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From quantitative protein complex analysis to disease mechanism

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ABSTRACT

Interest in the field of cilia biology and cilia-associated diseases – ciliopathies – has strongly increased over the last few years. Proteomic technologies, especially protein complex analysis by affinity purification-based methods, have been used to decipher various basic but also disease-associated mechanisms. This review focusses on some selected recent studies using affinity purification-based protein complex analysis, thereby exemplifying the great possibilities this technology offers.

Cilia are hair-like organelles with motile and sensory functions protruding from the cell surfaces of different cell types in many organisms (Leroux, 2007). They can be structurally divided into five different functional units: basal body, transition zone, axoneme, ciliary membrane, and ciliary tip (Fliegauf, Benzing, & Omran, 2007). The nomenclature distinguishes between two different categories of cilia, motile and non-motile cilia (Inglis, Boroevich, & Leroux, 2006). The axonemes of motile cilia mostly consist of nine microtubule doublets surrounding a central pair of singlets, whereas the non-motile cilia normally lack a central pair of microtubules (D'Angelo & Franco, 2009; Fliegauf, Benzing, & Omran, 2007). Additionally motile cilia have inner and outer dynein arms attached to the microtubule doublets. Using ATP as a source of energy, these motor proteins walk their way on the neighbouring doublet, thereby causing overall bending of the cilium (Afzelius, 2004; Marshall, 2008).

Because cilia are located on almost all polarized cell types of the human body, cilia-related disorders – ciliopathies – can affect many organ systems (van Reeuwijk, Arts, & Roepman, 2011). Ciliopathies can either involve single organs or can occur as multi-systemic disorders with phenotypically variable and overlapping disease manifestation (Fliegauf, Benzing, & Omran, 2007). Leber congenital amaurosis (LCA), a ciliary disease restricted to the eye, is the most common cause of congenital blindness in infants and children (den Hollander et al., 2007). Bardet–Biedl syndrome (BBS) on the other hand can affect many organs with symptoms including obesity, retinal degeneration and polycystic kidneys (Fliegauf, Benzing, & Omran, 2007; Nachury et al., 2007; Tobin & Beales, 2009). Understanding of the phenotypic overlap between BBS and other ciliary disorders has drawn attention to the similarities between traditionally discrete clinical disorders such as nephronophthisis (NPH), Joubert syndrome (JBTS), and Meckel–Gruber syndrome (MKS), offering the possibility that mechanistic insights gleaned from some ciliopathies might illuminate the ethiopathology of other ciliopathies (Garcia-Gonzalo et al., 2011; Zaghloul & Katsanis, 2009). The perception of proteins acting as elements in cell-specific structural as well as regulatory protein networks may explain how single gene mutations produce very complex cellular aberrations or disease phenotypes, or why others are restricted to a single organ (Boldt et al., 2009). It may also provide cues on why a given disease may be restricted to specific organs or cells in the body. As true for most proteins, ciliary proteins are organized in larger protein complexes and networks, which are dynamically assembled in a cell- and context-specific fashion (Blacque, Cevik, & Kaplan, 2008). Differential gene expression, alternative splicing, post-transcriptional regulation via micro-RNAs as well as post-translational regulatory mechanisms such as protein modifications and compartmentalization generate spatio-temporal patterns and compositions, which in turn result in context dependent protein compositions, complexes and organelles (Cao et al., 2012; Jenkins

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et al., 2009; Lancaster, Schroth, & Gleeson, 2011; Merbs et al., 2012; Zhang et al., 2002). Shared by cilia of different tissues and organs, specific protein repertoires and regulatory circuits result in specific functional properties (such as motor protein driven motion of cilia (Kobayashi & Takeda, 2012; Waters & Beales, 2011)). This is likely to define the degree of susceptibility to a perturbation of a given functional property (for example motility) shared by some cilia but not others that may then result in a disease-associated defect in tissues harbouring cilia with these properties (Takeda & Narita, 2012).

To date, knowledge of the composition, wiring, dynamics and associated signalling pathways of the corresponding molecular building blocks and associated protein networks remain very limited, despite the dramatic increase in efforts to study them. In this mini review we will discuss the application of modern proteomic analytical strategies and their importance for the investigation of functional aspects in ciliary disease mechanisms.

In 2007 homozygosity mapping led to the identification of mutations in the LCA5 locus, coding for the protein Lebercilin (den Hollander et al., 2007). To get first insights into the function of Lebercilin, we performed SF-TAP (Strep Flag-tandem affinity purification (Gloeckner et al., 2007)) analysis to detect the constituents of its interactome (see Fig. 1a). We found Lebercilin to be associated with ciliary proteins like two dynein light chain isoforms, indicating a link to the ciliary transport machinery. In a second, more recent study we analyzed the interactome of Lebercilin by a novel method (see Fig. 1b), combining sensitive one-step affinity purification with quantitative mass spectrometry (Boldt et al., 2011). By omitting the second purification step, we were able to minimize the loss of transient or weakly bound interaction partners.

To achieve this sensitive determination of the protein complex components, cells labelled by stable isotopes (SILAC: stable isotope labelling by amino acids in cell culture (Ong et al., 2002)) were transfected either with wild type SF-TAP-tagged Lebercilin or with the empty SF-TAP vector as a control. The protein complexes were then isolated by Strep-affinity purification and analyzed by LC–MSMS (liquid chromatography coupled to tandem mass spectrometry). In the mass spectrum the differentially labelled peptides of each sample can be distinguished by a defined mass shift induced by the isotopic label. Using the software MaxQuant (Cox & Mann, 2008) the relative abundance of proteins within each sample was determined and the ratios, as well as the significance for enrichment, compared to the control sample were calculated. By applying this more sensitive approach, we were able to detect proteins of the intraflagellar transport (IFT) to be connected to Lebercilin, further strengthening the hypothesis of it being involved in the ciliary transport machinery. Introduction of LCA-associated mutations into the protein sequence of Lebercilin led to loss of this connection, suggesting that the mechanism by which mutations in the LCA5 gene cause LCA is the loss of connectivity to the IFT. To validate this finding *in vivo*, a LCA5 knockout mouse was generated, which showed a disturbed architecture of photoreceptor outer segments. Although IFT proteins localized normally to the connecting cilium, rhodopsin and opsin transport to the outer segments was disrupted and the light-induced translocation of arrestin as well as transducin was clearly impaired within this mouse model. This demonstrates the importance of Lebercilin for the IFT along the connecting cilium in photoreceptors. The fact that the IFT is impaired indicates the severity of the loss of Lebercilin for the ciliary transport and explains the disturbed architecture of the outer segments. This study demonstrated that combining powerful tools for the purification of protein complexes with modern quantitative mass spectrometry-based methods opens up new possibilities. Especially in combination with a mouse model as well as functional cellular assays these tools can give valuable insights into the disease mechanism (Fig. 1c).

The two above mentioned studies show further that there are clear differences between tandem affinity purification (TAP) and one-step affinity purification. The major advantage of SF-TAP, or similar TAP approaches, is the quick detection of stable complexes. Thereby these methods can be applied for medium to high throughput studies. The one-step affinity purification allows a more sensitive detection of interaction partners compared to

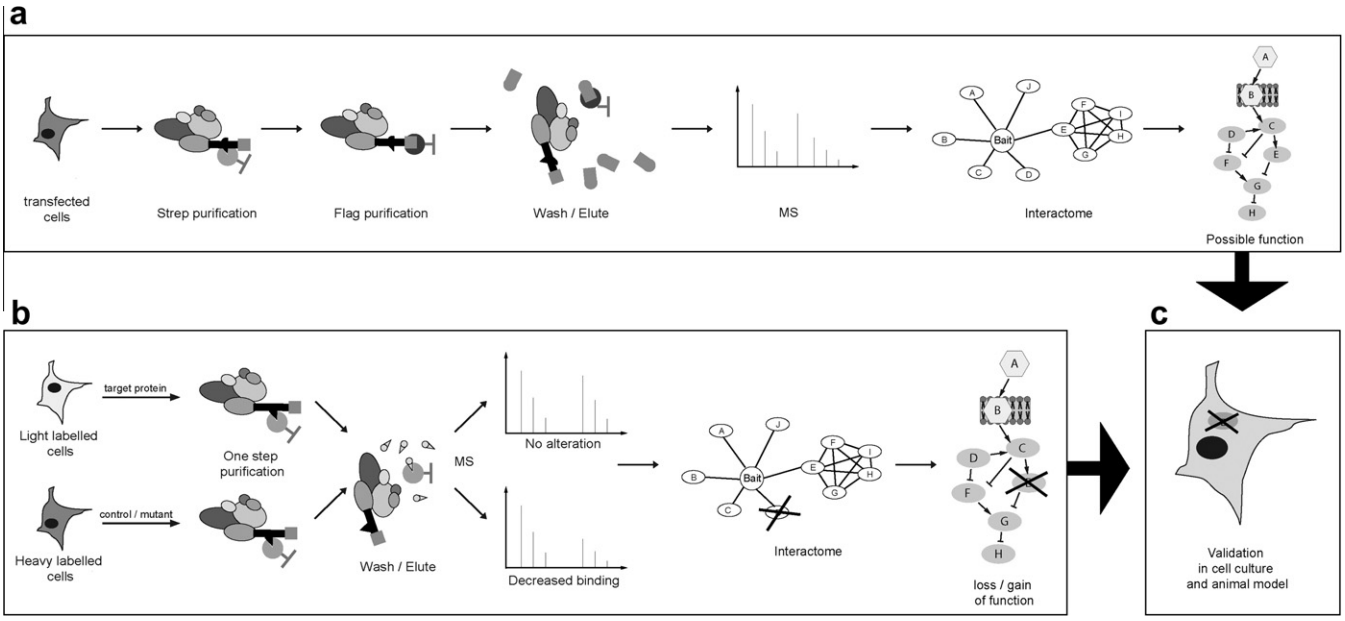

Fig. 1. From protein complex analysis to cellular function or disease mechanism by the application of affinity-based protein complex analysis. (a) Qualitative analysis of protein complexes by SF-TAP. The protein of interest, fused to the SF-TAP tag is used to isolate the protein complex by two-step purification strategy. The identity of the protein complex components is determined by mass spectrometry. Based on the complex components, the function of the protein of interest can be exerted. (b) Quantitative analysis of protein complexes. The protein complex of the target protein, compared to a control is analyzed by one-step purification combined with SILAC. Alternatively the same experiment can be performed comparing the interactome of a mutated form of the protein to the wild type protein. Possible alterations give insights into the disease mechanism of a mutation. (c) Validation of the data from a and b can be done in cell culture and animal models.

TAP, where only the core interacting proteins can be purified. The identification of more labile and transient complex components can be the result. This is, however, accompanied by the co-purification of hundreds to thousands of non-specific contaminants, which need to be subtracted by the application of clearly more labour intensive quantitative approaches like SILAC.

These examples are only two of many studies employing the power of affinity purification-based protein complex analysis technologies. The SF-TAP approach (see Fig. 1a) for example was used for the identification of novel interactors of ciliopathy-associated proteins by Coene et al. (2011). The authors analyzed the interactome of RPGRIP1 (retinitis pigmentosa GTPase regulator-interacting protein 1) and RPGRIPL (retinitis pigmentosa GTPase regulator-like protein) and identified NEK4 (never in mitosis A-related kinase 4) as a novel interaction partner for both proteins. The down-regulation of NEK4 resulted in impairment of cilia stability and assembly showing its importance for ciliary function. This study again showed that the analysis of protein complexes can improve the understanding of the molecular function of a protein and of its regulatory networks.

A further interesting approach of protein complex analysis was performed by Mukhopadhyay et al. (2010). The authors used stable clonal cell lines expressing LAP-tagged TULP3 (tubby like protein 3) for characterization of the interactome of TULP3 by TAP purification coupled to mass spectrometry. The results of the complex purification showed the association of TULP3 with proteins of the IFT complex A. Further functional studies revealed that WDR19, IFT140 and IFT122 form an IFT-A core complex that binds TULP3 and thereby provides ciliary access to the protein. The authors further showed that the phosphoinositide-binding properties of TULP3 promote the trafficking of G protein-coupled receptors (GPCRs) to the primary cilium.

An excellent study by Chih et al. (2012) described the analysis of a membrane-associated protein complex consisting of nine proteins (B9 complex, derived from proteins carrying the B9 domain) that is located at the transition zone of the primary cilium. Interestingly, seven of those nine protein complex components are mutated in ciliopathies. In this study the complex components were identified by TAP coupled to LC–MSMS. Functional follow-up experiments showed that disruption of this complex led to increased diffusion of ciliary membrane proteins into the cilium. This highlights that this complex is essential for building up or keeping up this barrier and thereby is responsible for the selective localization of membrane proteins within the cilium.

Most protein complex analysis studies concentrate on model systems like cell lines to purify and analyse protein complexes. Although these are valuable tools to identify complex components and alterations within those, the use of cell lines possesses some drawbacks. In a perfect world, each complex should be purified from the tissue of interest. Protein interactions due to tissue-specific expression or post-translational modification of proteins might not be detected in cell lines. The purification of complexes from their native tissue, however, is limited by the availability of specific antibodies that are suitable for the efficient enrichment of protein complexes as well as the amount of tissue sample. Furthermore, the necessity of quantification remains. As SILAC is a metabolic labelling technique it is not applicable to most organisms. Protein complex analysis approaches need to be combined with alternative quantification techniques like chemical labelling-based introduction of isotopes or software based approaches for label-free quantification (Bantscheff et al., 2007). Due to the technical challenges of protein complex analysis from native tissue, the well-established identification of complexes from cell lines followed by the functional *in vivo* validation will most likely remain a commonly used workflow.

Although the technologies described here were successfully employed in many studies, of which only few are presented here, one major limitation of all affinity-based protein complex analysis methods remains the fact that sub-modules within a complex cannot be elucidated. In general a mixture of complexes formed by the bait is detected. The composition of the single sub-complexes remains unclear, even though the integration of publically available protein–protein interaction data can result in a more detailed picture. Up to date the approaches possibly shedding light on this issue are very time consuming, like absolute quantification-based determination of the stoichiometry (Schmidt et al., 2010) or depend on high amounts of material like destabilization of complexes by detergent in combination with blue native gel-electrophoresis (Klodmann et al., 2010). The development of novel types of mass spectrometers as well as data analysis software might help to develop new and feasible approaches, suitable to solve sub-complex structures at a higher throughput.

The field of ciliopathies has been of great interest in the last few years. A variety of links between cilia and morphogen pathways have been published and the number of publications covering the field of cilia biology and ciliopathies is increasing dramatically (Beales & Jackson, 2012). The studies mentioned here show that the application of modern proteomic methods to detect and quantitatively compare protein complexes is a powerful tool to investigate disease, as well as basic biological mechanisms within this organelle. Proteomics studies just as genetic interaction studies at different levels of throughput have informed us about the physical connections and functional hierarchy in ciliary interaction networks, which unveiled important knowledge about the molecular pathogenesis of ciliopathies. For further information regarding the possibilities protein network analysis, we would like to refer to a more comprehensive review, published by van Reeuwijk, Arts, and Roepman (2011). It is however important to note that protein complex and protein network analysis can only be the basis for further investigations that, at the end may lead to the understanding of a disease and offering the starting point for therapy.

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