayed a weak biotin signal. This signal was negligible compared to the significant increase observed when the APEX2 and DAAO labeling substrates were added together and incubated simultaneously (**Fig. 23AC**). The intensity of the ciliary biotin signal successively increased when BT or DTBT were incubated together with D-Met for progressively longer time points (**Fig. 23C** and **Sup.-Fig. 7B**).

Simultaneous incubation of BT and D-Met with increasing exposure time resulted in a gradual increase in the biotinylation signal. The intensity of the ciliary biotin signals increased with longer exposure, peaking at 25 min before decreasing (**Fig. 23C**). This indicates that while prolonged exposure to both substrates enhances labeling, there is an optimal time frame beyond which the efficiency declines.

Substrate availability and membrane permeability could be bottlenecks for proper biotinylation, as the enzymes might have different catalysis dynamics (Callender & Dyer, 2015). To test the effect of sequential substrate incubation, cells were first incubated with one substrate for 30 min to allow sufficient time for availability, followed by a time course with progressively longer incubation of the second substrate. Additionally, a single substrate pre-incubation time course was performed to estimate the required uptake times for sufficient labeling by each substrate (**Fig. 23B**, based on **Sup.-Fig. 8**).

A detailed analysis of pre-incubation times showed that the ciliary biotin signal was highest when BT was pre-incubated for shorter durations followed by immediate D-Met addition. Conversely, prolonged D-Met pre-incubation followed by a fixed BT incubation time resulted in diminished signal intensity. This suggests that optimal pre-incubation times for each substrate are critical for maximizing labeling efficiency (**Fig. 23B**). In detail, the results showed the following:

30-minute substrate pre-incubation followed by second substrate time course:

Cells were pre-incubated with BT for 30 min followed by D-Met, resulting in approximately twice the signal intensity compared to the corresponding swapped conditions where D-Met was incubated first (**Fig. 23B**, top grey area and below; and **Sup.-Fig. 8**, top half). Labeling levels similar to the exogenous H₂O₂-catalyzed condition were achieved after 5 min of D-Met addition to 30 min BT pre-incubated cells. Ten minutes of D-Met exposure exceeded these levels, and 30 min resulted in twice the median standard labeling value.

Substrate pre-incubation time course followed by 16-minute substrate exposure:

When D-Met was pre-incubated at various time points with a fixed BT labeling exposure time of 16 min, overall biotinylation gradually decreased with longer exposure to the pre-incubated substrate before addition of the second substrate (**Fig. 23B**, below second grey area; and **Sup.-Fig. 8**, bottom half). This indicated that D-Met is the limiting factor for robust labeling in primary cilia. Notably, a 30 min D-Met pre-incubation resulted in about half the ciliary biotin signal compared to a 5 min pre-incubation. This suggested that early availability of D-Met did not improve labeling efficiency; instead, prolonged exposure without the APEX substrate led to poorer performance. For the biotin tyramide (BT) pre-incubation time course with a fixed 16 min addition of D-Met, a different trend was observed (**Fig. 23B**, second grey area; and **Sup.-Fig. 8**, bottom half).

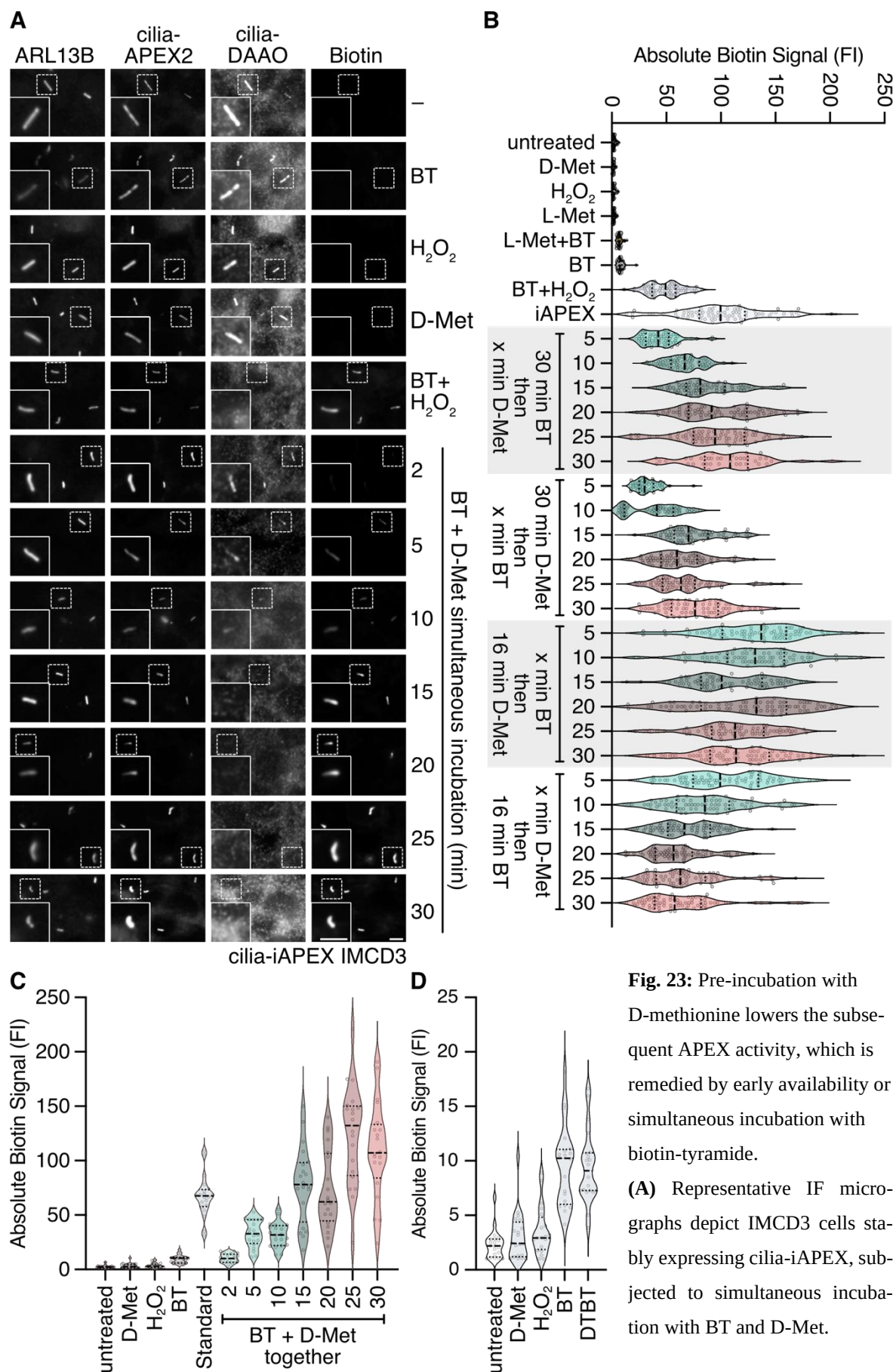


Fig. 23: Pre-incubation with D-methionine lowers the subsequent APEX activity, which is remedied by early availability or simultaneous incubation with biotin-tyramide.

(A) Representative IF micrographs depict IMCD3 cells stably expressing cilia-iAPEX, subjected to simultaneous incubation with BT and D-Met.

APEX labeling was executed by incubating cells for 30 minutes with 500 μ M BT and 10 mM D-Met; in the negative controls, one or the other substrate was omitted. Standard APEX labeling controls involved the addition of 1 mM H_2O_2 for 3 minutes alone or after 30 minutes of incubation with BT. Untreated cells are marked with (-). Primary cilia were visualized via ARL13B antibody staining, while cilia-DAAO was marked using FLAG staining. Cilia-APEX2 expression was observed through GFP fluorescence. Biotin was detected using fluorescent streptavidin. Scale bar = 5 μ m. **(B)** Violin plots show the CiliaQ quantification of absolute ciliary biotin fluorescence intensity based on experimental data from **Sup.-Fig. 8**. The quartiles are represented by dotted lines surrounding the median indicated by a dashed line. $n = 77$ per condition. **(C, D)** Violin plot as described before showing experimental data from (A) and **Sup.-Fig. 7**. The Y-axis of the violin plot in (D) was modified to show the biotin signal of the control conditions of (B) in more detail. $n = 20$ cilia per condition.

When BT was pre-incubated at various time points, followed by a fixed 16 min D-Met incubation, the biotinylation signal remained relatively consistent across all BT pre-incubation times. This consistency suggests that the limiting factor is the availability of D-Met rather than BT. The results indicated that pre-incubation with BT alone does not significantly affect the labeling efficiency as long as D-Met is available during the subsequent fixed incubation period. Thus, robust labeling in primary cilia is more dependent on the timely addition of D-Met after BT pre-incubation.

In summary, the results highlight the importance of substrate incubation timing in the cilia-iAPEX labeling system. BT pre-incubation followed by D-Met consistently yielded higher biotinylation signals compared to the reverse order. Simultaneous incubation showed a clear time-dependent increase. Overall, these findings emphasize the need for precise control of substrate exposure times to achieve robust and efficient labeling in primary cilia.

Comparison of Substrate Order and Labeling in Separate and Fused iAPEX Constructs

Upstream BT availability was also tested in the context of cilia-iAPEX^{fused}. Compared to cilia-iAPEX, there were drastic differences in performance. Ciliary biotinylation was twice as high with separate enzymes compared to the fusion protein at all time points tested (**Fig. 24A**). Biotinylation rose with longer time of D-Met exposure, whereas the increase in signal in cilia-iAPEX^{fused} remained far below the values of the separate enzymes (**Fig. 24BC**). Nevertheless, a cilia-restricted biotinylation could be achieved with the help of the fusion construct, which emphasized the flexible possibilities and functionality of the iAPEX system.

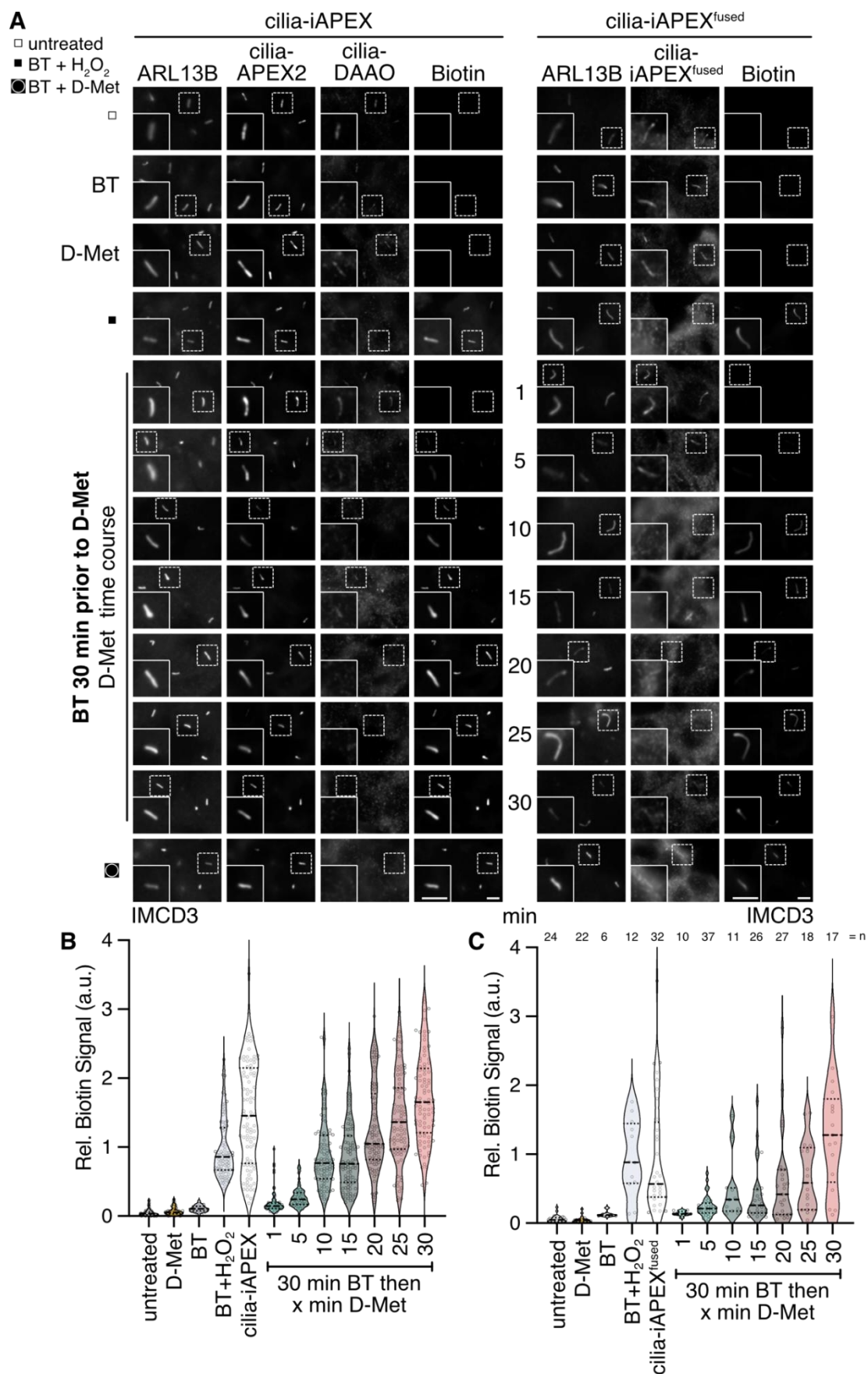


Fig. 24: Separated enzymes are superior to enzyme fusion in the biotinylation of cilia.

(A) IF micrographs of IMCD3 cells harboring stable expression of cilia-iAPEX or cilia-iAPEX^{fused}. Images depict before (untreated, □) or after APEX proximity labeling, with standard labeling using 500 μM BT and 1 mM H₂O₂ (■, 2 min) or DAAO-dependent labeling using BT and 10 mM D-Met (▣). (B, C) Quantification of CiliaQ-obtained relative ciliary biotin fluorescence intensity (each individual cilia biotin signal normalized to ARL13B cilia mask signal) represented as violin plots. Dashed line shows the median surrounded by dotted lines showing the quartiles. The terms "cilia-iAPEX" and "cilia-iAPEX^{fused}" denote DAAO-dependent labeling. For cilia-iAPEX n = 90 cilia (BT only control n = 43); for cilia-iAPEX^{fused} heterogeneous n was obtained as indicated by numbers above the individual violin plots.

Based on these results, further experiments were carried out with simultaneous incubation of the substrates, as the prolonged pre-incubation of one of the substrates had no significant positive effect on the labeling reaction. In addition, separate enzymes were preferred to the fusion construct.

3.2.4 Copper-Catalyzed Azide-Alkyne Cycloaddition After Proximity Labeling Allows Flexible Selection of Chemical Handles

Copper-catalyzed azide-alkyne cycloaddition (CuAAC), widely known as "click chemistry", is a highly specific and efficient reaction that covalently links azides to alkynes. Alkyne-phenol (or alkyne-tyramide) can be used as a substrate by APEX2 for PL (Fig. 25). The compound is smaller compared to the conventional biotin tyramide which allows passage through live yeast cells with intact cell wall (Li *et al*, 2020b). Following the APEX2-catalyzed reaction, click chemistry can be performed *in vivo* or post-fixation, enabling the attachment of various azide-functionalized probes, such as biotin or fluorophores, for downstream visualization and enrichment analysis of proteins that were in proximity to the enzyme within living cells. The APEX2-mediated proximity labeling was performed in living cells using alkyne-phenol (Alk-ph) as a substrate, followed by the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reactions. Two different azides, biotin-azide and azide-fluor545, were used to evaluate the efficiency and specificity of the labeling (Fig. 25).

Cells were labeled with alkyne-phenol in the presence or absence of D-methionine (D-Met) or hydrogen peroxide (H₂O₂) and subsequently reacted with biotin-azide (Fig. 25A). The labeled proteins were detected using streptavidin-NL557 and visualized by immunofluorescence. The ciliary marker ARL13B was used to identify cilia. In the absence of Alk-ph, no significant biotin signal was detected, confirming the specificity of the CuAAC reactants to

the alkyne-phenol substrate. The presence of alkyne-phenol alone did not yield a biotin signal in cilia, indicating no basal activity of APEX2 in the absence of an H_2O_2 source. When exogenous H_2O_2 was used on the Alk-ph incubated cells, an exclusively ciliary biotin signal was observed. Therefore, APEX2 was able to use the substrate to label proteins in its vicinity. Equally, when D-Met was incubated together with Alk-ph to perform DAAO-catalyzed *in situ* production of H_2O_2 , this allowed APEX2-mediated Alk-ph labeling of proximal ciliary proteins with comparable biotin signal.

Since biotin-azide as a click component requires an additional step involving fluorescently labeled streptavidin for visualization, azide-fluor545 was tested to enable direct visualization of the CuAAC reaction with a fluorescent label (**Fig. 25B**). Similar to the biotin-azide results, the absence of alkyne-phenol led to no detectable fluor545 signal, confirming the specificity of the reaction. Adding both substrates required for proximity labeling in the iAPEX system (Alk-ph + D-Met), a strong fluor545 signal was detected exclusively in cilia, demonstrating successful labeling and direct visualization of the CuAAC reaction.

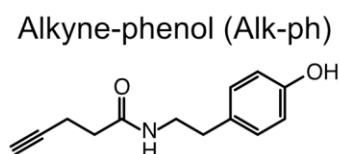
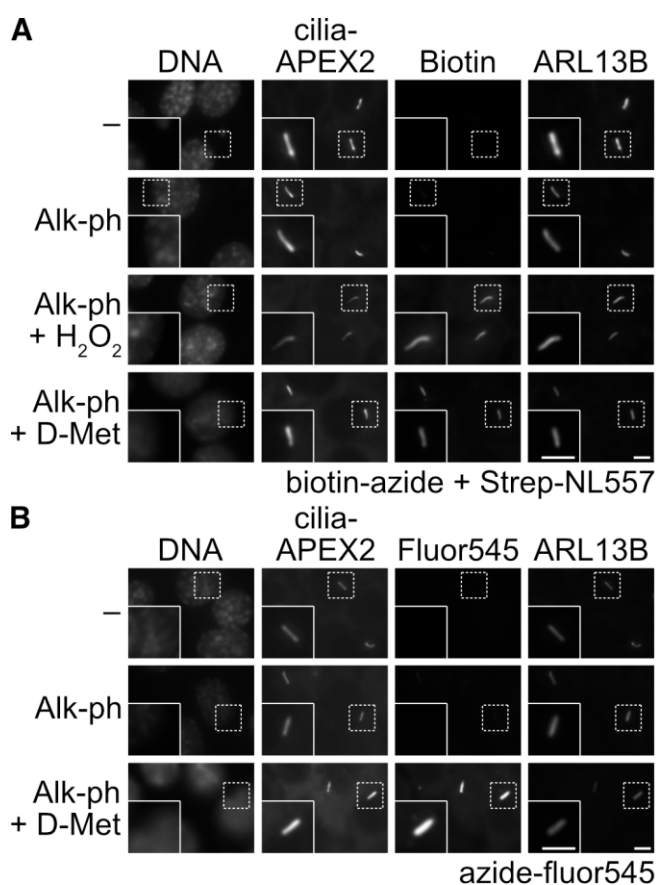


Fig. 25: The APEX2 alkyne-phenol substrate facilitates flexibility in post-labeling chemical handle modification.

IF micrographs of IMCD3 cells stably expressing cilia-iAPEX. Images depict untreated (-) or after APEX proximity labeling using substrates demarcated next to the images. 500 μM Alkyne-phenol (Alk-ph, shown above) was incubated on cells for 30 min. Either 1 mM H_2O_2 was added afterwards for 3 min or 10 mM D-Met was incubated simultaneously. Post-fixation cells were click-reacted with biotin-azide or azide-fluor545 for IF detection.

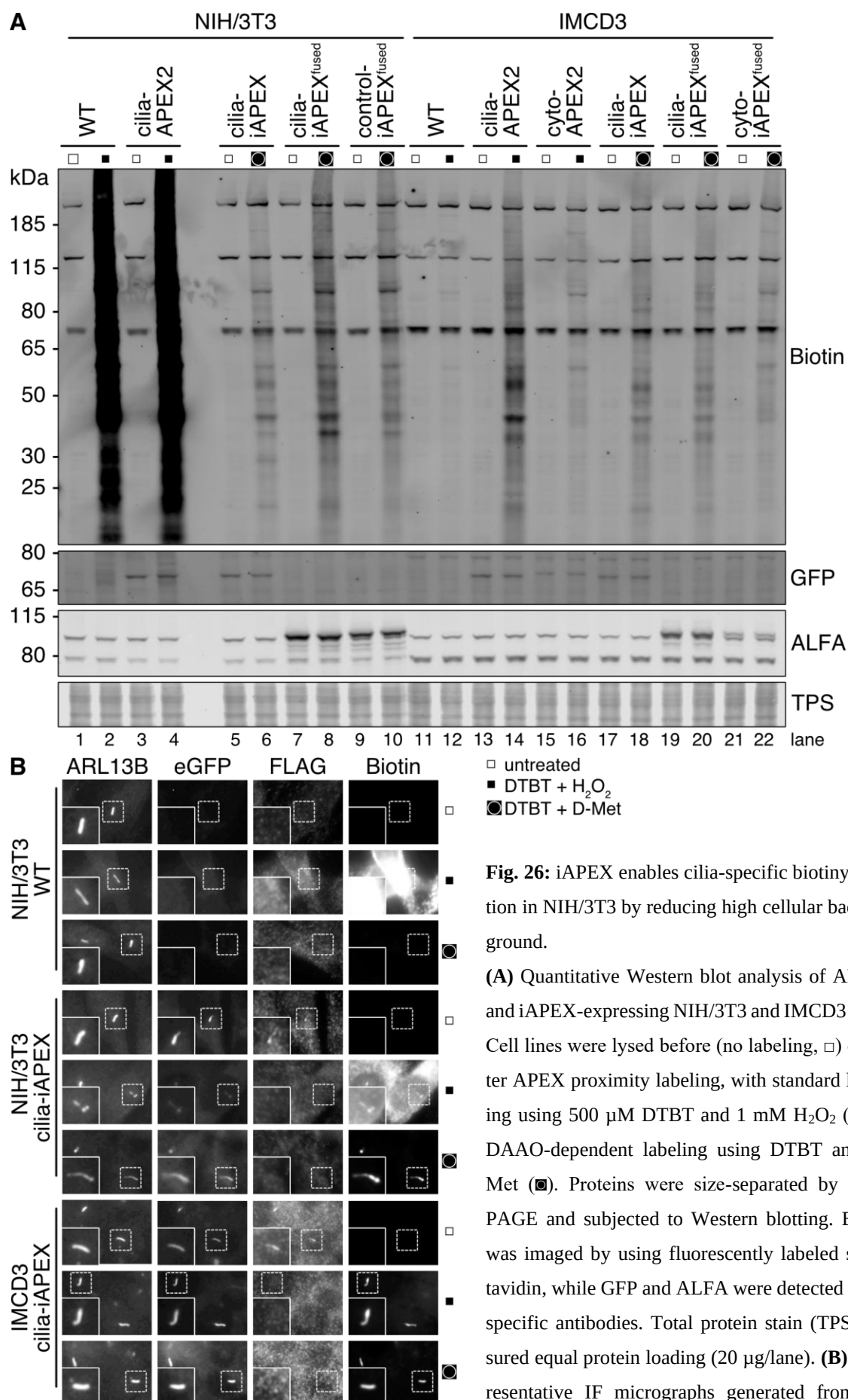
Together, these results indicate that using alkyne-phenol as a substrate for APEX2 proximity labeling, followed by CuAAC with biotin-azide or azide-fluor545, allows for specific and efficient visualization of labeled proteins. Both, exogenously added H₂O₂ and D-Met resulted in efficient ciliary labeling. The use of azide-fluor545 provides a direct and robust method for detecting labeled proteins in cilia.

3.2.5 Extending the iAPEX System to Mouse Fibroblasts

Once the substrate-dependent mechanisms were determined and the iAPEX system in IMCD3 cells was better understood, the initial question of this chapter could be addressed:

Can the iAPEX two-component system be introduced into previously inaccessible cell types to unravel their primary cilia proteomes using proximity labeling?

To answer this question, NIH/3T3 mouse fibroblast cells were selected for the generation of iAPEX lines. The WT cells showed substantial background labeling upon exposure to H₂O₂ and DTBT in WB and IF analysis; independent of the presence of the APEX2 enzyme (**Fig. 26A**, lanes 2 and 4; **Fig. 26B** NIH/3T3 WT micrographs). Remarkably, quantitative WB analysis of labeled samples from iAPEX NIH/3T3 cells revealed discernable biotinylation patterns (**Fig. 26A**, lanes 6, 8 and 10) comparable to those obtained by labeling in IMCD3 cells expressing the same system (**Fig. 26A**, lanes 18, 20, 22) or the classical APEX2 equivalents (**Fig. 26A**, lanes 14 and 16). IF micrographs obtained from the same experiment displayed clear and specific labeling in primary cilia in NIH/3T3 cilia-iAPEX cells when incubated with DTBT and D-Met. DTBT incubation with the addition of exogenous H₂O₂ enabled the labeling of primary cilia in these cells, albeit with a lower specificity due to the simultaneous intensive cellular biotinylation. As in IMCD3 cilia-iAPEX cells, individually added substrates to NIH/3T3 cilia-iAPEX cells did not exhibit an increase in cellular or ciliary biotin signal (**Sup.-Fig. 9**).



same samples used for the Western blot analysis presented in (A). Images illustrate biotin labeling in NIH/3T3 and IMCD3 iAPEX cell lines, alongside an NIH/3T3 wild-type (WT) control. Primary cilia were visualized using the ARL13B antibody, while cilia-DAAO was detected using the FLAG antibody. Cilia-APEX2 was visualized via GFP fluorescence, and biotin was detected using fluorescent streptavidin. Scale bar = 5 μ m. An extended version that includes independent substrate incubations is shown in **Sup.-Fig. 9**.

3.2.6 Quantitative Primary Cilia Proteomics Using the iAPEX system

iAPEX Analysis in Murine Kidney IMCD3 Cell Line

To test the primary cilia protein isolation using the improved iAPEX labeling method, streptavidin affinity chromatography was performed with labeled iAPEX samples to be finally analyzed by TMT-10plex-LC-MS3 (for method see page **91**). Since the two-component system permits classical APEX2 labeling, the same IMCD3 cilia-iAPEX cell line was incubated with BT and either

- DAAO substrate D-Met was added to catalyze H_2O_2 production to test the iAPEX labeling or
- exogenous H_2O_2 was added to test the classical APEX2 labeling.

May *et al*, 2021 introduced a cilia-removed IMCD3 cilia-APEX2 control cell line to control for non-ciliary proteins by deleting the centriolar distal appendage protein CEP164 (IMCD3 *Cep164*^{-/-} cilia-APEX2). This cell line together with an IMCD3 control-iAPEX cell line (myristylation-deficient cilia-DAAO (therefore cytosolic) and cilia-localized APEX2) were used as controls. All samples were prepared in duplicate or triplicate (**Fig. 27A**, scheme of cell line comparison in **Sup.-Fig. 10**) and the APEX2 expression of the individual cell lines was verified by GFP. In the control-iAPEX line, standard labeling was omitted because the expressed construct harbors a cilia-localized APEX2. After streptavidin chromatography, eluates were tested against total samples for enrichment of biotinylated material by quantitative WB analysis. Cilia-iAPEX samples were further tested for specific isolation of ciliary proteins compared to controls (**Fig. 27BC**). As previously observed, iAPEX labeling showed lower biotinylation signal compared to labeling by addition of H_2O_2 , both in total signal and after isolation, with controls showing even lower signals. Nevertheless, a twofold increase of isolated cilia-specific proteins (e.g., IFT88, IFT57, IFT20 and acTub) in the iAPEX samples compared to APEX2 was achieved (**Fig. 27C**).

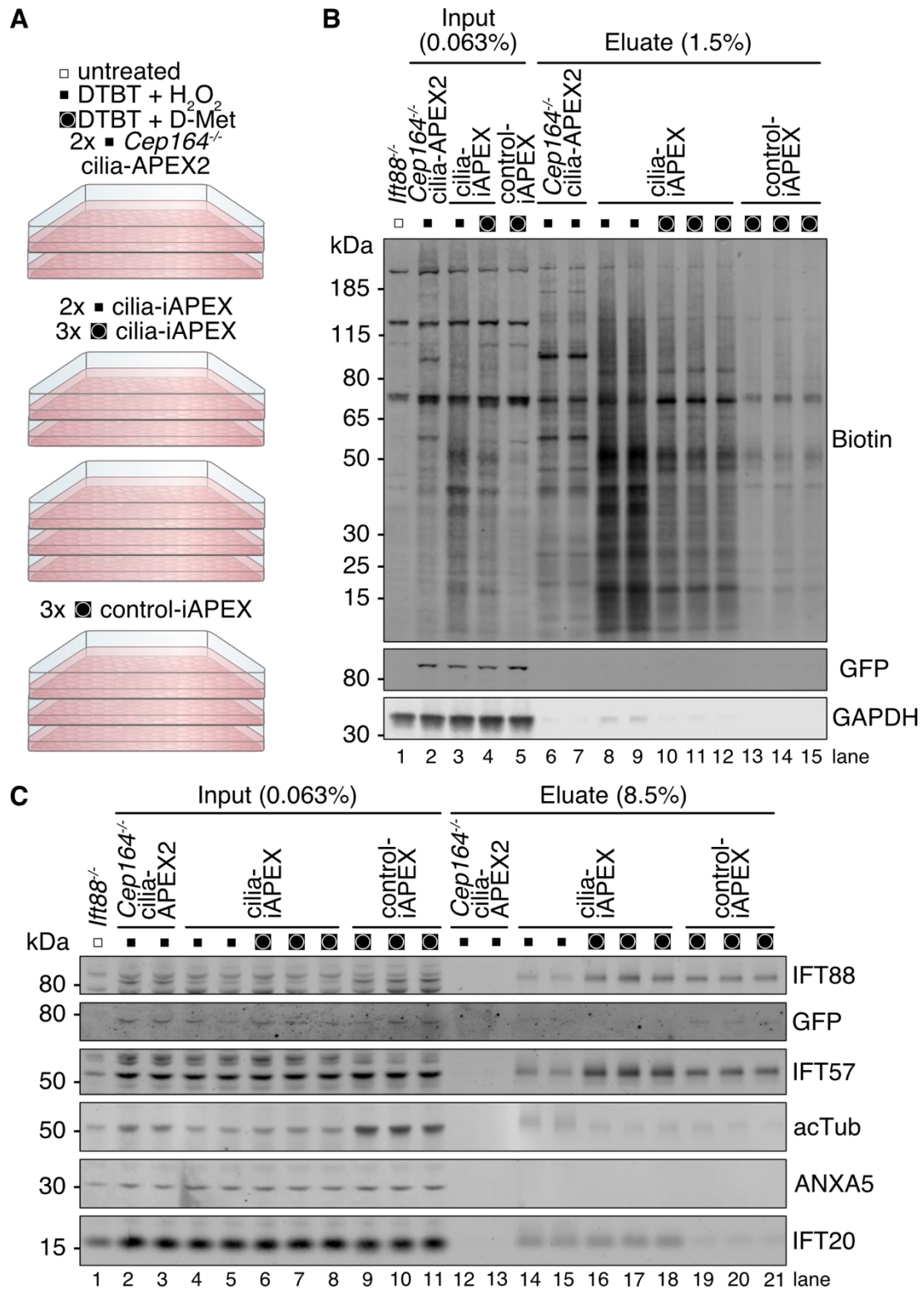


Fig. 27: Cilia-iAPEX isolates from IMCD3 cells exhibit stark enrichment of biotinylated material comprised of multiple cilia-related proteins.

(A) Schematic of cell culture plate count for each PL condition described in (B, C). Cell seeding numbers and affinity chromatography details are described in **Tab. 1**. **(B, C)** Quantitative Western blot analysis of samples from the same experiment was conducted on IMCD3 cells expressing either cilia- or control-iAPEX alongside control cell lines. IMCD3 *Ift88*^{-/-} served as a negative control for labeling (□). Additionally, a cilia-APEX2

expressing IMCD3 cell line with a *Cep164*^{-/-} background was included as a control for absence of primary cilia, employing standard labeling conditions (using DTBT and H₂O₂ (■)). Labeling dependent on DAAO was performed using DTBT and D-Met (■). Cell lines were lysed both before and after APEX proximity labeling, followed by isolation of biotinylated proteins by streptavidin affinity chromatography. Chromatography input and competitive eluates were separated based on size using SDS-PAGE and were subsequently analyzed by WB. Biotin was visualized using fluorescently labeled streptavidin, while specific antibodies were used for detecting indicated proteins. Equal protein loading was confirmed using GAPDH and Annexin (20 µg/lane for input). The percentage in brackets indicates the amount analyzed from the 10% eluate fraction obtained from total isolates. Beads were incubated with 32 mg protein.

Surprisingly, the control-iAPEX cell line showed increased IFT88 and IFT57 signals comparable to those of the cilia-iAPEX cell line, although ciliary biotinylation was less evident in the complementary IF analysis of the experiment (**Sup.-Fig. 11**). Annexin V was probed to verify specific isolation of primary cilia proteins.

As the samples showed sufficient isolation and primary cilia specificity, a proteomics analysis was conducted. For this purpose, the eluates prepared in **Fig. 27** were tryptically digested. The resulting peptides were 10-plex TMT labeled and analyzed by LC-MS3 (for method details see page **91**). The result was quantitative multiplex proteome data, which provided information on the statistically significant enrichment of cilia proteins of the cilia-APEX2 versus cilia-iAPEX samples compared to their respective controls. The reproducibility of each replicate was shown by a hierarchical cluster analysis of the relative individual protein abundance in each sample (**Sup.-Fig. 12**). Plotting the cluster means in a parallel coordinates plot highlighted protein groups that were more strongly represented in the cilia-APEX2 replicates compared to the iAPEX equivalents (**Sup.-Fig. 12B**; e.g., clusters 3 and 4). Certain clusters (especially clusters 28 to 32) were prominent in the controls and cilia-APEX2, but underrepresented in cilia-iAPEX. The enrichment scores for proteins in clusters 26 and 27 were twice as high in the cilia-iAPEX samples as in the cilia-APEX2 samples, whereas they were virtually absent in the controls. A closer look at these clusters revealed the proteins to be strong candidates and confirmed cilia proteins (**Fig. 28A**). A further statistical analysis was performed to compare the significance of the enrichment score differences in samples with cilia-localized PL enzymes (cilia-APEX2 or -iAPEX) to those without cilia (cilia-APEX2 in *Cep164*^{-/-} background) samples (**Fig. 28BC**). The criteria for potential cilia protein candidates and verified cilia proteins were the same as previously mentioned (see results chapter page **103**) and are indicated in **Fig. 28**. After applying the filter, 12.08% (721 proteins) significantly enriched cilia protein candidates out of a total of 5967 quantified

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proteins could be identified for cilia-APEX2, of which about one-fourth (24.4%, 176) were verified to be cilia-associated based on the manually curated gold cilia standard list. Among the 11.71% less abundant proteins, 4.15% (29 out of 699) were depleted cilia proteins (**Fig. 28B**). These figures were comparable to the cilia-iAPEX samples (10.68% depleted with 4.71% cilia proteins; 30 out of 637), which, on the other hand, showed an almost 10% increase in confirmed enriched cilia candidates (31.79%, 213 out of 670; from 11.23% enriched candidates) (**Fig. 28C**). When tested against the control-iAPEX instead of the cilia-removed *Cep164*^{-/-} cilia-APEX2 cell line, the enrichment of potential ciliary candidates dropped significantly to 7.39% (441), of which still one-fourth (25.85%, 114) were cilia proteins. Though this comparison almost doubled the number of depleted cilia proteins to 8.27% (33 out of 399) (**Sup.-Fig. 13A**). When compared to the cilia-ablated control, control-iAPEX exhibited 35.35% enrichment of which about one-fourth (23.79%, 133 out of 559) were cilia proteins; of which only 6.2% (38 out of 613) were depleted (**Sup.-Fig. 13B**). The quantitative WB analysis of protein enrichment in **Fig. 27**, combined with the overlapping relative protein abundance heatmap data for control-iAPEX in cilia cluster 2 (**Fig. 28A**), indicated ciliary labeling in the control cell line. While the IF analysis did not provide clear evidence of visible biotinylation in the cilia (**Sup.-Fig. 11**), the proteomic data suggested that the control-iAPEX cell line might have basal cilia-APEX2 activity potentially due to cellular levels of H₂O₂ in the cilium or diffused H₂O₂ from mislocalized DAAO in the cytoplasm.

The top 15 enriched hits from the IMCD3 cilia-iAPEX proteomics dataset (labeled cilia-iAPEX condition over cilia-APEX2 *Cep164*^{-/-}) can be found in **Tab. 2**. The majority of listed proteins are prototypical cilia proteins which were described before (Mick *et al*, 2015; May *et al*, 2021a). The most abundant hits were linked to ciliary protein trafficking, mainly intra-flagellar transport (IFT) protein complex B subunits (IFT20, IFT46, IFT57, IFT74, IFT81, IFT88) as well as the ADP-ribosylation factor-like protein 13B (ARL13B) (Humbert *et al*, 2012) and Leucine zipper transcription factor-like protein 1 (LZTFL1) (Seo *et al*, 2011). INPP5E and GPR161 are ciliary membrane associated signaling molecules (Chávez *et al*, 2015). FAM149A function has not been investigated yet, but mutations in the closely related FAM149B have been linked to Joubert syndrome (Maurya & Sengupta, 2021). Also associated with Joubert syndrome, CEP41 is involved in axoneme polyglutamylation and regulates the ciliary entry of TTLL6, a polyglutamylase (Lee *et al*, 2012; He *et al*, 2018).

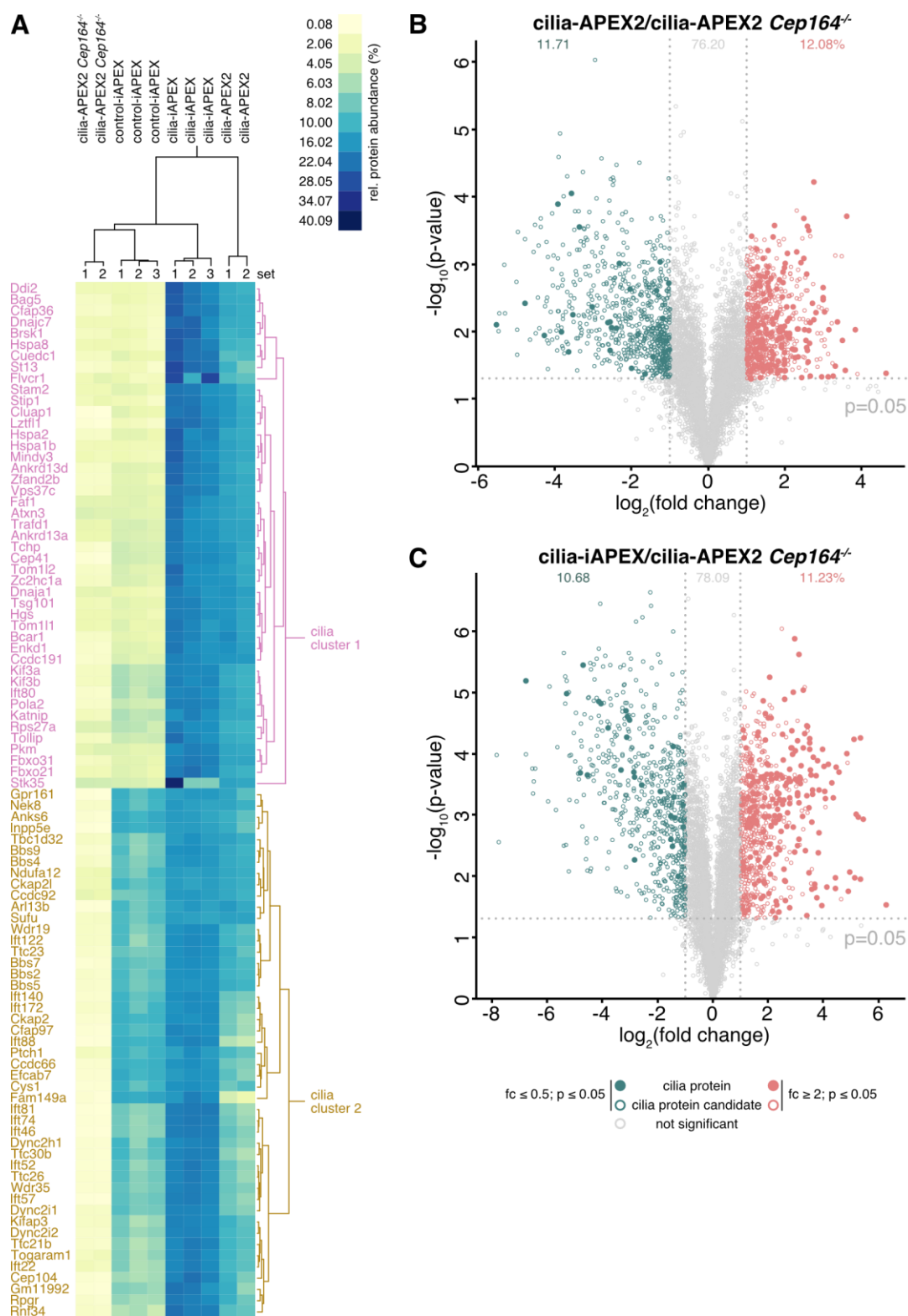


Fig. 28: Quantitative primary cilia proteomics using iAPEX outperforms conventional APEX2-based system.

(A) For the cilia-APEX2 and cilia-iAPEX-TMT experiment (data sourced from samples detailed in **Fig. 27**), a hierarchical two-way cluster analysis was performed using Ward's minimum variance method. In this analysis, the heatmap columns representing individual samples within each condition set were clustered based on the identified proteins listed in the rows. Relative protein abundance was calculated by dividing the individual TMT signals by the sum of the signals from all samples, with a color legend in the upper right corner indicating

the percent abundance. Highlighted clusters contain cilia proteins. The entire hierarchical clustering can be found in **Sup.-Fig. 12. (B, C)** Volcano plots were generated to visualize the aforementioned proteomics data. The plots depict proteins plotted against the negative logarithm (base 10) of two-sample t-test p-values (y-axis) and the logarithm (base 2) of the TMT fold change (x-axis) between conditions cilia-APEX2 and cilia-APEX2 in a *Cep164*^{-/-} background (A), or cilia-iAPEX with the identical control condition (B). Points in the top quantiles denote proteins with significant statistical significance ($p \leq 0.05$) and substantial fold changes (green for $fc \leq 0.5$; red for $fc \geq 2$). Filled circles highlight cilia proteins matched against a manually curated cilia gold standard set. Thresholds for significance are demarcated by a horizontal dashed gray line ($p = 0.05$) and vertical lines (fold change ≤ 0.5 ; ≥ 2), with non-conforming proteins displayed in gray. The percentages at the quadrant tops indicate the proportion of proteins meeting the specified criteria, out of the total 5967 quantified proteins. For further analyses, volcano plots showcasing distinctions among other tested conditions are provided in **Sup.-Fig. 13.**

Tab. 2: Top 15 enriched hits from IMCD3 cilia-iAPEX proteomics by fold change of labeled cilia-iAPEX condition over cilia-APEX2 *Cep164*^{-/-} in descending order

Gene	Description	Cluster	p value (Welch's t-test)	fold change
CLUAP1	Clusterin-associated protein 1	26	0.02897	76.87212
IFT81	Intraflagellar transport protein 81 homolog	27	0.00116	43.79194
IFT46	Intraflagellar transport protein 46 homolog	27	0.00005	40.55798
LZTFL1	Leucine zipper transcription factor-like protein 1	26	0.01122	40.26457
IFT57	Intraflagellar transport protein 57 homolog	27	0.00107	38.04914
IFT88	Intraflagellar transport protein 88 homolog	27	0.00095	36.35851
INPP5E	Phosphatidylinositol polyphosphate 5-phosphatase type IV	27	0.01060	35.25842

Continued on next page.

Top 15 enriched hits from IMCD3 cilia-iAPEX proteomics by fold change of labeled cilia-iAPEX condition over cilia-APEX2 *Cep164*^{-/-} in descending order (continued)

Gene	Description	Cluster	p value (Welch's t-test)	fold change
IFT20	Intraflagellar transport protein 20 homolog	25	0.00006	34.57095
GPR161	G-protein coupled receptor 161	27	0.00845	30.72230
CEP41	Centrosomal protein of 41 kDa	26	0.01750	29.94063
GM11992	Uncharacterized protein C7orf57 homolog	27	0.00014	29.10619
IFT74	Intraflagellar transport protein 74 homolog	27	0.00010	28.98979
FAM149A	Protein FAM149A	27	0.02346	24.80356
CFAP97	Cilia- and flagella-associated protein 97	27	0.00020	23.68816
ARL13B	ADP-ribosylation factor-like protein 13B	27	0.00016	22.08133

Identification of Potential Primary Cilia Candidate Proteins

Next, the extensive proteome list was analyzed for previously undescribed cilia proteins that were significantly enriched in the data set ($p\text{-Value} \leq 0.05$, fold change ≥ 2). The proteins of interest (POI) shown in **Tab. 3** were identified and further analyzed.

Tab. 3: Potential cilia protein candidates found enriched in the IMCD3 iAPEX dataset (ratio cilia-iAPEX/cilia-APEX2 *Cep164*^{-/-})

POI	Description	Cluster	p value (Welch's t-test)	fold change
ANKRD13D	Ankyrin repeat domain-containing protein 13D	26	0.00001	9.89457
C19ORF44 homolog	Uncharacterized protein C19orf44 homolog	25	0.00025	4.94511
CKAP2L	Cytoskeleton-associated protein 2- like	27	0.00108	3.71714
CUEDC1	CUE domain-containing protein 1	26	0.00026	9.04982
CUEDC2	CUE domain-containing protein 2	10	0.00039	6.71116
ENSA	Alpha-endosulfine	4	0.00143	4.21176
EYA1	Eyes absent homolog 1	25	0.00658	3.88639
FBXO21	F-box only protein 21	26	0.00111	5.96270
MINDY1 (FAM63A)	Ubiquitin carboxyl-terminal hydro- lase MINDY-1	25	0.00143	4.28997
MINDY3 (FAM188A)	Ubiquitin carboxyl-terminal hydro- lase MINDY-3	26	0.00003	6.24433
MINDY4 (FAM188B)	Probable ubiquitin carboxyl-termi- nal hydrolase MINDY-4	24	0.00027	10.13987
PICK1	PRKCA-binding protein	24	0.03900	5.07731
PSKH1	Serine/threonine-protein kinase H1	21	0.00030	8.91127
ZFAND2B	AN1-type zinc finger protein 2B	26	0.00022	11.52872

To determine the localization of the POIs, each was cloned from either mouse RNA-transcribed or plasmid-derived cDNA into a viral expression vector (pTS100, see page **60** and supplementary **Tab. 6**). The vector harbors cilia-APEX2 expression as a fluorescent cilia marker, followed by an IRES cassette regulating polycistronic expression of the POI which is fused to a small C-terminal ALFA-tag for improved detection by single domain antibody staining. From the 14 potential hits, 11 could be successfully cloned (see supplementary **Tab. 6**).

IMCD3 WT cells were transiently transfected with the generated plasmids, fixed after one day of serum deprivation and examined for POI localization using fluorescently coupled ALFA nanobody. Primary cilia were visualized by cilia-APEX2 and ARL13B. Additionally, CEP164 staining was included to complete the visualization of the cilium-basal body complex. Excitingly, from the 11 proteins analyzed, five proteins showed cilia-associated localization with heterogeneous signal intensities (**Fig. 29**). C19ORF44 homolog showed preferential localization at the basal body. CKAP2L, CUEDC1, MINDY3 and PSKH1 were clearly observed in the cilium, with Mindy3 showing only very low signal, so that primary cilia projecting into empty space had to be imaged. I concluded that iAPEX labeling was able to detect low abundance proteins that would otherwise be masked by exogenous H₂O₂-generated cellular noise.

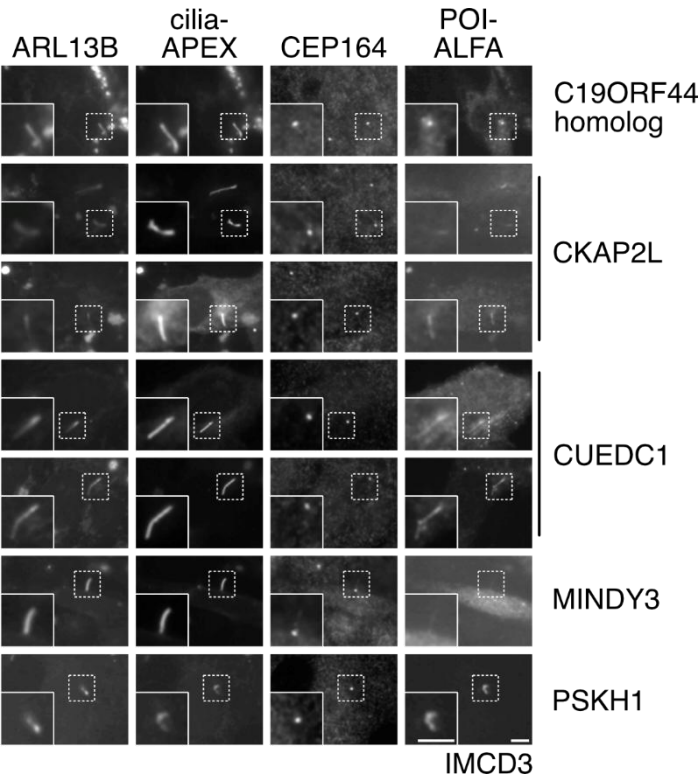


Fig. 29: Validation of primary cilia localization for proteins previously unlinked to cilia.

The representative IF micrographs illustrate IMCD3 wild-type cells transiently transfected with plasmids expressing both cilia-eGFP-APEX2 and various potential primary cilia-associated proteins. Upon fixation, primary cilia were visualized using antibodies targeting ARL13B and CEP164, while the protein of interest (POI) was stained with an ALFA antibody. Scale bar = 5 μ m.

iAPEX Analysis in Murine Fibroblast NIH/3T3 Cell Line

Since the IMCD3 control-iAPEX cell line unexpectedly enriched cilia proteins, the use of such a control cell line was omitted in the further investigation of the iAPEX NIH/3T3 cells. Owing to the stereoselectivity of DAAO and the need for dual incubation with substrates, alternative controls could be found. By incubating D-Met and DTBT simultaneously, ciliary proteins could be labeled, while D-Met alone was added as a substrate control that did not lead to biotinylation, but gave proteomic cell background noise. Finally, L-Met was incubated with DTBT to cover background noise generated by basal APEX2 activity without additional H₂O₂. Thus, the cilia-iAPEX system could be fully tested by incubating NIH/3T3 cilia-iAPEX with the different compound sets (**Fig. 30A**). Biotinylated proteins were clearly enriched for the DTBT and D-Met treated triplicates (**Fig. 30B**, lanes 5-7), as well as DTBT and L-Met incubated samples (**Fig. 30B**, lanes 11-13). Here, the ciliary proteins IFT88 and IFT57 could only be isolated with DTBT- and D-Met-treated samples (**Fig. 30B**, lanes 11-13), with minor isolation of IFT88 from samples incubated with L-Met (**Fig. 30B**, lanes 17-19). The D-Met single incubation was only able to visibly enrich endogenous biotin-associated carboxylases (**Fig. 30B**, lanes 8-10) and accordingly showed no enrichment of ciliary proteins (**Fig. 30B**, lanes 14-16).

With the samples generated in **Fig. 30**, another 10-plex TMT-LC-MS3 analysis could be performed, with the expectation of obtaining cilia-exclusive proteomic data from NIH/3T3, as has been successfully done previously for IMCD3 cells (**Fig. 28**). In contrast to the previous MS analyses, a control cell line in the form of a mislocalized PL enzyme or a cilia-ablated cell line was omitted here, which is reflected in the high overlap of protein abundance observed in the hierarchical cluster analysis (**Sup.-Fig. 14A**). The parallel coordinates plot demonstrated distinct clustered protein abundance patterns across the various experimental conditions tested in the NIH/3T3 iAPEX cell line (**Sup.-Fig. 14B**). Multiple clusters showed stable abundance levels across all conditions (e.g., clusters 6-12). In contrast, clusters 25-27 exhibited gradually increasing abundance levels from almost no signal in the omitted BT (D-Met) condition to some signal in the L-Met condition (BT present) and with increased signal in the labeled condition. Proteins falling into groups 28, 29 and 31 to 33 in particular achieved the highest relative abundance in the iAPEX labeled samples with almost no signal in the other conditions (cilia clusters 1-4 in **Fig. 31A**; original cluster order 31-33 then 28).

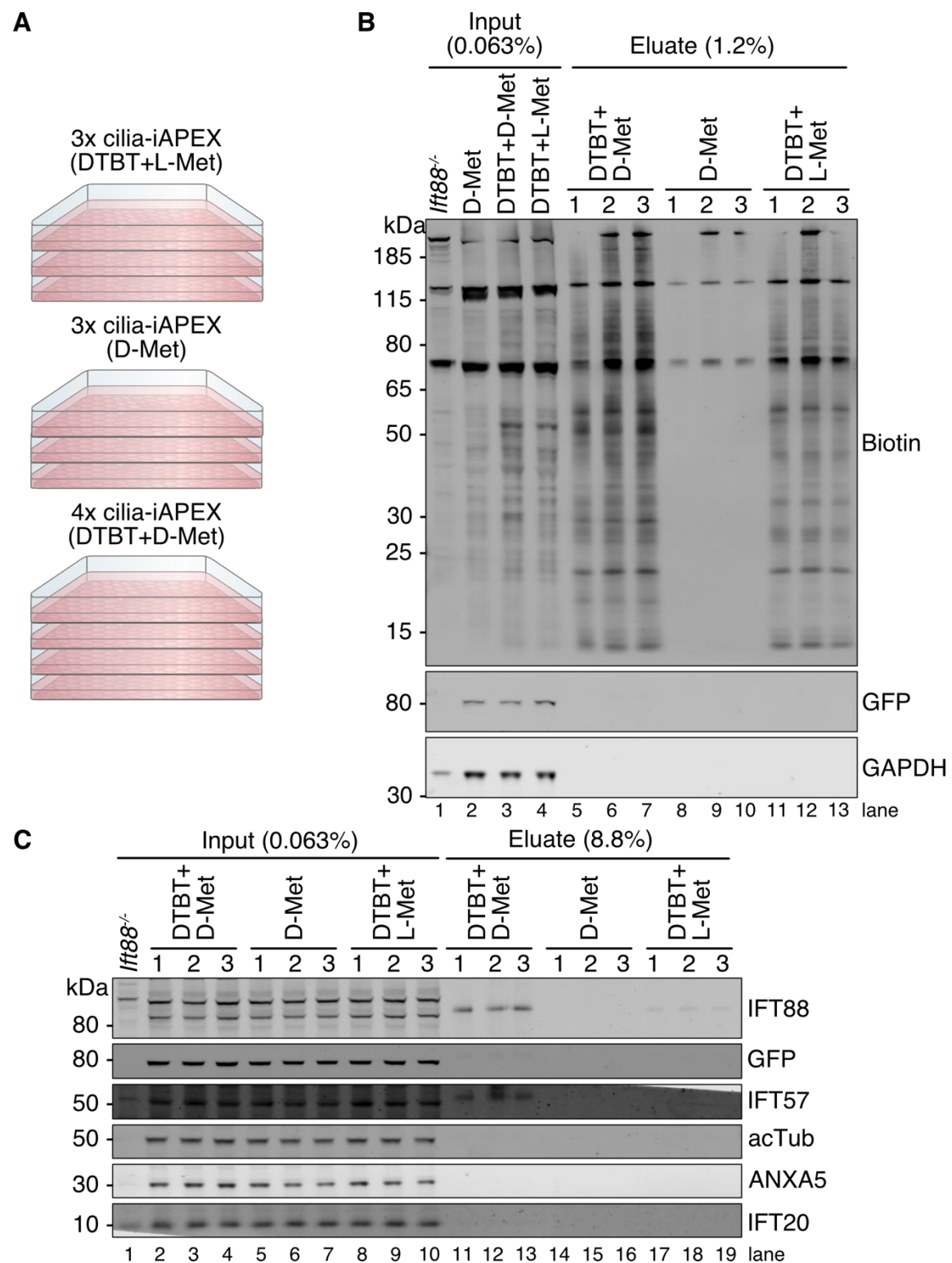


Fig. 30: Cilia-iAPEX isolates from NIH/3T3 cells display specific enrichment of biotinylated cilia-related proteins.

(A) A schematic of the cell culture plate count for each PL condition described in (B, C) is shown. Details on cell seeding numbers and affinity chromatography handling can be found in **Tab. 1**. **(B, C)** Quantitative Western blot analysis was conducted on NIH/3T3 cells expressing cilia-iAPEX. IMCD3 *Ift88*^{-/-} control cells served as a control for IFT88 antibody. DAAO-dependent labeling was performed using DTBT with either L- or D-

Met, with L-Met used to assess DTBT labeling background and D-Met alone as a labeling negative control. Cell lysates were analyzed before and after APEX proximity labeling, followed by SDS-PAGE separation and subsequent Western blotting. Biotin was detected using fluorescently labeled streptavidin, while specific antibodies were employed for other protein detection. Equal protein loading was confirmed with GAPDH and Annexin (20 µg/lane for input), and the percentage in brackets indicates the amount analyzed from the 10% eluate fraction obtained from isolates. Beads were incubated with 32 mg protein.

These clusters had a large number of confirmed cilia proteins and even included some of the candidates tested in **Fig. 29**, where cilia localization in IMCD3 cells could be detected (see **Tab. 4**). The most abundant proteins by fold change in the NIH/3T3 cilia-iAPEX dataset can be seen in **Tab. 5**. Similar to the IMCD3 top list (see **Tab. 2**), ciliary membrane-associated proteins (PTGS2 and NPHP3) and proteins involved in ciliary protein trafficking (ARL13B, CLUAP1, IFT81, IFT57, IFT88 and TTC30B (IFT70b)) were the most abundant. Notably, a high number of collagens (COL3A1, COL4A1 and COL16A) was found among the most enriched hits.

Statistical analysis of the proteomic data difference of cilia-iAPEX and the “D-Met only” samples exhibited a mostly right shifted volcano plot towards significantly enriched potential cilia candidates (**Fig. 31B**). About 7.15% (434) of the total 6068 identified proteins were located in the upper right quadrant, of which almost one third (29.72%, 129) were confirmed cilia proteins. The number of significantly downregulated cilia candidate proteins was negligible, with a proportion of only 18 proteins. A similar picture emerged for the difference between cilia-iAPEX and the stereoselective substrate control (**Fig. 31C**). Here, the control caused a leftward shift of the enriched proteins causing them to fall below the difference threshold, resulting in only 2.11% significantly enriched proteins. However, of these 129 proteins, three quarters (73.64%, 95) were confirmed cilia proteins. A comparison with the “D-Met only” control once again showed that BT alone generated background noise (**Sup.-Fig. 15**). Interestingly, hierarchical cluster analysis impressively demonstrated that cilia-iAPEX PL exclusively produced highly enriched candidates that were virtually absent in the used controls. It was shown that cilia-DAAO-generated and thus cilia-restricted H₂O₂ in combination with cilia-APEX2 could specifically label proteins in NIH/3T3 cells that were previously inaccessible to APEX PL.

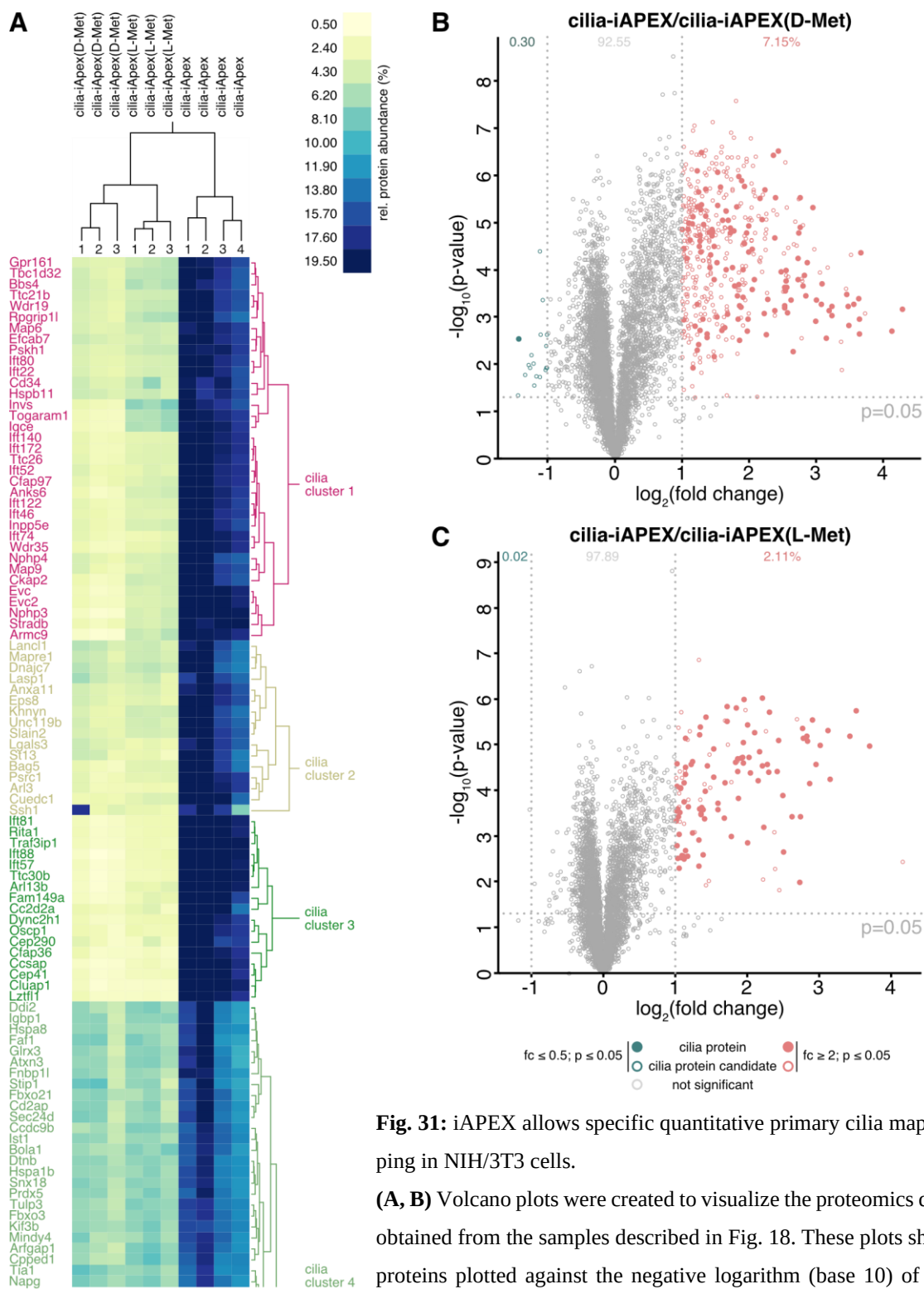


Fig. 31: iAPEX allows specific quantitative primary cilia mapping in NIH/3T3 cells.

(A, B) Volcano plots were created to visualize the proteomics data obtained from the samples described in Fig. 18. These plots show proteins plotted against the negative logarithm (base 10) of the two-sample t-test p-values (y-axis) and the logarithm (base 2) of the TMT fold-change (x-axis). Conditions compared include incubation of cilia-iAPEX with regular substrates (D-Met + DTBT) compared to D-Met alone (D-Met) (**A**), and cilia-iAPEX with regular substrates (D-Met + DTBT) compared to a stereoselective cilia-iAPEX control (L-Met + DTBT) (**B**). The dots in the upper quadrants indicate proteins with statistical significance ($p \leq 0.05$) and substantial fold changes (green for $fc \leq 0.5$; red for $fc \geq 2$). The filled circles further indicate cilia proteins benchmarked against a gold standard. Significance thresholds are indicated by a horizontal dashed gray line ($p = 0.05$) and vertical lines ($fc \leq 0.5$; ≥ 2),

with unmatched proteins shown in gray. The percentages in the upper quadrants indicate the proportion of proteins meeting the specified criteria out of a total of 6068 quantified proteins. An extended clustering can be seen in **Sup.-Fig. 14**. A volcano plot shown in **Sup.-Fig. 15** highlights the background noise generated by BT incubation alone. **(C)** A hierarchical two-way cluster analysis using Ward's minimum variance method was performed on the data from the cilia-iAPEX-TMT experiments performed in NIH/3T3 cells in **Fig. 30**. Columns representing individual samples within each condition set were clustered based on the identified proteins listed in the rows. Relative protein abundance was calculated by dividing individual TMT signals by the sum of signals from all samples, with the upper right corner displaying a color legend for percent abundance. The clusters shown include cilia proteins.

Tab. 4: IMCD3 candidates found in clusters in the cilia-iAPEX NIH/3T3 proteomics dataset

POI	Cluster	p value (Welch's t-test)	fold change (ratio cilia-iAPEX/ cilia-iAPEX (D-Met))
Cuedc1	32	0.00001	4.10319
Fbxo21	28	0.00025	1.65757
Mindy1 (Fam63a)	21	0.00004	1.69233
Mindy3 (Fam188a)	29	0.00012	2.67872
Mindy4 (Fam188b)	28	0.02086	1.76486
Pskh1	31	0.00058	3.61008

Tab. 5: Top 15 enriched hits from NIH/3T3 cilia-iAPEX proteomics by fold change of labeled cilia-iAPEX condition over cilia-iAPEX (D-Met only) in descending order

Gene	Description	Cluster	p value (Welch's t-test)	fold change
Ptgs2	Prostaglandin G/H synthase 2	27	0.00068	19.45638
Ift88	Intraflagellar transport protein 88 homolog	33	0.00200	17.50186
Rcn1	Reticulocalbin-1	27	0.00083	13.27699
Armc9	LisH domain-containing protein ARMC9	31	0.00004	12.71296
Cluap1	Clusterin-associated protein 1	33	0.00229	12.50019
Ttc30b	Tetratricopeptide repeat protein 30B	33	0.00108	12.45688
Ccsap	Centriole, cilia and spindle-associated protein	33	0.00041	12.35958
Arl13b	ADP-ribosylation factor-like protein 13B	33	0.00053	11.74971
Col4a1	Collagen alpha-1(IV) chain	27	0.00005	11.45353
Ift57	Intraflagellar transport protein 57 homolog	33	0.00150	11.17226
Cep41	Centrosomal protein of 41 kDa	33	0.00060	11.10395
Nphp3	Nephrocystin-3	31	0.00032	10.98739
Col3a1	Collagen alpha-1(III) chain	27	0.00025	10.87097
Col16a1	Collagen alpha-1(XVI) chain	27	0.00326	10.35897
Ift81	Intraflagellar transport protein 81 homolog	33	0.00093	9.42232

Comparative Analysis of IMCD3 and NIH/3T3 Proteomes

When comparing the significantly enriched proteins of the iAPEX datasets from IMCD3 and NIH/3T3 cells against the manually curated Cilia Gold Standard (CGS) list of core ciliary components, iAPEX identified a total of 191 shared ciliary proteins across both cell lines (**Fig. 32**). Specifically, the IMCD3 iAPEX analysis identified 155 ciliary proteins, with 77 of these overlapping with the CGS. In contrast, the NIH/3T3 iAPEX dataset revealed 255 proteins, with 36 overlapping with the CGS. Notably, 78 proteins were shared among the IMCD3 iAPEX, NIH/3T3 iAPEX, and CGS, indicating a core set of ciliary proteins consistently identified across both cell types. The proteomic overlap of 18 proteins between IMCD3 and NIH/3T3 datasets, not present in the CGS, suggests potential cell-type-specific ciliary components that were not captured in the gold standard. Interestingly, the NIH/3T3 dataset exhibited a larger number of unique candidate hits (201 proteins) compared to the IMCD3 dataset (133 proteins), which could be indicative of cell-type-specific proteomic profiles not yet described in the CGS list which covers candidates found in the IMCD3 proteome (see methods page **93**). A GO term enrichment analysis revealed distinct and overlapping proteomic landscapes (**Sup.-Fig. 16**). In the section unique to IMCD3s, the proteins were found to show significant enrichment in processes related to cellular component organization, microtubule cytoskeleton organization, and the mitotic cell cycle. In contrast, the unique NIH/3T3 section was primarily associated with extracellular matrix organization, cell adhesion, and signaling pathways such as collagen-activated tyrosine kinase receptor signaling. The shared set of proteins between both cell types highlighted common processes such as intraciliary transport and microtubule-based movement.

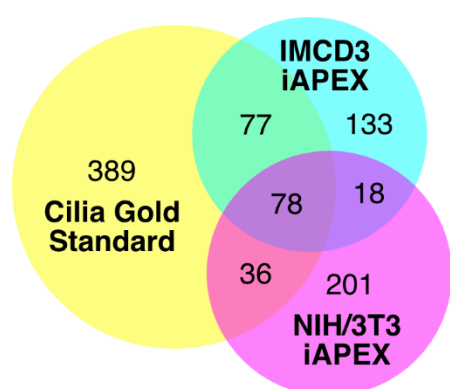


Fig. 32: iAPEX analyzed cilia proteomes of IMCD3 and NIH/3T3 cells show distinct overlap with cell specific differences.

Venn diagram depicting proteomic overlap of iAPEX-proximity labeled IMCD3 and NIH/3T3 cells against a manually curated cilia gold standard list. Ciliary hits were determined by comparing cilia-iAPEX labeled condition to *Cep164*^{-/-} cilia-APEX2 control in IMCD3 (with $p \leq 0.05$ & fold change ≥ 3.3) and cilia-iAPEX(D-Met) control in NIH/3T3 cells (with $p \leq 0.05$ & fold change ≥ 2.3).

Further, a comparative Gene Set Enrichment Analysis (GSEA) was conducted on the proteomic data, with results visualized through ridge and dot plots (**Fig. 33**). The analysis was

designed to evaluate the differentially distributed enrichment profiles of protein sets and their respective gene ratios between cilia-iAPEX and controls (*Cep164*^{-/-} cilia-APEX2 in IMCD3 cells and cilia-iAPEX(D-Met) in NIH/3T3 cells) by assessing the overrepresented Gene Ontology (GO) terms related to biological processes and cellular compartments.

In IMCD3 cells (**Fig. 33A**), GSEA revealed a pronounced enrichment of primary cilia-associated GO terms, indicating the effectiveness of the iAPEX approach in capturing proteins specifically localized to or associated with the cilia. The ridge plot shows that the majority of enriched GO terms are strongly associated with ciliary functions, while the dot plots highlight significant high gene ratios, underscoring the abundance of these cilia-related proteins within the dataset. The consistent enrichment of these terms across multiple biological processes and compartments reaffirms the specificity of the iAPEX method in targeting ciliary proteins.

In contrast, the NIH/3T3 dataset (**Fig. 33B**) also demonstrated significant enrichment in cilia-related GO terms, reaffirming the reliability of iAPEX for ciliary proteome studies across different cell lines. However, a notable difference could be observed in the broad spread of enrichment density across GO terms with overall lower fold change particularly for the cilia-related terms in NIH/3T3 compared to IMCD3 cells. Selection of *Cep164*^{-/-} cilia-APEX2 or no labeling (D-Met only) control for the iAPEX setup might have affected the broadening as well as the observed background profile differences seen in the depleted terms between IMCD3 and NIH/3T3.

Overall, the GSEA results from IMCD3 and NIH/3T3 iAPEX datasets demonstrated the enrichment of primary cilia-associated proteins, validating the effectiveness of the iAPEX technique in proteomic studies of ciliary structures. The lack of mitochondrial background in IMCD3 cells highlights the method's specificity, while the differences in depleted terms between the cell types provide additional insights into the unique proteomic profiles captured in each context.

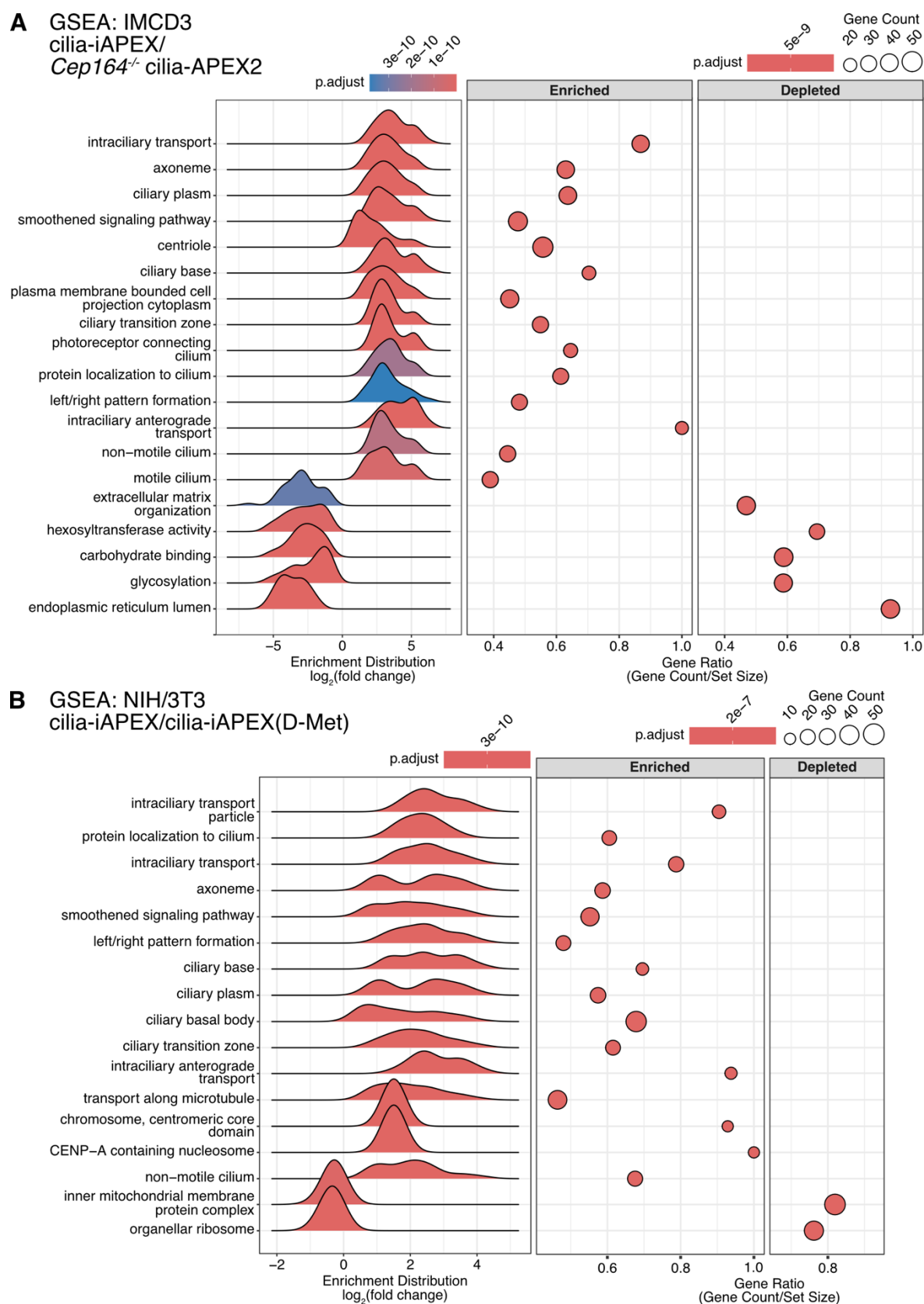


Fig. 33: Primary cilia-associated GO terms dominate IMCD3 and NIH/3T3 iAPEX-generated proteomic data sets.

(A) Ridge and dot plot representation of Gene Set Enrichment Analysis (GSEA) results from cilia-iAPEX proteomic data in IMCD3 cells. Protein fold changes between cilia-iAPEX and *Cep164*^{-/-} cilia-APEX2 were calculated, sorted in descending order, and used to generate a ranked list. GSEA was performed, and the top scoring enriched and depleted GO terms for biological processes and cellular compartments were plotted. The gene count represents the number of genes associated with each GO term, while the gene ratio indicates the percentage of significantly correlated genes relative to the total number of genes linked to that process or compartment. (B) GSEA results as in (A) for NIH/3T3 cilia-iAPEX data compared to cilia-iAPEX(D-Met) control.

3.2.7 Lentivirus-Mediated iAPEX System Integration Across Diverse Cell Types

With the effectiveness of the iAPEX system in NIH/3T3 cells confirmed, I aimed to integrate the iAPEX transgenes into other cell types without relying on the availability of a Flp-In locus. The cell types of interest are described on page 111.

To achieve this, lentiviral vectors containing the dual components of the iAPEX system within a multicistronic cassette were developed by me. The virus work, as well as the generation and evaluation of cell lines, were conducted by Lea Sanwald under my supervision. She has granted permission to present this data here to showcase the potential of the vectors developed.

Lentiviruses were produced and used to infect wild-type renal epithelial cells (IMCD3), myoblast precursors (C2C12), and preadipocytes (3T3-L1) (see methods section page 81). After infection, the cells were cultured, trypsinized, and sorted into GFP-positive pools based on their GFP (APEX2) fluorescence intensity using fluorescence-activated cell sorting (FACS) (see page 77).

Sorted cells were further cultivated and assessed for the expression of individual iAPEX components (**Fig.A**). The results showed that virally transduced IMCD3 and 3T3-L1 cells exhibited similar GFP-APEX2 expression levels (**Fig.A**, lanes 2 and 4), while C2C12 cells displayed reduced GFP expression (**Fig.A**, lane 3), yet comparable ALFA-DAAO expression to 3T3-L1 cells (**Fig.A**, lanes 3 and 4). Here, the viral GFP-APEX2 construct had a smaller molecular weight due to the removal of viral *attB* linker sites within the cassette. As the cilia-iAPEX Flp-In construct was generated in the first phase of the project, it lacks an ALFA tag for detecting DAAO (**Fig.A**, lane 1), making direct comparison with the viral

system difficult. Attempts to use a FLAG tag instead were made, since both constructs exhibit the tag, but these did not yield successful results. Next, the biotinylation efficiency of all viral cell lines was evaluated by performing APEX PL. Non-labeled, BT-incubated, iAPEX-labeled (BT + D-Met), and standard-labeled (BT + H₂O₂) samples were compared (**Fig.B**). BT alone resulted in basal labeling (**Fig.B**, lanes 5-8), which was substantially enhanced with additional substrate (**Fig.B**, lanes 9-16). iAPEX labeling produced distinguishable signals (**Fig.B**, lanes 9-12), whereas classical labeling in the problematic cell types (C2C12 and 3T3-L1) led to indistinguishable, non-specific biotinylation (**Fig.B**, lanes 15 and 16). This non-specific biotinylation with H₂O₂ and BT was also observed in corresponding immunofluorescence analysis (**Fig.C**).

Using the iAPEX system, such non-specific biotinylation was avoided (similar to NIH/3T3 Flp-In cells in **Fig. 26**), and biotinylation was confined to the primary cilium. Thus, the iAPEX two-component system can be integrated into various previously inaccessible cells using lentiviruses to explore their primary cilia proteomes.

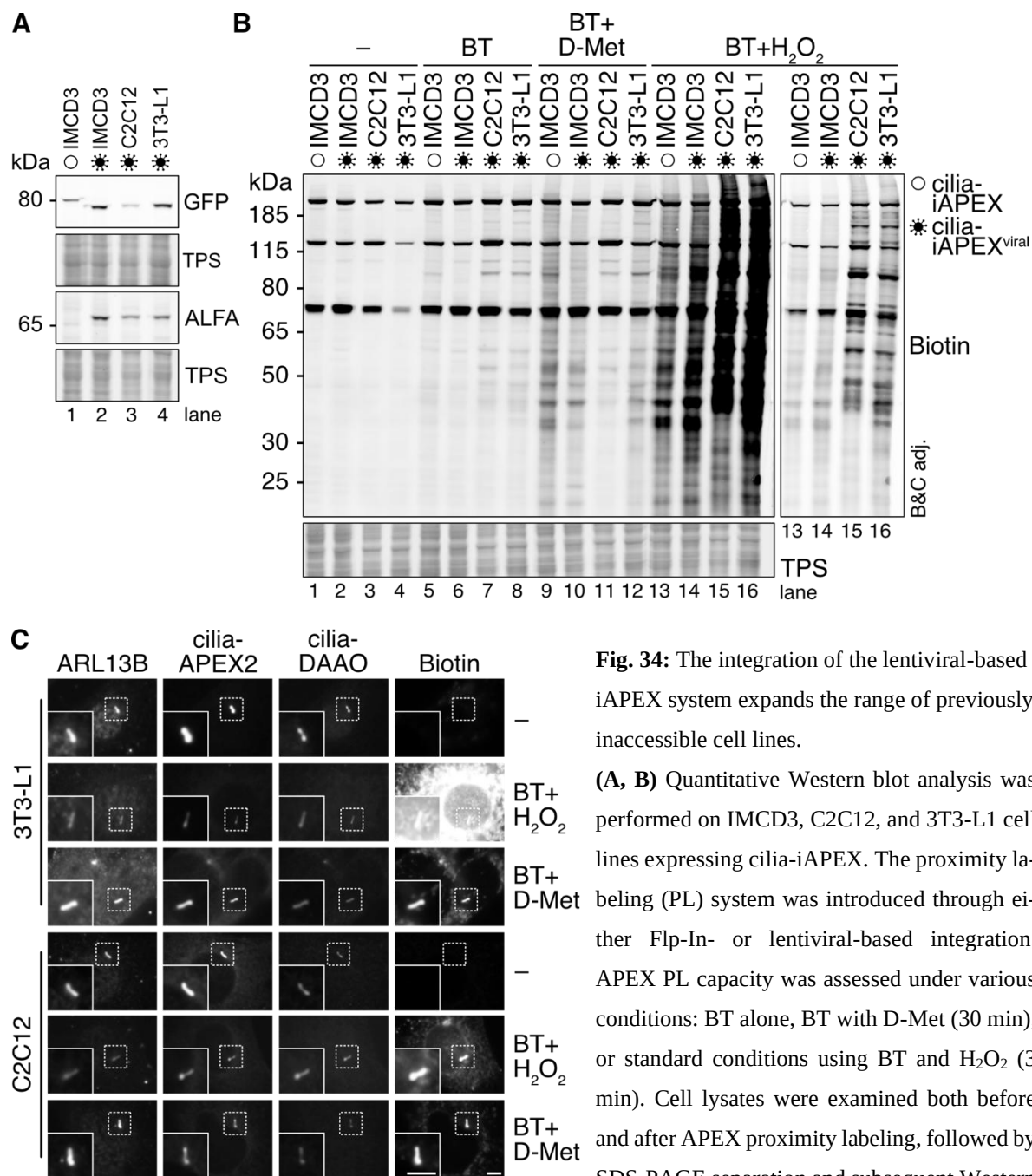


Fig. 34: The integration of the lentiviral-based iAPEX system expands the range of previously inaccessible cell lines.

(A, B) Quantitative Western blot analysis was performed on IMCD3, C2C12, and 3T3-L1 cell lines expressing cilia-iAPEX. The proximity labeling (PL) system was introduced through either Flp-In- or lentiviral-based integration. APEX PL capacity was assessed under various conditions: BT alone, BT with D-Met (30 min), or standard conditions using BT and H₂O₂ (3 min). Cell lysates were examined both before and after APEX proximity labeling, followed by SDS-PAGE separation and subsequent Western

blotting. Biotin was visualized using fluorescently labeled streptavidin, while specific antibodies were utilized for GFP and ALFA detection. TPS was employed to confirm equal protein loading (25 μg/lane). (C) Representative IF micrographs corresponding to the experiments depicted in (A) and (B) are presented. Following proximity labeling, cells were fixed, and primary cilia were visualized using antibodies specific to ARL13B. FLAG immunostaining was employed to detect cilia-DAAO. Biotinylation was visualized using fluorescently labeled streptavidin, while cilia-APEX2 was detected through GFP fluorescence. Scale bar = 5 μm. Data generated by Lea Sanwald and generously contributed for this thesis.

4. DISCUSSION

Primary cilia are present on virtually every cell in the body at some point during their development. These antenna-like cell protrusions play a crucial role in signal transduction pathways, development, and tissue homeostasis (Wheway *et al*, 2018). Understanding the variability in ciliary proteomes among different cell types is key to uncovering the functional specializations of these compartments. Therefore, establishing a comprehensive understanding of core ciliary proteins and their tissue-specific variants is fundamental to gain valuable insights into the molecular basis of ciliopathies and pave the way for future therapeutic interventions (Mill *et al*, 2023).

Proximity labeling (PL) techniques such as these mediated by engineered ascorbate peroxidase APEX2 and kinetically engineered biotin ligase TurboID have revolutionized the study of subcellular proteomes by enabling the selective labeling of proteins in close proximity to a tagged enzyme, followed by their identification and quantification through mass spectrometry (Liu *et al*, 2021a). These techniques are particularly powerful for investigating the proteomic landscape of primary cilia, given their ability to capture transient and spatially restricted proteins (Arslanhan *et al*, 2020). However, caution is advised when using PL techniques for interactome studies, as the diffusive nature of the reactive species of both techniques does not exclusively map physically interacting proteins (Kang & Rhee, 2022). A direct comparison of both PL methods was challenging due to their mechanistic differences in substrate preferences, enzyme kinetics and the amino acid targets on the proximal proteins. In the following the advantages and limitations of the two compared PL techniques are discussed.

4.1 Advantages and Limitations of APEX2 and TurboID

APEX2 and TurboID are two prominent techniques used for proximity labeling in cellular and molecular biology, each offering distinct advantages and limitations. Both are commonly used in combination with a biotin derivative to label proximal proteins which allows subsequent enrichment by streptavidin affinity chromatography prior to LC-MS/MS analysis (Kang & Rhee, 2022).

APEX2 is renowned for its rapid labeling capabilities and high temporal resolution, allowing (subcellular) proteomic mapping within living cells (Kalocsay, 2019). In the presence of hydrogen peroxide (H₂O₂), the peroxidase has the ability to activate aromatic molecules (e.g., ascorbic acid, various tyramides) through one-electron oxidation into short-lived reactive biotin phenoxyl radicals, which rapidly modify tyrosines in the enzyme's surrounding (Lam *et al*, 2015). The labeling of proximal proteins has been estimated to occur in seconds (Dumrongprechachan *et al*, 2021). An important structural characteristic of APEX2 includes its partially solvent-exposed heme binding pocket and peripheral aromatic substrate binding site, which both contribute to weak substrate specificity (Sharp *et al*, 2004; Martell *et al*, 2012; Lam *et al*, 2015). This makes APEX2-mediated PL highly adaptable for addressing various cellular challenges, such as selectively labeling extracellular or membrane-bound proteins using substrates that cannot pass through the cell membrane, or targeting nucleic acids through the development of diverse chemical probes (Rhee *et al*, 2013; Lee *et al*, 2017; Padrón *et al*, 2019; Zhou *et al*, 2019; Li *et al*, 2020b).

For APEX2 PL biotin tyramide (BT) is widely used in mammalian cell experiments, as the phenol part of tyramine is the chemical moiety to react with the peroxidase leaving biotin as a chemical handle for enrichment (Kang & Rhee, 2022).

Desthiobiotin-tyramide (DTBT), consisting of a sulfur-free biotin analogue, can instead be used for competitive biotin elution with a streptavidin matrix to minimize the amount of endogenously present biotin-associated proteins. These remain on the matrix when excess biotin displaces the DTBT-containing sites with weaker affinity. In addition, DTBT has been shown to enhance the signal in LC-MS/MS analysis due to improved elution efficiency (Lee *et al*, 2017). BT has been shown to generate two populations of modified peptides, namely unoxidized thioether groups and those that are oxidized to sulfoxide or sulfone during the radical APEX reaction, which interferes with the acquisition of mass spectra (Kang & Rhee, 2022).

BT can penetrate through multiple cellular membranes, for instance to label the mitochondrial matrix proteome (Rhee *et al*, 2013). Though its moderate permeability makes working with more complex membrane structures found in organisms challenging (e.g., *Drosophila* (Mannix *et al*, 2019), *C. elegans* (Reinke *et al*, 2017), *S. saccharomyces* (Hwang & Espen-

shade, 2016)). To overcome this limitation, various strategies have been used, such as compromising structural cell integrity through the use of detergents, enzymatic digestions or genetic manipulations that cause drastic changes in the system to be analyzed.

The biotin moiety can be changed to a smaller lipophilic alkyne group, which offers significantly better membrane permeability and allows click chemistry-based conjugation to an enrichment handle of choice (Li *et al*, 2020b; Liang *et al*, 2023).

In contrast, the biotin ligase TurboID exclusively catalyzes the activation of biotin through ATP which is then spontaneously released to react with nucleophiles on proximal proteins, preferentially lysines. Proximity biotinylation happens on the minute scale in TurboID compared to hours in its predecessor BioID (Branon *et al*, 2018). TurboID is valued for its inherently non-cytotoxic labeling process using exogenous biotin compared to APEX2 necessity for cytotoxic H₂O₂ addition (Cho *et al*, 2020; Sato, 2022).

4.1.1 Differences in Primary Cilia Proteome Coverage of APEX2 and TurboID

The enhanced temporal resolution of TurboID inspired me to create a genetically engineered cell line expressing TurboID targeted to primary cilia, allowing for a comprehensive comparison with the previously established cilia-APEX2 system (May *et al*, 2021a). TurboID has been shown to achieve efficient labeling in as little as 10 minutes, with an increase in subcellular proteome depth of up to 46% when the incubation time was extended to an hour (Branon *et al*, 2018). Indeed, I observed a consistent increase in signal with prolonged biotin incubation in my experiments (**Fig. 9**). However, while Branon *et al* (2018) reported a significant enhancement in proteome depth with extended labeling times, but also noted a substantial rise in non-specifically labeled proteins. Consequently, I set the labeling time for cilia-TurboID to 30 minutes and compared it to the 3-minute labeling time of cilia-APEX2 in my study (**Fig. 10**). This duration was chosen to allow parallel handling to APEX2 conditions which were performed with a 30 min BT preincubation and to give TurboID sufficient time to generate activated biotin molecules while also testing the temporal resolution. Although both techniques proved effective for primary cilia proteomics, cilia-APEX2 outperformed cilia-TurboID in capturing a broader spectrum of ciliary proteins with less contamination from non-ciliary regions and in a significantly shorter time frame (**Fig. 11**). The cilia-APEX2 proximity labeling resulted in stronger and more localized signals, in contrast to cilia-TurboID, which showed more cytosolic biotinylation, indicating lower specificity

(**Fig. 9**). This observation aligns with Branon *et al* (2018), who suggested that with increased labeling time, proximal nucleophiles may become saturated with biotin, allowing TurboID-generated biotin-AMP to diffuse farther and biotinylate distal, non-specific proteins. This is particularly important as identifying low-abundance ciliary proteins might become challenging when obscured by background signals in TurboID labeling. Compared to APEX2, TurboID covered a higher number of potential cilia candidates not yet covered by the Cilia Gold Standard list (**Fig. 12**) potentially due to a potentially broader biotinylation scope through lysine modification. Though the higher background labeling would require stringent data filtering to discern true ciliary components against the non-ciliary background.

4.1.2 Proteome Bias Through Differential Amino Acid Targets

Quantitative proteomic analysis reveals that the exposure of tyrosine (Tyr) or lysine (Lys) residues on proteins is often linked to greater labeling intensity by PL enzymes (Lee *et al*, 2017). Tyr is a common amino acid in proteins, making up an average of 3.2% of residues in native structures, and can be located either on the surface or buried within the hydrophobic core (Moinpour *et al*, 2020). Notably, protein surfaces that interact with small molecules, nucleic acids, or protein partners are often enriched in Tyr, making these residues particularly suitable for site-specific modifications (Sato, 2022). Tyr can be masked by a handful of post-translational modifications (PTMs), with phosphorylation, sulfation, and adenylation specifically targeting its hydroxyl group, while halogenation and nitration affect adjacent carbon residues (Griswold-Prenner *et al*, 2023).

Lysine, an essential amino acid, comprises approximately 6% of the human proteome and is predominantly located on protein surfaces due to its positively charged side chain (Shannon & Weerapana, 2015; Hacker *et al*, 2017). This positioning allows lysine to contribute significantly to protein stability through ionic interactions and hydrogen bonding. With its long, flexible side-chain and terminal ϵ -amino group, lysine's high reactivity makes it a frequent target for a multitude of post-translational modifications (PTMs) (Azevedo & Saiardi, 2016).

The Western blot results of TurboID-labeled samples indicated a potential bias in the identification of ciliary candidates (**Fig. 10**). Acetylation, a prominent lysine PTM, may hinder TurboID biotin conjugation on tubulin. Similarly, since tyrosine can also undergo various PTMs, these modifications could affect APEX2 labeling efficiency on other ciliary targets.

4.1.3 Complementing Strengths of the Proximity Labeling Methods

In my hands, APEX2 offered high specificity, low background noise, and superior temporal resolution compared to TurboID (**Fig. 11**). However, combining APEX2 and TurboID could provide complementary proteomic insights, as TransitID (TRafficking ANalysis by Sequentially Incorporated Tags for IDentification) has shown to some extent (Xu *et al*, 2023). This method allowed for the mapping of dynamic protein trafficking in living cells by performing two PL reactions in tandem using biotin as substrate for TurboID and a phenolic compound with a chemical linker as substrate for APEX2. By employing alkyne tyramide mediated click chemistry and fluorescein-based affinity chromatography, endogenous biotin-containing proteins could be mitigated and at the same time enabled the detection of dual-labeled proteins. After performing tryptic digestion, fluorescein-labeled and biotinylated peptides could be separated. The use of the flowthrough containing the biotinylated peptides in a subsequent second chromatography would allow the isolation of the other truly labeled peptide population from the ciliary proteome. Combining the two distinct enriched peptide populations could ensure precise identification of labeled peptides while minimizing background noise. This approach could improve both coverage and specificity in proteomic analysis by utilizing the different amino acid preferences of the reactive species involved in the PL reactions.

4.1.4 *In vivo* Capabilities of APEX2 and TurboID

Proximity labeling studies gain traction in *in vivo* studies due to differences observed in biotinylation patterns obtained from mouse tissue compared to mouse-derived cell lines (Kim *et al*, 2021).

TurboID, while exhibiting strong PL activity in both cell culture systems and *in vivo* models, has been associated with background labeling even before additional biotin is introduced (May *et al*, 2020; Kubitz *et al*, 2022). This basal biotinylation, driven by free biotin in commercial culture media, can impair the function and detection of adjacent bait proteins, potentially through direct biotinylation or disruption of cellular homeostasis (Garloff *et al*, 2023). Furthermore, the enzyme's basal activity can deplete endogenous biotin in expressing cells, negatively affecting organismal development (Branon *et al*, 2018; Oura *et al*, 2022; Kanzler *et al*, 2022; Garloff *et al*, 2023). To mitigate these effects, restricting exogenous biotin in culture media or food supplementation is essential when using TurboID in *in vivo*

models, as uncontrolled biotin levels complicate the identification of true positive candidates in complex systems.

In contrast, APEX2 has been utilized in mice to study various spatial proteomes (Liu *et al*, 2020; Dumrongprechachan *et al*, 2021; Hobson *et al*, 2022) and to map neurons by electron microscopy (Joesch *et al*, 2016; Zhang *et al*, 2019). The enzyme's rapid biotinylation effectively captures dynamic proteomic changes, but its reliance on exogenous H₂O₂ poses significant challenges for *in vivo* studies. While H₂O₂ serves as a signaling molecule at low levels, at supraphysiological levels the compound can cause oxidative distress (Sies, 2020). Of note, oxygenated room temperature medium was found to induce higher endogenous H₂O₂ production than medium at 37 °C, demonstrating the importance of handling the culture dishes promptly during *in cellulo* proximity labeling experiments to ensure minimizing H₂O₂-induced cellular responses (Eid *et al*, 2024).

4.2 Integration of D-Amino Acid Oxidase to APEX2 Systems Could Close the Gap to *in vivo* Labeling

The in this work presented iAPEX system addressed the limitation of APEX2 PL by incorporating cilia-localized D-amino acid oxidase (DAAO)-catalyzed *in situ* H₂O₂ production. DAAO oxidizes D-amino acids to their corresponding α -keto acids in the presence of FAD and molecular oxygen (O₂), producing equimolar concentrations of H₂O₂, α -keto acid, and ammonium (NH₄⁺) (Pollegioni & Molla, 2011; Kiss & Ferenczy, 2019). In the case of D-alanine (D-Ala), oxidation by DAAO yields pyruvate and ammonium as co-products, which have been postulated to only have a negligible effect on already abundant intracellular levels in cells, whereas a striking fold increase in intracellular levels of H₂O₂ is observed (Cooper & Plum, 1987; Sorrentino *et al*, 2020; Ghaffari Zaki *et al*, 2021; Kalinichenko *et al*, 2023).

This localized and controlled D-amino acid-dependent H₂O₂ generation allowed for precise cilia-APEX2-mediated labeling (**Fig. 15**), significantly improving specificity bypassing off-target oxidation reactions caused by H₂O₂-scavenging endogenous heme peroxidase (Davies *et al*, 2008). These peroxidases can compete with APEX2 for labeling reagents (e.g., BT and exogenous H₂O₂) supplied in excess to the cell culture media (Martell *et al*, 2017; Mick, 2019; Han *et al*, 2019; Tan *et al*, 2020; Eid *et al*, 2024). These observations are consistent with my findings, where certain cell types (e.g., NIH/3T3, 3T3-L1, and C2C12) previously inaccessible to APEX2 showed off-site labeling that interfered with the localized labeling of

primary cilia, in contrast to the unproblematic labeling seen in IMCD3 cells. Additionally, I could observe a wave-like pattern distribution of fluorescent resorufin in the Amplex Ultra-Red live-cell labeling reactions catalyzed by exogenously added H_2O_2 (**Fig. 22**). To reduce background in imaging, Martell *et al* (2017) recommended pre-treatment with 10 mM H_2O_2 to inhibit endogenous peroxidase activity before APEX2 labeling, taking advantage of the enzyme's enhanced resistance to H_2O_2 (Lam *et al*, 2015).

The Amplex UltraRed oxidation assay has a long and well-documented history as a sensitive peroxidase-based method for detecting H_2O_2 in various biological systems (Komlodi *et al*, 2021). This method was first introduced in 1997, where it was employed as a highly sensitive fluorescent micro-assay to measure H_2O_2 release from activated human leukocytes (Mohanty *et al*, 1997). The assay's utility was further demonstrated in 2006, when it was adapted for screening DAAO inhibitors (Brandish *et al*, 2006). In this application, differently cell-permeable inhibitors were screened for intracellular DAAO-catalyzed H_2O_2 production which was sensed by extracellular horseradish peroxidase (HRP) through oxidation of Amplex to resorufin. This allowed a direct readout for inhibitor efficacy and permeability—essentially utilizing a peroxidase as a tool for direct H_2O_2 detection. In 2017, Martell *et al* (2017) used the Amplex oxidation to specifically visualize the localization of APEX2 activity within cells. Of note, to achieve accurate distinction of localized activity, both the cells and reagent mixes had to be kept cold, as the oxidized product's fluorescence could diffuse away from the site of activity or even leak from the cells (Martell *et al*, 2017; Han *et al*, 2019); an observation I also made during microscopy of living cells when room temperature solutions were used, but which could be circumvented by using cold buffers.

More recently, Eid *et al* (2024) introduced APEX2 as a novel sensor for directly measuring H_2O_2 fluctuations using plate reader-based assays, with Amplex or luminol fluorescence serving as direct readouts of H_2O_2 levels. In this context, the fluorescence produced was cytosolic, allowing for a broader measurement of H_2O_2 turnover. Notably, the authors reported a significant increase in Amplex-based H_2O_2 turnover by APEX2 when cytosolic peroxiredoxins Prx1 and Prx2 were depleted, highlighting the impact of endogenous scavengers on H_2O_2 detection. Additionally, the thioredoxin reductase (TrxR) inhibitor auranofin selectively enhanced APEX2-catalyzed H_2O_2 turnover in cell types with high endogenous TrxR levels, while no change was observed in cell lines with low TrxR expression. This suggests that cell type-specific profiles of endogenous H_2O_2 scavenging proteins may influence APEX2 labeling efficiency.

4.2.1 Hydrogen Peroxide-induced Peroxidase Inhibition

Previous research has shown that wild-type pea APEX is highly sensitive to H_2O_2 inhibition, with both reversible and irreversible forms documented (Nakajima & Yamazaki, 1987; Arnao *et al*, 1990; Hiner *et al*, 2000; Lam *et al*, 2015). Likewise, horseradish peroxidase (HRP), a mechanistically similar peroxidase to APEX (Škulj *et al*, 2024), was shown to be inactivated through three primary pathways: reaction with excess H_2O_2 (Chance, 1949; Hiner *et al*, 1996; Morales-Urrea *et al*, 2023), occlusion by polymeric products (Nakamoto & Machida, 1992; Morales Urrea *et al*, 2018), and heme destruction by reactive radicals (Ator & Ortiz de Montellano, 1987; Ator *et al*, 1987; Mao *et al*, 2013).

Irreversible inhibition involves covalent modification of the heme, while reversible inhibition traps the heme cofactor in a catalytically inactive state slowing down the oxidation rate (Valderrama, 2010). Kinetic experiments performed by Lam *et al* (2015) revealed that APEX was prone to reversible and irreversible inhibition, whereas APEX2 due to its A134P mutation showed to be susceptible only to the latter. Under conditions of rapid phenolic compound oxidation (1 mM H_2O_2 and 1.4 mM guaiacol) over longer timescales (3-6 minutes), both enzymes eventually ceased activity, with attempts to reverse APEX2 inhibition by further substrate addition proving unsuccessful. Nevertheless, APEX2 has been shown to be resistant to reversible H_2O_2 inhibition beyond 5 mM H_2O_2 . Notably, pre-treatment of APEX or APEX2 with a brief burst of high H_2O_2 (10 mM for 1 minute) did not reduce their subsequent activity (Lam *et al*, 2015).

This is in line with the data I presented, where 3 minutes of labeling with exogenously added H_2O_2 peaks at 3 min, whereas APEX2 exhibits no increase in signal by the 9-minute mark (**Fig. 9**). In the iAPEX setup, APEX2 did not exhibit clear inhibition by prolonged exposure to the substrates (**Fig. 24**), though longer exposure to H_2O_2 prior to BT addition led to overall decreased biotinylation potentially due to slowed down APEX2 enzyme activity (**Fig. 23**). The close proximity of the iAPEX enzymes might further risk the irreversible inactivation of APEX2 in the presence of substrate abundance over longer time which might lead to overall decreased labeling turnover (**Fig. 24**). Notably, the amount of H_2O_2 produced by DAAO in NIH/3T3 cells has been shown to not exceed 50-100 nM when using D-Alanine as substrate. Here, the available O_2 was suggested to be the limiting factor for H_2O_2 production with concentrations in cell culture media at 200 μM and even less available O_2 in live

animals (13-15 μM) (Matlashov *et al*, 2014). Altered kinetics of either enzyme due to phenoxyl radical damage cannot be ruled out (Kang & Rhee, 2022).

4.2.2 D-Amino Acid-dependent DAAO Kinetics

In my study, I could demonstrate that APEX2 proximity labeling (PL) can be linked to nearby H_2O_2 production catalyzed by D-amino acid oxidase (DAAO), which in turn is influenced by the choice of DAAO substrate and its concentration (**Fig. 19** and **Sup.-Fig. 6**), as well as the timing of substrate addition in relation to iAPEX substrates (**Fig. 23** and **Fig. 24**). The timing and order of substrate incubation significantly impacted the biotinylation efficiency in the iAPEX system. For instance, a short pre-incubation with biotin tyramide (BT) before D-methionine (D-Met) addition resulted in enhanced labeling efficiency, though longer pre-incubations negatively affected the overall signal produced (**Fig. 23**). Longer D-Met pre-incubations on the other hand led to stronger decrease in obtained labeling, potentially due to slowing down the APEX2 kinetic as discussed before.

The stereoselectivity of DAAO, combined with its amino acid promiscuity, enables the *in situ* generation of H_2O_2 from a broad range of D-amino acids, offering strict control over the labeling process. The choice of D-amino acids plays a critical role in modulating DAAO-catalyzed H_2O_2 production within the cell. In mammalian cells, amino acid transporters are broadly distributed across subcellular spaces, including the plasma membrane (Palacín *et al*, 1998), inner mitochondrial membrane (Monné *et al*, 2019), and lysosomal membranes (Pisoni & Thoene, 1991; Bröer & Gauthier-Coles, 2021). In my work, D-methionine (D-Met) outcompeted D-alanine (D-Ala) as DAAO substrate in IMCD3 primary cilia APEX-based PL, since concentrations as low as 250 μM could produce a specific ciliary biotinylation signal. At 1 mM the D-Met concentration showed significantly higher biotinylation compared to much higher D-Ala concentrations (**Fig. 19** and **Sup.-Fig. 6**). In line with my findings, D-Met has been shown to produce significantly faster and more robust H_2O_2 sensor signals in multiple cancer and endothelial cell lines compared to the same concentration (1 mM) of other D-amino acids, which generated lower and slower signals (Erdogan *et al*, 2021). A significant benefit of using D-Ala as a substrate for DAAO is that its oxidative deamination produces pyruvate, a metabolite that is readily processed by cellular metabolic pathways (Dixon & Kleppe, 1965; Molla *et al*, 2012). Additionally, D-Ala has been shown to influence gluconeogenesis in the murine kidney by modulating the circadian rhythm (Sakai *et al*, 2024). In contrast, the oxidation of D-Met generates 2-keto-4-methylthiobutyric

acid (KMB or Keto-Met) (Gordon, 1965) the α -oxo analogue of methionine, which can be transaminated (by transaminases or amino acid donors) into L-Met, providing a usable methionine source for the organism (Langer, 1965; Scislowski & Pickard, 1993; Becquet *et al*, 2023).

It was demonstrated that transmembrane transport of D-amino acids can be a rate-limiting factor for DAAO activity, influenced by the specific amino acid and the potential competition from L-amino acids for uptake (Erdogan *et al*, 2021). When comparing two hydrophobic amino acids at a 5 mM concentration, D-phenylalanine did not limit DAAO activity, in contrast to D-Ala (Den Toom *et al*, 2023). Furthermore, H₂O₂ production was found to be D-Ala concentration-dependent, with detectable levels starting at 1-2 mM in NIH/3T3 cells and at 4 mM in HeLa Kyoto cells (Matlashov *et al*, 2014). This suggests that different cell lines may have varying D-Ala uptake rates, which could explain the differences in sensor response. Increasing the D-Ala concentration further led to more cells responding, but did not consistently elevate the rate of H₂O₂ production. Above 4 mM D-Ala concentration in NIH/3T3 cells, Matlashov *et al* (2014) observed that the H₂O₂ production was slightly inhibited and hypothesized that this could be due to substrate-dependent inhibition of the DAAO enzyme. *In vitro* experiments demonstrated a competitive inhibitory effect of the reaction product, α -keto acid, on enzyme activity for both immobilized *Rhodotorula gracilis* DAAO (RgDAAO) (Butò *et al*, 1994) and pig kidney-purified DAAO (Moreno *et al*, 1996). This inhibition was observed when the product was not gradually removed, and no additional substrate was added. However, it is important to note that the α -keto acids were tested at concentrations higher than those typically achieved through catalysis (Butò *et al*, 1994).

For future proximity labeling based experiments, I found that either simultaneous incubation of both substrates or short pre-incubation of BT before D-Met maximized biotinylation signals, with an optimal time frame of 10-15 minutes to reach comparable levels achieved by exogenously added H₂O₂ for 3 min (**Fig. 23** and **Fig. 24**).

4.2.3 Does Proximity Labeling Benefit from DAAO proximity to APEX2?

By positioning DAAO in proximity to APEX2, I demonstrated that the iAPEX system is a versatile tool for labeling specific cellular sub-proteomes, such as the primary cilium (for model see **Fig. 16**; **Fig. 18**). To explore the role of enzyme proximity in this system, I tested both a fusion construct of APEX2 and DAAO, as well as a setup where the enzymes were

localized separately within the cilia (**Fig. 17**). Interestingly, the APEX2-DAAO fusion construct exhibited decreased labeling efficiency compared to the configuration where the enzymes were separated but still localized within the cilia. Despite the reduced efficiency in the fused setup, both configurations were able to achieve specific compartmentalized biotinylation in response to substrate incubation (**Fig. 24**).

In contrast, when DAAO was localized cytosolically with APEX2 still residing in cilia (control-iAPEX), it failed to produce distinguishable ciliary signals in immunofluorescence (**Fig. 21**). Though ultimately by mass spectrometry, some enrichment of ciliary proteins was observed with cytosolic DAAO, likely due to partial diffusion of H₂O₂ (**Fig. 27** and **Fig. 28**). To explore the H₂O₂ diffusion inside cells, Eid *et al* (2024) used APEX2 as a sensor for DAAO-generated H₂O₂. The authors showed that the presence of competing H₂O₂ scavengers (e.g., peroxiredoxins) restricted the diffusion radius of H₂O₂ in the cell. Therefore, a system was tested in which the “transmission” range of H₂O₂ could be chemically changed by dimerization of the participating enzymes. The chemical approximation of the enzymes allowed an increase in the detected H₂O₂ signal, responding to lower levels of H₂O₂ compared to separate enzymes. In this context, the non-proteinogenic amino acid D-norvaline, an isomer of valine, was utilized as a potent substrate to activate DAAO-catalyzed chemogenetic H₂O₂ production in CA1 pyramidal neurons in acute brain slices (Kalinichenko *et al*, 2023). In these neurons, the application of D-Ala caused less effective transmission of excitatory signals. Using higher amounts of D-norvaline increased H₂O₂ generation rates to the point that non-dimerized (distal) enzymes exhibited a response (Eid *et al*, 2024). My data suggested that indeed titrating the D-amino acid concentration for H₂O₂ production is key for regulating the APEX2-mediated response and that selection of an optimal D-amino acid can enhance the resulting labeling (**Fig. 19** and **Fig. 23**).

Comparing the proximity dependence of enzymes in fluorescence-based assays with proximity labeling presents a challenge. In Amplex-based fluorescence assays, the oxidized fluorescent dye diffuses away from the APEX2 active site and is eventually released from the cell body (Martell *et al*, 2017). However, in H₂O₂ production coupled with vicinal proximity labeling, the generated radical species may potentially affect the enzymes themselves by covalent attachment to key amino acid residues. The attachment could reduce or even diminish enzyme activity through induced conformational changes or steric hindrance (Hawkins & Davies, 2001).

4.2.4 DAAO Active Site

Rhodotorula gracilis D-amino acid oxidase (RgDAAO) and mammalian DAAO orthologues, while sharing three conserved residues in their active sites (namely, Tyr223, Tyr238, and Arg285) (Boselli *et al*, 2004), exhibit significant kinetic and structural differences (Boselli *et al*, 2007; Pollegioni *et al*, 2007). RgDAAO lacks an active site lid, relying instead on the Tyr238 side chain for substrate interaction, leading to broader substrate specificity and superior catalytic efficiency compared to mammalian DAAOs (Boselli *et al*, 2002; Pollegioni *et al*, 2002; Sacchi *et al*, 2006). Specifically, RgDAAO has a much higher turnover rate (k_{cat}) and lower Michaelis constant (K_m), even for bulky substrates, due to its faster substrate-product exchange (Pollegioni *et al*, 1993, 2007). In contrast, mammalian DAAOs are limited by a slower product release step (Porter *et al*, 1977; Pollegioni *et al*, 2007). These structural and kinetic differences have practical implications for the iAPEX system, which utilizes DAAO-catalyzed oxidation for APEX2-mediated proximity labeling. The fusion of DAAO with APEX2 resulted in observed reduced labeling activity (**Fig. 24**). A possible explanation could be the premature formation of phenoxyl radicals by APEX2 induced by endogenous H_2O_2 and the subsequent labeling of the tyrosine Tyr238 in the active site of DAAO, which could slow down or even inhibit further H_2O_2 production. This is further reflected by the ability of the fusion construct to biotinylate proximal proteins albeit with a slowed down trend considering the multiple time points tested (**Fig. 24**). It should be noted that no DAAO peptides could be detected in the conducted mass spectrometric analyses, whereas 40 unique APEX2 peptides could be identified in the IMCD3 dataset.

Even in the event of DAAO active site tyrosine labeling, which could potentially hinder amino acid catalysis, H_2O_2 production may only slow down rather than cease entirely. To mitigate the risk of covalent modification of active site tyrosines, substrate incubation should either be simultaneous or involve the sequential addition of D-amino acid followed by BT, allowing sufficient substrate binding. A brief incubation with D-Met prior to BT addition did not significantly alter the labeling output compared to the reverse order in the separate enzymes (**Fig. 23**). Another possible explanation for the observed lower labeling output could be the reduced stability of the bulky fusion construct or steric hindrance due to insufficient linker spacing between the two enzymes (Chen *et al*, 2013).

4.2.5 Mammalian D-Amino Acid Oxidases

For this study, we utilized *Rhodotorula gracilis* D-amino acid oxidase (RgDAAO), but it is important to consider background DAAO activity, as DAAO is present across a wide range of organisms, including fungi, mollusks, insects, fish, amphibians, reptiles, birds, and mammals (Pollegioni *et al*, 2007). In mammals, DAAO is predominantly expressed in the kidney, liver, and brain, with lower levels in the small intestine and neutrophilic leukocytes (Pollegioni *et al*, 2007; Koga *et al*, 2017; Pollegioni *et al*, 2018b). The kidney, particularly in mice, is the most DAAO-rich organ, with significant enzyme activity localized in the proximal tubule cells (Koibuchi *et al*, 1995). Notably, mouse liver is an exception, where DAAO is absent (Konno *et al*, 1997). To date, multiple mouse strains lacking DAAO activity have been established and utilized in various studies. The identification of the *ddY/dao*^{-/-} mouse strain, which expresses an inactive DAAO variant due to a G181A substitution, has been pivotal in understanding the enzyme's physiological roles (Konno & Yasumura, 1983; Du *et al*, 2018). Mutant mice exhibited abnormally high levels of D-amino acids in their urine, suggesting that DAAO in the liver, kidney, urinary apparatus, and colon is crucial for eliminating these amino acids.

For effective future proximity labeling using the iAPEX system in the brain, understanding the role of endogenous DAAO in the central nervous system (CNS) is crucial. DAAO has been shown to catabolize endogenous D-serine (D-Ser), a neuromodulator and N-methyl-D-aspartate receptor (NMDAR) co-agonist essential for neural circuit development (Hashimoto *et al*, 1993; Strick *et al*, 2011; Sacchi *et al*, 2018; Szilágyi *et al*, 2018). D-Ser distribution in the CNS closely mirrors DAAO expression in both rodents and humans (Sacchi *et al*, 2018). According to the "serine shuttle" model, D-Ser is primarily produced in neurons, then shuttled to astrocytes, where DAAO regulates its concentration, indirectly influencing NMDAR activation (Wolosker, 2011). Notably, the activity of mammalian DAAO is finely regulated by its interaction with the protein pLG72, which serves as a negative effector to maintain D-Ser levels in the brain (Sacchi *et al*, 2008; Pollegioni *et al*, 2018a; Murtas *et al*, 2022).

4.3 Primary Cilia Proteomic Differences in IMCD3 and NIH/3T3 Cells

The iAPEX system identified a core set of 191 shared ciliary proteins across both IMCD3 and NIH/3T3 cell lines, highlighting a common ciliary proteome. However, significant cell-type-specific differences were observed between the ciliary proteomes of these two cell lines (**Fig. 32** and **Sup.-Fig. 16**). Proteins unique to IMCD3 cells were predominantly associated with microtubule organization, cell cycle regulation, and cytoskeletal processes, whereas those unique to NIH/3T3 cells were enriched in extracellular matrix organization, cell adhesion, and notably, collagen-activated tyrosine kinase receptor signaling. The observed differences in the ciliary proteomes between IMCD3 and NIH/3T3 cells likely stem from the intrinsic characteristics and functional diversity of these cell types.

NIH/3T3 fibroblasts, as mesenchymal cells, engage in extracellular matrix component production (e.g., collagen types I and III) and tissue remodeling—processes that are fundamental to defining the architecture and topography of tissues and organs (Rahimi *et al*, 2022). The iAPEX analysis of the NIH/3T3 ciliary proteome showed enrichment in various collagen types (**Tab. 5**), which aligns with the role of these cells in extracellular matrix production. Type I collagen, in particular, has been shown to promote the elongation of primary cilia in 3T3-L1 adipocyte cells, a subtype of 3T3 fibroblasts, by down-regulating autophagy through the reduction of histone deacetylase 6 (HDAC6) activity (Xu *et al*, 2018). Additionally, type I collagen facilitates the nuclear translocation of Yes-associated protein (YAP), a key effector in the Hippo signaling pathway. This process promotes ciliary elongation by upregulating ciliogenesis-associated proteins such as IFT88 and KIF3a. The YAP-mediated elongation of primary cilia is crucial for effective cell migration, as silencing YAP or IFT88 diminishes both ciliary length and migration efficiency (Xu *et al*, 2019).

Additionally, fibroblasts like NIH/3T3 are key players in wound healing, a process requiring significant cellular rearrangement and matrix remodeling (Cialdai *et al*, 2022). Further, NIH/3T3 cells have the potential to differentiate into multiple lineages, including adipogenic, chondrogenic, and osteogenic pathways, although modified variants like Flp-In-3T3 cells showed a lack of differentiation potential (Dastagir *et al*, 2014). Importantly, these cells are highly heterogeneous, derived from whole mouse embryos (Todaro & Green, 1963), and can give rise to diverse subtypes with specialized functions within a single population (Rahimi *et al*, 2022).

In the developing aortic valve, primary cilia are observed on mesenchymal cells. These cilia are gradually lost as the cells differentiate into fibroblast-like cells responsible for collagen secretion. Using *Ift88* conditional knockout mice, it has been shown that loss of primary cilia in endocardial cells leads to excessive deposition of extracellular matrix (ECM), including overproduction of collagen I and the proteoglycan versican, resulting in a disrupted collagen structure and a degenerative, weakened valve phenotype. Therefore, cilia may serve to restrict the expression of the ECM, which affects the differentiation of aortic valve mesenchymal cells in a temporal manner (Toomer *et al*, 2019).

In contrast, IMCD3 cells are a valuable model to study the physiology of the inner medullary collecting duct (IMCD), particularly in relation to the mechanosensory functions of primary cilia. In organs like the kidney, liver, and pancreas, each epithelial cell carries a single primary cilium that acts as a mechanosensor, bending elastically under fluid flow (Roth *et al*, 1988; Schwartz *et al*, 1997). This bending is thought to influence epithelial cell proliferation and autophagy, essential for maintaining tissue architecture and physiological functions, though the underlying signaling events remain enigmatic (Delling *et al*, 2016; Morleo *et al*, 2023). Derived from an SV40 transgenic mouse, these cells retained key characteristics of the IMCD, including high transepithelial resistance and the ability to facilitate sodium transport. Notably, IMCD3 cells were shown to adapt to hypertonic environments, tolerating osmolalities up to 910 mosmol/kg H₂O by accumulating protective osmolytes such as inositol and sorbitol under osmotic stress (Rauchman *et al*, 1993; Sun *et al*, 1998).

Further analysis of the unique IMCD3 proteins led to the identification of potential primary cilia candidates (**Tab. 3**), whose ciliary localization was confirmed in IMCD3 cells (**Fig. 29**). Interestingly, some of these proteins were either absent in the NIH/3T3 dataset or did not show high enrichment (**Tab. 4**). In the following chapters, I will explore the structural properties, functions, and potential implications in cilia biology of the novel candidates that, for the first time, have shown primary cilia localization in IMCD3 cells.

4.3.1 Novel Candidate Proteins in IMCD3 Primary Cilia

Uncharacterized Protein (C19ORF44 homolog)

To date, the localization and function of the uncharacterized protein C19orf44 homolog (UniProt Accession Q922C1) remain unclear. A cancer-focused sub-interactome study of chromosome-specific open-reading frame genes has suggested that the C19orf44 homolog

may modulate spermatogenesis by disrupting its interaction with the testis-specific enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and may also be involved in metabolic reprogramming (Ershov *et al*, 2023). My study was the first to associate the C19orf44 homolog with primary cilia, using cilia-specific proximity labeling and immunofluorescence to confirm the protein's basal body localization (**Fig. 29**).

Cytoskeleton Associated Protein 2 Like (CKAP2L)

Cytoskeleton-associated protein 2-like (CKAP2L), also known as radmis ("radial fiber and mitotic spindle"), is recognized as a protein involved in the regulation of the mitotic spindle apparatus and the proper segregation of chromosomes during cell division (Yumoto *et al*, 2013; Kwon *et al*, 2024). Although only 16% amino acid identity is shared with its paralogue, cytoskeleton-associated protein 2 (CKAP2), CKAP2L was found to exhibit similar biochemical and functional characteristics. Particularly its conserved 'Cytoskeleton-associated protein 2, C-terminal' domain contains phosphorylation sites that have been shown to be critical for mitotic regulation in CKAP2 (Kwon *et al*, 2024). Specifically residues Thr685 and Ser690 were targets for phosphorylation by Cdk1/cyclin B1 and Aurora B kinase during mitosis (Hong *et al*, 2009; Kim *et al*, 2010).

Kwon *et al* (2024) have shown that the expression of human CKAP2L is tightly regulated throughout the cell cycle, with its levels being observed to peak during the G2/M phase. In the study, high expression levels have been detected in rapidly proliferating tissues such as the testis, intestine, and spleen, and CKAP2L was shown to be consistently present across various human cancer cell lines (lung, liver and colon). During mitosis, CKAP2L localized to the mitotic spindle apparatus, where it directly participated in the organization of spindle microtubules and ensured accurate chromosome alignment and segregation. In contrast, during interphase, CKAP2L was primarily localized in the nucleus.

In a murine study, CKAP2L's critical role in neurogenesis has been further elucidated. In neural stem/progenitor cells, murine CKAP2L localized to mitotic spindles and radial fibers, facilitating proper cell division and neurodevelopment (Yumoto *et al*, 2013). Overexpression of CKAP2L in cell lines such as HEK293 and neuroblastoma cells has been shown to excessively stabilize microtubules, leading to aberrant spindle formation and mitotic arrest. Further, CKAP2L had to be precisely regulated to ensure proper cell division and prevent oncogenic processes as was shown by the use of a ubiquitination-inhibited mutant (KEN-box mutant), which drastically enhanced protein stability *in vivo* altering cell cycle progression.

Conversely, the depletion of CKAP2L has been observed to disrupt mitotic spindle organization and result in chromosome mis-segregation, underscoring its indispensable role in mitosis (Yumoto *et al*, 2013). Mutations in CKAP2L have been associated with causing Filippi syndrome, an autosomal recessive genetic disorder characterized by several severe neurological manifestations, including microcephaly, intellectual disability, and seizures (Hussain *et al*, 2014). Recently, CKAP2L was implicated to be strongly functionally related to DNA damage induced apoptosis suppressor (DDIAS) in generalized genetic epilepsies (Lara *et al*, 2024).

Moreover, CKAP2L has been identified as a prognostic marker in several human cancers, including esophageal squamous cell carcinoma (Chen *et al*, 2022a), hepatocellular carcinoma (Liu *et al*, 2019), and lung adenocarcinoma (Xiong *et al*, 2019; Monteverde *et al*, 2021). In knockdown studies, reduced CKAP2L expression has been demonstrated to induce G2/M phase arrest, impair cell proliferation, and diminish the migratory capacity of cancer cells (Wang & He, 2020; Zhu *et al*, 2021; Li *et al*, 2021b, 2022).

None of the previous studies have investigated the localization of CKAP2L in primary cilia. In this work, I could demonstrate that CKAP2L localizes to primary cilia (**Fig. 29**). As discussed in chapter 1.2, primary cilia play a crucial role in cell cycle regulation by serving as a checkpoint that restricts cell cycle entry by locking the centriole to the plasma membrane (Izawa *et al*, 2015). Given the role of CKAP2L in microtubule dynamics, there is a strong basis to explore its potential association with primary cilia, as both structures are critically dependent on microtubule integrity (Anvarian *et al*, 2019). During quiescence, cells are in a non-dividing, resting state, where the primary cilium serves as a signaling hub that maintains the cell in this phase by modulating various signaling pathways (Venugopal *et al*, 2020). If CKAP2L is restricted from the cell or sequestered within the primary cilium during quiescence, this could be a mechanism to ensure that CKAP2L's role in promoting spindle assembly and mitotic progression is tightly controlled. By keeping CKAP2L levels low or inactive during quiescence, the primary cilium could effectively prevent the cell from prematurely entering the cell cycle, thus maintaining the integrity of the quiescent state.

Primary cilia are disassembled as cells transition from the G0 phase into the G1 until G2/M phase, allowing the centrioles to function in spindle formation during mitosis (Patel & Tsiokas, 2021). As cells prepare to exit quiescence and enter the cell cycle, the disassembly of the primary cilium could release CKAP2L, allowing it to accumulate and peak during the

G2/M phase, where it would then facilitate proper mitotic spindle formation and chromosome segregation (Kwon *et al*, 2024). This regulatory mechanism would add another layer of control over CKAP2L activity, linking it directly to the status of the primary cilium and the cell's readiness to divide. The ciliary localization of CKAP2L raises intriguing questions about its possible role in coordinating cilia disassembly with the onset of mitosis. Given the established role of CKAP2L in microtubule organization and spindle assembly, it is plausible that CKAP2L could be directly or indirectly involved in the processes that govern cilia disassembly, ensuring that cells are prepared for accurate chromosome segregation during mitosis (Yumoto *et al*, 2013; Kwon *et al*, 2024). Further, if CKAP2L is involved in the regulation of primary cilia, or *vice versa*, their dysfunction could disrupt neurogenesis or neuronal migration, processes that are essential during brain development (Hussain *et al*, 2014; Youn & Han, 2018). For example, if CKAP2L levels are not properly regulated by primary cilia during neurogenesis, this could lead to abnormal cell cycle progression, resulting in neurodevelopmental defects.

CUE Domain Containing 1 (CUEDC1)

CUE domain-containing 1 (CUEDC1) is characterized by its name giving domain, which is primarily known for its ubiquitin-binding properties critical in the ubiquitin-proteasome system and cellular homeostasis (Lim *et al*, 2019). Collective findings on CUEDC1 have revealed tissue-specific roles across various cancer types with involvement in tumor progression, metastasis, and the regulation of key signaling pathways.

In hepatocellular carcinoma (HCC), CUEDC1 has been identified as an oncogenic factor that supports tumor growth, migration, and invasion (Wang *et al*, 2024). The knockdown of CUEDC1 in HCC cells led to significant inhibition of these malignant behaviors, which are mediated through the activation of the transforming growth factor (TGF)- β /SMAD signaling pathway (Peng *et al*, 2022). This suggested that CUEDC1 normally acts to suppress TGF- β /SMAD signaling, preventing cell cycle arrest and apoptosis, thereby promoting tumor progression. This oncogenic role is contrasted by the function of CUEDC1 found in non-small cell lung cancer (NSCLC), where it acted as a tumor suppressor (Cui *et al*, 2020). In NSCLC, CUEDC1 inhibited epithelial-mesenchymal transition (EMT) and metastasis by stabilizing SMURF2, an E3 ubiquitin ligase that targets TGF- β for degradation, thereby downregulating the TGF- β /SMAD signaling pathway (Bai & Ying, 2020).

In breast cancer, particularly in estrogen receptor alpha (ER α)-positive subtypes, CUEDC1 emerged as a critical downstream target of ER α signaling (Lopes *et al*, 2018). CUEDC1 was shown to be essential for ER α -positive breast cancer cell proliferation, with its expression being regulated by the CUEDC1 Transcriptional Enhancer (CUTE) situated within the first intron of the CUEDC1 gene. The dependency of these cancer cells on CUEDC1 highlights its role in mediating estradiol-driven oncogenic processes, where its ubiquitin-binding function likely supports the regulation of key proteins involved in cell cycle progression and survival.

The dual role of CUEDC1 as oncogene or tumor suppressor in tissue-specific cancer contexts underscores the cell type-dependent functions of CUEDC1, where its impact on tumor progression can vary significantly.

Given that CUEDC1 interacts with SMURF2 in NSCLCs to stabilize it, this interaction could extend to the regulation of Sonic hedgehog (SHH) signaling in the context of primary cilia, as CUEDC1 was found to localize it in my study (**Fig. 29**). SMURF2 has been shown to poly-ubiquitinate both Smoothened (SMO) and Patched (PTCH1) depending on the state of the pathway (Li *et al*, 2018). In Shh ligand-induced endocytic turnover SMURF2 mediated the internalization and ultimately degradation of PTCH1 (Yue *et al*, 2014). Further, ubiquitin modification has been shown to be a requirement for G-protein coupled receptor removal from primary cilia (Shinde *et al*, 2020). CUEDC1 might influence the ubiquitination and degradation of SHH pathway components, mediated by SMURF2, thereby affecting SHH signaling outcomes.

The reactivation of SHH signaling has been implicated in the pathogenesis of tissue fibrosis in various organs, including the kidney, where SHH is typically silenced in adults (Zhou *et al*, 2016). This aberrant Shh activity is associated with upregulation of the sensor receptor PTCH1 and transcription factors GLI1 and GLI2, contributing to the progression of chronic kidney disease (CKD) through mechanisms such as fibroblast activation and epithelial-mesenchymal interaction (Zhou *et al*, 2014; Kramann *et al*, 2015). Notably, significant upregulation of *CUEDC1*, among other genes, in the kidneys of patients with kidney dysfunction was demonstrated (Nguyen *et al*, 2020). The upregulation of *CUEDC1* in this context suggests its oncogenic potential in hyperactive Shh signaling pathway and its contribution to renal fibrosis and CKD progression.

A potential involvement of CUEDC1 in TGF- β signaling at the cilium could be discussed, given that the TGF- β receptor complex localizes to primary cilia tips and exits via clathrin-mediated endocytosis for SMAD activation (Clement *et al*, 2013; Christensen *et al*, 2017; Mill *et al*, 2023). This spatial regulation of TGF- β signaling at the cilium suggests that CUEDC1 might play a role in modulating the stability or trafficking of the TGF- β receptor complex or associated signaling components, thereby influencing SMAD activation and downstream signaling pathways critical in various cellular processes including proliferation, differentiation and migration (Hata & Chen, 2016).

Ubiquitin Carboxyl-Terminal Hydrolase MINDY-3 (Mindy3)

MINDY3 (also known as C10ORF97 or CARP (“CARD containing protein”)) is a member of the MINDY family of deubiquitinating enzymes (DUBs), which are characterized by their unique structural fold and specificity for K48-linked polyubiquitin chains (Mevissen & Komander, 2017). MINDY3, like other members of this family, functions as an exo-DUB, selectively cleaving ubiquitin units from the distal end of K48-linked polyubiquitin chains. This specificity for K48 linkages is crucial because these chains are typically associated with targeting proteins for degradation via the proteasome, a process fundamental to maintaining cellular proteostasis (Grice & Nathan, 2016).

Structurally, MINDY3 contains a catalytic domain that is distinct from other known DUBs, with no significant homology to other protein families. This catalytic domain relies on conserved cysteine and histidine residues, which are essential for its enzymatic activity. The enzyme's unique structure and mechanism were elucidated through crystallographic studies, which revealed that structurally related MINDY1 activity is dependent on the precise alignment of these residues within the active site, and that substrate binding induces conformational changes necessary for catalysis (Abdul Rehman *et al*, 2016).

MINDY3 has been identified as a tumor suppressor, particularly in the context of non-small-cell lung cancer (NSCLC) (Shi *et al*, 2011). The protein is ubiquitously expressed in various human tissues, especially heart, skeletal muscle, kidney and liver. Suppression of MINDY3 expression in mammalian cell lines favored proliferation, whereas overexpression in multiple cancer cell lines inhibited cell proliferation, induced G1 cell cycle arrest, and promoted apoptosis (Liu *et al*, 2002; Shi *et al*, 2011). These effects were mediated, at least in part, by its interaction with key cell cycle regulators and its ability to modulate their activity, such as the stabilization of p27 through inhibition of Jun-activating domain-binding protein-1

(JAB1)-mediated nuclear export (Shi *et al*, 2011). The role of MINDY3 in apoptosis is linked to its caspase-associated recruitment domain (CARD), which is essential for the protein's ability to trigger cell death pathways (Park, 2019).

While the localization of ubiquitin-related enzymes, such as E3 ligases and DUBs, to the basal body of primary cilia is well-established, their presence within the cilia itself remains a topic of ongoing debate. Despite this, the role of DUBs in the processes of cilia assembly and disassembly has gained increasing recognition (Hossain & Tsang, 2019; May *et al*, 2021b). Additionally, several ciliary proteins have been linked to apoptosis, although the precise mechanisms underlying this relationship are yet to be fully elucidated (Pfaffmann & Gerhardt, 2022). In this context, the ciliary localization of MINDY3, as demonstrated in this work (**Fig. 29**), highlights its potential as a candidate for dual functions in both ciliary dynamics and apoptotic regulation.

Protein Serine Kinase H1 (Pskh1)

The protein serine kinase H1 (PSKH1) was first characterized in a study by Brede *et al* (2000), where it was identified as a novel serine kinase with localization to the Golgi apparatus, centrosomes, and the nucleus. This protein shares significant sequence similarity with the CaM kinase family but notably lacks a clear calmodulin-binding domain, suggesting a unique regulatory mechanism. PSKH1 was shown to be ubiquitously expressed and its enrichment in testicular tissue hinted at a role in spermatogenesis or other testis-specific functions. Intriguingly, PSKH1 demonstrated dual fatty acylation shown to be essential for its proper subcellular localization. Specifically, PSKH1 undergoes myristylation at glycine 2 and palmitoylation at cysteine 3, which directs it to the Golgi apparatus. When palmitoylation is disrupted, PSKH1 mutants were observed to disassemble the Golgi apparatus, leading to the redistribution of Golgi-associated proteins into a diffuse cytoplasmic pattern. This finding underscores the critical role of PSKH1 in maintaining Golgi integrity and highlights its involvement in intracellular membrane dynamics.

Beyond its role in the Golgi, PSKH1 localizes to nuclear splicing factor compartments (SFCs) during interphase, where it was shown to interact with serine/arginine-rich (SR) proteins, key regulators of RNA splicing. The interaction was dependent on both the catalytic domain and C-terminal region of PSKH1. Although PSKH1 did not directly phosphorylate these SR proteins, kinase overexpression led to a reorganization of SR proteins within the

nucleus, suggesting a non-catalytic role in modulating RNA processing and splicing (Brede *et al*, 2002).

In a study by Li *et al* (2015), PSKH1 was implicated in laterality defects associated with congenital heart disease (CHD). These defects are linked to disruptions in ciliary function, a process critical for normal organ positioning during embryonic development.

The most recent insights into the function of PSKH1 emerged from a large-scale genomic study by Maddirevula *et al* (2024), which identified mutations in *PSKH1* as the cause of a novel hepatorenal ciliopathy characterized by pediatric cholestasis and kidney dysfunction. The mutations found in *PSKH1* disrupted the protein's kinase activity, leading to the formation of abnormally long cilia with delayed anterograde and retrograde transport. Patient-derived fibroblasts displayed these ciliary defects, and a *Pskh1* mutant mouse model successfully recapitulated the human disease phenotype, providing strong evidence for the protein's critical role in ciliary biology.

These findings together with my data, demonstrating ciliary localization of PKSH1 (**Fig. 29**), position the protein within the proteomic network that governs cilia-dependent developmental processes.

4.4 Future Perspectives

Looking ahead, the future of this project involves expanding the application of the iAPEX dual-component system across a broader range of tissues and cell types, enabling a comprehensive comparison of proteomic landscapes in different biological systems. This expansion could lead to the discovery of novel ciliary proteins and signaling pathways unique to specific tissues. The successful integration of the iAPEX system into previously inaccessible cell types via lentivirus-mediated delivery (**Fig.**) is a promising step towards development of a cross-tissue cilia protein atlas. Integrating quantitative TMTpro 32-plex-based ([Thermo Fisher 2024](#)) or MS-based quantification by isotope-labeled cell-free products (MS-QBiC) 35-plex-based proteomics to the established iAPEX workflow could offer enhanced multiplexed insights into cell type-specific differences and identifying new biomarkers or therapeutic targets in one mass spectrometry run (Li *et al*, 2021a; Masuda *et al*, 2022). Advancing the iAPEX system for potential *in vivo* applications could facilitate precise studies of primary cilia in living organisms, deepening our understanding of their roles in health and disease.

In this work, various iAPEX substrates were successfully applied in cultured cells. However, *in vivo* applications may present challenges, such as reduced proximity labeling efficiency due to reduced substrate cell permeability or cellular responses to substrates; as was observed for D-alanine in pyramidal neurons (Kalinichenko *et al*, 2023). Recent developments suggest that improved substrates, like the click-chemistry reagent alkyne-phenol for APEX2 (Li *et al*, 2020b; Xu *et al*, 2023) and the non-proteogenic amino acid D-norvaline for DAAO (Kalinichenko *et al*, 2023), could enhance the system's performance. Alkyne-phenol has shown reduced cellular off-site labeling background (Xu *et al*, 2023), while D-norvaline's non-proteogenic nature ensures minimal interference with cellular amino acid metabolism, even if it were to undergo enzymatic racemization (Kalinichenko *et al*, 2023).

The selection of appropriate control cell lines remains a subject of debate. In this work, the cilia-ablated *Cep164*^{-/-} cilia-APEX2 expressing cell line proved effective in demonstrating the enrichment of primary cilia proteins in cilia-iAPEX labeled samples (**Fig. 28**). However, establishing reliable and genetically verified *Cep164*^{-/-} lines for all target cells would be costly and impractical. Especially for living organisms, such a control would be hardly conceivable, unless certain KO models were used as a control group in parallel, which could be done using a conditional knockout mouse for CEP164 (Siller *et al*, 2017). In such cases, the stereoselectivity of DAAO with L-Met provides a suitable alternative, covering only the biotin-tyramide-induced basal protein background (**Fig. 31**). Attempts to use a cytosolic cilia-mislocalized DAAO with cilia-localized APEX were not successful, as cilia proteins were still labeled, complicating enrichment analysis (**Fig. 28**). Given that DAAO-mediated H₂O₂ production and “transmission” depends on substrate concentration (Eid *et al*, 2024), the aforementioned control could be used when previous substrate titration for each cell line (or organism) ensured dependence on enzyme proximity and therefore local restriction of labeling. Alternatively, a cytosolic APEX2 and ciliary DAAO could allow cellular background labeling, though this approach presents challenges due to potential cellular H₂O₂ scavengers, which could prevent H₂O₂ diffusion and ultimately labeling.

Additionally, the use of DAAO in hypoxic tissues could be problematic, as DAAO consumes oxygen for catalysis (Pollegioni & Molla, 2011; Den Toom *et al*, 2023). Recently, an engineered DAAO variant with reduced oxygen requirements was developed, offering a potential solution for using iAPEX in hypoxic tissues *in vivo* (Erdogan *et al*, 2021).

To this end, DAAO-catalyzed H_2O_2 production was not directly measured in my study due to the complexity of establishing a trimeric construct that includes chemogenetic H_2O_2 sensors, such as HyPer (Belousov *et al*, 2006; Smolyarova *et al*, 2022), for stable cell line generation. However, a recently developed assay by Den Toom *et al* (2023) offers a more straightforward approach to monitor DAAO-catalyzed H_2O_2 production. This assay utilizes the Seahorse XFe analyzer to measure the oxygen consumption rate upon the addition of D-amino acids, capitalizing on the fact that DAAO consumes equimolar amounts of O_2 for each oxidized D-amino acid molecule.

In conclusion, this work has laid the foundation for advancing proteomic studies of primary cilia through the development and application of the iAPEX dual-component system. The successful integration of iAPEX into previously inaccessible cell types and its potential expansion into *in vivo* applications represent significant strides toward a comprehensive understanding of ciliary biology across diverse tissues. While challenges remain, particularly in adapting the system for *in vivo* studies and selecting appropriate controls, recent advancements in substrate development and assay techniques offer promising avenues for overcoming these obstacles. This work marks a crucial step in the ongoing effort to unravel the complex roles of primary cilia in health and disease, setting the stage for future discoveries in this dynamically evolving field.

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VIII. Acknowledgment of Contributions

The following table provides a detailed overview of experimental transparency and highlights the specific contributions made by others to the various aspects of the research conducted in this thesis.

Contributor	Experimental Contribution	Fig.
Mick Lab		
Chaumet, Valerie	Constructed the Gateway-compatible cilia-APEX2 dual-expression destination vector “pVC08”, which was used to generate expression vectors for Flp-In mediated cell line generation in this study	Fig. 6
	Established ExM methodology in the lab and provided assistance in expanding and imaging IMCD3 and RPE-1 cilia-iAPEX cell lines	Fig. 18
	Assisted in the development and execution of the Amplex UltraRed-based live-cell imaging setup for visualizing cilia-APEX2 oxidation	Fig. 22
Sanwald, Lea K.	Assisted in different cloning steps of multiple vectors in the study, especially the construction of vectors used for the validation of primary cilia localization	Fig. 29
	Established the lentivirus-mediated cilia-iAPEX system integration using my constructed lentiviral vectors and performed the cell line characterizations	Fig.
Schuster, Patrick	Cloned the dual-expression vector “pPS0099” expressing cilia-APEX2 and ALFA-IFT88 for improved detection of enriched IFT88 after streptavidin chromatography in native settings	Sup.-Fig. 5
EMBL Proteomics Core Facility (PCF) – paid proteomics service		
Haberkant, Per	Processed and analyzed all chromatography eluates generated in this study using TMT-based LC/MS ³	Fig. 11 Fig. 28 Fig. 31
Stein, Frank	Conducted bioinformatic analysis and pre-processing of identified peptide signal intensities, prior to my re-analysis	

Personal Acknowledgments

I would like to express my deepest gratitude to my PhD advisor, Prof. Dr. David Mick, for his unwavering support, guidance, and encouragement throughout my doctoral journey. His expertise and insight in primary cilia biology have been truly inspirational, especially when I was riddled with challenges in the multifaceted APEX projects. The ongoing exchange of ideas and the reassurances he provided after numerous experimental setbacks have been invaluable in shaping both my research and academic growth. Since day one as part of the Mick Lab, Prof. Dr. David Mick has inspired me with his passion and commitment for science. Certainly, I will miss the infamous question asked during each lab meeting:

“Science?” - Prof. Dr. David Mick

Thank you, David, for your mentorship and for having the patience to allow me to pursue a project that my heart was so deeply committed to.

I would like to extend my heartfelt thanks to my fellow lab members at the Mick Lab, Schrul Lab and van der Laan Lab. Everyone, from technicians to PhD peers, have been simply amazing. Thank you for supporting me throughout the course of my thesis. Whether it was through experimental support, sharing knowledge, or simply brightening my day by sharing the most random things. Any daily mundane lab task has been a joy with you!

I want to thank all the people that allowed me to become a true researcher with heart and smart:



To all my friends that hold a special place in my heart, thank you for supporting me even through the toughest times. I will forever be grateful for the immense love and support I have gotten in one of the most challenging periods of my life. Without you I wouldn't have been able to finish this milestone.

I want to thank the friends that paved my PhD way, Valerie Chaumet, Elena May, Lea Sanwald, Shanli Seyed Tarrah, Luisa Tremmel and Patrick Schuster. You are true heroes in and out of lab!

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I want to thank my partner, Gian Andrea Inkof, for his incredible patience and tolerance with me and my project and for allowing me to be myself during a truly eventful phase of my PhD. Every moment together made it easier for me to see things working out.

Finally, I would like to thank my family, who grounded me during the most turbulent phases and gave me the strength to chase my dreams.

IX. Publications

The following table provides a detailed overview of experimental transparency and highlights the specific contributions

Research Article

iAPEX: Improved APEX-based proximity labeling for subcellular proteomics using an enzymatic reaction cascade (pre-print; submission to Nature Methods pending)

Tommy J. Sroka, Lea K. Sanwald, Avishek Prasai, Josefine Hoeren, Karina von der Malsburg, Valerie Chaumet, Per Haberkant, Kerstin Feistel, David U. Mick (2025)

bioRxiv 2025.01.10.632381

doi: <https://doi.org/10.1101/2025.01.10.632381>

A cAMP signalosome in primary cilia drives gene expression and kidney cyst formation

Jan N. Hansen, Fabian Kaiser, Philipp Leyendecker, Birthe Stüven, Jens-Henning Krause, Fatemeh Derakhshandeh, Jaazba Irfan, **Tommy J. Sroka**, Kenley M. Preval, Paurav B. Desai, Michael Kraut, Heidi Theis, Anna-Dorothee Drews, Elena De-Domenico, Kristian Händler, Gregory J. Pazour, David J. P. Henderson, David U. Mick & Dagmar Wachten
EMBO rep (2022) 23: e54315

doi: <https://doi.org/10.15252/embr.202154315>

Review Article

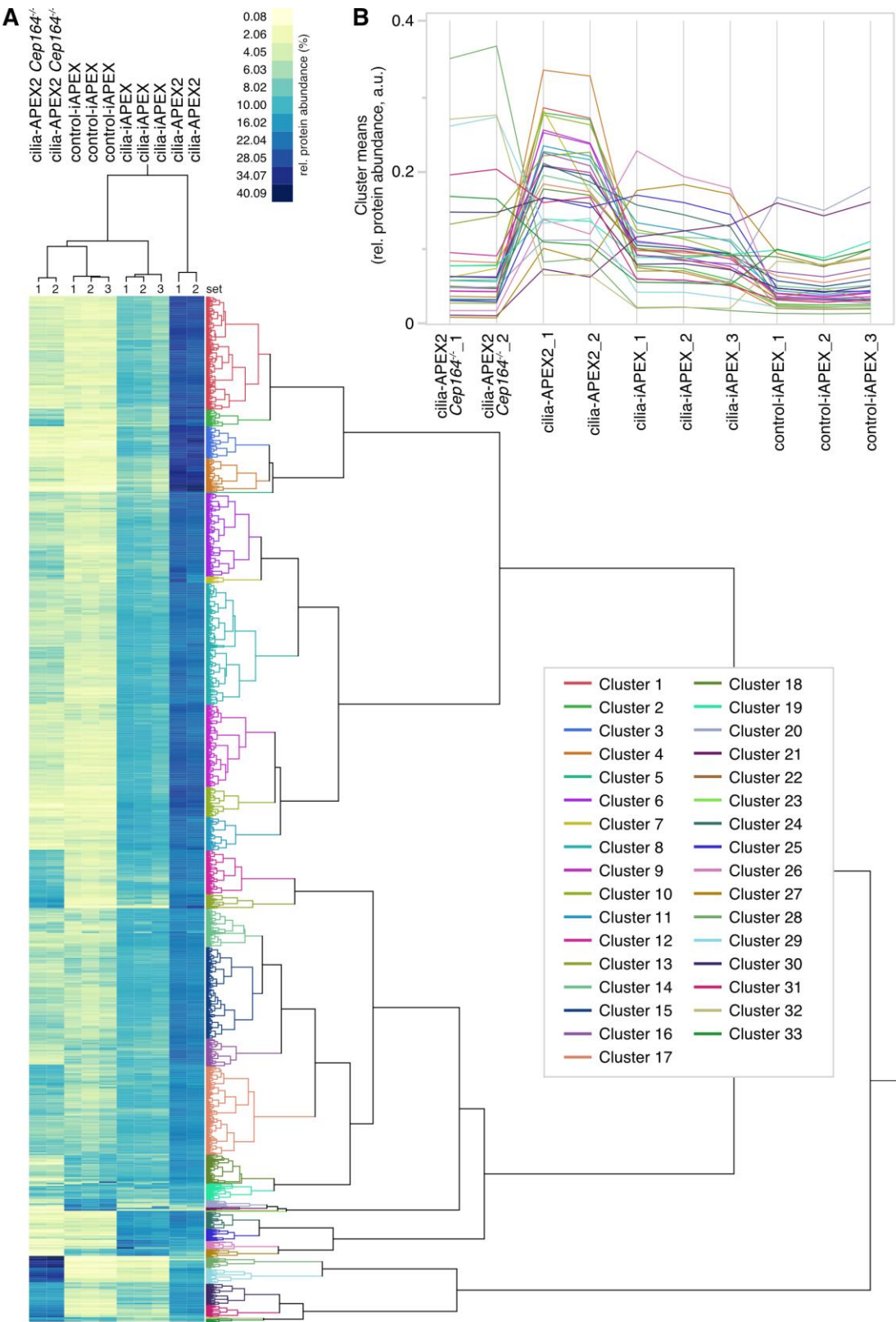
Phosphorylation and Ubiquitylation Regulate Protein Trafficking, Signaling, and the Biogenesis of Primary Cilia

Elena A. May, **Tommy J. Sroka** & David U. Mick

Front Cell Dev Biol. (2021) Apr 12:9:664279

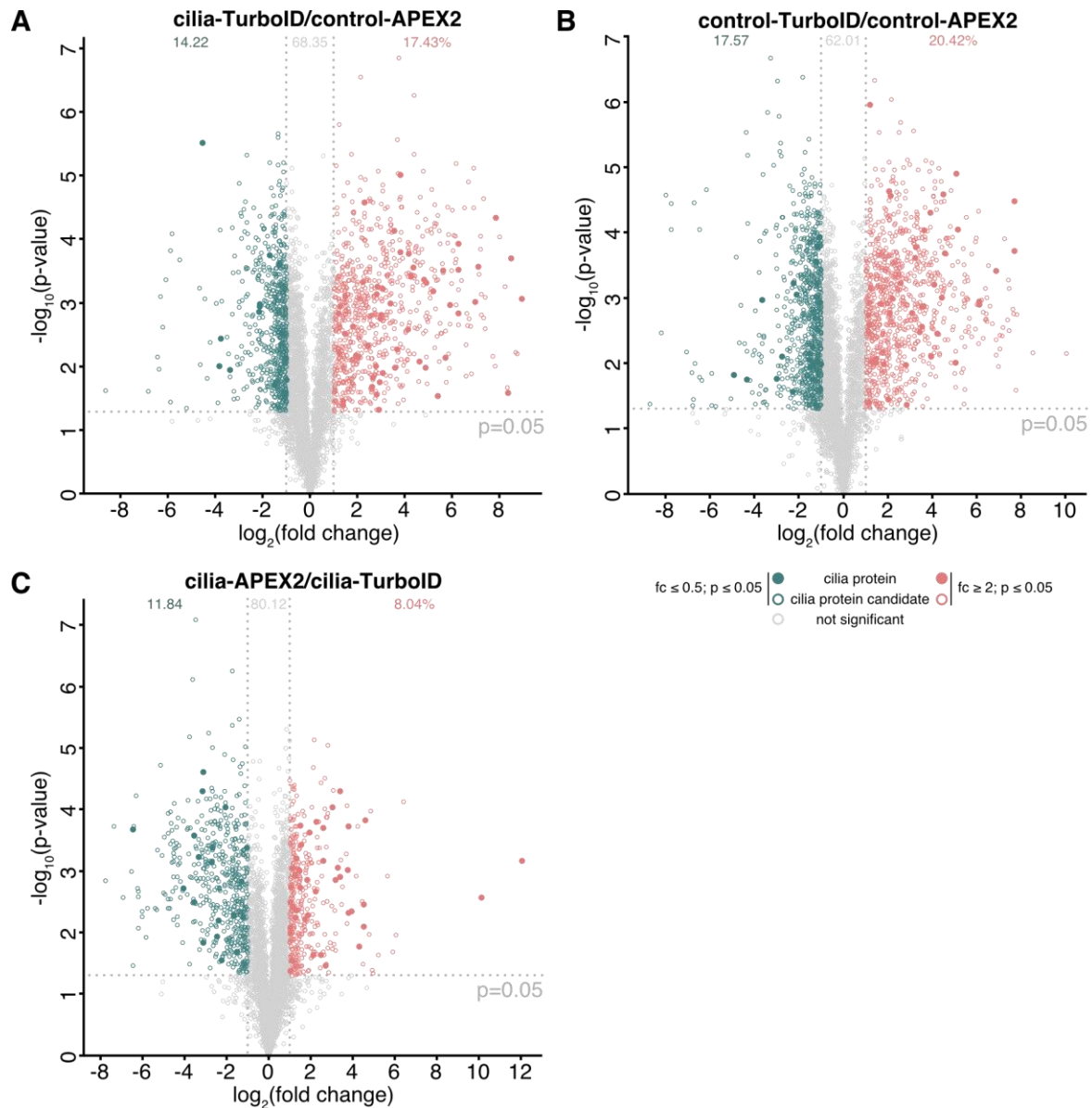
doi: <https://doi.org/10.3389/fcell.2021.664279>

X. APPENDIX



Sup.-Fig. 1: Extended hierarchical two-way clusters of conditional sets of quantitative primary cilia proteomics show high reproducibility of replicates.

(A) This supplementary figure shows the extended hierarchical clustering data presented in **Fig. 11A**. (B) Means analysis of the row dendrogram clusters highlights ciliary protein groups for cilia-APEX2 (notably clusters 6, 7, 14 and 25) and cilia-TurboID (notably clusters 4, 15, 19, 20, 21, 25, 28 and 29).



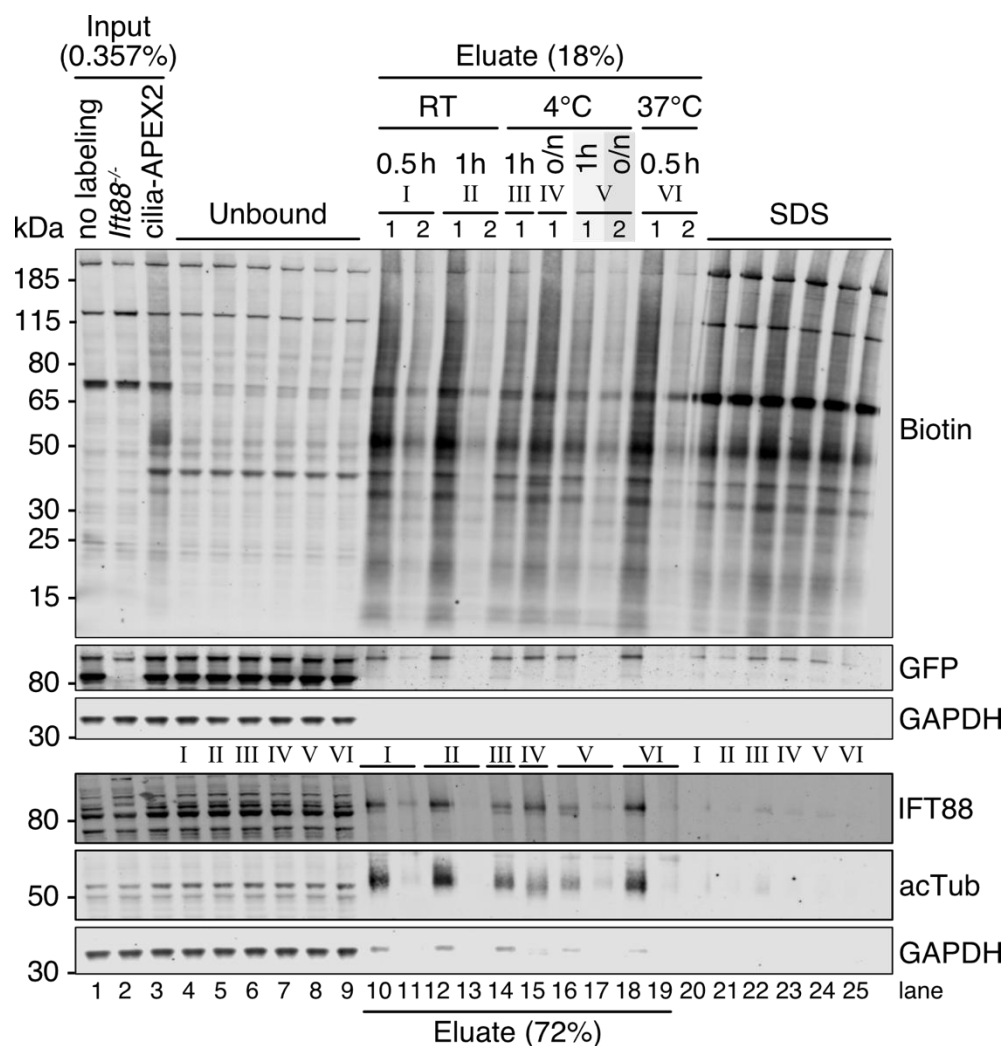
Sup.-Fig. 2: Control-TurboID creates a noisy cilia protein background that decreases mapping efficacy.

(A, B, C) This supplementary figure extends the data presented in Fig. **Fig. 11BC**, using the same representation and analysis methods.

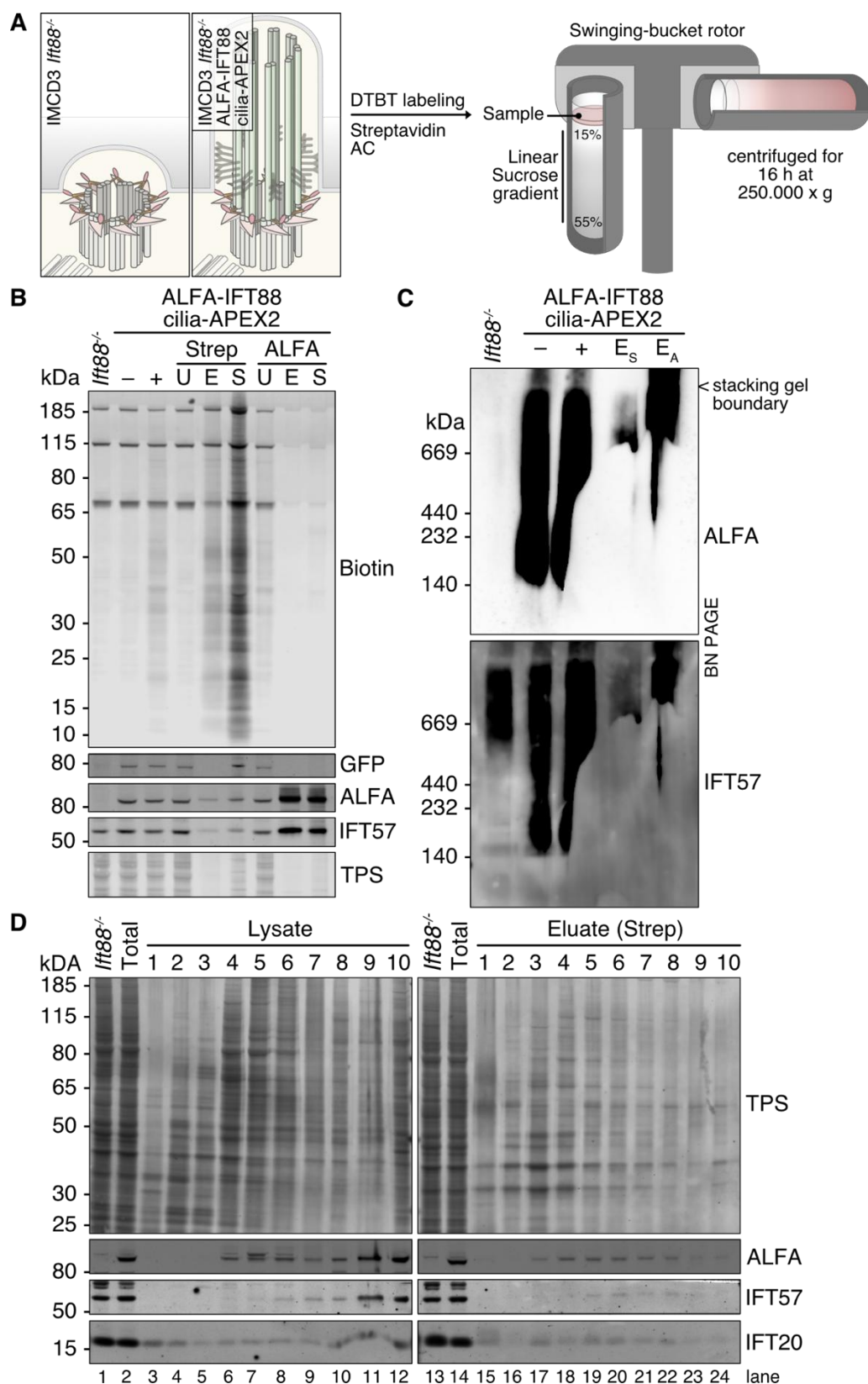


Sup.-Fig. 3: Gene Ontology (GO) enrichment analysis of unique and shared proteins from IMCD3 cilia-APEX2 and cilia-TurboID proteomics data.

Venn diagram sections from **Fig. 12**. The top section (cyan) represents 316 proteins unique to APEX2, the middle section (purple) indicates 38 proteins shared between both APEX2 and TurboID, and the bottom section (magenta) corresponds to 172 proteins unique to TurboID. The right panels display the GO enrichment analysis of the proteins in each section of the Venn diagram. The analysis covered biological processes, highlighting the most significant GO terms for each protein group. The length of the bars represents the GO term fold enrichment. The color intensity of the bars indicates different significance levels, with more intense colors corresponding to higher FDR values. The size of the dots represents the number of genes associated with each GO term.



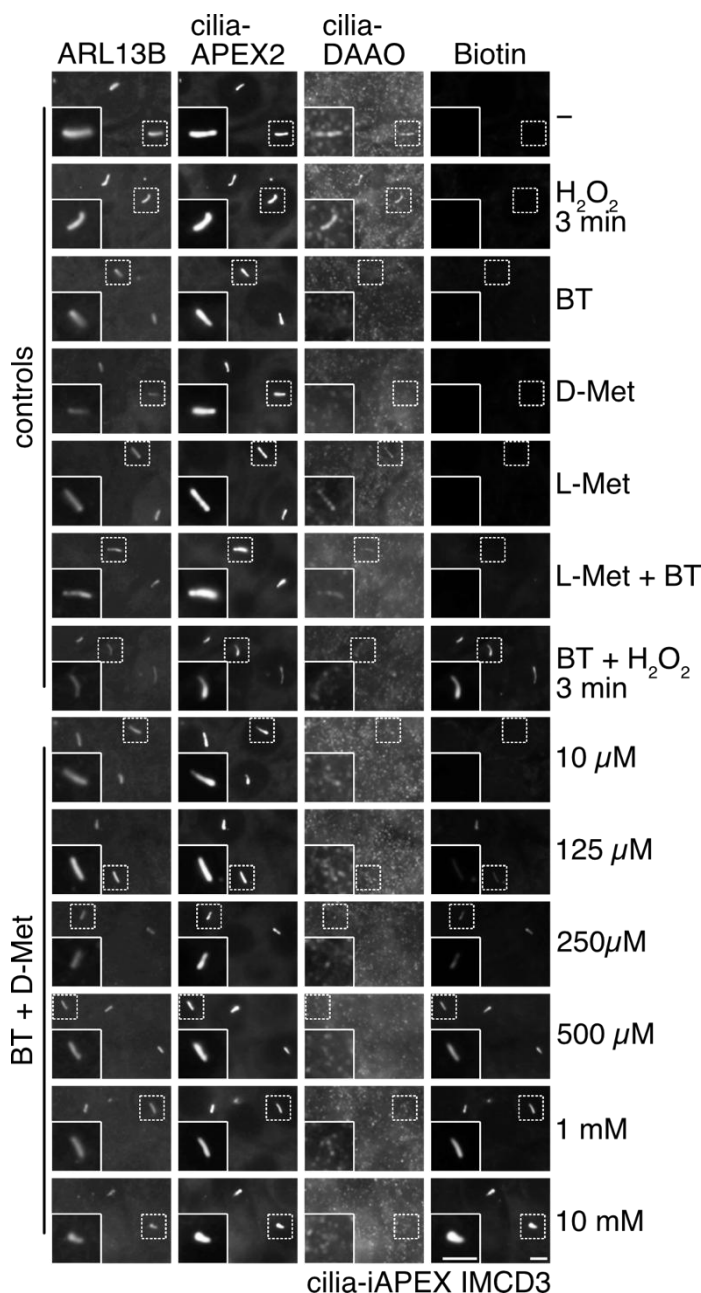
Sup.-Fig. 4: The APEX2 substrate desthiobiotin tyramide allows biotinylation of proteins and their subsequent isolation via streptavidin affinity chromatography and temperature-dependent competitive elution. A quantitative Western blot analysis was performed on proximity labeled IMCD3 cilia-APEX2 cell lysates (input), flow-throughs (unbound) and eluates. Non-labeled and *If88^{-/-}* controls were loaded. Biotin, GFP and GAPDH signals were detected on one membrane. Here, 18% of each eluate was analyzed. Signals for IFT88, acTub and GAPDH were detected on another membrane. Here, the remaining 72% of each eluate was analyzed. Roman numbering indicates various conditions of competitive elution with duration and temperature of the condition mentioned above the samples (o/n: overnight). Arabic numbers below indicate the elution step analyzed. All signals were obtained through primary antibodies and fluorescently-labeled secondary antibodies or fluorophore-coupled streptavidin. Input and unbound were 0.375% each (about 25 µg/lane). In total, 7 mg protein was incubated on the streptavidin beads.



Sup.-Fig. 5: DTBT proximity labeling coupled with streptavidin affinity capture under non-denaturing conditions and sucrose density gradient ultracentrifugation enables intact protein complex isolation.

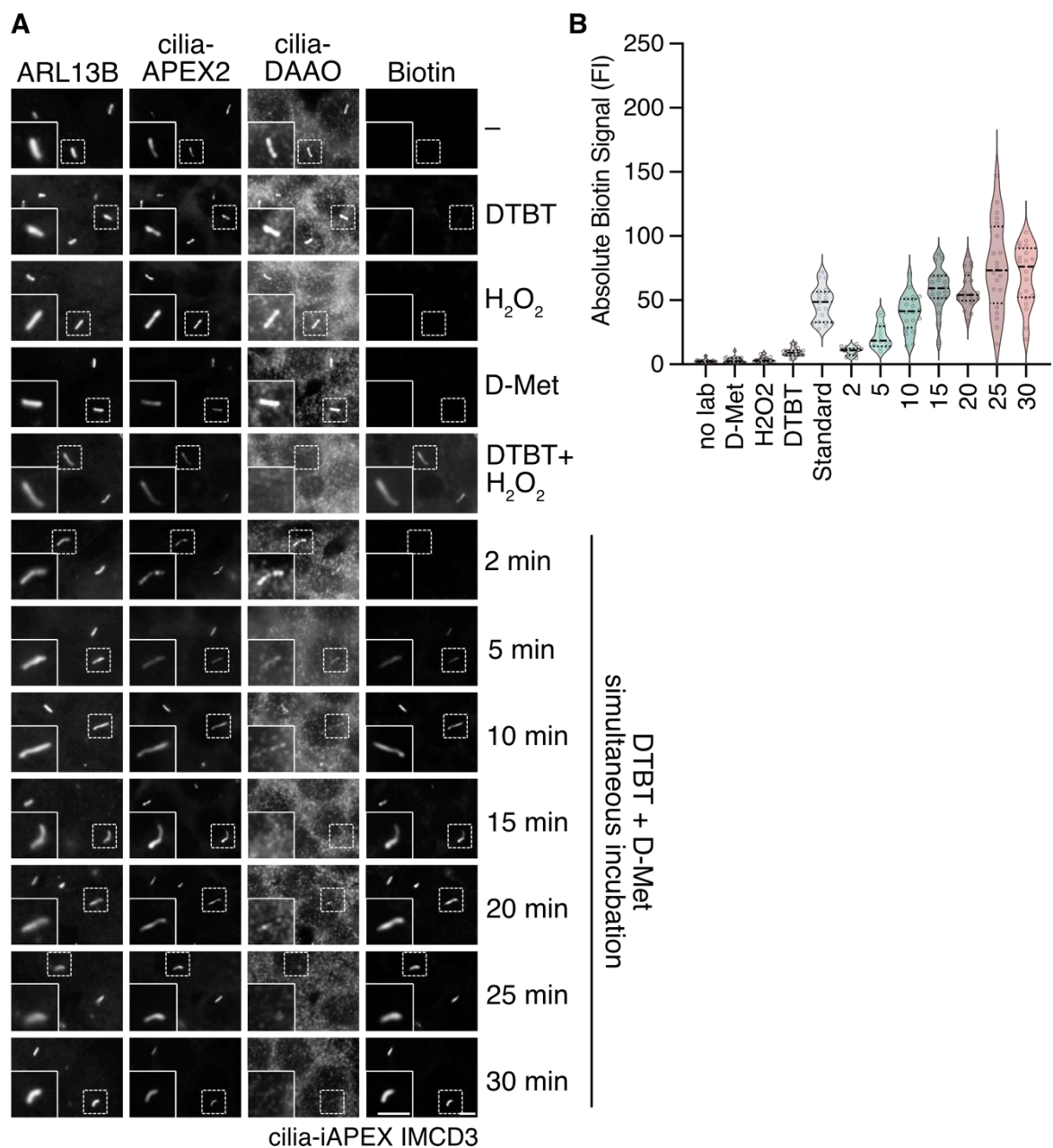
(A) Scheme of IMCD3 *Ift88*^{-/-} cells and rescue using dual expression vector harboring sequences for ALFA-IFT88 and cilia-APEX2. Proximity labeling using DTBT and H₂O₂ is carried out, followed by cell lysate generation, streptavidin chromatography and subsequent sucrose density gradient ultracentrifugation (SDGU).

Fractions of the gradient were loaded in (D). **(B)** Quantitative Western blot analysis of ALFA-IFT88 cilia-APEX2 IMCD3 cell lysates after DTBT proximity labeling (- not labeled, + labeled). *Ift88*^{-/-} cell lysate was used for IFT88 antibody verification. Lysate was halved and processed through either streptavidin or ALFA affinity chromatography (AC). Unbound (U) and SDS (S) samples were loaded. Fractions of the resulting eluates (E) were analyzed for ALFA-IFT88 and IFT57 isolation. **(C)** Remaining eluates from (B)—streptavidin (E_s) or ALFA (E_A) chromatography—were separated side by side with respective cell lysates in BN PAGE. The resulting Western blot result is depicted here, where ALFA (for IFT88) and IFT57 (re-decorated) were stained and visualized by HRP-coupled secondary antibodies and subsequent chemiluminescence. **(D)** Quantitative Western blot analysis of SDGU fractioned ALFA-IFT88 cilia-APEX2 lysate and streptavidin AC eluate fractions against total input and *Ift88*^{-/-} lysate. Fractions were loaded from top (15% sucrose) to bottom (55% sucrose) (order 1-10). ALFA, IFT57 and IFT20 were stained and recognized by fluorescently-labeled secondary antibodies.



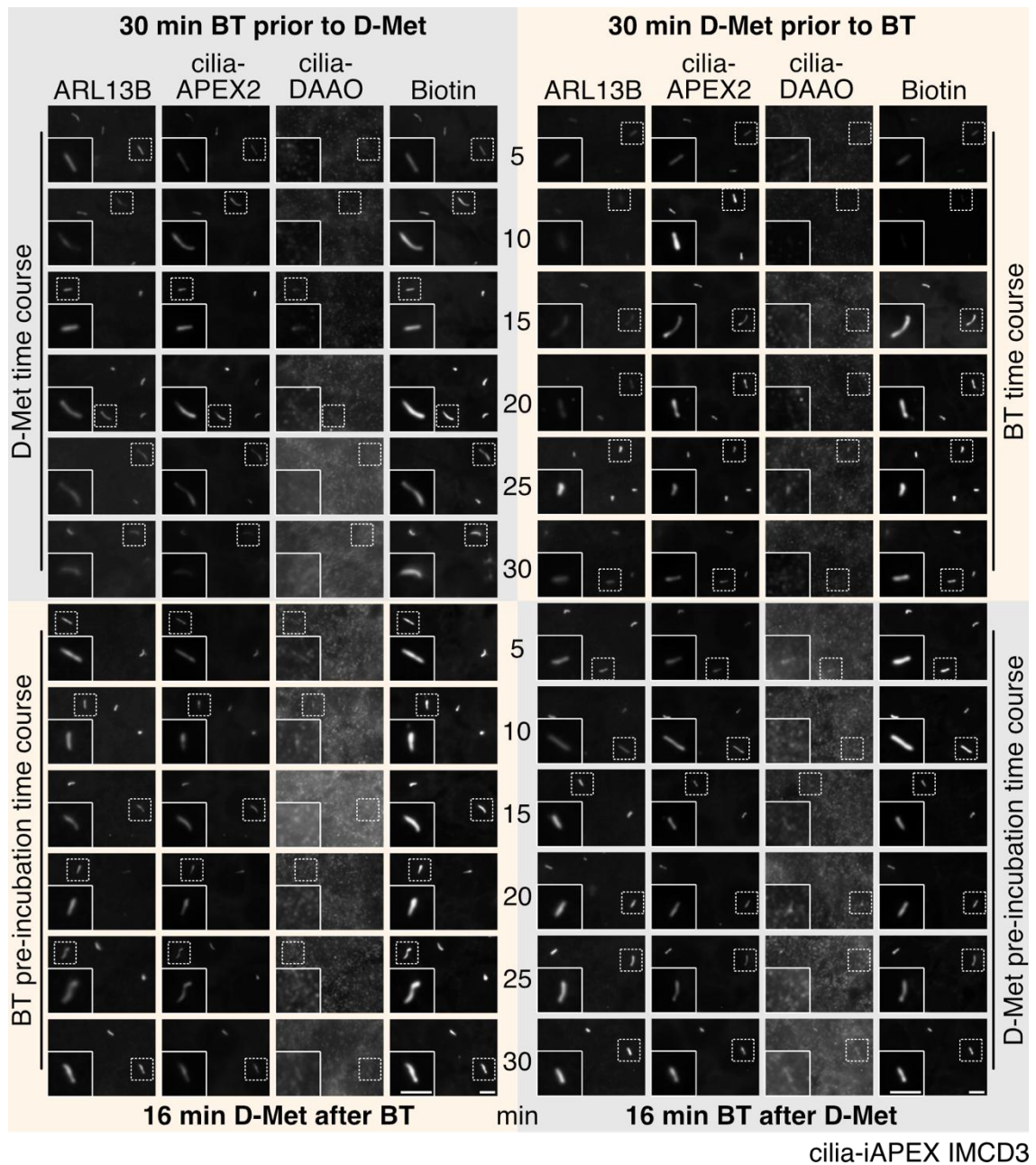
Sup.-Fig. 6: Micromolar D-methionine titration demonstrates concentration boundary for effective DAAO-dependent biotinylation.

Representative IF micrographs of D-methionine titration in IMCD3 cells stably expressing cilia-iAPEX shown in **Fig. 19C**. APEX labeling was performed by incubating cells with 500 μ M biotin tyramide and varying concentrations (range from 10-10⁴ μ M) of D-methionine for 30 min. To compare to standard APEX labeling, cells were treated with BT followed by 3 min incubation of H₂O₂. Additionally, L-methionine and respective labeling substrates alone were used as negative controls. Cells marked with (-) were left untreated. ARL13B antibody staining labeled primary cilia, while staining against FLAG marked cilia-DAAO. cilia-APEX2 was observed through GFP fluorescence. Biotin was detected by fluorescent streptavidin. Scale bar = 5 μ m.



Sup.-Fig. 7: Desthiobiotin tyramide is a suitable substrate for iAPEX proximity labeling.

(A) Exemplary IF images from experimental data shown in **Fig. 23D** of simultaneous incubation of desthiobiotin tyramide (DTBT) and D-Met in IMCD3 cells stably expressing cilia-iAPEX. APEX labeling was performed by incubating cells for 30 min with 500 μ M DTBT and 10 mM D-Met; omitting one or the other substrate for negative controls. Standard APEX labeling controls included of the addition of 1 mM H₂O₂ for 3 min alone or after 30 min BT incubation on cells. Cells marked with (-) were left untreated. ARL13B antibody staining labeled primary cilia, while staining against FLAG marked cilia-DAAO. cilia-APEX2 was observed through GFP fluorescence. Biotin was detected by fluorescent streptavidin. Scale bar = 5 μ m. **(B)** Quantification of absolute ciliary biotin fluorescence intensity based on experimental data from (A) obtained by Cilia Q (see page 93) shown as violin plots. The quartiles are shown as dotted lines surrounding the median represented by a dashed line. n = 20 cilia per condition.



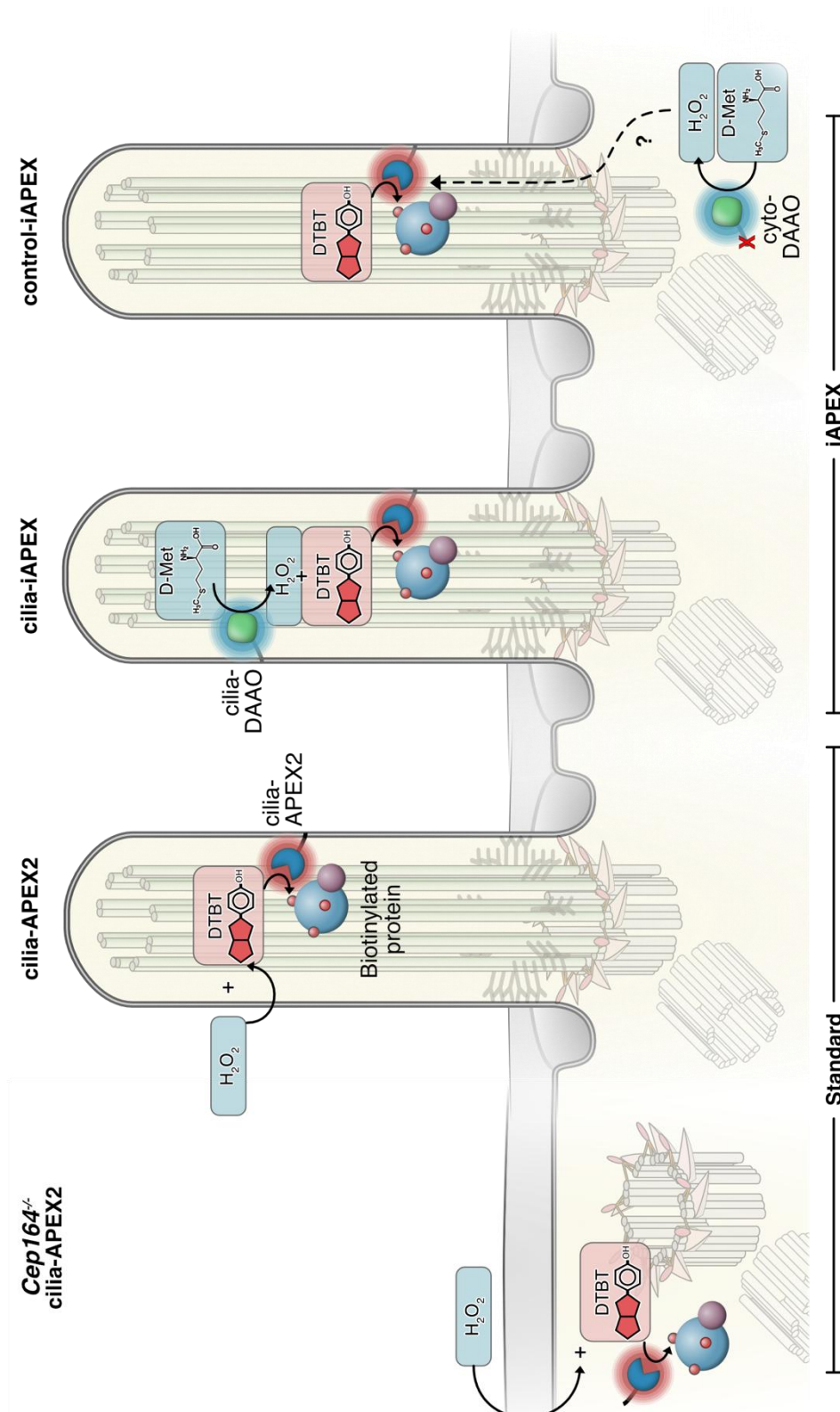
Sup.-Fig. 8: Different substrate incubation times influence ciliary biotinylation.

Representative IF images of various incubation combinations of BT and D-Met in cilia-iAPEX IMCD3 cells. Data is shown in **Fig. 23B**. APEX labeling was performed by incubating cells for different times and in different orders with 500 μ M BT and 10 mM D-Met; data shown here was produced together with data from sub-figure 4. Primary cilia were stained by antibodies targeted against ARL13B and FLAG (for cilia-DAAO). GFP fluorescence marked cilia-APEX2. Biotin was detected by fluorescently labeled streptavidin. Scale bar = 5 μ m.

	ARL13B	eGFP	FLAG	Biotin	DTBT	H ₂ O ₂	D-Met
NIH/3T3 WT					-	-	-
					-	+	-
					+	-	-
					+	+	-
					-	-	+
					+	-	+
NIH/3T3 cilia-iAPEX					-	-	-
					-	+	-
					+	-	-
					+	+	-
					-	-	+
					+	-	+
IMCD3 cilia-iAPEX					-	-	-
					-	+	-
					+	-	-
					+	+	-
					-	-	+
					+	-	+

Sup.-Fig. 9: Biotinylation is not initiated when substrates are incubated independently.

Extended version of **Fig. 26** including micrographs of independent substrate incubations.



Sup.-Fig. 10: Schematic of the analyzed APEX proximity labeling processes.

Detailed scheme of APEX2 expressing IMCD3 cell lines either with or without the DAAO component. Source of H_2O_2 is external for standard labeling conditions, whereas DAAO allows production *in situ*. The primary cilia are ablated in the *Cep164*^{-/-} cell line, so that cilia-APEX2 is not localized to the cilia, although the cilia-

targeting signal is intact. Proximity labeling is induced in the presence of H_2O_2 and DTBT as peroxidase substrate for radical formation and subsequent covalent modification of nearby proteins.

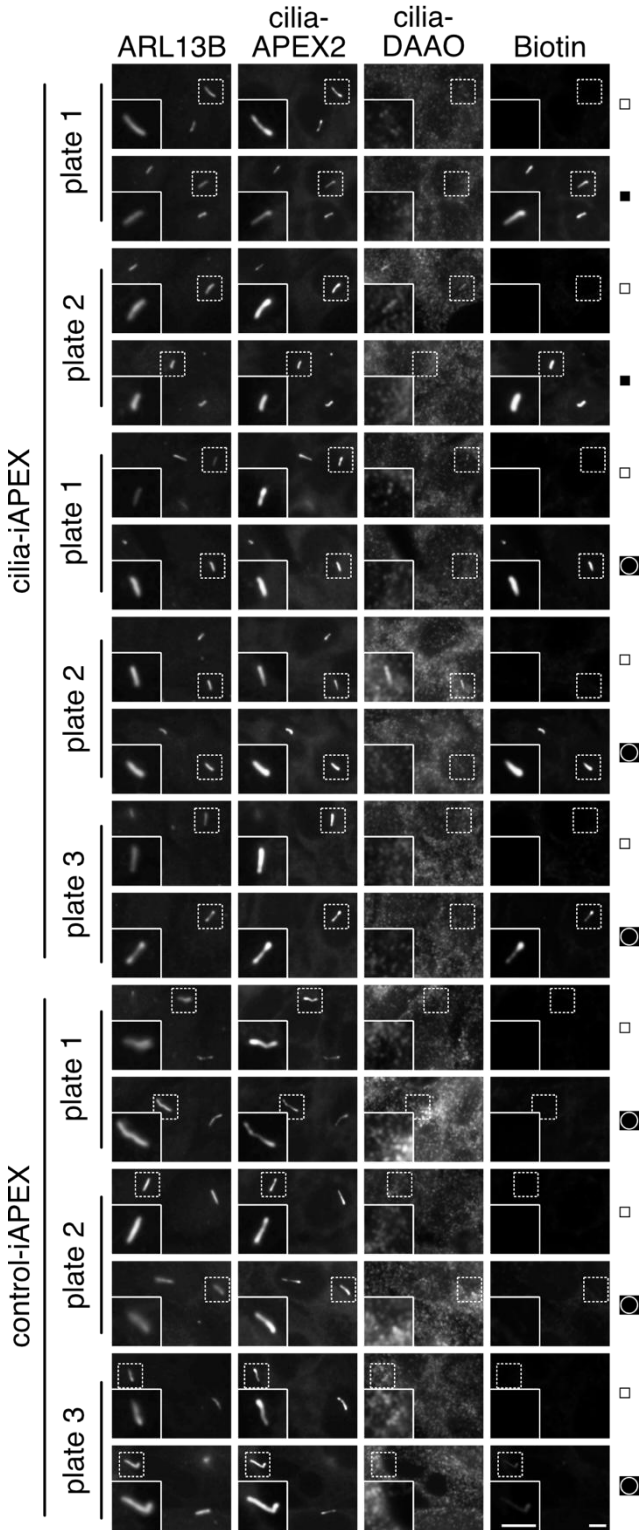
- untreated

■

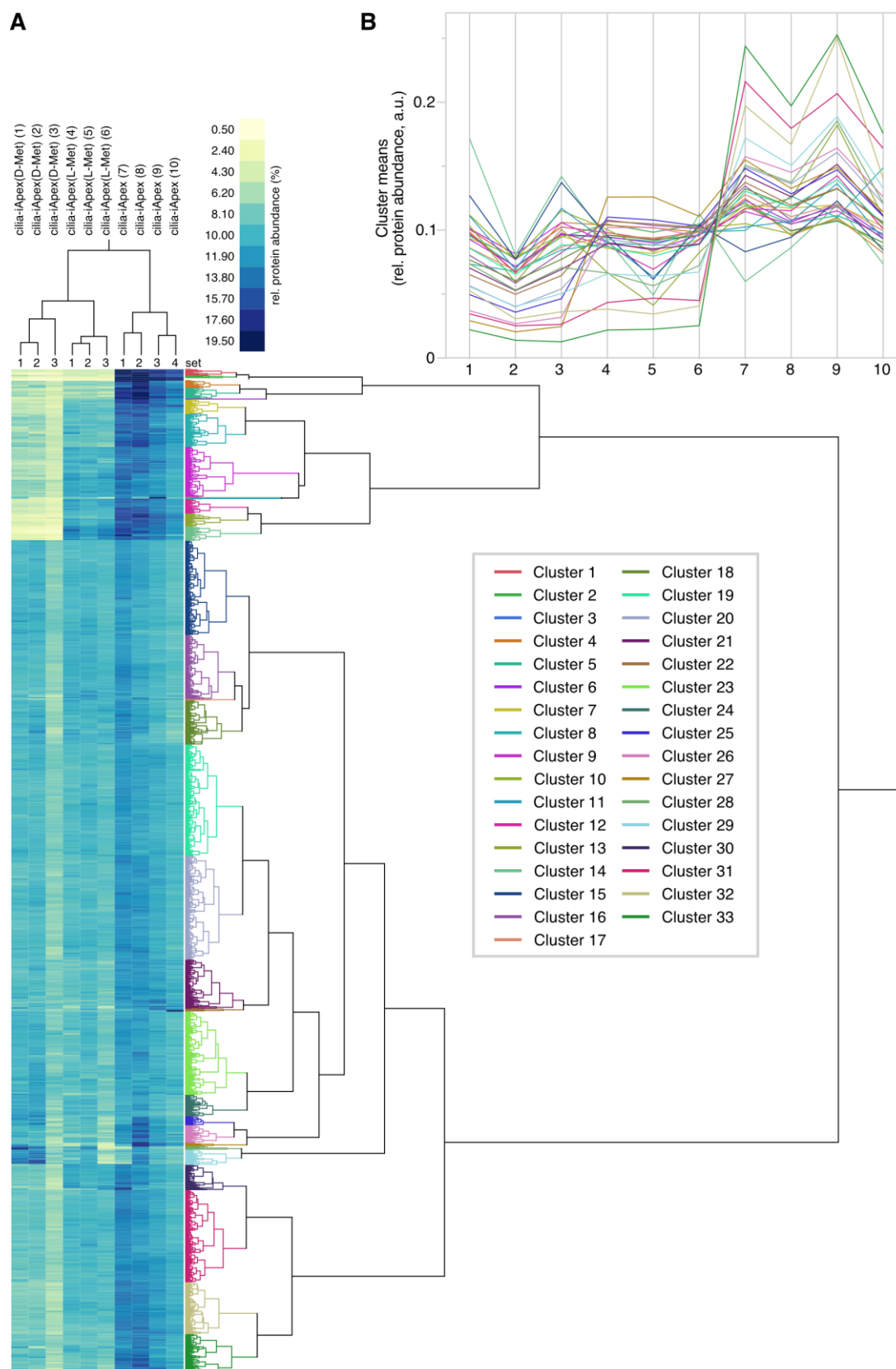
DTBT + H_2O_2

●

DTBT + D²-Met

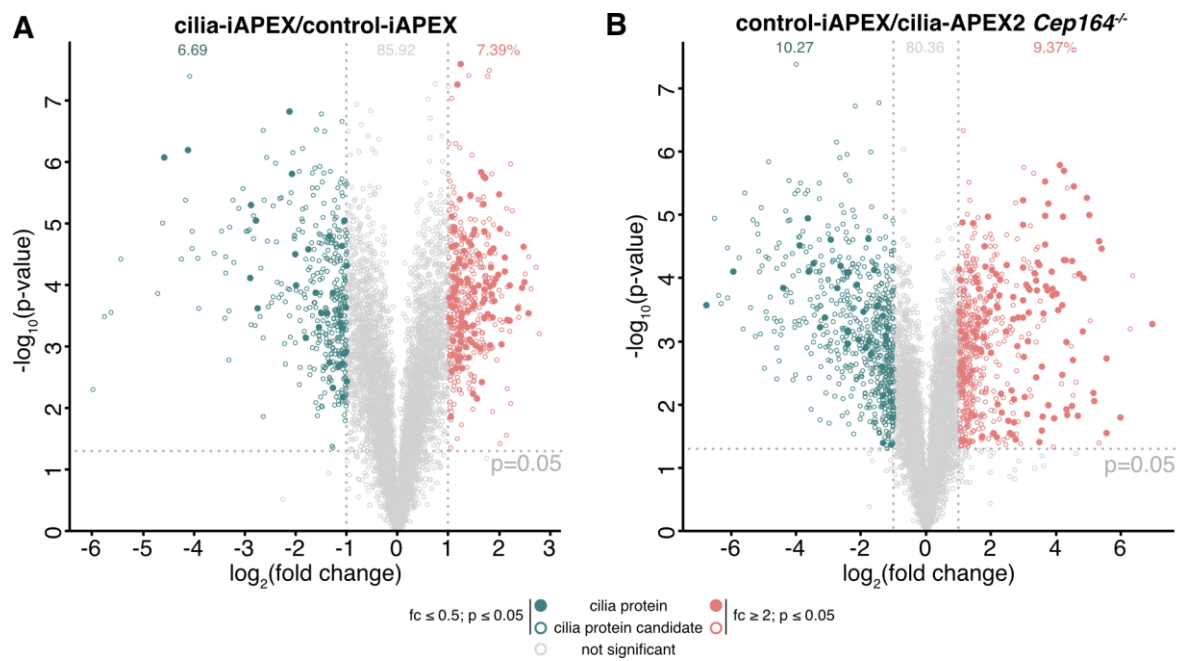


Sup.-Fig. 11: Control-iAPEX shows no clear ciliary biotinylation compared to cilia-iAPEX. Complementary IF micrographs of experimental data shown in **Fig. 27**. Shown are IMCD3 cells harboring stable expression of cilia- or control-iAPEX. Cells were fixed before (no labeling, □) or after APEX proximity labeling, with standard labeling using 500 μ M BT and 1 mM H_2O_2 (■, 3 min) or iAPEX labeling using DTBT and 10 mM D-Met (●, 30 min together). Primary cilia were visualized using the ARL13B antibody. Cilia-APEX2 was visualized via GFP fluorescence, while cilia-DAAO was detected using the FLAG antibody. Biotin was detected using fluorescently labeled streptavidin. Scale bar = 5 μ m.



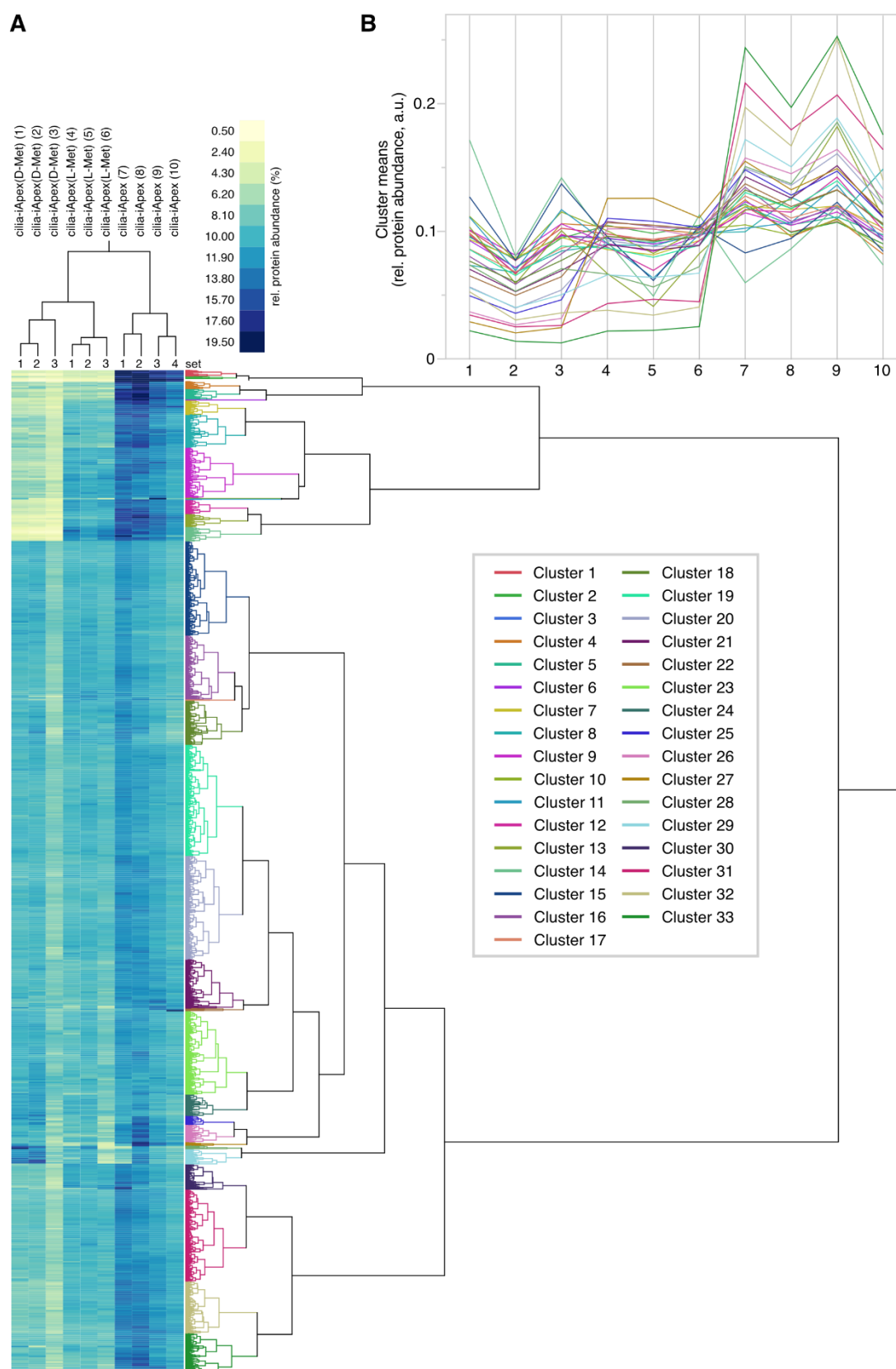
Sup.-Fig. 12: Extended hierarchical two-way clusters of iAPEX quantitative primary cilia proteomics samples show high reproducibility of replicates and similarity to cilia-APEX2 pattern.

(A) Extended hierarchical clustering of data presented in **Fig. 28A**. (B) Means analysis of the row dendrogram clusters highlights group patterns for all conditions. Clusters with a low protein count (< 10) were excluded from the plot.



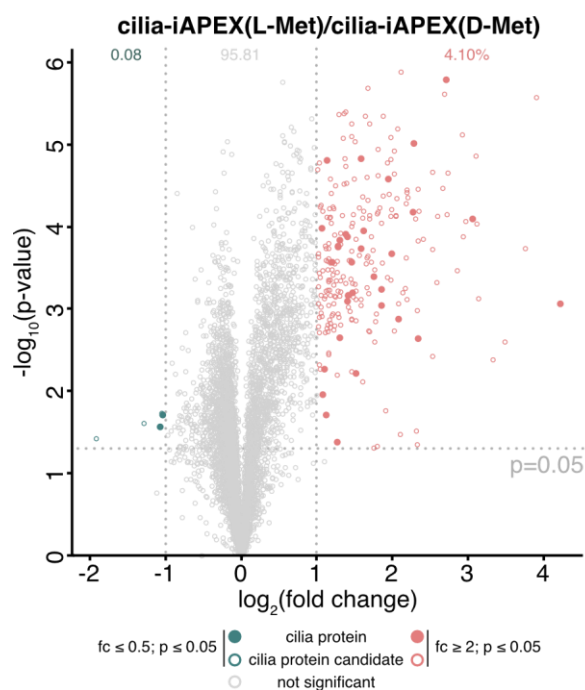
Sup.-Fig. 13: Control-iAPEX is not a suitable control as the cilia proteins are quantified to a comparable extent to cilia-iAPEX, reducing the enrichment effect achieved by *Cep164*^{-/-}.

(A, B) This supplementary figure expands upon the data depicted in **Fig. 28**, utilizing the same representation and analytical approaches.



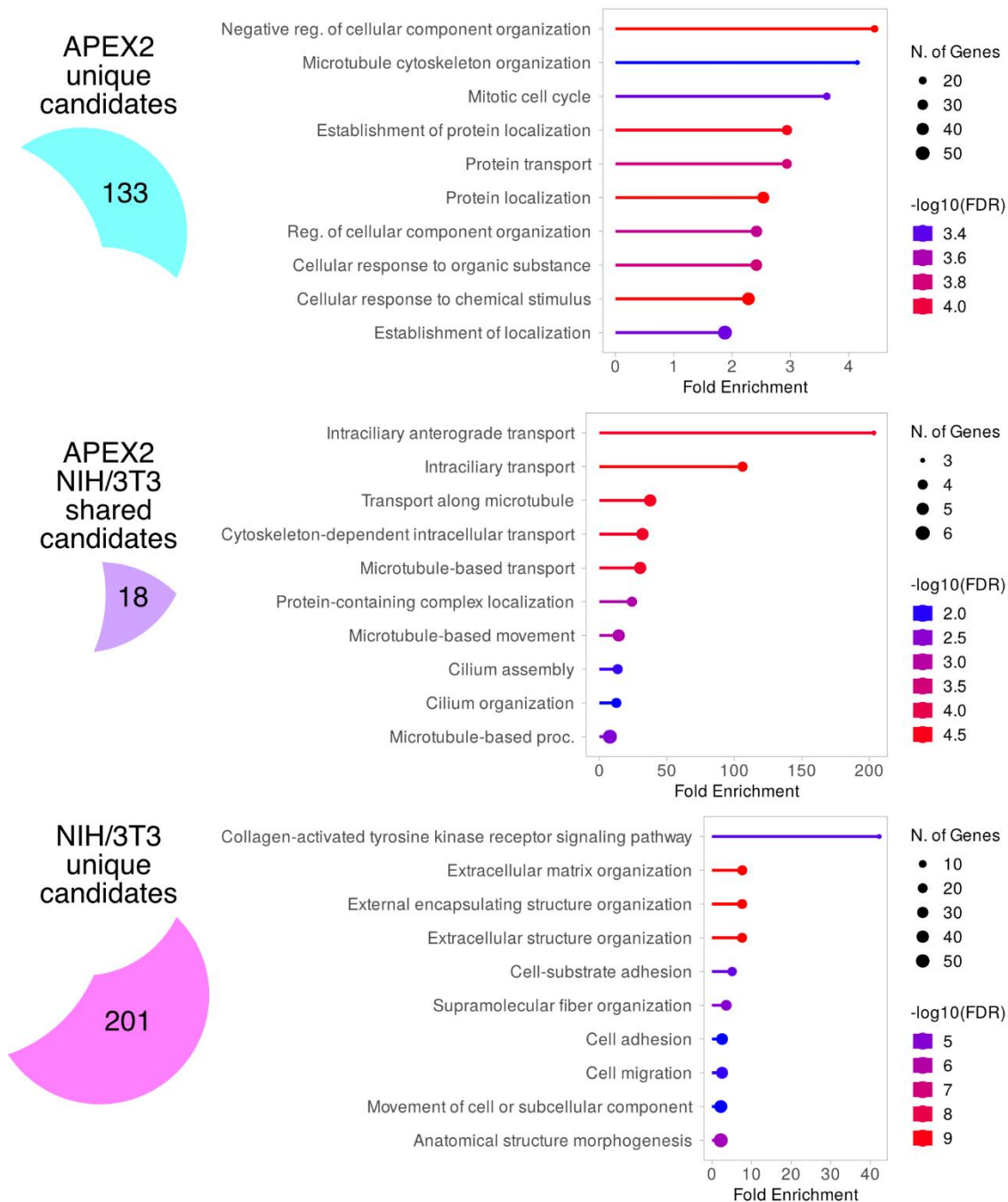
Sup.-Fig. 14: Extended hierarchical two-way clustering of iAPEX quantitative primary cilia proteomics in NIH/3T3 cells reveals significant ciliary protein enrichment.

(A) Extended hierarchical clustering of data presented in **Fig. 31A**. (B) Means analysis of the row dendrogram clusters highlights group patterns for all conditions. Clusters with a low protein count (< 10) were excluded from the plot.



Sup.-Fig. 15: Proteomic background noise generated by biotin tyramide incubation alone.

The volcano plot supplements the data shown in **Fig. 31**, employing similar methods for representation and analysis.



Sup.-Fig. 16: Gene Ontology (GO) term analysis of cilia-iAPEX proximity labeling candidates from IMCD3 and NIH/3T3 cells.

Venn diagram sections from **Fig. 32**. The top section (cyan) represents 133 proteins unique to IMCD3 candidates, the middle section (purple) indicates 18 proteins shared between both IMCD3 and NIH/3T3 (excluding core ciliary proteins), and the bottom section (magenta) corresponds to 201 proteins unique to NIH/3T3 cells. The right panels represent the GO enrichment analysis results of the proteins in each individual sectional dataset. The analysis depicted biological processes with the most significant GO terms for each protein group. The length of the bars represents the GO term fold enrichment. The color intensity of the bars indicates different significance levels, with more intense colors corresponding to higher FDR values. The size of the dots represents the number of genes associated with each GO term.

Tab. 6: List of plasmids generated during the thesis work

Abbr.	Plasmid Name	Backbone	Insert	Insert from	Primer Pair(s) (for, rev)	Bacterial Host	Bacterial Resistance	Mammalian Resistance	Enzymes	Method
pTS001	pENTR4-Kozak-Nphp3(1-200)	pDaM611	Nphp3 (1-200)	pDaMC182 (pB.TATA.Nphp3-GFP-APEX2)	oTS001 oTS002	MACH1	KanR	/	NcoI, XbaI	Classic
pTS002	pENTR4-Kozak-Nphp3.G2A(1-200)	pDaM611	Nphp3.G2A (1-200)	pDaMC209 (pB.tCMV.Nphp3.G2A-GFP-APEX2)	oTS003 oTS002	MACH1	KanR	/	NcoI, XbaI	Classic
pTS003	pEF5B-CMVΔ6-DEST-MiniTurbo-V5	pDaM681	MiniTurbo-V5	C1(1-29)-miniTurbo-V5_pCDNA3	oTS006 oTS005	DB3.1	AmpR CamR	BlastR	Bsp119i	Classic
pTS004	pEF5B-CMVΔ6-DEST-TurboID-V5	pDaM681	TurboID-V5	C1(1-29)-TurboID-V5_pCDNA3	oTS007 oTS005	DB3.1	AmpR CamR	BlastR	Bsp119i	Classic
pTS005	pEF5B-CMVΔ6-Nphp3(1-200)-MiniTurbo-V5	pTS003	Nphp3 (1-200)	pTS001	/	MACH1	AmpR	BlastR	LR clonase	Gateway
pTS006	pEF5B-CMVΔ6-Nphp3(1-200)-TurboID-V5	pTS004	Nphp3 (1-200)	pTS001	/	MACH1	AmpR	BlastR	LR clonase	Gateway
pTS007	pEF5B-CMVΔ6-Nphp3.G2A(1-200)-MiniTurbo-V5	pTS003	Nphp3.G2A (1-200)	pTS002	/	MACH1	AmpR	BlastR	LR clonase	Gateway
pTS008	pEF5B-CMVΔ6-Nphp3.G2A(1-200)-TurboID-V5	pTS004	Nphp3.G2A (1-200)	pTS002	/	MACH1	AmpR	BlastR	LR clonase	Gateway

Continued on next page.

List of plasmids generated during the thesis work (continued)

Abbr.	Plasmid Name	Backbone	Insert	Insert from	Primer Pair(s) (for, rev)	Bacterial Host	Bacterial Resistance	Mammalian Resistance	Enzymes	Method
pTS009	pEF5B-CMVΔ6-Nphp3(1-200)-GFP-MiniTurbo-V5	pTS003	Nphp3(1-200)-GFP	pMN1713 (pENTR NPHP3(1-200)-GFP-NoStop)	/	MACH1	AmpR	BlastR	LR clonase	Gateway
pTS010	pEF5B-CMVΔ6-Nphp3(1-200)-GFP-TurboID-V5	pTS004	Nphp3(1-200)-GFP	pMN1713 (pENTR NPHP3(1-200)-GFP-NoStop)	/	MACH1	AmpR	BlastR	LR clonase	Gateway
pTS011	pEF5B-CMVΔ6-Nphp3.G2A(1-200)-GFP-MiniTurbo-V5	pTS003	Nphp3.G2A(1-200)-GFP	pDaMC110 (pENTR-Nphp3.G2A.EGFP-NoStop)	/	MACH1	AmpR	BlastR	LR clonase	Gateway
pTS012	pEF5B-CMVΔ6-Nphp3.G2A(1-200)-GFP-TurboID-V5	pTS004	Nphp3.G2A(1-200)-GFP	pDaMC110 (pENTR-Nphp3.G2A.EGFP-NoStop)	/	MACH1	AmpR	BlastR	LR clonase	Gateway
pTS058	pGEX-N-GST-HRVC-FLAG-DAAO-NES	pDaM715	DAAO	AAV2-SypHer2-DAAO-NES	oTS081 oTS082	MACH1	AmpR	/	EcoRI, NotI	Classic
pTS059	pENTR4-FLAG-DAAO-NES	pDaM611	FLAG-DAAO	pTS058	oTS083 oTS084	MACH1	KanR	/	NcoI, NotI	Classic
pTS060	pEF5B-CMVΔ6-FLAG-DAAO-NES	pDaM681	FLAG-DAAO	pTS059	/	MACH1	AmpR	BlastR	LR clonase	Gateway
pTS062	pEF5B-CMVΔ6-mNphp3(1-200)-FLAG-DAAO-NES	pTS060	mNphp3(1-200)	pDaMC182 (pB.TATA.Nphp3-GFP-APEX2)	oTS085 oTS086	MACH1	AmpR	BlastR	EcoRI	Classic

Continued on next page.

List of plasmids generated during the thesis work (continued)

Abbr.	Plasmid Name	Backbone	Insert	Insert from	Primer Pair(s) (for, rev)	Bacterial Host	Bacterial Resistance	Mammalian Resistance	Enzymes	Method
pTS066	pEF5B-FRT-EF1aCCCC-mNphp3 (1-200)-NotI-FLAG-DAAO-STOP- eGFP-APEX2	pDaM300	Nphp3 (1-200)- NotI- FLAG- DAAO- NES	pTS068	/	MACH1	AmpR CamR	BlastR	LR clonase	Classic
pTS067	pENTR4-EcoRI-NotI-FLAG-DAAO- NES	pDaM611	EcoRI- NotI- FLAG- DAAO- NES	pTS058	oTS088 oTS089	MACH1	KanR	/	NcoI, NotI	Classic
pTS068	pENTR4-mNphp3(1-200)-NotI-FLAG- DAAO-NES	pTS067	mNphp3 (1-200)	pDaMC182 (pB.TATA.Nphp 3-GFP-APEX2)	oTS090 oTS091	MACH1	AmpR	/	EcoRI	Classic
pTS069	pEF5B-FRT-EF1aCCCC-mNphp3 (1-200)-NotI-FLAG-DAAO-NES- CMVΔ6-Nphp3-eGFP-APEX2	pVC08	mNphp3 (1-200)- NotI- FLAG- DAAO- NES	pTS068	/	MACH1	AmpR	BlastR	LR clonase	Gateway
pTS070	pENTR4-mNphp3(1-200)-NotI-FLAG- DAAO-NES-no-stop	pDaM611	mNphp3 (1-200)- NotI- FLAG- DAAO- NES- no_stop	pTS069	oTS090 oTS094	MACH1	KanR	/	NcoI, NotI	Classic

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List of plasmids generated during the thesis work (continued)

Abbr.	Plasmid Name	Backbone	Insert	Insert from	Primer Pair(s) (for, rev)	Bacterial Host	Bacterial Resistance	Mammalian Resistance	Enzymes	Method
pTS082	EF5B-CMVΔ6-Nphp3(1-200)-GFP-APEX2-Puro	pDaM181	Puro-mycin	pDaM562	oTS102 + oTS103, oTS104 + oTS105	MACH1	AmpR	PuroR	/	Fast Cloning
pTS083	pB.tCMV.DEST-APEX2-Puro	pTS082	Gateway cassette (att sites, ccdB, CamR)	pDaM608	/	DB3.1	AmpR CamR	PuroR	BP clonase	Gateway
pTS084	pB.tCMV.mNphp3(1-200;G2A)-GFP-APEX2-Puro	pTS082	mNphp3 (1-200; G2A)	/	DaM-P417 DaM-P418	MACH1	AmpR	PuroR	/	Mutagenesis
pTS085	pENTR4-mNphp3(1-200;G2A)-NotI-FLAG-DAAO-NES	pTS067	mNphp3 (1-200; G2A)	pDaMC209 (pB.tCMV.Nphp3.G2A-GFP-APEX2)	oTS101 oTS091	MACH1	KanR	/	EcoRI	Classic
pTS086	pEF5B-FRT-EF1aCCCC-mNphp3 (1-200;G2A)-NotI-FLAG-DAAO-NES-CMVΔ6-Nphp3-eGFP-APEX2	pVC08	mNphp3 (1-200; G2A)-NotI-FLAG-DAAO-NES	pTS085	/	MACH1	AmpR	BlastR	LR clonase	Gateway
pTS087	pEF5B-FRT-tCMV-mNphp3(1-200)-NotI-FLAG-DAAO-ALFA-APEX2-Puro	pTS082	NotI-FLAG-DAAO-ALFA	pTS067	oTS106 + oTS107, oTS108 + oTS109	MACH1	AmpR	PuroR	/	Fast Cloning

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List of plasmids generated during the thesis work (continued)

Abbr.	Plasmid Name	Backbone	Insert	Insert from	Primer Pair(s) (for, rev)	Bacterial Host	Bacterial Resistance	Mammalian Resistance	Enzymes	Method
pTS088	pEF5B-FRT-ICMV-mNphp3 (1-200;G2A)-NotI-FLAG-DAAO-ALFA-APEX2-Puro	pTS087	mNphp3 (1-200; G2A)	/	DaM-P417 DaM-P418	MACH1	AmpR	PuroR	/	Mutagenesis
pTS093	pEF5B-FRT-ICMV-mNphp3(1-201)-NotI-FLAG-DAAO-ALFA-APEX2-Blast	pTS087	Blasticidin	pDaM181	oTS114 + oTS115, oTS116 + oTS117	/	AmpR	BlastR	/	Fast Cloning
pTS094	pEF5B-FRT-ICMV-mNphp3 (1-201;G2A)-NotI-FLAG-DAAO-ALFA-APEX2-Blast	pTS088	Blasticidin	pDaM181	oTS114 + oTS115, oTS116 + oTS117	/	AmpR	BlastR	/	Fast Cloning
pTS095	pEF5B-FRT-ICMV-mNphp3(1-201)-NotI-FLAG-DAAO-ALFA-APEX2-cPuro	pTS087	working Puro-mycin	pDaM634	oTS114 + oTS115, oTS116 + oTS117	MACH1	AmpR	PuroR	/	Fast Cloning
pTS097	pEF5B-FRT-ICMV-mNphp3(52-201)-GFP-APEX2	pDaM181	/	/	oTS120_ new, oTS121	/	/	/	/	Mutagenesis
pTS098	pEF5B-FRT-ICMV-mNphp3(96-201)-GFP-APEX2	pDaM181	/	/	oTS122 oTS123_ new	/	/	/	/	Mutagenesis
pTS100	pLVX-cilia-eGFP-APEX-IRES-cilia(opt)-FLAG-DAAO-ALFA-NES	/	/	/	/	Stbl3	AmpR	/	/	Synthesis

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List of plasmids generated during the thesis work (continued)

Abbr.	Plasmid Name	Backbone	Insert	Insert from	Primer Pair(s) (for, rev)	Bacterial Host	Bacterial Resistance	Mammalian Resistance	Enzymes	Method
pTS106	pEF5B-CMVΔ6-mNphp3(1-200)-eGFP-APEX2-EF1αCCCC-ALFA-IFT88	pVC08	ALFA-IFT88	pPS0099 (pENTR4-ALFA-IFT88)	/	DH5a	AmpR	BlastR	LR clonease	Gateway
pTS107	pEF5B-CMVΔ6-mNphp3(1-200)-mCherry-APEX2-EF1αCCCC-ALFA-IFT88	pVC07	ALFA-IFT88	pPS0099 (pENTR4-ALFA-IFT88)	/	DH5a	AmpR	BlastR	LR clonease	Gateway
pTS108	pLVX-cilia-eGFP-APEX-IRES-fCTS-FLAG-DAAO-ALFA	pTS100	fCTS	oligo anneal	oTS134 oTS135	Stbl3	AmpR	/	NotI	Classic
pTS109	pLVX-eGFP-CEP104-APEX2-IRES-Nphp3(1-200;codonopt.)-FLAG-DAAO-ALFA	pTS100	CEP104	pDaM830	oTS138 oTS139	Stbl3	AmpR	/	SpeI, SphI	Classic
pTS110	pLVX-Nphp3(1-200)-eGFP-APEX2-IRES-CEP104-FLAG-DAAO-ALFA	pTS100	CEP104	pDaM830	oTS140 oTS141	Stbl3	AmpR	/	NotI	Classic
pTS111	pLVX-Eb1-eGFP-APEX2-IRES-Nphp3(1-200;codonopt.)-FLAG-DAAO-ALFA	pTS100	Eb1	pDaM825	oTS142 oTS143	Stbl3	AmpR	/	SpeI, SphI	Classic
pTS112	pLVX-Eb3-AausFP1-APEX2-IRES-Nphp3(1-200;codonopt.)-FLAG-DAAO-ALFA	pTS100	Eb3	pDaM822	oTS144 oTS145	Stbl3	AmpR	/	SpeI, SphI	Classic
pTS113	pLVX-CMV_Nphp3(1-200)-eGFP-APEX2-IRES-Nphp3(1-200;codonopt.)-FLAG-DAAO-ALFA	pTS100	CMV+ Spacer	pc3.1-mNPHP3-bPAC-mCherry	oTS136 oTS137	Stbl3	AmpR	/	NsiI, SpeI	Classic
pTS114	pEF5B-FRT-EF1αCCCC-mNphp3(1-200)-NotI-FLAG-DAAO-NES-CMVΔ6-Nphp3-HyPer-APEX2	pVC08	HyPer7	pCS2+HyPer7 (pDaM836)	oTS146 oTS147	MACH1	AmpR	BlastR	NdeI, NcoI	Classic

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List of plasmids generated during the thesis work (continued)

Abbr.	Plasmid Name	Backbone	Insert	Insert from	Primer Pair(s) (for, rev)	Bacterial Host	Bacterial Resistance	Mammalian Resistance	Enzymes	Method
pTS115	pLVX-cilia-eGFP-APEX-T2A-fCTS-FLAG-DAAO-ALFA	pTS108	/	/	/	Stbl3	AmpR	/	MluI	Classic
pTS116	pLVX-cilia-eGFP-APEX-T2A-fCTS-FLAG-DAAO-ALFA-no_stop	pTS115	no stop mutation	/	oTS156 oTS157	Stbl3	AmpR	/	/	Mutagenesis
pTS124	pLVX-Nphp3(G2A;1-200)-eGFP-APEX-T2A-fCTS-FLAG-DAAO-ALFA	pTS115	mNphp3 (1-200; G2A)	/	oTS154 oTS155	Stbl3	AmpR	/	/	Mutagenesis
pTS128	pLVX-cilia-eGFP-APEX2-IRES-Pskh-ALFA	pTS100	Pskh	pDaM887	oTS182 oTS183	Stbl3	AmpR	/	NotI, XmaI	Classic
pTS129	pLVX-cilia-eGFP-APEX2-IRES-Pit-pnm2-ALFA	pTS100	Pitpnm2	pDaM888	oTS198 oTS199	Stbl3	AmpR	/	Gibson	Gibson
pTS130	pLVX-cilia-eGFP-APEX2-IRES-Fam63a(Mindy1)-ALFA	pTS100	Fam63a	pDaM890	oTS200 oTS201	Stbl3	AmpR	/	NotI, XmaI	Classic
pTS131	pLVX-cilia-eGFP-APEX2-IRES-Fbxo21-ALFA	pTS100	Fbxo21	pDaM891	oTS192 oTS193	Stbl3	AmpR	/	Gibson	Gibson
pTS132	pLVX-cilia-eGFP-APEX2-IRES-Ankrd13d-ALFA	pTS100	Ankrd13d	pDaM892	oTS170 oTS171	Stbl3	AmpR	/	NotI, XmaI	Classic
pTS133	pLVX-cilia-eGFP-APEX2-IRES-Ckap2l-ALFA	pTS100	Ckap2l	pDaM893	oTS172 oTS173	Stbl3	AmpR	/	NotI, XmaI	Classic
pTS134	pLVX-cilia-eGFP-APEX2-IRES-Eya1-ALFA	pTS100	Eya1	pDaM894	oTS174 oTS175	Stbl3	AmpR	/	NotI, XmaI	Classic
pTS135	pLVX-cilia-eGFP-APEX2-IRES-C19orf44_homolog-ALFA	pTS100	C19orf44 homolog	pDaM895	oTS188 oTS189	Stbl3	AmpR	/	Gibson	Gibson

List of plasmids generated during the thesis work (continued)

Abbr.	Plasmid Name	Backbone	Insert	Insert from	Primer Pair(s) (for, rev)	Bacterial Host	Bacterial Resistance	Mammalian Resistance	Enzymes	Method
pTS136	pLVX-cilia-eGFP-APEX2-IRES-Fam63b (Mindy2)-ALFA	pTS100	Fam63b	pDaM896	oTS176 oTS177	Stbl3	AmpR	/	NotI, XmaI	Classic
pTS137	pLVX-cilia-eGFP-APEX2-IRES-Fam188a (Mindy3)-ALFA	pTS100	Fam188a	pDaM897	oTS178 oTS179	Stbl3	AmpR	/	NotI, XmaI	Classic
pTS138	pLVX-cilia-eGFP-APEX2-IRES-Cuedc1-ALFA	pTS100	Cuedc1	pDaM898	oTS190 oTS191	Stbl3	AmpR	/	Gibson	Gibson
pTS139	pLVX-cilia-eGFP-APEX2-IRES-Pick1-ALFA	pTS100	Pick1	pDaM899	oTS180 oTS181	Stbl3	AmpR	/	NotI, XmaI	Classic
pTS140	pLVX-cilia-eGFP-APEX2-IRES-Ensa-ALFA	pTS100	Ensa	mouse standard cDNA	oTS160 oTS161	Stbl3	AmpR	/	NotI, XmaI	Classic
pTS141	pLVX-cilia-eGFP-APEX2-IRES-Zfand2b-ALFA	pTS100	Zfand2b	mouse standard cDNA	oTS168 oTS169	Stbl3	AmpR	/	NotI, XmaI	Classic
pTS142	pLVX-cilia-eGFP-APEX2-IRES-Cuedc2-ALFA	pTS100	Cuedc2	mouse standard cDNA	oTS184 oTS185	Stbl3	AmpR	/	Gibson	Gibson

Tab. 7: List of purchased or already available plasmids that were used in this study

Abbr.	Plasmid Name	Bacterial Host	Bacterial Resistance	Mammalian Resistance	From/Made by
pDaM181	pB.tCMV.Nphp3-GFP-APEX2	MACH1	AmpR	BlastR	David Mick
pDaM209	pB.tCMV.Nphp3.G2A-GFP-APEX2	MACH1	AmpR	BlastR	David Mick
pDaM300	pB.TATAmutant.FRT.DEST-GFP-APEX2	MACH1	AmpR	BlastR	David Mick
pDaM466/pVC07	pEF5B-FRT-EF1aCCCC-mNG-GS-TeV-FLAG-Gm11992-Myc-DDK-CMVΔ6-Nphp3-mCherry-APEX2	DB3.1	AmpR	BlastR	Valerie Chaumet
pDaM482	pc3.1-mNPHP3-bPAC-mCherry	MACH1	AmpR	NeoR	gifted by Dagmar Wachten
pDaM483	C1(1-29)-TurboID-V5_pCDNA3	MACH1	AmpR	NeoR	Addgene (Plasmid #107173)
pDaM484	C1(1-29)-miniTurbo-V5_pCDNA3	MACH1	AmpR	NeoR	Addgene (Plasmid #107174)
pDaM611	pENTR4	DB3.1	KanR	/	David Mick
pDaM681	pEF5b CMVd6pro DEST	DB3.1	AmpR, CamR	BlastR	David Mick
pDaM715	pGEX6P1-N-FLAG	MACH1	AmpR	/	Addgene (Plasmid #119759)
pDaM722/pVC08	pB-FRT-CMVΔ6-NPHP3-eGFP-APEX2-HSV and EF1α-TATA-DEST-BGHstop	DB3.1	AmpR, CamR	BlastR	Valerie Chaumet
pDaM825	pEGFP-C2-EB1Δ-GFP	MACH1	KanR	NeoR	gifted by Carsten Janke

Continued on next page.

List of purchased or already available plasmids that were used in the study (continued)

Abbr.	Plasmid Name	Bacterial Host	Bacterial Resistance	Mammalian Resistance	From/Made by
pDaM830	pEGFP-C1-CEP104	MACH1	KanR	NeoR	gifted by Elif Nur Firat-Karalar
pDaM836	pCS2-HyPer7	MACH1	AmpR	/	gifted by Leticia Prates-Roma
pDaM887	pSport1-Pskh1	MACH1	AmpR	/	Dharmacon: MMM1013-202805603
pDaM888	pYX-Asc-Pitpnm2	MACH1	AmpR	/	Dharmacon: MMM1013-202733108
pDaM889	pBluescriptR-Tmem67	MACH1	AmpR	/	Dharmacon: MHS6278- 202807241
pDaM890	pYX-Asc-Fam63a (Mindy1)	MACH1	AmpR	/	Dharmacon: MMM1013-202798055
pDaM891	pCMV-SPORT6-Fbxo21	MACH1	AmpR	/	Dharmacon: MMM1013-202763402
pDaM892	pYX-Asc-Ankrd13d	MACH1	AmpR	/	Dharmacon: MMM1013-202797782
pDaM893	pCMV-SPORT6.1-Ckap21	MACH1	AmpR	/	Dharmacon: MMM1013-202805167
pDaM894	pYX-Asc-Eya1	MACH1	AmpR	/	Dharmacon: MMM1013-202799148

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List of purchased or already available plasmids that were used in the study (continued)

Abbr.	Plasmid Name	Bacterial Host	Bacterial Resistance	Mammalian Resistance	From/Made by
pDaM895	pCMV-SPORT6-C19orf44 homolog	MACH1	AmpR	/	Dharmacon: MMM1013-202762689
pDaM896	pYX-Asc-Fam63b (Mindy2)	MACH1	AmpR	/	Dharmacon: MMM1013-202859658
pDaM897	pCMV-SPORT6-Fam188a (Mindy3)	MACH1	AmpR	/	Dharmacon: MMM1013-202765455
pDaM898	pCR4-TOPO-Cuedc1	MACH1	AmpR	NeoR	Dharmacon: MMM1013-211691912
pDaM899	pCMV-SPORT6-Pick1	MACH1	AmpR	/	Dharmacon: MMM1013-202770481
pPS0099	pENTR4-ALFA-IFT88	MACH1	KanR	/	Patrick Schuster

Tab. 8: List of oligonucleotides designed and used in this study

Abbr.	Position	Oligonucleotide Name	Oligonucleotide Sequence (5'-3')
oTS001	forward	Gibson_Kozak_Nphp3 (1-200)_for	TACAAAAAAGCAGGCTCCACGCCACCATGGGTACCGCCTCTTCC
oTS002	reverse	Gibson_Kozak_Nphp3 (1-200)_rev	TTTGTACAAGAAAGCTGGGTGACCCCTGTGCTTGGAGACG
oTS003	forward	Gibson_Kozak_Nphp3 (1-200)_G2A_for	TACAAAAAAGCAGGCTCCACGCCACCATGGGTACCGCCTCTTCC
oTS005	reverse	Gibson_MiniTurbo/TurboID_rev	GTTAGGGATAGGCTTACCTTTTATAGGTGCTGCCAGGCC
oTS006	forward	Gibson_MiniTurbo_for	AGTCTAGAGGGCCCGGGTTTCATATGATCCCCTGCTGAACGCT
oTS007	forward	Gibson_TurboID_for	AGTCTAGAGGGCCCGGGTTTCATATGAAAAGACAATACTGTGCCTCTG
oTS081	forward	Gibson_EcoRI_DAAO_fwd	TGACGACAAAGCGATCCCCGGGTGGTAGTGGTCACAGC
oTS082	reverse	Gibson_NotI_DAAO_rev	TCGTCAAGTCAGTCACGATGCTTACAGGGTCAGCCGCTC
oTS083	forward	Gibson_NcoI_Flag_fwd	TACAAAAAAGCAGGCTCCACATGGACTACAAAGACGATGACGAC
oTS084	reverse	Gibson_NotI_DAAO_Entry_rev	GGTCTAGATATCTCGAGTGCTTACAGGGTCAGCCGCTC
oTS085	forward	Gibson_EcoRI_NPHP3_fwd	AGCTGGCTCCAGTGTGGTGGATGGGTACCGCCTCTTCC
oTS086	reverse	Gibson_EcoRI_NPHP3_rev	AACTTGTGATATCTGCAGACCCCTGTGCTTGGAGACG
oTS088	forward	Gibson_EcoRI_NotI_ATG_FLAG-DAAO-NES_fwd	TACAAAAAAGCAGGCTCCACGAAATTCGCGCCGCATGGACTACAAAGACGATGACG
oTS089	reverse	Gibson_FLAG-DAAO-NES_NcoI_rev	GGTCTAGATATCTCGAGTGTTACAGGGTCAGCCGCTC
oTS090	forward	Gibson_NcoI_Nphp3_fwd	ACAAAAAAGCAGGCTCCACGATGGGTACCGCCTCTTCC
oTS091	reverse	Gibson_Nphp3_NcoI_rev	TTGTAGTCCATGCGGGCCGACCCCTGTGCTTGGAGACG
oTS094	reverse	Gibson_NotI_DAAO_no_stop_rev	GGTCTAGATATCTCGAGTGCAGGGTCAGCCGCTCCAG

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List of oligonucleotides designed and used in this study (continued)

Abbr.	Position	Oligonucleotide Name	Oligonucleotide Sequence (5'-3')
oTS101	forward	Gibson_NcoI_Nphp3_G2A_fwd	ACAAAAAAGCAGGCTCCACGATGGCTACCGCCTCTTCC
oTS102	forward	PuroR_pDaM563_insert_ampli_fwd	GGAACCTTCCTTGGCCACTGAGTACAAAGCCTAC
oTS103	reverse	pDaM181_pDaM209_plasmid_ampli_rev	GGCCAAAGGAAGTTCCTATACCTTCTAGAGAAATAGG
oTS104	reverse	PuroR_pDaM563_insert_ampli_rev	GAGCTCGAATTGTGCTCAGGCTCCTGGTTTTC
oTS105	forward	pDaM181_pDaM209_plasmid_ampli_fwd	GCACAAATTCGAGCTCGGTACCTTTAA
oTS106	reverse	pTS067_FLAG_DAAO_ALFA_reverse fastcloning	GGGCTCGGTCAGCCTCCTCCTCAGCTCCTCCTCCAGCCTGCTGGGGCTCTCCCTAGCTGCG
oTS107	forward	pTS067_FLAG_DAAO_forward_fastcloning	CCGGTCGCCACCATGGACTACAAAGACGATGACG
oTS108	forward	pTS082_ALFA_attB2_forward_fastcloning	GGAGGCTGACCGAGCCCGGATCCACCGGATCTAGAGACC
oTS109	reverse	pTS082_Nphp3_C_ALFA_reverse fastcloning	CATGGTGGCGACCGGTCCGGAGCCACCGGAAC
oTS114	reverse	bGH_insert_rev	CATCCCCAGCATGCCTGCTATTGTCTTCC
oTS115	forward	bGH_backbone_resistance_fwd	GGCATGCTGGGGATGCGGTGGGCTCTATGG
oTS116	reverse	Amp_backbone_resistance_rev	CCGTGTCGCCCTTATTCCTTTTGTGCGGC
oTS117	forward	Amp_insert_fwd	ATAAGGGCGACACGGAAATGTTGAATACTC
oTS120	reverse	Nphp3_52-201_rev	AGAGCCTGGGCCCATATCAACTTTTGTACAAACTTGTGATATCTGCAGAAATTCC
oTS121	forward	Nphp3_52-201_fwd	ATGGCCCCAGGCTCTCTGCCCGGTGCGGCA
oTS122	forward	Nphp3_96-201_fwd	ATGATTTTCCGCGTATCTAAGAATCAAGAACTCCTCAGC

List of oligonucleotides designed and used in this study (continued)

Abbr.	Position	Oligonucleotide Name	Oligonucleotide Sequence (5'-3')
oTS123	reverse	Nphp3_96-201_rev	TACGCGGAAAAATCATATCAACTTTTTTGTACAAACTTGTGTG
new			
oTS134	forward	fCTS_anneal_1	GGCCCTATGTGTCTCGTTTGTCTGCTGTTTAAGAAAAAGCAAAACCAGAAAAATAAAACCAGAACGC
oTS135	reverse	fCTS_anneal_2	GGCCGCGTCTCGTTTATTTTCTGCTTTTCTTAAACCAGCAGCAACGAGACACATAG
oTS136	forward	CMV_in_pTS100_fwd	GCAATGCATGTGATGCGGTTTTTGGCAGTAC
oTS137	reverse	CMV_in_pTS100_rev	GCAACTAGTGCTAGCCAGCTTGGGTCTC
oTS138	forward	SpeI_eGFP-CEP104_fwd	CGGACTAGTATGTTGAGCAAGGGCGAGGAGC
oTS139	reverse	eGFP-CEP104_SphI_rev	ATAGCATGCGCGCTTGGCGTACGTCCTGC
oTS140	reverse	NotI_CEP104_fwd	AATGGGGCCGGCGGCTTGGCGTACGTCCTGC
oTS141	forward	CEP104_Noti_rev	GTGGCGGCCGCTCTATGCCCCACAAGATTGGATTGTAGTCG
oTS142	forward	SpeI_EGFP-Eb1_fwd	CGAACTAGTATGTTGAGCAAGGGCGAGG
oTS143	reverse	eGFP-Eb1_SphI_rev	GCGGCATGCATACTCTTCTTGTTCCTCCTGTGG
oTS144	forward	SpeI_Eb3-AausFP1_fwd	CGGACTAGTATGGCTGTCAATGTGTACTCC
oTS145	reverse	Eb3-AausFP1_SphI_rev	GGTGCATGCTGCATATGCGGTTTTTGCAATCG
oTS146	forward	NcoI_HyPer_fwd	CCACCATGGGCATGCACCTGGCTAATGAGG
oTS147	reverse	HyPer_NdeI_rev	CCTCATATGATCGCAGATGAAGCTAACACC
oTS154	forward	pTS100_mutate_Nphp3_G2A_fwd	ACTAGTATGGCTACCGCCTCTTCCCTGGTCTC
oTS155	reverse	pTS100_mutate_Nphp3_G2A_rev	AGAGGCGGTAGCCATACTAGTGATCTGAGTCC

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List of oligonucleotides designed and used in this study (continued)

Abbr.	Position	Oligonucleotide Name	Oligonucleotide Sequence (5'-3')
oTS156	forward	pTS115_mutate_stop_fwd	GCTGACCGAGAAACTCGAGCGACCGCGTCTG
oTS157	reverse	pTS115_mutate_stop_rev	CGCTCGAGTTTCTCGGTCAGCCTCCTCCTCAGC
oTS160	forward	Ensa_NotI_fwd	ATAGCGGCCGCATGTCCAGAAACAAGAAAGAAACC
oTS161	reverse	Ensa_XmaI_rev	AGACCCGGGCTCAACTTGGCCACCCGC
oTS168	forward	Zfand2b_NotI_fwd	ATACCGCGCCGCATGGAGTTTCCGGACCTCG
oTS169	reverse	Zfand2b_XmaI_rev	ATACCCGGGGCACAGGCTGCAGTTGG
oTS170	forward	Ankrd13d_NotI_fwd	ATAGCGGCCGCATGGTACAGTTGGTACTCCAGTATCG
oTS171	reverse	Ankrd13d_XmaI_rev	AATCCCGGGGTGCTCTGTGAGTGACAGC
oTS172	forward	Ckap21_NotI_fwd	ATAGCGGCCGCATGGTGGGGCCCGC
oTS173	reverse	Ckap21_XmaI_rev	AGTCCCGGGTGATTCAAGGACTTCAAAAGCCCAGTG
oTS174	forward	Eya1_NotI_fwd	ACAGCGCCGCATGCAGGATCTAACCAGCCCG
oTS175	reverse	Eya1_XmaI_rev	ACACCCGGGCAGGTACTCTAATTCCAAGGCATGATGC
oTS176	forward	Mindy2_NotI_fwd	ATAGCGCCGCATGGAGAACAGCCCTGAC
oTS177	reverse	Mindy2_XmaI_rev	ACACCCGGGCAAAATCACACAGCTATTTTTC
oTS178	forward	Mindy3_NotI_fwd	ATAGCGCCGCATGTCCGAAGTGACCAAAGAGC
oTS179	reverse	Mindy3_XmaI_rev	CGTCCCGGGGTTGAGCGAAGGACAGCGG
oTS180	forward	Pick1_NotI_fwd	ATAGCGCCGCATGTTTGCAGACTTAGACTATGACATCG
oTS181	reverse	Pick1_XmaI_rev	CGTCCCGGGGAGTCACACCAGCTTC

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List of oligonucleotides designed and used in this study (continued)

Abbr.	Position	Oligonucleotide Name	Oligonucleotide Sequence (5'-3')
oTS182	forward	Pskh_NotI_fwd	ATAGCGGCCGCATGGGCTGTGGACAAAG
oTS183	reverse	Pskh_XmaI_rev	TAACCCGGGGCCGTTATACTGCTGTTGATAGCG
oTS184	forward	Cuedc2_Gib_NotI_fwd	ATCCCGGGCCCATCTAGACGCGGCCGCATGGAGTTGGAGAGGATC
oTS185	reverse	Cuedc2_Gib_XmaI_rev	CCTCCTCCAGCCTGCTGGGCCCCGGGTGGAAAGCGGTACTTTCT
oTS188	forward	C19orf44_homolog_Gib_NotI_fwd	ATCCCGGGCCCATCTAGACGCGGCCGCATGGCTTCCACAAAGAAGAC
oTS189	reverse	C19orf44_homolog_Gib_XmaI_rev	CCTCCTCCAGCCTGCTGGGCCCCGGGTGATTCCGAGGTGGACAG
oTS190	forward	Cuedc1_Gib_NotI_fwd	ATCCCGGGCCCATCTAGACGCGGCCGCATGACCAAGCTTGTTCGC
oTS191	reverse	Cuedc1_Gib_XmaI_rev	CCTCCTCCAGCCTGCTGGGCCCCGGGTGTCCTTCTCTTAGGGC
oTS192	forward	Fbxo21_Gib_NotI_fwd	ATCCCGGGCCCATCTAGACGCGGCCGCATGGCGTCGGTAGCGGGG
oTS193	reverse	Fbxo21_Gib_XmaI_rev	CCTCCTCCAGCCTGCTGGGCCCCGGGTGCGGCTGTGTCTCCTTTGC
oTS198	forward	Pitpnm2_Gib_NotI_fwd	ATCCCGGGCCCATCTAGACGCGGCCGCATGATCATAAAGGAATACCGGATTCCCC
oTS199	reverse	Pitpnm2_Gib_XmaI_rev	CCTCCTCCAGCCTGCTGGGCCCCGGGTGGGTCCCGTGGCTGC
oTS200	forward	Fam63a_NotI_fwd	ATAGCGGCCGCATGGAACAAACCTCAGACTGAG
oTS201	reverse	Fam63a_XmaI_rev	ACACCCGGGGAGCAGAACACACAGTCTGACTC
DaM-P417	forward	/	GTTGATATGGCTACCGCCTCTTCCCTGGTCT
DaM-P418	reverse	/	AGAGGGGTAGCCATATCAACTTTTGTGTAC