A high-resolution subcellular map of proteins in ciliated cells

Authors

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Abbreviations

AI - artificial intelligence

AX – axoneme

BB - basal body

BP - biological process

CC - cellular composition

DSB - double-strand break

FE - function evidence

FT - fallopian tube

GO - gene ontology

HIER - heat-induced epitope retrieval

HPA - Human Protein Atla

HPP - Human Proteome Project

HRP - horseradish peroxidase

IgE – immunoglobulin E

IHC - immunohistochemistry

MF - molecular function

mIHC - multiplex immunohistochemistry

PCD - primary ciliary dyskinesia

PE - protein evidence

PKA – protein kinase A

QC - quality control

ROI - regions of interest

RT - room temperature

scRNA - single-cell RNA

TMA - tissue microarray

TZ - transition zone

Abstract

Motile cilia are complex structures regulated by thousands of genes and are essential for various physiological functions such as respiration and reproduction. Dysfunction can lead to severe conditions, underscoring the need for a deeper understanding of the specific ciliary compartments at a molecular level. Here, we developed a stringent high-throughput workflow combining multiplex immunohistochemistry and automated image analysis to investigate protein expression and subcellular localization in motile ciliated cells across five different tissues: nasopharynx, bronchus, fallopian tube, endometrium, and cervix. We characterized 187 proteins in different regions of ciliated cells and were able to divide these proteins into seven distinct expression clusters based on the regional distribution of the proteins. Interestingly, a large proportion of the studied proteins had previously only limited information on the exact molecular function, and several proteins were mapped to motile cilia for the first time, providing novel spatial evidence of their localization. We also identified several proteins that differed in expression between the investigated tissues, suggesting that not all motile cilia proteins are consistently expressed. Our presented approach holds promise for mapping the entire motile cilia proteome in health and disease, forming the basis for further understanding of underlying mechanisms of ciliopathy, and a first step towards applications in precision diagnostics and healthcare.

Introduction

Cells are not organized in a random manner, but instead form complex organ systems where molecules, cellular compartments and cell types are spatially distributed based on cellular function. Proteins often execute their functions in specific subcellular locations, which is crucial for maintaining various cellular processes such as cell growth, motility, and signaling[1]. To understand the role of proteins and their biochemical functions within the cell, knowledge about the spatial localization from both a cellular and subcellular perspective is necessary[2]. With the goal to map all human proteins, the Human Protein Atlas (HPA) project has built one of the world's largest biological knowledge resources, available as an open-access database (www.proteinatlas.org). The HPA integrates both transcriptomics data and spatial antibody-based proteomics[3], and within this effort, >15,000 proteins have been mapped to all major human normal and cancer tissues using classical immunohistochemistry (IHC). The resource of more than 10 million manually annotated high-resolution histological images provides important information on spatial expression patterns of proteins from a body-wide and cell type-specific perspective, but the possibilities to distinguish subcellular patterns are limited. IHC is primarily used to study one protein at a time, but an increasing number of methods utilize multiplexed IHC (mIHC), enabling novel insights into co-expression and tissue or cell type heterogeneity. Spatial proteomics technologies are of utmost importance to characterize the intrinsic subcellular functions of human proteins[4], as has been demonstrated in multiple large-scale studies[2,5]. To increase the resolution of the Tissue resource in the HPA, we have in the most recent update of the database (HPA v24) implemented a novel protocol for studying protein localization in single-cell types, cell states and subcellular structures in human tissue samples, utilizing mIHC-based fluorescence imaging. With the help of single-cell transcriptomics and an iterative staining-stripping method, antibody panels have been developed to profile tissues more in detail. As mIHC is more quantitative than regular IHC due to higher dynamic range, the images are also better suited for automated image analyses efforts, limiting potential bias due to subjectivity during manual annotation[5,6].

Here, we have focused on the important subcellular organelle cilium (plural cilia), a filamentous hair-like cellular membrane protrusion, whose assembly (ciliogenesis) and structure requires at least 2,000 genes[7]. There are two forms of cilia in human cells - primary cilia and motile cilia. Motile cilia are present in different organs where they aid in transporting cells or particles, and their function is crucial for e.g. reproduction, respiration, L/R body

asymmetry, or brain development and function. Defects in genes related to motile cilia have been linked to motile ciliopathies, contributing to a large spectrum of diseases in various tissues, including chronic respiratory problems, infertility, and hydrocephalus[8]. The most common ciliopathy, primary ciliary dyskinesia (PCD), is characterized by impaired ciliary movement. PCD is usually caused by autosomal recessive mutations, although it can be caused by autosomal dominant and X-linked mutations in rare cases[9,10]. Despite advancements in understanding PCD in the last 20 years, its diagnosis remains challenging, and treatments are lacking[11]. Studying motile cilia genes can provide us with fundamental insights into ciliary function and structure, being a first step towards understanding the underlying molecular mechanisms of ciliopathies (e.g., situs abnormalities)[12]. Structurally, the cilium features several structurally unique regions, such as the basal body, which anchors the cilium to the cell and serves as a microtubule organizing center; the transition zone, a specialized region at the ciliary base that regulates protein trafficking and acts as a diffusion barrier; and the axoneme, the central ciliary skeleton that extends from the centrioles in the basal body and traverses the transition zone and that is composed of microtubule doublets and associated motor proteins that mediate ciliary movement[13,14]. Since these ciliary compartments are small and attached to each other and underlie dynamic changes, the optimal way to study them, i.e., their architecture, is through image- and microscopy-based methods[15].

In the present investigation, we aimed to develop a novel high-throughput workflow for quantifying cilia proteins at subcellular resolution using mIHC and automated image analysis. Based on signal overlap with a panel of marker proteins outlining the axoneme, the transition zone, the basal body, the cytoplasm, and the nucleus of ciliated cells, the spatial subcellular localization of 187 motile cilia proteins was mapped across five different tissue types from both respiratory and reproductive tracts (nasopharynx, bronchus, fallopian tube, endometrium and cervix). The detailed information at the tissue, cellular, and subcellular levels offered new insights into the presumed functions of these proteins in motile cilia, establishing a valuable resource for future research in cilia biology. Moreover, our approach, combining spatial proteomics with automated image analysis, holds significant potential for large-scale studies investigating motile cilia in both healthy and diseased tissues.

Results

Development of a multiplex panel for motile ciliated cells

In this project, a multiplex immunohistochemistry (mIHC) panel was developed with the goal to study the subcellular localization of proteins to the different complex structures of ciliated cells across different tissue types, leveraging publicly available data from the Human Protein Atlas (HPA, www.proteinatlas.org)[3](Figure 1A). A dedicated tissue microarray (TMA) was created, including five tissue types known to harbor motile ciliated cells in both the respiratory and reproductive tracts: nasopharynx, bronchus, fallopian tube (FT), endometrium, and cervix (Figure 1B; Supplementary Table 1). To precisely determine the localization of candidate proteins using an iterative mIHC workflow, a fixed panel of five antibodies outlining different subcellular structures of motile ciliated cells was developed (Figure 1C). The panel was used together with each of the candidate proteins, which for each microscopic slide was replaced by a new antibody for another candidate protein (Figure 1D). This design allowed for high-throughput characterization of a large number of proteins across multiple tissue samples.

The fixed panel was designed to target five distinct subcellular structures of ciliated cells: the axoneme (AX), the transition zone (TZ), the basal body (BB), the cytoplasm, and the nucleus (Figure 2A). The selection of markers and antibodies was based on multiple criteria, including literature descriptions (e.g. UniProtKB), reliability of antibodies available in the HPA, IHC staining patterns in ciliated cells across the tissues of interest (Figure 2D), and compatibility with the mIHC workflow. The final panel was based on the following proteins: DNAH9 (Dynein Axonemal Heavy Chain 9), a component of the motile cilia axoneme[16]; NPHP4 (Nephrocystin 4) localized in the TZ[17–19]; CROCC (Rootletin), the main structural protein of the ciliary rootlet[20]; AGR3 (Anterior Gradient Protein 3), described in mice as essential for regulating ciliary beat frequency[21]; and the transcription factor FOXJ1 (Forkhead Box Protein J1), the key transcription factor regulating motile cilia formation[13,22] (Figure 2B; Supplementary Table 2). To validate the panel antibodies, particularly those targeting ciliary compartments (AX, TZ, and BB), we performed confocal microscopy at 63X magnification (Figure 2C). Given the complex structure of the cilium, no overlap was observed between these three markers, reinforcing confidence in the panel. This validation enabled the development of image analysis pipelines and the definition of regions of interests (ROIs) based on the signals obtained from the fixed panel, corresponding to the five distinct subcellular localizations in ciliated cells (Supplementary Figure 1E).

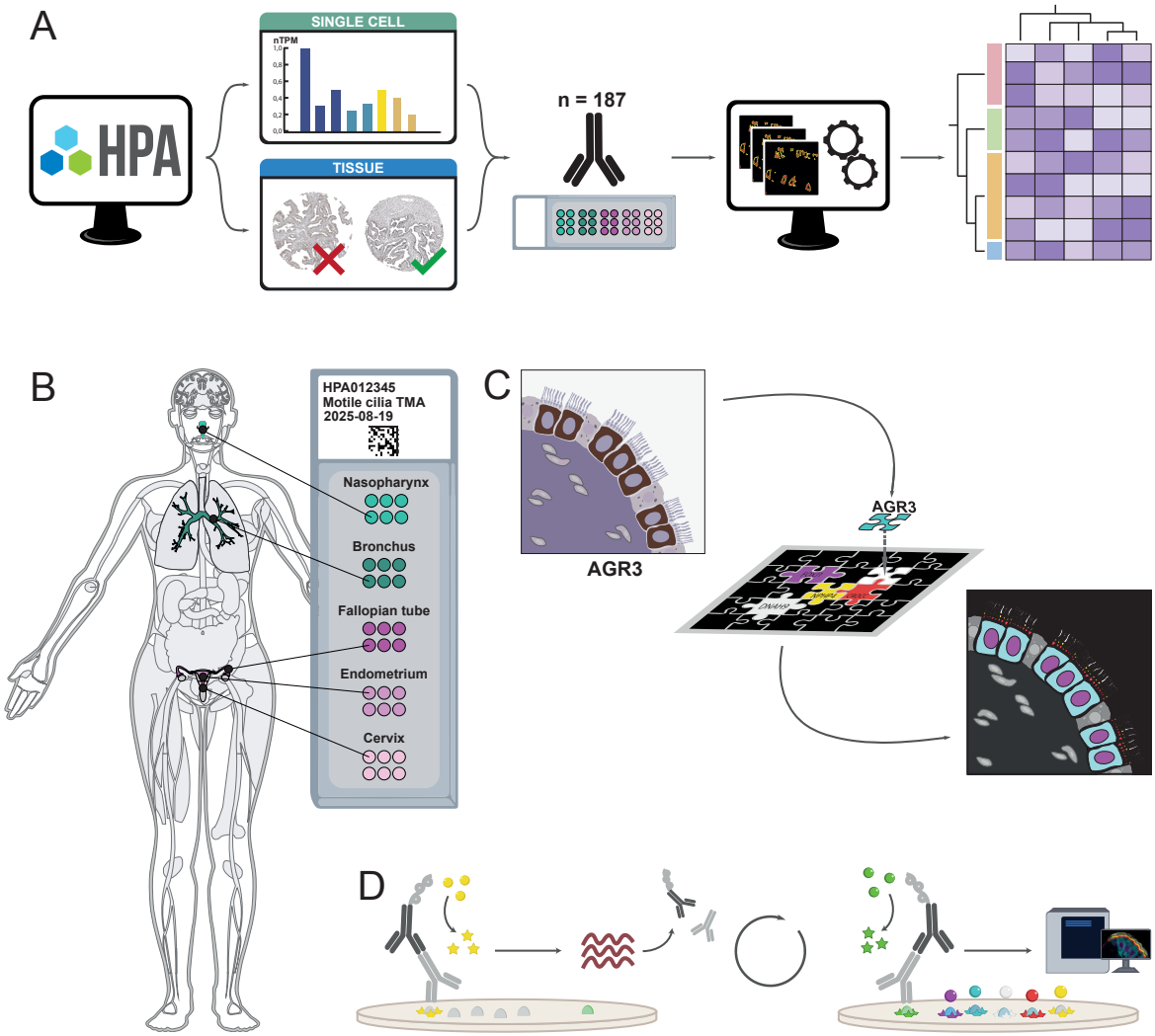

Figure 1: Experimental design. **A)** Selection of protein candidates from the Human Protein Atlas (www.proteinatlas.org) using both the Single Cell and Tissue resources, allowing the identification of candidates expressed in ciliated cells with a reliable antibody (n=187). These candidates were stained alongside the fixed antibody panel. Each acquired image was automatically analyzed to determine the expression profile, followed by clustering analysis. **B)** Five different tissues were collected and organized into a tissue microarray (TMA), consisting of two airway tissues (nasopharynx and bronchus) and three reproductive tissues (fallopian tube, endometrium, cervix). For each tissue, six cores from three patients were included. **C)** The fixed panel antibodies were selected based on a literature review and IHC staining comparisons available in the Tissue resource of the HPA, here exemplified by the protein AGR3, showing staining in the cytoplasm of ciliated cells. Antibodies with non-overlapping signals targeting different subcellular compartments of ciliated cells: nucleus (purple), cytoplasm (cyan), basal body (BB, red), transition zone (TZ, yellow), and axoneme (AX, white) were combined into a multiplex immunohistochemistry (mIHC) panel. **D)** Schematic representation of the mIHC

workflow, with the first and last cycles illustrated. The method relies on iterative cycles, each separated by heat-induced antibody stripping.

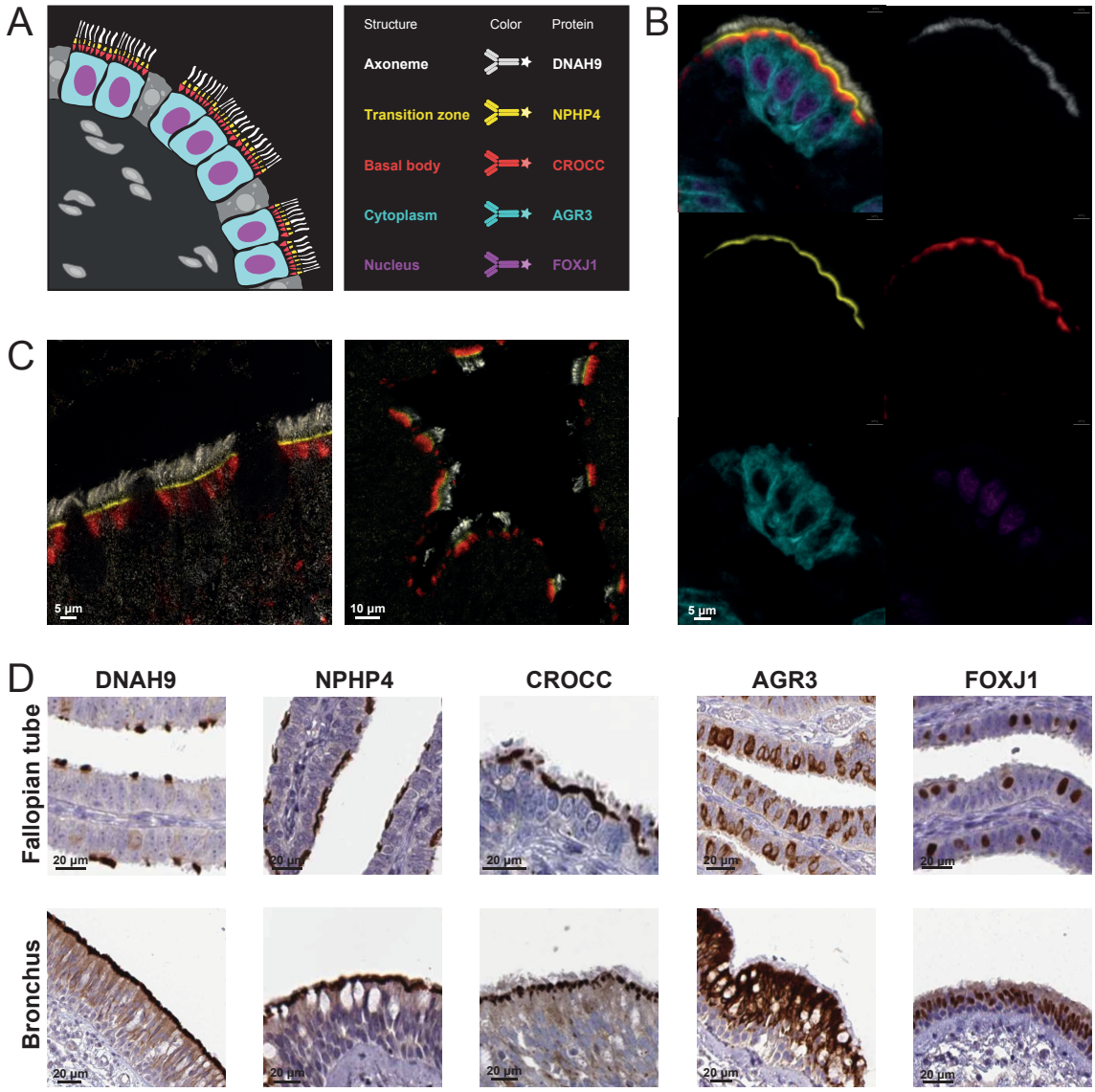

Figure 2: Validation and characterization of the fixed antibody panel. **A)** Schematic overview of the fixed panel used for mIHC stainings. The mIHC panel included five antibodies targeting proteins expressed in specific subcellular structures of ciliated cells: DNAH9 for the axoneme (AX, white), NPHP4 for the transition zone (TZ, yellow), CROCC for the basal body (BB, red), AGR3 for the cytoplasm (cyan), and FOXJ1 for the nucleus (purple). **B)** Staining of the 5-plex fixed panel in ciliated cells of the fallopian tube. Top left: all five antibodies combined; top right: AX; middle left: TZ; middle right: BB; bottom left: cytoplasm; bottom right: nucleus. **C)** Confocal microscopy images. Confocal microscopy was used to validate the markers targeting ciliary structures. Non-overlapping signals representing the AX (white), TZ (yellow), and BB (red) are shown in the nasopharynx (left) and

fallopian tube (right) at 63X resolution. **D)** IHC images from the HPA. Antibodies included in the panel were selected based on their staining patterns and reliability scores in the Tissue resource of the HPA.

Subcellular mapping of protein expression in ciliated cells

The selection of candidate proteins to be stained alongside the fixed panel was performed using both the Tissue and Single Cell resources of the HPA (Figure 1A). Candidate proteins were identified based on their normalized RNA expression levels in ciliated cell clusters from the Single Cell resource, prioritizing those with higher expression in ciliated cells compared to other cell types present in the studied tissues (Supplementary Figure 2). IHC staining patterns were examined across the five tissues, and antibody reliability was assessed based on the stringent criteria for determining specificity of antibodies in the Tissue resource of the HPA[23]. A final list of 187 candidate proteins expected to be expressed in motile ciliated cells was compiled for staining with mIHC (Supplementary Table 3).

Hierarchical clustering and functional annotation of ciliated cells proteins

A total of 187 proteins were stained using the mIHC panel in motile ciliated cells across five different tissues. In order to provide a quantitative and systematic assessment of expression levels, an automated image analysis workflow was developed to quantify pixel intensity values for each analyzed protein and assess the subcellular localization of the staining patterns in relation to the fixed antibody panel (Supplementary Figure 1). To determine the overlap between each candidate protein and a specific area of the ciliated cells, the analysis method defined ROIs based on each panel marker individually. Pixel intensity values within these ROIs were then extracted from the images, allowing quantification of colocalization for each specific subcellular structure (Supplementary Figure 1). To account for multiple images per protein per ROI and per tissue, a median pixel intensity value was calculated per protein and tissue type for each ROI, generating an expression profile across the dataset. The resulting quantitative protein expression levels were subsequently used for hierarchical clustering, which identified seven distinct protein clusters (Figure 3A). Clustering was also performed on ROI per tissue type, revealing that AX and TZ regions consistently grouped together across all tissues. In contrast, BB, cytoplasm, and nucleus displayed more heterogeneous localization patterns, with the cytoplasm and BB exhibiting the highest variability across tissues. Gene ontology (GO) enrichment analysis was performed for each cluster across the three GO categories: biological process (BP), molecular function (MF) and cellular component (CC). This analysis aimed to validate the expression patterns across different subcellular localizations and provide insights

into the potential functions of the protein within each cluster (Figure 3B; Supplementary Table 4). Although the number of proteins per cluster varied, ranging from n=7 in Cluster 1 to n=94 in Cluster 2, GO terms were retrieved for each cluster, despite some candidate proteins remaining poorly categorized (Supplementary Table 4). To address gaps in functional annotation, the Human Proteome Project (HPP)[24] recently introduced a ranking system that classifies proteins based on the strength of existing functional evidence. This system ranges from Function Evidence 1 (FE1), where at least one function has been well described, to Function Evidence 5 (FE5), where functional annotations are uncertain or nonexistent[25]. We integrated these scores into our dataset (Supplementary Table 5) to assess the functional characterization of the proteins included in our study. Interestingly, just over a quarter (27%, n=51) of the candidate proteins stained alongside the panel fall into the FE1 category, indicating that at least one of their functions has been described. However, the majority (73%, n=136) remain poorly or partially characterized, with limited or no functional annotations (FE2 to FE5). To further examine the relationship between protein function and clustering, we analyzed the distribution of functional evidence scores across clusters (Supplementary Figure 3).

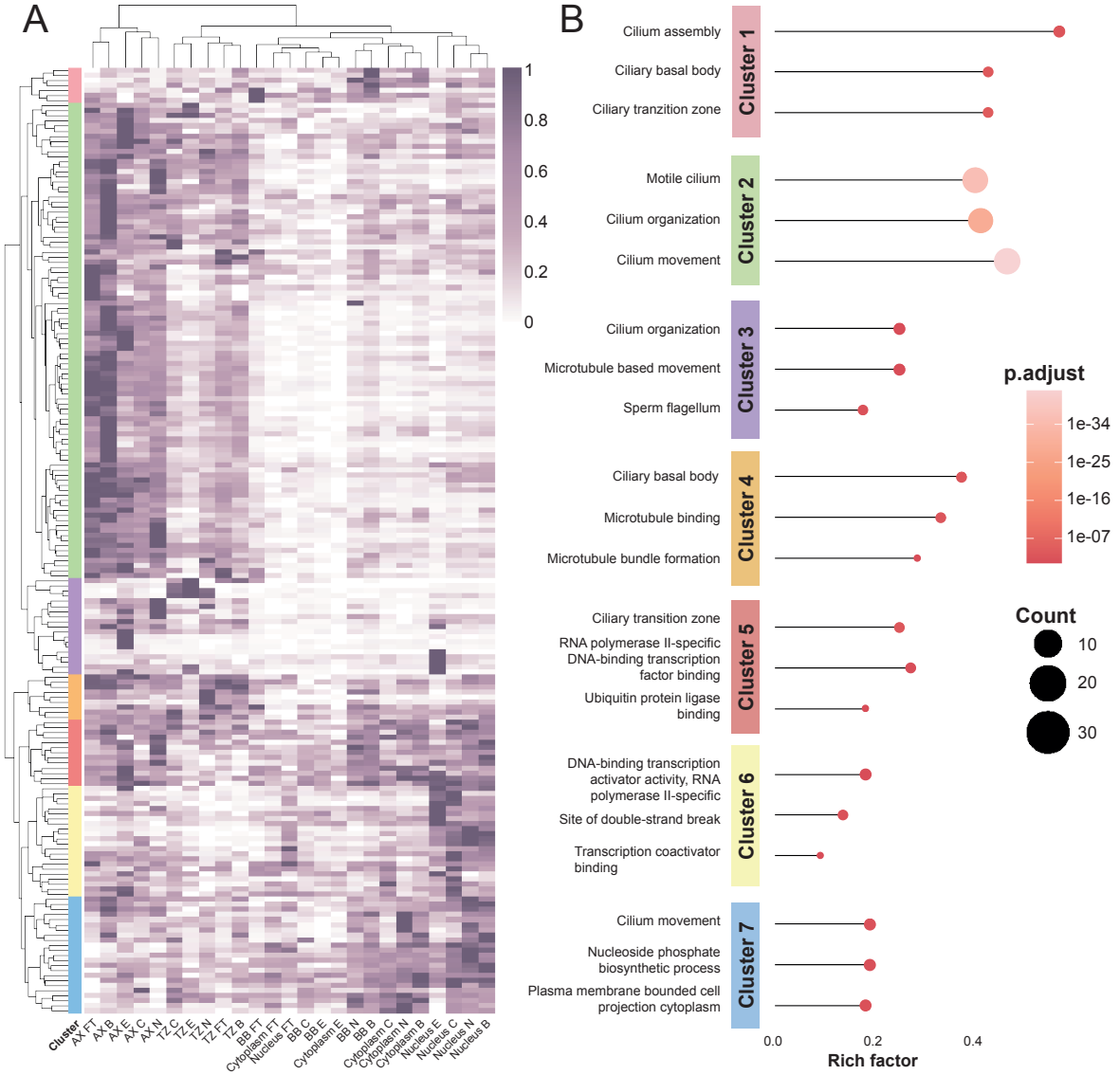

Figure 3: Expression Patterns, Clustering Analysis, and Gene Ontology Enrichment. **A)** A heatmap was generated based on expression levels obtained from image analysis for each candidate protein across different subcellular localizations and tissues (Fallopian tube (FT), Bronchus (B), Endometrium (E), Cervix (C), and Nasopharynx (N). Hierarchical clustering was applied to both rows and columns. Interestingly, the columns were primarily clustered according to subcellular localization rather than tissue type, particularly for ciliary structures such as the axoneme (AX) and transition zone (TZ). Row clustering identified seven distinct clusters based on the expression patterns of the 187 candidate proteins. **B)** Bubble plot showing the top three Gene Ontology (GO) terms of the three categories (BP, MF and CC) per cluster, with enrichment levels represented by the rich factor i.e. the proportion of input genes annotated to a specific term relative to the total genes associated with that term.

Cluster 1: Basal body and ciliary core

Cluster 1 (n = 7, primarily expressed in the BB, Figure 3A), was strongly linked to cilia, as indicated by its top GO terms: cilium assembly, ciliary basal body, and ciliary transition zone (Figure 3B). This suggests that proteins in this cluster play a central role in ciliary function. One notable example is MDM2, which localized to the BB of the cilia in all five tissues and showed a strong concordance between image observation and image analysis results (Figure 4A). Interestingly, none of the 65 GO terms associated with MDM2 in our analysis; including response to magnesium ion, cellular response to estrogen stimulus, positive regulation of intracellular protein transport, and negative regulation of cell cycle G1/S phase transition; are directly related to cilia or their structure. Although MDM2 is an FE1 protein with at least one well-characterized function, its role in ciliated cells remains unclear.

Cluster 2: Axonemal and motile cilia proteins

Cluster 2 harbored the largest number of proteins (n = 94), with the top GO terms: motile cilium, cilium organization, and cilium movement, indicating a strong association with motile cilia rather than cilia in general (Figure 3B). The proteins in this cluster were primarily expressed in AX across all five analyzed tissues (Figure 3A). One example is NME9 (NME/NM23 family member 9, also known as TXNDC6 or TXL-2) (Figure 4B). While NME9 is highly expressed in multiple tissues, with the highest mRNA level detected in the testis, previous studies have shown that it localizes to airway epithelial cilia as a microtubule binding protein[26]. Here, we demonstrated that NME9 is not restricted to the airway epithelium but is also present in the motile cilia of three female reproductive tissues, where it exhibited similar staining intensity. Another protein in Cluster 2 is LRRC73 (Leucine rich repeat containing 73) (Figure 4B). The mIHC analysis indicated that LRRC73 was equally expressed in AX and BB but absent in TZ, with a consistent pattern across all five tissues. However, no GO terms were identified for LRRC73 in our analysis, nor are functional annotations available in UniProt, and it is ranked as a FE5 protein (Supplementary Table 4 and 5). A third example is DPCD (Deleted in primary ciliary dyskinesia homolog) (Figure 4B), a protein implicated in the function or formation of ciliated cells. Its highest gene expression was observed in the testis, and previous studies have shown it is expressed in sperm[27]. Our analysis confirmed that DPCD was strictly localized to AX of motile cilia in all five tissues, with stronger expression in airway epithelial cilia compared to those in the female reproductive tract. In humans, DPCD interacts with RUVBL1 and RUVBL2, proteins known to be crucial for the initiation of ciliogenesis of primary cilia[28–30]. RUVBL1, which fell into Cluster 7, was detected in the cytoplasm and

nucleus of ciliated cells but not in the ciliary structure (Figure 5C), in line with previous findings. GO terms identified for RUVBL1 include dynein axonemal particle, TFIID-class transcription factor complex binding, and ADP binding (Supplementary Table 4). Notably, *Ruvbl1* knockdown in mice leads to axoneme structural defects, further supporting its role as a ciliopathy-related protein essential for ciliary integrity[30]. Finally, KIAA2012, a poorly characterized protein with no known function (FE5) (Figure 4B; Supplementary Table 5) was also found in this cluster. Our data revealed that it localized to AX of motile cilia across all five tissues, with similar expression levels throughout.

Cluster 3: Ciliary and flagellar motility proteins

The top GO terms for Cluster 3 (n= 19), were cilium organization, microtubule-based movement, and sperm flagellum (Figure 3B), highlighting its association with ciliary and flagellar structures. One notable protein in this cluster is MS4A8 (Membrane spanning 4-domains A8, also known as MS4A8b) (Figure 4C), a member of the MS4A protein family, that shares sequence and structural homology with the IgE receptor CD20[31]. Here, we demonstrated that MS4A8 is specifically localized to AX of motile ciliated cells across all five tissue types. MS4A8 has been ranked FE4 and interestingly also lacks previous evidence at the protein level (Supplementary Table 5). Our findings provide the first spatial evidence of MS4A8 protein expression, offering a valuable insight into its potential role in motile cilia function.

Cluster 4: Dual localization in axoneme and transition zone

Cluster 4 (n = 9, with no FE1 proteins, Supplementary 3), presented a more complex expression pattern, with overrepresentation in both the axoneme and TZ (Figure 3A). The top GO terms identified were ciliary basal body, microtubule binding, and microtubule bundle formation (Figure 3B), highlighting the potential structural roles of these proteins in cilia. One key protein in this cluster was SPAG6 (Sperm associated antigen 6) (Figure 4D), which is strongly linked to microtubule bundle formation, cilium organization, and cilium movement (Supplementary Table 4). SPAG6 is crucial for maintaining normal ciliary structure and function, and its loss has been associated with brain edema in mice[32,33]. While SPAG6 deficiency leads to male infertility, female mice remain fertile, although fertilization is delayed, suggesting an impact on the motile cilia in the oviduct[34]. In this study, SPAG6 showed widespread expression across the whole cell in bronchus and nasopharynx, while its localization was more restricted to AX and TZ in endometrium and cervix (Figure 4D). Another protein in this cluster is LRRC45 (Leucine rich repeat containing 45), which was primarily localized to AX, as well as TZ and

BB (Figure 4D). Previously described in primary cilia, defects of LRRC45 are thought to alter ciliary length and beat frequency[35,36]. Here, we showed its presence and specific localization in motile cilia across both the respiratory and reproductive tract. However, our GO term analysis did not identify any functional annotation for LRRC45, and it is classified as FE5 (Supplementary Table 5), indicating that its precise role remains to be elucidated.

Cluster 5: Ciliated proteins with diverse localization

In Cluster 5 (n = 13), protein expression patterns were distributed across the entire ciliated cell (Figure 3A). The GO terms identified in this cluster were ciliary transition zone, RNA polymerase II-specific DNA-binding transcription factor binding, and Ubiquitin protein ligase binding, suggesting a broad functional involvement in cellular activity (Supplementary Table 4). One example is RIIAD1 (Regulatory subunit of type II PKA R-subunit domain containing 1), which was predominantly localized in AX, TZ, cytoplasm and nucleus across all examined tissues (Figure 5A). Although RIIAD1 remains poorly characterized, studies have reported its upregulation in breast cancer tumors[37,38]. Notably, our GO term analysis did not identify any functional annotations for this protein, which is classified as a FE5 protein (Supplementary Table 4). Another protein in this cluster is SGMS2 (Sphingomyelin synthase 2), which was detected in all targeted locations in the ciliated cells across all tissues, although significantly lower presence was observed in BB in the reproductive tissues (Figure 5A). SGMS2 is classified as a FE1 protein (Supplementary Table 5), indicating strong evidence for a molecular function. While our GO term analysis identified phosphotransferase activity for other substituted phosphate groups as a related term (Supplementary Table 4), additional annotations, including nucleoplasm, plasma membrane, and sphingomyelin synthase activity, suggest broader functional roles.

Cluster 6: Nuclear and cytoplasmic proteins

Cluster 6 (n = 22) consisted of proteins primarily localized to the cytoplasm and nucleus (Figure 3A), as supported by the top three GO terms: DNA-binding transcription activator activity, RNA polymerase II-specific, site of double-strand break, and transcription coactivator binding (Figure 3B). Notably, 45% of the proteins (n=10) in this cluster are classified as FE1, indicating strong evidence of the molecular function (Supplementary Figure 3). One key protein of this cluster is WRAP53 (WD repeat containing antisense to TP53) (Figure 5B), classified as FE1 (Supplementary Table 5). The GO term enrichment identified the site of double-strand break as a related function (Supplementary Table 4), aligning with its known role in DNA damage

response. WRAP53 is recruited to the site of a double strand break (DSB), where it facilitates the recruitment of other DNA repair proteins[39]. Here, we showed that WRAP53 was predominantly expressed in the nucleus of ciliated cells across all tissues. Another protein in this cluster is SAMHD1 (SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1) (Figure 5B), also classified as FE1 (Supplementary Table 5). Like WRAP53, SAMHD1 is associated with the GO term site of double-strand break (Supplementary Table 4). The present study revealed its nuclear localization in ciliated cells across all five tissues, reinforcing its potential role in DNA repair mechanisms.

Cluster 7: mixed localization with ciliary and cytoplasmic functions

The final cluster, Cluster 7 (n = 23), exhibited less specific localization patterns compared to other clusters, with proteins predominantly found in the cytoplasm and the nucleus (Figure 3A). GO term analysis identified key biological functions, including cilium movement, nucleoside phosphate biosynthetic process, and plasma membrane bounded cell projection cytoplasm (Figure 3B). Among the proteins in this cluster, RUVBL1 was previously mentioned with proteins from cluster 2 (Figure 5C) and classified as FE1 (Supplementary Table 5). Another protein in this cluster is CFAP73 (Cilia and flagella associated protein 73), classified as FE4 (Supplementary Table 5), which was primarily expressed in the cytoplasm and, to a lesser extent, in the nucleus across all tissues (Figure 5C). GO analysis linked CFAP73 to essential ciliary processes, including cilium movement, axonemal dynein complex assembly, and cilium organization (Supplementary Table 4), suggesting a role in ciliary structure and function.

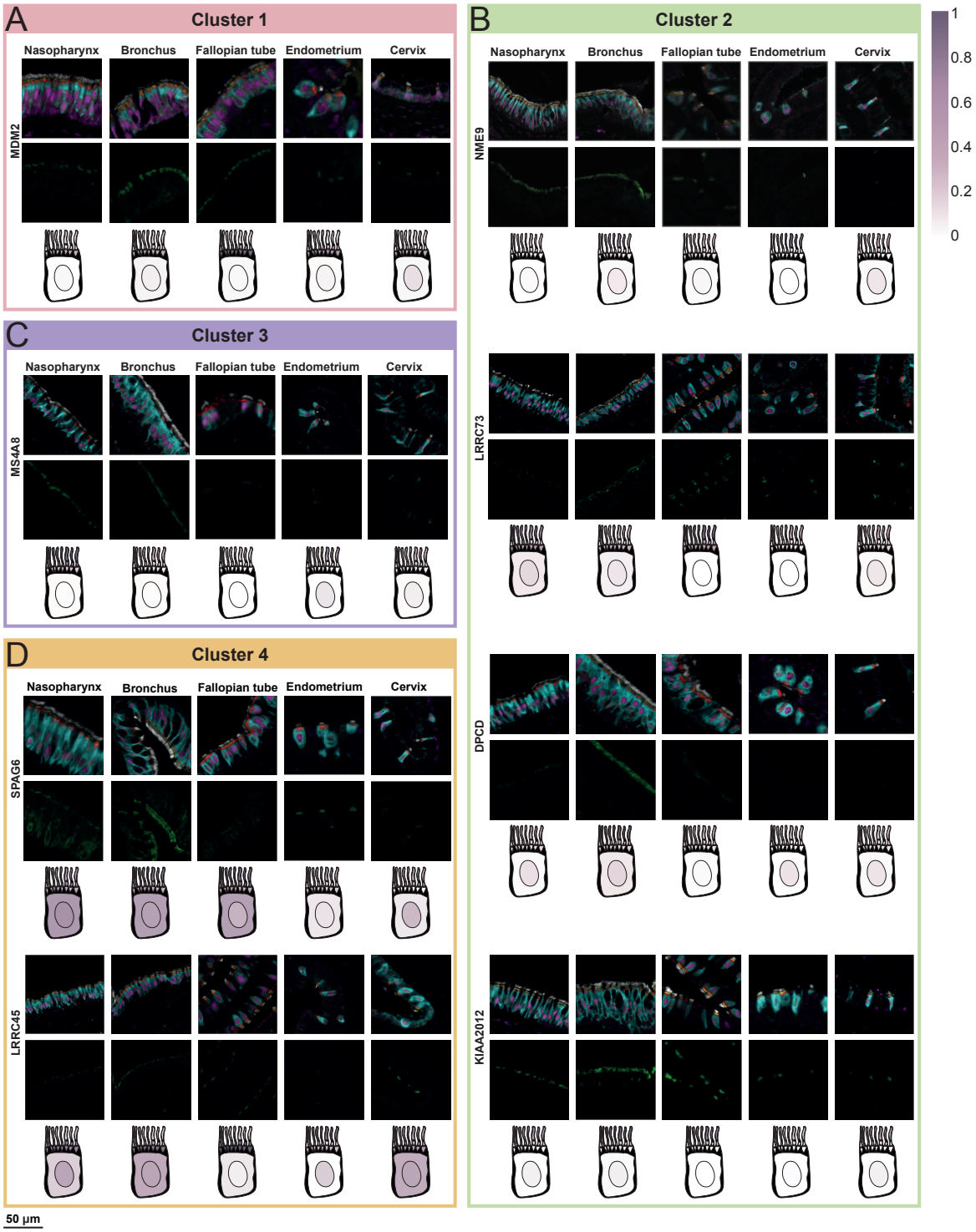

Figure 4: mIHC staining of representative proteins from each cluster across different tissues. The top row of images shows the composite staining, including the fixed panel and the candidate protein. The bottom row displays only the candidate protein (in green). Below the images, a schematic representation of a ciliated cell illustrates the expression levels obtained from image analysis for each subcellular localization. A) Cluster 1: MDM2 (BB) B) Cluster 2: NME9 (AX), LRRC73 (AX and BB),

DPCD (AX), and KIAA2012 (AX). C) Cluster 3: MS4A8 (AX). D) Cluster 4: SPAG6 (whole cell in bronchus and nasopharynx; AX and TZ in endometrium and cervix) and LRRC45 (AX, TZ and BB).

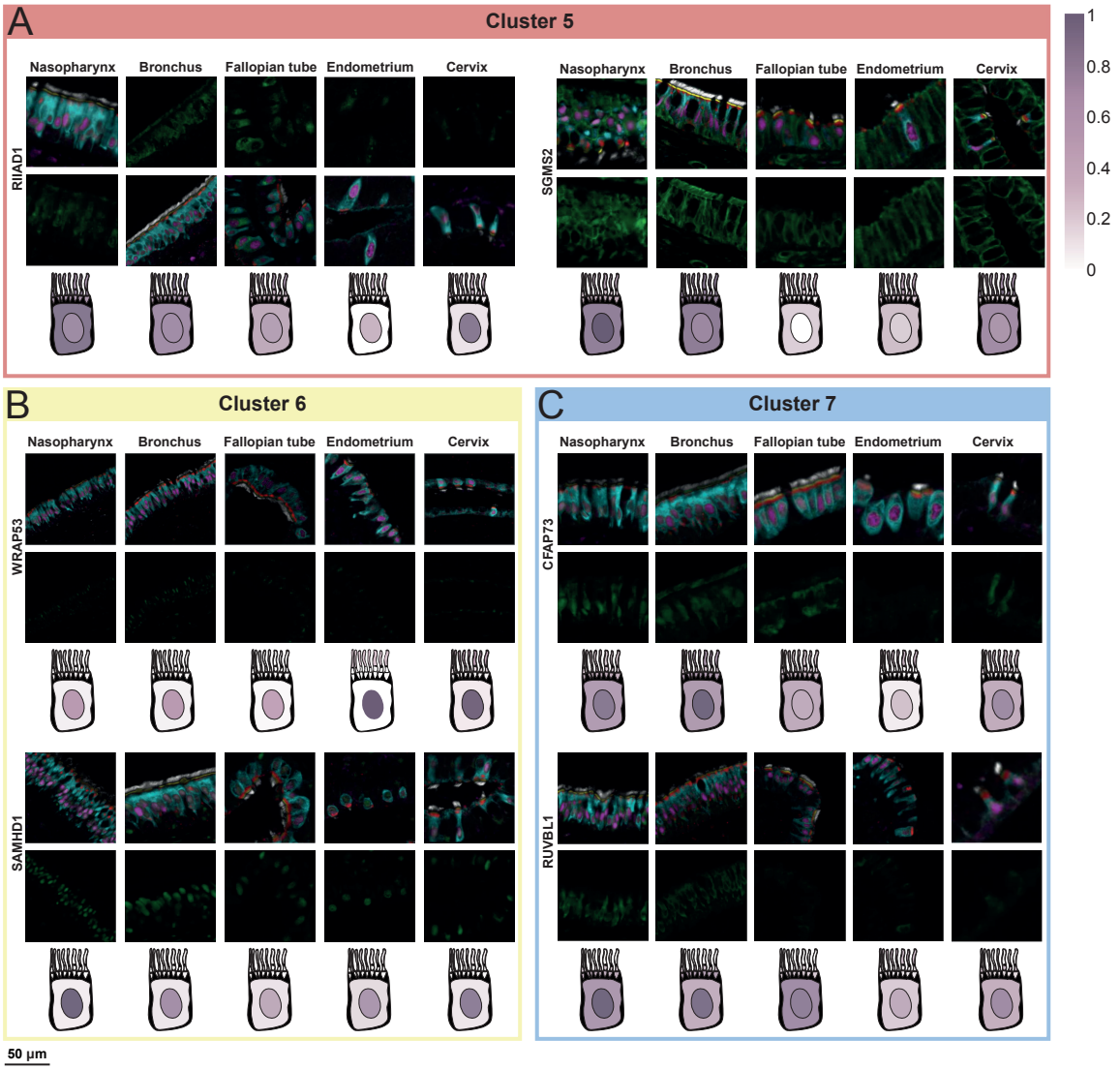

Figure 5: mIHC staining of representative proteins from each cluster across different tissues. The top row of images shows the composite staining, including the fixed panel and the candidate protein. The bottom row displays only the candidate protein (in green). Below the images, a schematic representation of a ciliated cell illustrates the expression levels obtained from image analysis, the darker the color, the higher the expression level. **A**) Cluster 5: RIIAD1 (AX, TZ, cytoplasm and nucleus) and SGMS2 (whole cell). **B**) Cluster 6: WRAP53 (nucleus) and SAMHD1 (nucleus). **C**) Cluster 7: CFAP73 (cytoplasm and nucleus) and RUBBL1 (cytoplasm and nucleus).

Discussion

This study introduces a comprehensive mIHC and automated image analysis workflow to investigate the subcellular localization of proteins in motile ciliated cells at a high resolution. To our knowledge, this is the first approach to combine high-resolution imaging with the potential for high-throughput analysis of a large set of proteins across multiple tissue types. The novel workflow was successfully used to spatially map 187 proteins in ciliated cells across five human tissues from both respiratory and female reproductive tracts, based on analysis of the overlap of each of the candidate proteins with a fixed 5-plex panel outlining distinct subcellular compartments of motile ciliated cells. The validated panel allowed us to localize and quantify each of the candidate proteins within five distinct subcellular compartments of ciliated cells: the nucleus, cytoplasm, and three different ciliary regions defined by ciliary structures localizing to these: AX, TZ, and BB[14], using an automated image analysis workflow.

The 187 candidates proteins studied in the present investigation were selected using two key resources of the Human Protein Atlas (HPA): i) the Single Cell resource, to ensure a minimum transcript-level expression in ciliated cells compared to other cell types present in the studied tissues, and ii) the Tissue resource, to prioritize candidates with reliable antibodies showing positive IHC staining in ciliated cells in at least one of the five analyzed tissues included in the study. This selection process enabled us to study not only well-characterized proteins in cilia (such as CCDC39[40,41], and DNAH7[42,43]) but also a broader set of proteins with no previous description in ciliated cells (such as KIAA2012).

While the majority of the proteins encoded by the human genome have been credibly identified, most proteins still lack a specifically defined function based on experimental evidence. One of the major goals of the international consortium HPP is to gain a solid understanding of at least one function for every protein at the molecular level[24]. As a result, the HPP has introduced the definition of function evidence (FE) scores that takes into consideration GO terms, described as FE1-FE5 according to the level of evidence for a molecular function[25]. These new FE scores have been implemented for each protein encoded by the human genome. Interestingly, a majority (73%) of the candidate proteins characterized in our study showed FE2-FE5 scores, indicating that a full understanding of the molecular function is still lacking. Since spatial localization is a first step towards understanding molecular function, our detailed data presenting a specific localization to ciliated cells with a subcellular resolution gives further

insights into a presumed function of these proteins. The spatial localization data constitutes a starting point for further functional studies using e.g. model systems, to determine the roles of these proteins in each of the analyzed tissue types.

For further functional analysis, we also performed a hierarchical clustering analysis of the candidate proteins for each of the analyzed tissues using quantitative protein localization data generated by automated image analysis. The clustering analysis revealed that protein localization was largely conserved across tissues, particularly for AX and TZ, suggesting a shared spatial distribution for these proteins. Similarly, candidate proteins clustered based on their expression patterns, resulting in seven distinct expression groups. While some clusters exhibited clear localization patterns, such as cluster 2, with proteins mainly expressed in the axoneme, or cluster 6, in the nucleus; others, like cluster 5, displayed a more dispersed expression profile. Nevertheless, the clustering analysis allowed us to group the proteins according to expression patterns, highlighting numerous poorly described proteins that shared a similar expression profile with well-described cilia markers, thus indicating a similar molecular function. We also performed a GO analysis for each of the expression clusters, indicating a clear overrepresentation of ciliary functions, thus validating our strategy.

In the present investigation, we could for the first time visualize the detailed subcellular localization of proteins expressed in motile ciliated cells for several proteins not previously described in the context of cilia biology. One example is KIAA2012, a poorly described protein located in the axoneme of motile cilia in both respiratory and female reproductive tissues. Based on its subcellular location, it can be anticipated that this protein may contribute to cilia motility and thereby has the potential to cause primary ciliary dyskinesia (PCD) where the function of motile cilia is impaired, a syndrome causing a wide range of symptoms and diseases[8]. Another not well studied protein is RIIAD1, previously shown to be upregulated in breast cancer tumors, but without a known molecular function. The name of the protein suggests that it regulates a subunit of protein kinase A (PKA), involved in endocrine signaling and previously described to play a crucial role in regulating ciliary beating frequency[44]. Here, we show that this protein is generally expressed across the whole ciliated cell, both in the cell body and the axoneme, in all five analyzed tissues. Interestingly, scRNA levels in other analyzed tissues in the Single Cell resource of the HPA show that expression is dominated in ciliated cells, followed by early spermatids, and cone photoreceptor cells. As mature sperm contain flagella, with a similar molecular function related to motility as ciliated cells[45], and

photoreceptor cells contain specialized sensory cilia[46], the mRNA expression levels together with the spatial localization pattern suggests that this protein may be crucial for regulating and supporting key ciliary functions. In addition to KIAA2012 and RIIAD1, our study included numerous other proteins that lack a description of molecular function or were not previously described in the context of cilia biology, including MS4A8 which previously only has been described at the transcript level. Here, we could provide the first spatial evidence that this protein is expressed in the axoneme of ciliated cells.

The selection of proteins included in the present investigation was mostly focusing on conserved motile ciliary proteins present in all the analyzed tissues, but we did identify several proteins where the pattern of expression differed between respiratory and reproductive tissues. This shows that our workflow has the potential of cross-tissue comparison and that motile cilia may have different proteomes in different human tissue compartments, a finding that may explain phenotypic diversity in motile ciliopathy patients. Further studies using our novel strategy for mapping the entire motile cilia proteome in health and disease are expected to give unprecedented insights into the field of cilia biology.

In the rapidly expanding field of spatial biology, a multitude of methods focus on multiplexed imaging of proteins. Here, we used a medium-plex workflow, building a fixed panel of antibodies outlining five different subcellular structures of motile ciliated cells. While higher-plex methods allow for studying more cell types and structures within a specific tissue, our pipeline instead has the advantage of mapping proteins in a high-throughput manner, allowing for proteome-wide studies. The strategy presented here holds promise for mapping the entire motile cilia proteome in health and disease, forming the basis for further understanding of underlying mechanisms of ciliopathy, and a first step towards applications in precision diagnostics and healthcare.

Methods

Panel development and candidate selection

A 6-plex panel was built to target five structures of the ciliated cell: axoneme, transition zone (TZ), basal body (BB), cytoplasm, and nucleus. Protein markers and corresponding antibodies have been selected based on i) literature search (e.g. UniProtKB), ii) antibody IHC validation score on HPA, iii) IHC staining patterns in ciliated cells in nasopharynx, bronchus, fallopian tube (FT), endometrium, and cervix, and iv) compatibility in the multiplex immunohistochemistry (mIHC) panel and workflow. Each antibody was first tested with a single-plex run to ensure that it works with the OPAL detection system. Then, each antibody was tested in each position of the 6-plex, except the third location reserved for the candidate protein, to find the best location for each antibody and ensure a trustable staining pattern and a stable panel (Supplementary Table 2). To validate the three markers targeting the cilium, confocal microscopy at 63X was conducted using Leica STELLARIS 5 (Leica Microsystems, Illinois, US).

Ethics and tissue preparation

Human tissue samples from five tissue types were obtained from Uppsala University Hospital, Sweden, and collected within the Uppsala Biobank organization. All samples were anonymized in accordance with the approval and advisory report of the Uppsala Ethical Review Board (ref nos. 2002-577, 2005-388, 2007-159). The tissue microarray (TMA) was generated as previously described[47] and comprised five distinct tissues: fallopian tube, endometrium, cervix, nasopharynx, and bronchus (Figure 1B). Each tissue was represented by six one mm diameter cores, with duplicate cores from three individuals. Patient information is provided in Supplementary Table 1. The TMA was sectioned into 4-µm sections using a waterfall microtome (Microm H355S, ThermoFisher Scientific, Fremont, CA) and mounted onto SuperFrost Plus slides (Thermo Fisher Scientific, Fremont, CA). The slides were dried at room temperature (RT) overnight, baked at 50°C for 12–24 hours, cooled, and stored at RT until further use.

Slide pretreatment

To remove paraffin and rehydrate tissue cores, a ST5010 Autostainer XL (Leica Biosystems, Baden-Württemberg, Germany) was used with the following protocol: Xylene (5 min), Xylene (5 min), Xylene (1 min), absolute ethanol (3 min), absolute ethanol (3 min), 96% ethanol (3

min), 96% ethanol + 0.3% H_2O_2 (5 min), 80% ethanol (3 min), and dH_2O (30 sec). The slides were then stored in deionized water until antigen retrieval.

Antigen retrieval was performed to expose protein epitopes for antibody binding. Slides were subjected to heat-induced epitope retrieval (HIER) in pH 6 buffer (Agilent Technologies Inc., Santa Clara, CA, USA) at 125°C for 4 min using a Biocare Medical Decloaking Chamber™ PLUS (DC2008INTL). After cooling to 90°C, the slides were rinsed in deionized water (1–2 min) and transferred to TBS-Tween wash buffer (Thermo Fisher Scientific, TA-999-TT, Waltham, MA, USA) until further processing. To reduce autofluorescence, slides were incubated in a bleaching buffer containing 1.5% hydrogen peroxide, 0.2 M glycine, and 1X TBS-Tween (Thermo Fisher Scientific, TA-999-TT) in 50-mL Falcon tubes. Tubes were rotated in LED light at RT for 1 hour using a Stuart SRT9D roller mixer. Slides were subsequently washed in TBS-Tween and stored until further use.

Multiplex immunofluorescence

For multiplex immunofluorescence (mIF) staining, one slide per candidate protein was used. Each staining cycle proceeded as follows: Tissue sections were blocked with UV Blocking Buffer (10 min), incubated with primary antibody (30 min), rinsed in TBS-Tween, incubated with HRP-conjugated secondary antibody (10 min), and washed again in TBS-Tween. Slides were then incubated with the cycle-specific OPAL™ fluorophore (Akoya Biosciences) for 10 min, rinsed, and subjected to HIER (90°C, pH 6 buffer, 20 min). The order of the Opals and panel markers from first to last cycle was: OPAL690 for CROCC, OPAL620 for DNAH9, OPAL520 for candidate protein (changed for each slide), OPAL570 for AGR3, OPAL480 for NPHP4, and OPAL-DIG-780 for FOXJ1. After each cycle, slides were rinsed and stored in wash buffer. The final step involved incubating slides with OPAL 780 fluorophore-conjugated anti-DIG antibody (1:125 in Antibody Diluent) for 1 hour, followed by staining with DAPI (1:1000, Invitrogen, D1306, Thermo Fisher Scientific) for 5 min. Slides were rinsed, mounted with Invitrogen ProLong™ Glass Antifade Mountant, covered, and cured overnight. Finally, slides were digitized at 40X magnification using PhenoImager (Akoya Biosciences).

Image analysis

A manual quality control was performed on all tissue cores, ensuring representativity of the staining pattern, presence of ciliated cells and normal tissue histology. Tissue cores that did not pass this quality control were not included in the data analysis. Automated image analysis was

conducted in ImageJ Fiji[48] to generate quantitative data. TIFF image stacks were processed per TMA core (Supplementary Figure 1A). Each stack contained separate images for panel marker proteins, DAPI, and autofluorescence. Images were converted to 8-bit, and DAPI/autofluorescence images were discarded (Supplementary Figure 1B). Segmentation was performed using 3-level Multi Otsu thresholding[49], where only the highest threshold level (brightest pixels) was used (Supplementary Figure 1C). If segmentation failed for any marker, the stack was excluded from further analysis. For each panel marker image, segmentation masks were generated (Supplementary Figure 1D), defining regions of interest (ROIs) corresponding to each marker and candidate protein (Supplementary Figure 1E). ROIs were used to extract pixel values from candidate protein images by measuring the pixel value within each ROI, including mean pixel intensity, maximum intensity, minimum intensity, and total ROI area per core.

Data analysis

Data analysis was performed using R 4.4.2. Median intensity values were calculated per ROI (axoneme, TZ, BB, cytoplasm, nucleus) for each protein and tissue, identifying predominant colocalization patterns. Cores excluded during manual annotation were omitted from median calculations. Data were normalized via Min-Max scaling per protein and slide across all tissues, with values rescaled between 0 and 1. Heatmaps were generated using the pheatmap package (v1.0.12). Gene Ontology (GO) enrichment analysis was conducted using clusterProfiler (v4.14.6) and the human gene reference database org.Hs.eg.db (v3.20.0), retrieved on 2025-02-25. Only GO terms with $p < 0.05$ were retained. The top three terms per cluster were selected based on the highest number of associated proteins. The Protein Evidence (PE) and Function Evidence (FE) scores for the candidate proteins were downloaded from the HPP portal (https://hppportal.net/) on 2025/02/13.

Data availability

Out of the 187 proteins, 149 were made available on the atlas in for the latest version (v25). The remaining 38 will be available for the next update of HPA during 2025.

Code availability

The script for image analysis (Macros) and data analysis (R) used for data analysis and visualization will be available on GitHub at time of submission:
github.com/LindskogLab/Subcellular-map-of-proteins-in-ciliated-cells

Supplementary information

Supplementary Figure 1: Image analysis workflow

Supplementary Figure 2: mRNA expression levels across different cell types based on scRNA sequencing data for the 187 candidates included in the study

Supplementary Figure 3. Bar plot showing the distribution of FE scores across each expression cluster.

Supplementary Table 1: Patient information for all tissue samples included in the TMA

Supplementary Table 2: Information for the antibodies included in the fixed panel

Supplementary Table 3: Information for the antibodies corresponding to the 187 protein candidates included in the study

Supplementary Table 4: Protein expression levels based on automated image analysis, and results based on gene ontology enrichment for the 187 protein candidates included in the study

Supplementary Table 5: Protein Evidence (PE) and Function Evidence (FE) scores from the HPP for the 187 candidate proteins included in the study

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Author contributions

Conceptualization: CL and LM; Data curation: FB and LM; Formal Analysis: FB and LM; Funding acquisition: CL and MU; Investigation: FH, LM, JNH, FB; Methodology: CL, FB, LM and FH; Project administration: CL and LM; Resources: CL and LM; Image analysis: FB, FH, LM; Supervision: CL, LM, FH; Validation: FB, LM, and CL; Visualization: FB and LM; Writing – original draft: FB, LM and CL; Writing – review & editing: FB, LM, CL, FH, JNH and MU. All authors have read and approved the final version of the manuscript.

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