Spatial Characterization of the Human Centrosome Proteome Opens Up New Horizons for a Small but Versatile Organelle

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After a century of research, the human centrosome continues to fascinate. Based on immunofluorescence and confocal microscopy, an extensive inventory of the protein components of the human centrosome, and the centriolar satellites, with the important contribution of over 300 novel proteins localizing to these compartments is presented. A network of candidate centrosome proteins involved in ubiquitination, including six interaction partners of the Kelch-like protein 21, and an additional network of protein phosphatases, together supporting the suggested role of the centrosome as an interactive hub for cell signaling, is identified. Analysis of multi-localization across cellular organelles analyzed within the Human Protein Atlas (HPA) project shows how multi-localizing proteins are particularly overrepresented in centriolar satellites, supporting the dynamic nature and wide range of functions for this compartment. In summary, the spatial dissection of the human centrosome and centriolar satellites described here provides a comprehensive knowledgebase for further exploration of their proteomes.

1. Introduction

The centrosome, which consist of two centrioles embedded in a protein matrix, referred to as the pericentriolar material, is the major microtubule-organizing center in animal cells. As such, it has well established functions in cellular architecture, polarity and organization, cell adhesion and motility, as well as cell division, but scientific achievements continue to reveal a growing array of additional roles for this small compartment.[1,2] Recent findings point to its ability to coordinate cellular processes and dynamically respond to molecular cues by serving as a compact hub, where proteins can interact at high concentrations.[3] The current understanding of the different functions carried out at the site of the centrosome have been reviewed extensively,[4] and cellular dysfunctions caused by aberrations in centrosome architecture with pathological implications have been described.[2,5,6] Centriolar satellites are membraneless granules that, in most cell types, cluster and move around centrosomes and primary cilia.[7,8] They were originally assigned a major function in microtubule-dependent transport of components to and from these compartments. In agreement, centriolar satellites share many proteins with centrosomes, sometimes being considered their sub-structures, and are important for centrosome-related functions. However, in recent studies, centriolar satellites have also been emerging as regulators of multiple other cellular processes, some of which are independent of centrosomes and cilia. Similarly, centrosome and cilia are not fully dependent on centriolar satellites.

Subcellular compartmentalization is a hallmark of the eukaryotic cell. Confinement of molecules to specific compartments is what enables multiple cellular processes to occur in parallel, in isolated or enriched environments, where optimal physiological conditions can be achieved locally. To understand cellular behavior, knowledge of the underlying molecular processes and their constituents is essential. One approach that has proven successful for this purpose is the exploration of organellar proteomes, which enables a systematic evaluation of the phenotypic space of the cell as a whole.[9,10] Around the turn of the 21st century, several efforts were made to study the protein composition and interactions of the centrosome, as well as the centriolar...
satellites.\textsuperscript{[6,11–14]} Together, these studies identified and validated a large number of proteins localized to these compartments, with numerous examples involved in human disease.

As part of the HPA effort to resolve the spatial distribution of the complete human proteome across cells and tissues,\textsuperscript{[10,15]} we here present a comprehensive proteomics dissection of the human centrosome and centriolar satellites, using immunofluorescence and confocal microscopy. Although an already near-complete coverage of the centrosome proteome has been suggested,\textsuperscript{[16]} we reveal over 300 additional proteins localized to the centrosome and/or centriolar satellites. Among the newly identified candidates are two novel networks of phosphatases and ubiquitination proteins, respectively, with no previous evidence for localization to the centrosome. This contributes to establish the role of the centrosome as a signaling center in human cells, for precise spatiotemporal coordination of cellular processes.

2. Results and Discussion

The Cell Atlas of the HPA aims to systematically map the expression and spatiotemporal distribution of all proteins in human cells. Using immunofluorescence and confocal microscopy, we have localized 434 proteins to the centrosome and/or centriolar satellites (Table S1, Supporting Information, HPA version 18, excluding proteins with location reliability score uncertain, https://v18.proteinatlas.org/search/subcell_location:Centrosome%2CMicrotubule,organizing.center;Enhanced%2CSupported%2CApproved). For 306 of these that show a distinct staining of one or two dots in the microtubule organizing region, the annotation centrosome has been used (Figure 1A), while for 128 of these that show a more diffuse staining pattern in this region, the annotation centriolar satellite has been used (Figures 1B). However, as satellite clusters tend to overlap with the centrosome, this annotation may include proteins that additionally localize to the centrosome. The identified centrosome and centriolar satellite proteins correspond to almost 4% of all proteins localized within the HPA Cell Atlas (HPA version 18, N = 12 073). Notably, a large majority (78%, N = 339) of these proteins have no previous experimental evidence for being localized to these compartments according to data in the GO database, thus contributing considerably to the desirable complete proteomic characterization of the centrosome and centriolar satellites.

Functional enrichment analysis of the 434 proteins localizing to the centrosome and centriolar satellites generated 51 significantly enriched GO terms (Bonferroni p-value < 0.01, GO_BP_FAT category) (Table S2, Supporting Information, only including proteins associated with a GO term in the database). The major clusters of enriched terms describe known functions associated with the centrosome such as cell cycle, cellular and protein organization, and movement and morphogenesis (Figure 1C).

A comparison of RNA levels show that the average expression of genes encoding proteins localizing to the centrosome and/or centriolar satellites is slightly, but significantly, lower across human cell lines and tissues compared all other genes in the HPA Cell Atlas (t-test, p-value < 2.2e-16) (Figure S1, Supporting Information). This difference becomes more evident for genes encod-
Figure 2. A) Confocal images of immunofluorescently stained centrosome and centriolar satellite proteins with no previous evidence for localization to these compartments. The protein of interest is shown in green, the nucleus in blue, and the cytoskeleton (tubulin) in red. The scale bar indicates 10 µm. Proteins are stained in the cell lines A-431 (FAM45A, C9orf40, RABL2A, CLHC1, NUDT17, CCDC57), MCF7 (KIAA1211L), RH30 (AMN1), U-251 MG (CAPS2) and U-2 OS (CDR1, C1orf74, TTC6, PLEKHS1, INTS6L, RFPL4AL1, GRAPL, CVQW3, PLEKHG3). For information about cell lines and the antibodies used see www.proteinatlas.org. Functional STRING interaction network analysis for the centrosome proteome based on experimental evidence. Nodes are colored based on the presence of experimental evidence for localization to the centrosome in the UniProt database (Blue) or not (Orange). B) Network showing the interactions between known and novel centrosome proteins. For complete information about included proteins and their interactions, see Table S4, Supporting Information. C) Subnetwork of six proteins involved in Ubiquitination. FBXO9 localized to the centrosome in SiHa cell line. UBE2Q1 localized to the centrosome in U-2 OS cell line. KEAP1 localized to the centrosome in A-431 cell line. D) Subnetwork of six proteins involved in Phosphorylation. MTMR9 localized to the centrosome in U-2 OS cell line. INPP4B localized to the centrosome in U-251 MG cell line. I) CLHC1 localized to the centrosome in U-251 MG cell line. The protein of interest is shown in green, the nucleus in blue, and the cytoskeleton (tubulin) in red. The scale bar indicates 10 µm.

Among the newly identified centrosome and centriolar satellite proteins (N = 339), there are several uncharacterized proteins. For 24 of these proteins, such as CLHC1, INTS6L, and CAPS2, previous knowledge about subcellular location is lacking (Table S3, Supporting Information). Figure 2 shows 18 of these proteins that have been localized to the centrosome in at least two different human cell lines. Over half (N = 186) of all the newly identified centrosome proteins have externally derived experimental evidence for other subcellular locations according to the UniProt database. This may be a consequence of the multi-locational characteristics that can be technically challenging to detect or a matter of different subcellular resolution in the methods being used. According to data in the GO database, a majority of these proteins have also been found in protein-rich organelles such as the cytosol, nucleus, and in different membranes. For example, the brain-specific inhibitor of angiogenesis ADGRB2, the adherence junction protein PATJ and the Ca²⁺ independent protein kinase PRKCQ are all previously reported as being plasma membrane-associated. In our study, the localization of these proteins to centrosomes and/or centriolar satellites has been validated by the use of two independent antibodies that bind to different epitopes of the target protein.
Systematic characterization of protein–protein interactions provides molecular context and aids in the aspiration of complete functional characterization of proteins. To generate such context for the centrosome proteome, a network analysis using the STRING software was performed (Figure 2B). This revealed an interaction network covering 59% (N = 256) of the 434 proteins localized to centrosomes and centriolar satellites in this study, supporting their co-localization in cells (Table S4, Supporting Information). Indeed, a majority of the interactions include at least one of the 95 proteins in the dataset that were previously known to localize to centrosomes. As relatively close intracellular proximity is required for physical interaction to occur, interactions with externally confirmed centrosome proteins provides further support for centrosome location for 48% (N = 157) of the 339 new centrosome proteins identified in this study. Furthermore, two isolated subnetworks containing proteins with no previous evidence for localization to the centrosome were identified. One subnetwork contains seven proteins involved in ubiquitination, all interacting with each other and the adapter of the E3 ubiquitin-protein ligase complex (KLHL21), which is required during cytokinesis and has previously been experimentally localized to the mitotic spindle (Figure 2C). The link between the ubiquitin-proteasome system (UPS) and cellular division has been known since studies of mutations in the ubiquitin E1 activating enzyme showed that ubiquitination is important for continuous cell cycle progression. The connection between centrosome biology and UPS, as well as other cellular functions, has also been observed in recent studies. This has led to an emerging view of the centrosome as a potential hub for cell signaling, especially since the centrosome has been shown to harbor signaling molecules that are particularly dependent on the UPS. Additionally, comprehensive mapping of ubiquitination sites has revealed that many centrosome and centriolar satellite proteins are ubiquitinated at multiple sites. The second subnetwork contains a branch of six phosphatases and pseudo-phosphatases that all interact with the phosphatase Synaptojanin-1 (Figure 2D). The human protein phosphatome was recently characterized and classified, listing 189 known and predicted phosphatases. In the HPA Cell Atlas a large majority of these proteins are localized to the nucleus, cytosol or plasma membrane, while only six of the phosphatases are localized to the centrosome, five of which are interaction partners of Synaptojanin in the subnetwork. As dephosphorylation is a molecular process crucial in the regulation of many cellular signaling systems, this strengthens the suggested view of the centrosome as a signaling center.

With the emergence of techniques that enable the capture of spatial and temporal variations in protein expression, it has now become clear that subcellular localization is highly dynamic, with about half of the proteome being localized to multiple cellular compartments. Given the proposed fraction of >50% of the human proteome being multi-localizing, it is expected that on average 77% of the protein content of a given organelle is detected in at least one other organelle (note that due to multiple locations, the corresponding proteins are redundant when counting the multi-localization fraction for each organelle separately). Supporting the hypothesized role of the centrosome as an interactive hub tied to a myriad of cellular processes spanning far beyond cellular division, we can now demonstrate how this is particularly evident for proteins localized to centriolar satellites. Of the 434 proteins localized to the centrosome or centriolar satellites, 76% (N = 328) have been identified as multi-localizing proteins (MLPs) in the HPA, with a majority additionally being localized to the cytosol, nucleus, plasma membrane, or vesicles (Figure S3, Supporting Information). The pronounced multi-localization of these proteins may well relate to the role of the centrosome as a signaling hub to which proteins are recruited from other compartments, either for local interactions, sequestration or posttranslational modifications. Interestingly, a significantly higher fraction of MLPs are observed among proteins that localize to the centriolar satellites (85%, N = 109, p-value 3.4E-4) as compared to the proteins at the site of the centrosomes (72%, N = 219). A hypergeometric test was performed to investigate if the groups of MLPs localizing to the centrosomes and the centriolar satellites, respectively, are significantly over-represented among MLPs in certain organelles within the cell. This analysis revealed significant over-representation among MLPs in broader set of cellular structures for the MLPs of the centriolar satellites, including vesicles, cytosol, mitotic spindle, microtubules, cytokinetic bridge, and midbody ring (Figure 3, Table S5, Supporting Information). In contrast, the MLPs of the centrosomes display a more restricted localization pattern, only being significantly over-represented among MLPs of the nucleoplasm and cytosol. Although an unambiguous distinction between centrosomes and centriolar satellites cannot be made solely based on staining patterns, this finding is in agreement with satellites being highly dynamic structures, involved in several function independent of centrosomes and cilia.

3. Concluding Remarks

Fifteen years ago, the first large-scale study was published that used a combination of complementary proteomics strategies to explore localization, turnover, and abundances of proteins localized to the centrosome in human lymphoblast cells. In this study, the authors managed to identify 47 out of 60 proteins that where then listed in the literature as being associated with the centrosome during interphase, as well as 90 novel proteins. Eventually, a number of proteins were further stratified based on their preference for localization to mother or daughter centrioles. This work laid an important ground for the continued work toward a complete characterization of the human centrosome proteome, with special emphasis on the need for technical validation and the use of complementary approaches. We now present a centrosome proteome that is about four times as large as initially believed. Noteworthy, other efforts have been made throughout the years, but to our knowledge, no one has focused solely on the proteome of the centrosome alone but rather on the centrosome as part of the larger machinery that enables mitosis to occur. We now present an expanded view of the human centrosome proteome, with spatial resolution, covering 434 proteins. We have identified over 300 new proteins that localize to this essential cellular organelle, of which almost half are supported by experimentally confirmed interactions with previously known centrosome proteins. We have also identified two functional networks of novel centrosome proteins involved in ubiquitination and phosphorylation, respectively. In addition, we demonstrate how dynamic protein localization is particularly evident for
proteins localizing to centriolar satellites. While we support a cautious discussion about the completeness of this proteome, and acknowledge the need for further independent validation,[12,13,25] the dataset presented here have greatly expanded the centrosome and centriolar satellite proteome, laying a foundation for further exploration of their cellular functions.

4. Experimental Section

Antibodies: The antibodies used in this study were generated within the HPA project and designed to target as many different isoforms of the target protein as possible. Antigen fragments were used for affinity purification and validation and quality assessment for sensitivity and lack of cross-reactivity were performed according to HPA standard procedures.[26] A subset of the antibodies have been validated by co-localization in HeLa cell lines stably expressing green fluorescent protein-tagged target protein or using small interfering RNA for knockdown of RNA expression. Expression data from application-specific validation and detailed descriptions of laboratory procedures are available at v18.proteinatlas.org.

Immunofluorescence: Cells were allowed to attach overnight (at 37 °C and 5% CO₂) in a 96-well glass bottom plate (Greiner, Kremsmuenster, Austria). The plate had been coated with fibronectin (VWR, US) at 12.5 µg mL⁻¹ in PBS. Shortly, the cells were fixed with 4% paraformaldehyde and permeabilized with Triton X-100. For detailed information about subsequent procedures for fixation, permeabilization, and immunostaining of cells, see previous publications.[10,27] Images were manually acquired using a Leica SP5 laser scanning confocal microscope (DM6000CS) equipped with a 63x HCX PL APO 1.40 oil CS objective (Leica Microsystems, Mannheim, Germany) and connected to the software LAS AF (LAS AF 2.6.0 BETA build 6964, Leica Microsystems). Images were acquired in four sequential steps with the following scanning settings: 16-bit, 600 Hz, line average 2, pixel size 0.08 µm. Images were colored and assembled as RGB with the software ImageJ1.46r (National Institutes of Health, USA). All images were manually annotated for 33 discrete subcellular locations, each of which was given a reliability score. Detailed descriptions of the annotations are available at v18.proteinatlas.org/about/assays,annotation.

RNA Sequencing: RNA was extracted from the cells using the RNeasy kit (Qiagen), generating high quality total RNA (i.e., RIN > 8) that was used as input material for library construction with Illumina TruSeq Stranded mRNA reagents (Illumina). Duplicate samples for each cell line were sequenced on the Illumina HiSeq2500 platform. Raw sequences were mapped to the human reference genome GrCh37 and further quantified using the Kallisto software,[28] generating normalized transcript per million (TPM) values for all genes covered by Ensembl version 88.38. TPM values for genes were generated by summing up TPM values for the corresponding transcripts generated by Kallisto. Genes with a TPM value greater than 1 were considered as expressed. The RNA expression data used here is available at v18.proteinatlas.org.

Data Analysis: To generate the list of broader functions associated with the centrosome proteome, gene set redundancy across GO-terms was overcome by using the Cytoscape application Enrichment map.[29]
Violin plots for comparison of RNA levels (Figures S1 and S2, Supporting Information) were generated using R/Bioconductor and p-values calculated using a t-test. Proteins with location reliability score uncertain (for more information on scoring system see v18.proteinatlas.org) were excluded from the analysis, generating a background of HPA localization data including N = 11,618 proteins.

Data Availability: All data generated and used in this study are available online at v18.proteinatlas.org.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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