**Comparative Super-r­­esolution Mapping of Basal Feet Reveals a Modular, but Distinct Architecture in Primary and Motile Cilia**

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**SUMMARY**

Molecular architecture analysis *in situ* of organelles and protein assemblies is essential to understand the role of individual components, their cellular function and to engineer new molecular functionalities. Through a super-resolution-driven approach, here we characterize the organization of the ciliary basal foot, an appendage of basal bodies whose main role is to provide a point of anchoring to the microtubule cytoskeleton. Quantitative image analysis shows that the basal foot is organized into three main regions linked by elongated coiled-coil proteins, revealing a conserved modular architecture in primary and motile cilia, but showing distinct features reflecting its specialized functions. Using domain-specific BioID proximity labelling and super-resolution imaging, we identify CEP112 as a basal foot protein and other candidate components of this assembly, aiding future investigations on the role of basal foot across different cilia systems.

**INTRODUCTION**

 Primary and motile cilia are cellular structures playing a fundamental role in signalling, flow sensing and force generation through beating (Reiter and Leroux, 2017). During the process of ciliogenesis, centrioles migrate toward the cell membrane and undergo molecular changes to become basal body, the structural base of primary and motile cilia, which elongates axonemal microtubules to extrude a cilium. The basal body extends from its microtubule wall a conical structure named the basal foot, a “supramolecular” assembly that is thought to originate from subdistal appendages, the nine-fold symmetrical structures on the mother centriole. The architecture of the basal foot and how it differs in primary and motile cilia remains largely unclear.

The primary cilium plays an essential role as the cell’s antenna by sensing and transducing mechanical, chemical and light signals from the extracellular environment into the cell, such as fluid flow, pressure, vibration, and signalling molecules such as Hedgehog and Wnt among others (Anvarian et al., 2019; Singla and Reiter, 2006). In the primary cilium, basal foot proteins, in concert with their centrosomal proximal end pool, function to keep the primary cilium submerged in the cytoplasm by linking the basal body to Golgi thereby limiting ectopic Shh signal transduction activation (Galati et al., 2016; Mazo et al., 2016). In addition to Shh signalling, the basal foot in primary cilia has also been linked to TGF beta signalling, albeit indirectly (Monnich et al., 2018). The basal foot of the primary cilium has multiple copies per basal body (Figure 1A) and is thought to originate from nine (or less depending on the cell type) subdistal appendages, mother centriole-associated structures contributing to the organization of the interphase microtubule (MT) array (Chong et al., 2020; Paintrand et al., 1992; Uzbekov and Alieva, 2018).

Motile cilia on the other hand beat in coordination on the surface of specialized epithelia, generating the propulsive force required for mucociliary clearance of environmental pollutants, bacterial and viral infections in the airway, to move ependymal fluid in the brain or to transport eggs across the fallopian tubes (Lucas et al., 2020; Reiter and Leroux, 2017). Within a multiciliated cell, motile cilia beating coordination depends on the linkage and alignment of hundreds of motile cilia basal bodies through the sub-apical MT network and their basal feet. This cellular alignment of basal bodies/basal feet is termed rotational polarity (Clare et al., 2014; Kunimoto et al., 2012; Marshall and Kintner, 2008; Mitchell et al., 2007; Vladar et al., 2012).

In airway multiciliated cells, loss of basal feet results in disruption of the microtubule apical network, irreversible disorientation of basal bodies and lack of motile cilia coordination (Herawati et al., 2016). In mice, this leads to respiratory manifestations consistent with primary ciliary dyskinesia (PCD) (Kunimoto et al., 2012), a rare human rare disease characterized by chronic airway infections, bronchiectasis, and frequently associated with conductive hearing loss, male infertility and heart malformations (Lucas et al., 2020). Despite the basal foot’s critical role in primary and motile cilia function, its molecular organization remains to be elucidated.

In mammalian cells, the subdistal appendages, which are considered the cognate structures of the basal feet, appear by transmission electron microscopy (TEM) as thin, conical-shaped structures with a round tip, which are linked to the centrosomal barrel by two axonemal microtubule triplets (Paintrand et al., 1992; Sorokin, 1968; Uzbekov and Alieva, 2018; Winey and O'Toole, 2014). However, less is known about the basal foot structure and composition in primary and motile cilia, and no consensus has yet been reached on its nomenclature, such that this assembly has been named differently depending on the study and cell type analysed (e.g. satellite arms (Sorokin, 1968), basal feet (Albrecht-Buehler and Bushnell, 1980), and subdistal appendages (Mazo et al., 2016)).

Several proteins have been assigned to the basal foot and subdistal appendages in mammalian cells through fluorescence microscopy and/or immuno-EM (Ninein (NIN) (Mogensen et al., 2000), Odf2/Cenexin (Ishikawa et al., 2005) (Kunimoto et al., 2012) (Nakagawa et al., 2001), Cc2d2a (Veleri et al., 2014), CEP170 (Guarguaglini et al., 2005), Galactin-3 (Clare et al., 2014), ε-Tubulin (Chang et al., 2003), Centriolin (CNTRL) (Gromley et al., 2003), Trichoplein (TCHP) (Ibi et al., 2011), CEP128 (Mazo et al., 2016), CEP19 (Gupta et al., 2015), CCDC120 and CCDC68 (Huang et al., 2017). Odf2/Cenexin is a fibrillar protein related to the intermediate filament (IF) superfamily that plays a critical role in basal foot assembly: lack of the basal foot-specific Odf2/Cenexin isoform results in loss of entire basal foot structure (Kunimoto et al., 2012; Tateishi et al., 2013). Odf2/Cenexin interacts with Tchp, an IF-binding protein implicated in NIN recruitment to subdistal appendages (Ibi et al., 2011). NIN and CEP170 have been implicated in microtubule anchoring and nucleation (Delgehyr et al., 2005; Welburn and Cheeseman, 2012). Previously, CEP19 (Gupta et al., 2015) and CC2D2A (Veleri et al., 2014) have been assigned to subdistal appendages, with the latter shown to play a critical role in their assembly.

 Although information has accumulated on individual proteins, to date a comprehensive and quantitative view of the molecular architecture of the basal foot and the orientation of its components is still lacking. Moreover, it remains unknown how basal foot organization changes in basal bodies of different cilia to accommodate its specific functional and structural requirements. Here, we resolved the structure of the basal foot *in situ* using super-resolution microscopy, leveraging 3D-structured illumination microscopy (3D-SIM) for high-throughput positional mapping, and stochastic optical reconstruction microscopy (STORM) for highest localization precision (Liu et al., 2020; Sydor et al., 2015).

Our analysis revealed a modular architecture composed of three main regions linked by elongated proteins NIN and CNTRL. This modular architecture is conserved in motile and primary cilia, but partly organized differently within modules, suggesting that distinct structural features have been adapted in different cilia types. By using domain specific information gathered from BioID proximity labelling and super-resolution microscopy, we identified CEP112 as a basal foot protein.

Altogether, our study provides a comprehensive molecular map of the basal foot and identifies candidate components of this structure for future studies investigating basal foot structure and function in different cilia systems.

**RESULTS**

***Basal Foot has a Modular Architecture in the Primary Cilium***

To determine the molecular architecture of the basal foot *in situ*, we first focused on primary cilia in immortalized retinal pigment epithelia 1 (hTERT-RPE1) cells, a cellular model characterized by robust ciliation and apparent homogenous ciliary structure by fluorescence microscopy. We reasoned that 3D-SIM resolution power (~125 nm lateral and ~250 nm axial) (Gustafsson, 2000; Schermelleh et al., 2008; Sydor et al., 2015) was sufficient to assign proteins to the basal foot or to other ciliary regions. To test this possibility, we first examined the distribution of NIN, a basal foot protein reported to have different sub-populations at the basal body (Ou et al., 2002). Using 3D-SIM, we clearly distinguished three pools of NIN, localizing at 1) the basal body proximal end, 2) the daughter centriole, and 3) the basal foot, with the latter population forming a ring at the distal end of the basal body (Figure 1B).

Once our primary imaging strategy was validated, we imaged all reported basal foot and subdistal appendage proteins using as reference marker polyglutamylated-Tubulin (Glut-TUB), a modification of centriolar microtubules serving as a proxy for mother centriole axonemal microtubules perimeter (diameter= ~200 nm) (Anderson, 1972; Edde et al., 1990), and quantitatively mapped proteins positions relative to the center of the basal body (Figures 1C and S1). Since 3D-SIM resolution is maximal in plane, end-on and side-views were preferentially selected from hundreds of micrographs (Figure 1D).

 Notably, 3D-SIM mapping showed that basal foot proteins are clustered into spatially separated regions relative to the centriole in the radial direction (Figure 1E, Table S1). NIN and CEP170 are the most distant from the mother centriole centre (248±16 nm and 237±25 nm, respectively), consistently with their association with microtubules (Delgehyr et al., 2005; Mogensen et al., 2000; Welburn and Cheeseman, 2012), therefore this was termed the microtubule-anchoring region. Most basal foot proteins are clustered with ODF2, a component critical for basal foot assembly (Kunimoto et al., 2012; Tateishi et al., 2013) (ODF2: 155±16 nm; CEP128: 139±15 nm; CEP19: 162±15 nm; CNTRL: 153±18 nm; TCHP: 135±15 nm; Galectin-3: 185±36 nm). Since this region contains ODF2, it was termed scaffolding region. Interestingly, our imaging map shows a gap where basal body connects to the basal feet; this was termed basal body anchoring region. To facilitate comparison of basal feet in different cilia systems, we named these regions (basal body anchoring, scaffolding and MT anchoring regions) as region I, II and III, relative to their distances from the basal body. Among the proteins previously assigned to subdistal appendages/basal foot, ε-tubulin, CCDC120 and CCDC68 could not be reliably detected at the basal foot with commercial antibodies, while TCHP, a protein thought to be associated only with subdistal appendages, was also located at the basal foot in primary cilia (Ibi et al., 2011).

To ensure correct assignment of proteins to the basal foot, we then measured the position of these proteins along the axoneme relative to the proximal end of the basal body, which we refer to as the axial distance (Figure 1F, Table S1). As expected, most basal foot proteins were distributed in the same axial region, which includes both basal foot and distal appendage region (209-284 nm), except for CC2D2A, which was located significantly above this region (333±22 nm) and below transition zone labeled by RPGRIP1L, a *bona fide* transition zone protein part of Y-links (393± 90 nm; Figure 1F) (Shi et al., 2017).

In summary, 3D-SIM imaging suggests a modular architecture of basal foot in primary cilia, with its components clustered in spatially distinct regions associated with different structural roles such as basal body anchoring, scaffolding and microtubule organization (Figure 1G).

***Domain Mapping Analysis of Basal Foot Proteins in The Primary Cilium***

Given that basal foot components were organized in distinct regions, we hypothesized that some proteins must be connecting these different regions together as molecular linkers, either as pearls on a string or elongated proteins, to ensure basal foot structural integrity (Mennella et al., 2014). Linkers were likely to be high-molecular weight, elongated coiled-coil proteins, which are particularly enriched in centrosome and cilia, similarly to centrosomal proteins of the pericentriolar material (PCM) (Lawo et al., 2012; Mennella et al., 2012).

To test this hypothesis, we labelled specific protein domains with antibodies or GFP to identify their position within the basal foot (Figure 2A, Table S1). Interestingly, we found that region II protein ODF2 did not elongate into region III, despite its role in basal foot assembly (middle domain (MD): 143±16 nm; C-terminus domain (CTD): 155±16 nm; Figure 2B). In contrast, CNTRL linked regions II and III by extending over a distance of ~87 nm (CTD: 153±18 nm; MD: 205±15 nm; GFP-CNTRL: 240±19 nm) (Figure 2C). Similarly, CEP128 showed an extended organization, but not as far from the basal body as CNTRL (CTD: 139±15 nm; GFP-CEP128: 203±16 nm; Figure 2D). Conversely, region III protein CEP170 did not extend inwards towards region II, consistent with its lack of predicted coil-coiled sequences (CTD: 237±25 nm; MD: 231±22 nm, N-terminus domain (NTD): 244±22 nm; Figure 2E). Surprisingly, NIN CTD and NTD showed similar distance from the basal body, despite its extensive predicted coiled-coil sequences and possible elongated organization in subdistal appendages (Delgehyr et al., 2005) (CTD: 248±16 nm; NTD: 253±25 nm; Figure 2F).

Altogether, the domain radial mapping of basal foot components in the primary cilium identified CNTRL as a bridging protein connecting regions II and III together (Figure 2G). Axial analysis also confirmed the localization of these protein domains in the same axial region, suggesting their *bona fide* associations with the basal foot (Figure 2H).

***NIN has an Elongated, Looping Distribution Stabilizing Bridging Protein CNTRL***

Radial distribution analysis of NIN suggested a similar localization of its CTD and NTD in region III of the basal foot. Since NIN contains several coiled-coil predicted sequences, this result was rather unexpected, leading us to further characterize its distribution. As domain-specific anti-NIN antibodies were not commercially available, we devised a strategy where GFP was inserted at different positions along NIN coiled-coil sequence to map their position (GFP-NIN, aa 197, aa 764, aa 1460 and aa 1647; Figure 3A). To confirm successful translation of GFP-NIN proteins, we first performed Western Blot of cell extracts from transfected RPE-1 cells (Figure 3B). Next, we used 3D-SIM imaging to examine the localization of GFP-NIN constructs and their potential effect on endogenous NIN function. Our data show that GFP-NINs were efficiently recruited to the basal body and daughter centriole, suggesting normal targeting (Figure 3C). In addition, their expression did not affect CEP170 localization, indicating that GFP-NINs recruit CEP170 normally (Mazo et al., 2016) (Figure S2). When GFP-NINs were then used for NIN domain distribution analysis in RPE-1 cells, we observed that NIN has a partially elongated distribution, looping back towards region II (Figures 3D and 3E, Table S2), suggesting a role in bridging region II and III.

To test this further, we examined the role of NIN in recruiting and/or stabilizing basal foot proteins by looking at the localization of ODF2, CEP128, CNTRL and CEP170 proteins upon NIN siRNA-mediated knockdown. The efficiency of NIN knockdown in ciliated RPE-1 cells was confirmed via Western Blot and 3D-SIM imaging (Figures 3F and 3G). Interestingly, co-localization analysis of NIN and basal foot proteins revealed that NIN siRNA impacted differently basal foot proteins (Figures 3G and 3H). As expected, NIN knockdown abolished CEP170 recruitment to region III of the basal foot, consistent with previous reports (Mazo et al., 2016). However, while NIN depletion did not affect CEP128 recruitment, it led to a significant reduction in the recruitment of CNTRL, and, to a lesser extent ODF2, indicating a functional role in recruitment and/or stabilization of these region II proteins (Figures 3I).

Altogether, our data show that the basal foot in primary cilia is organized into distinct structural regions linked by specific coiled-coil proteins: region III is the microtubule-anchoring/nucleation region made of CEP170 and NIN; region II consists of most known basal foot proteins (TCHP, CEP128, ODF2, CEP19 and CNTRL), and region I anchors basal foot to the basal body, but its composition is not yet well characterized. CNTRL and NIN are the main proteins linking region II and III, while CEP128 is mainly elongated within region II.

Of note, basal feet in primary cilium show a similar architecture to subdistal appendages, suggesting that their structure remains largely conserved during the transition from centrioles to basal bodies in primary cilia (Chong et al., 2020; Kashihara et al., 2019) (Figure S3, Table S3).

***BIO-ID Proximity Mapping Identified CEP112 as a Component of Basal Foot in Primary Cilium***

As our map pointed to a lack of proteins in the region close to the basal body wall (region I, Figure 2G), we reasoned that there are unidentified basal foot proteins that connect the basal body to the rest of basal foot. We hypothesized that these proteins would be located in close proximity to the CTD of CEP128, which was one the closest domain to the basal body microtubule wall according to the domain mapping analysis (Figure 2D). To test this possibility, we performed BioID proximity-dependent biotinylation, a technique identifying potential protein interacting partners located within approximately ~10 nm of the bait in living cells (Gupta et al., 2015; Roux et al., 2018). These experiments were performed in HEK293 cells expressing CEP128-BirA\* under serum starvation condition to induce ciliation (Figure 4A). As expected, CEP128 CTD proximity mapping identified many centrosomal, ciliary and satellite proteins, together with components of different cytoskeletal structures, including actin (e.g. WASH complex) and several intermediate filaments proteins (Figure 4B).

To select candidates proteins of basal foot region I, we used several selection criteria including 1) confirmed direct interaction with CEP128 CTD by FLAG-Immuno Precipitation; 2) previously identified centrosomal localization or predicted coiled coil/filamentous structure; 3) proximity by BioID with other region II proteins and 4) proteins in close proximity to CEP128 CTD and NTD by analysis of published BirA\*-CEP128 BioID dataset (Gupta et al., 2015) (Figure 4C). Among the proteins shortlisted as candidates (Figure S4), we focused on CEP112, a protein enriched in predicted coiled-coil sequences (Figure 4D), which was previously assigned to the centrosome (Jakobsen et al., 2011). CEP112 was identified in BioID experiments in close proximity of both centriolar marker CEP135 and region II basal foot marker CNTRL (Figure S4).

Immunolabeling of CEP112 revealed a distribution consistent with that of a basal foot protein, being positioned radially close to the basal body center (CTD: 134±13 nm; MD: 145±18 nm, Table S1), with a second population at the daughter centriole (Figures 4E and 4F), and axially in the same region of other basal foot proteins (CTD: 190±41 nm; MD: 195±44 nm; Figure 4G). CEP112 mapping shows that its average position is similar to that of CEP128 CTD, suggesting that CEP112 is part of region II (Figures 4H).

To examine CEP112 role in basal foot assembly, we generated a HEK293 knock-out (KO) line by CRISPR-Cas9 deletion (Figure 5A) and confirmed its absence by Western Blot analysis and immunolocalization (Figures 5B and 5C). Analysis of basal foot proteins recruitment, ciliogenesis or cilia length, showed however that lack of CEP112 did not have an impact on these processes (Figures 5C and 5E). Altogether, our data show that CEP112 is a basal foot protein and suggest potential unique functions in the basal foot beyond a structural role that needs further investigation.

***Basal Foot in Motile Cilium has a Conserved Modular Architecture, but Distinct Organization within Modules***

We next asked whether the architecture of the basal foot was conserved in motile cilia. Since in this specialized ciliary type, the basal feet play an important structural role by linking motile cilia together to ensure coordinated beating, we hypothesized that it is likely organized differently relative to primary cilia (Kunimoto et al., 2012; Tateishi et al., 2013). We chose airway multiciliated cells as our cellular model, which can be differentiated from human nasal basal cells in an air liquid interphase (ALI). Each differentiated multiciliated cell has about 200 to 300 basal bodies, each with one basal foot, thereby allowing a large number of basal foot measurements compared to cells with a primary cilium (Figure 6A). To assign the position of basal foot proteins *in situ* in motile cilia*,* we used POC1B as a reference marker, a component of the centriole/basal body wall (Le Guennec et al., 2020; Sydor et al., 2018; Venoux et al., 2013). Since there is only one basal foot per basal body in motile cilia, basal foot proteins appeared in the end-on view as individual diffraction-limited spots that can be resolved by 3D-SIM, rather than a ring as observed in primary cilia (Figures 6A and 1B).

3D-SIM imaging map data showed that basal foot molecular architecture is only partially conserved between motile and primary cilia (Figure 6B, Table S4). Similar to primary cilia, NIN and CEP170 are located in region III, together with γ-Tubulin and NEDD1, components of the γTuRC microtubule nucleating complex (Kollman et al., 2011) (NIN CTD: 188±29 nm, NTD: 177±32 nm; CEP170 CTD:191±30 nm, NTD: 194±33 nm; γ -Tubulin: 206±33 nm; NEDD1: 197±41 nm; note that γ-Tubulin and NEDD1 position could not be measured in primary cilia due to the presence of a second abundant population of γTuRCs in the PCM (Luders et al., 2006)). In addition, motile cilia basal foot region II components CEP128 (162±25 nm), ODF2 (158±16 nm) and CEP112 (165±28 nm) showed similar distances from the centriole wall to their primary cilia counterparts. Interestingly, both CNTRL CTD and MD were located in region III, further away from the basal body (180±23 nm and 200±24 nm, respectively) suggesting a distinct organization of this protein in motile cilia.

3D-SIM mapping also revealed unique features of other basal foot proteins in motile cilia (Figures 6C and S5). CEP112 was observed either as a two dots/arch like distribution, or as a complete ring in cells that appeared not fully differentiated, suggesting a dynamic distribution of CEP112 during multiciliogenesis (Figure 6C). CEP19, on the other hand, forms a complete ring more like distal appendages proteins in fully differentiated cells (Figure S5). Lastly, TCHP exhibited a filamentous distribution that did not allow accurate measurements (Figure S5).

To better understand the differences in distribution of basal foot proteins in motile cilia, we examined basal foot architecture using transmission electron microscopy (TEM). Consistent with 3D-SIM quantitative image analysis, TEM micrographs of the basal feet in human airway multiciliated cells suggested both structural similarities and differences relative to primary ciliated cells (Figure 6D). The basal foot appeared as a conical structure (height= ~130 nm and width= ~200 nm) attached to the basal body by three microtubule triplets and displays several electron-dense regions, including a bulky tip, the basal foot cap, consistent with previous reports (Figure 6D) (Clare et al., 2014; Kunimoto et al., 2012; Sorokin, 1968). Similar to primary cilia, the basal foot cap in motile cilia appeared as a round structure with anchored microtubules (Figure 6D, asterisk). In addition, the motile cilia-associated basal foot shows fibrils connecting the cap to the central region (Figure 6D, open arrowhead) and two spherical structures, symmetrically positioned on both sides of the tip (Figure 6D, closed arrowhead). In contrast to primary cilia, the basal foot in motile cilia is connected to the basal body by three tall, electron-dense arches originating each from a separate axonemal microtubules triplet (Figure 6D, arrow), with one side of the arch connecting to the A-tubule and one to the C-tubule.

Correlation of radial distances measured from TEM images with the ones obtained from 3D-SIM protein spatial map provided further insights on the *in situ* molecular organization of the basal foot in motile cilia (Figures 6B, 6D and 6E). First, the average distribution of region II proteins ODF2, CEP112 and CEP128 was in close proximity of the arches extending from the microtubule triplets (arch’s tip radial distance: 150±12 nm), indicating a potential scaffolding role of the arches to region II proteins in the basal foot of motile cilia. We also noticed that CEP112 formed a two-dot distribution pattern by 3D-SIM imaging, supporting the prediction that CEP112 localizes within the same region at the base of the basal foot (Figure 6C).

Second, region III proteins CEP170, NIN and γTuRC proteins NEDD1 and -Tubulin were located approximately in the electron-dense basal foot cap region, together serving their suggested microtubule anchoring and nucleation functions (basal foot cap radial distance: 233±15 nm).

Third, the distance of CNTRL from the basal body center in 3D-SIM was consistent with that of the electron-dense, symmetrical spherical structures of the basal foot in TEM (Figure 6D, closed arrowhead) (spherical structure radial distance: 190±15 nm; 3D-SIM radial distance: CNTRL CTD and MD: 180±23 nm and 200±24 nm, respectively), which suggested its localization at this distinct structure. Since CNTRL distribution appeared not fully resolved by 3D-SIM, showing as a bar across the basal foot height, we then used STORM, which reaches a resolution of ~25 nm, to resolve its localization (Rust et al., 2006). STORM imaging unambiguously showed that CNTRL was distributed into two main symmetrical populations at the basal foot in motile cilia, located on opposite sides of its longitudinal axis (Figure 6F). Although the average radial distance and distribution pattern of CNTRL in fluorescence imaging appeared to mirror those of the spherical EM structures, future correlative EM-fluorescence studies will be required to confirm whether they are indeed part of the same basal foot structure.

**DISCUSSION**

In this study, we present a comprehensive super-resolution molecular map of the basal foot in primary and motile cilia. Our spatial analysis of basal foot proteins shows that it has an architecture characterized by three main protein regions linked by elongated coiled-coil protein assemblies.

This molecular organization of primary cilia basal foot (Figure 7A) is largely conserved in its cognate structures in cycling cells, the subdistal appendages, which recent studies have shown to present a dynamic architectural organization (Chong et al., 2020). Our mapping data and previous studies suggest a model where ODF2 functions as a scaffold of the central region (II), which is then required for region III assembly. ODF2 is a highly insoluble protein that forms fibrils (Donkor et al., 2004), which has been shown to be required for basal foot formation (Kunimoto et al., 2012; Tateishi et al., 2013) and recruitment of CEP128, TCHP, CNTRL, NIN and CEP170 to subdistal appendages (Ibi et al., 2011; Mazo et al., 2016; Nakagawa et al., 2001). The close proximity between ODF2 and CEP128 in region II of basal foot is consistent with evidence that CEP128 is an ODF2-interacting protein and plays a role in ODF2 organization and subdistal appendages formation (Chong et al., 2020; Kashihara et al., 2019; Mazo et al., 2016). It is important to note that ODF2 harbours a second population at the transitional fibers/distal appendages, structures residing distal to the basal foot that cannot be resolved by 3D-SIM (Chong et al., 2020).

Our data also indicate that CEP128 has an elongated distribution, extending towards the bridging region between region II and III. This observation can account, at least in part, for its suggested role in NIN recruitment and centriolar MT stabilization, possibly via its interaction with NIN, a MT-associated protein found in region III furthest from the basal body wall (Kashihara et al., 2019; Mazo et al., 2016). The distribution of CEP128, ODF2 and CNTRL domains along the basal foot longitudinal axis is consistent with those reported for subdistal appendages by Kashihara and colleagues, providing further evidence of conservation in the molecular architecture between subdistal appendage and primary cilia basal foot.

Our domain map reveals that CNTRL forms elongated protein assemblies linking regions II and III along the longitudinal axis of the basal feet. On the other hand, Ninein loops back from region III to region II, and it is important for the recruitment and/or stabilization of CNTRL and, to a lesser extent, ODF2, in addition to its known role in recruitment of CEP170. Altogether, our quantitative analysis extends previous studies (Mazo et al., 2016), where NIN was found dispensable for recruitment of region II proteins such as CNTRL, CEP128 and ODF2 and support a model in which major basal foot components, including ODF2, CEP128, CNTRL and NIN, stabilize one another, collectively contributing to basal foot structural integrity, as opposed to a model in which each protein is recruited sequentially downstream of the other.

BioID and super-resolution imaging mapping experiments presented here led to the identification of CEP112 as a basal foot protein localized in region II. Our data show that CEP112 does not play a major structural role in primary cilia, but do not exclude its potential role in anchoring a small subset of microtubules as in subdistal appendages (Chong et al., 2020), in signal transduction, similar to that of CEP128 in primary cilia (Monnich et al., 2018), or a role in multiciliated cells. Interestingly, two patients with infertile sperm have been recently identified with mutations in CEP112 (Sha et al., 2020), suggesting that this protein might be important for cilia and/or flagella function.

BioID proximity map of CEP128 CTD provided an interesting candidate list of basal foot proteins, which include multiple intermediate filament proteins (SYNM, INA and IFF02) pointing to a role in basal foot assembly, possibly as region I proteins. Previous studies have shown that a 2D circular mesh network of IF forms in close association with basal bodies around the basal foot region in motile cilia. This IF network precedes basal foot formation, and it is cross-linked with the MT apical network in mouse epithelial multiciliated cells, hinting at a potential role of this type of filament in structure and/or function of basal foot (Herawati et al., 2016; Tateishi et al., 2013). It has been previously proposed that ODF2, one of the closest proteins to the wall and a self-interacting protein that forms a fibrillar structure, might belong to a new type of intermediate filament protein family (Donkor et al., 2004). Interestingly, TCHP, a reported component of subdistal appendages and basal foot, which our data show to be located close to the basal body wall in primary cilia, is also a keratin IF scaffold protein (Ibi et al., 2011). It remains to be addressed whether these proteins are components of region I, play a scaffolding role in basal foot assembly or interact as part of a network of filaments with other cytoskeletal structures.

Lastly, the basal foot map in primary cilia also clarifies the distribution of reported basal foot proteins such as CC2D2A, TCHP and CEP19. We show that CC2D2A and TCHP are not classic basal foot proteins, a notion supported by studies of the transition zone region (Chih et al., 2011; Garcia-Gonzalo et al., 2011), and that CEP19 resides above the basal foot at a distance from the basal body consistent with the measurements from Kanie et al. (d=332±30nm (our data); d=372.6±16.4nm (Kanie et al., 2017). This observation is supported by recent evidence suggesting that CEP19 forms a functional complex with FOP and CEP350, distal appendages proteins required for an early step of ciliogenesis (Kanie et al., 2017; Mojarad et al., 2017; Nishijima et al., 2017).

The modular architecture of basal foot in motile cilia, although conserved, displays an overall more compact organization of region II and III proteins compared to that in primary cilia (Figure 7B). Regions II proteins CEP112, CEP128 and ODF2 appear in close proximity of the tall arches originating from microtubule triplets detected by EM, suggesting a scaffolding role of these electron-dense structures, which connect the basal body to the basal foot region II proteins. These motile cilia basal foot-associated structures, and their attachment to three triplet microtubules most likely provide a robust molecular anchor of the basal foot to sustain mechanical pulling forces present during ciliary beating. It remains unclear whether a similar shorter arch structure is present in the basal foot of primary cilia.

In summary, our study provides a subdiffraction view into the conserved, yet distinct, molecular architecture of basal foot in primary and motile cilia, highlighting a fine-tuned assembly of basal foot components adapted to serve different cilia functions.

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**AUTHOR CONTRIBUTIONS**

Q.P.H.N. designed and conducted experiments, collected and analysed the data, made figures and wrote the first draft of the manuscript. Z.L. did STORM experiments, analysed CEP112 KO ciliation data, wrote methods, contributed to figures and edited the manuscript. A.A, H.O., Z.L., W.F. and T.M. helped with airway multiciliated cells culturing system; A.A. did CEP112 KO clone selection. E.C., E.L. and B.R. performed BioID experiments. T.M. and W.F provided human nasal cells. V.M. conceived the project, designed experiments, analysed data, made figures and wrote the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing financial interests.

**MAIN FIGURE TITLES AND LEGENDS**

**Figure 1. 3D-SIM mapping reveals the molecular architecture of basal foot in primary cilia**

(A) Representative TEM micrographs of a primary cilium and its basal feet (BF, green arrowheads) in immortalized RPE-1 cells. BB: basal body; DC: daughter centriole. Scale bar represents 200 nm. (B) 3D-SIM volume maximum intensity projection of RPE-1 cell primary cilia labeled with anti-Ninein (NIN, green) and anti-glutamylated tubulin (Glut-TUB, red) antibodies. Note the three known subpopulations of NIN: 1) at the daughter centriole; 2) proximal ends of basal body; 3) basal feet (see arrowheads). Scale bar represents 1 μm. (C) 3D-SIM volume maximum intensity projection of ciliated RPE-1 cells labeled with anti-basal foot proteins (green) and anti-glutamylated tubulin (red) antibodies. Scale bar represents 1 μm. (D) Cartoon depiction of the strategy used to measure distances of basal foot proteins (green) in primary cilia. The radial distance, or distance from the centriolar center, was calculated either from end-on view by dividing each of the ring diameter measurements by two, or from side view by measuring the lateral distance of basal foot proteins positioned across the basal body. Axial distance was measured relative to the basal body proximal end. (E) Box plot of radial distances of basal foot proteins in primary cilia of RPE-1 cells (n=40). Region and color assignment of proteins was based on statistical analysis by one-way ANOVA and Tukey's multiple comparison test. Distance measurements of proteins not significantly different from at least one other protein were grouped into the same region. (F)Box plot of axial distances of basal foot proteins in primary cilia from RPE-1 cells (n=40). RPGRIP1L was used to label the transition zone. Statistical analysis was done by one-way ANOVA with Tukey's multiple comparison test. See also Figure S1 and Table S1. (G) Cartoon summary of basal foot molecular organization into 3 main regions: region I (basal body anchoring), region II (scaffolding) and region III (microtubule anchoring) in end-on (left) and side view (right). Region II and III are connected via a bridging region. See also Figure S3 and Table S3.

**Figure 2. Domain analysis of basal foot proteins in primary cilia**

(A) Linear maps representing protein polypeptide sequences showing the regions recognized by antibodies and the position of GFP insertion used in super-resolution imaging experiments (See also Figure S1). (B to F)Box plot of radial distances of different domains of ODF2 (B), CNTRL (C), CEP128 (D), CEP170 (E), and NIN (F) using antibodies and GFP-labelled proteins. Statistical analysis was done using Welch’s t-test and Tukey's multiple comparison test. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001, \*\*\*\* p<0.0001. Cartoons depicts domain radial distances of basal foot proteins. C: CTD, N: NTD, M: Middle Domain. (G)Summary domain analysis of radial distances of basal foot proteins in primary cilia of RPE-1 cells (n=40). Region and color assignment of proteins were based on statistical analysis by one-way ANOVA and Tukey's multiple comparison test. Distance measurements of proteins not significantly different from at least one other protein were grouped into the same region. (H)Summary domain analysis of axial distances of basal foot proteins in primary cilia from RPE-1 cells (n=40). RPGRIP1L was used to label the transition zone. Statistical analysis was done by one-way ANOVA with Tukey's multiple comparison test. See also Table S1.

**Figure 3. Domain analysis of NIN using GFP-insertion strategy and NIN role in basal foot assembly**

(A)Linear map representing NIN polypeptide sequence showing the GFP insertion sites of NIN fusion constructs. (B) Western blot of RPE-1 lysates from control untransfected cells (untr) or from transfected cells with different Ninein-GFP constructs for 72h. Membranes were probed with anti-GFP (Ninein: 248kDa) and anti-alpha tubulin antibodies. (C) 3D-SIM volume maximum intensity projection of RPE-1 cells expressing different Ninein-GFP constructs (72 hpt), labelled with anti-GFP (green) and anti-glutamylated tubulin (red) antibodies. Scale bar represents 1 μm. (D)Box plot of radial distances of NIN-GFP constructs in primary cilia of RPE-1 cells (n>23). Statistical analysis was done using Tukey's multiple comparison test. n.s non-significant, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001, \*\*\*\* p<0.0001. (E) Cartoon depiction of NIN regional localization. C: CTD, N: NTD, M: Middle Domain. (F)Western blot of RPE-1 lysate of cells untransfected (untr), or transfected with non-specific (siCTR) or NIN-targeting (siNIN) siRNA for 90h. Membranes were probed with anti-Ninein and anti-alpha tubulin antibodies. (G)3D-SIM volume maximum intensity projection of RPE-1 cell primary cilia transfected with non-targeting (siControl) and NIN-targeting (siNIN) siRNAs (90hpt), labeled with anti-basal foot proteins (CEP170, CNTRL, ODF2 and CEP128, green), anti-NIN (red) and anti-Glut-TUB (blue) antibodies.Scale bar represents 1 μm.(H)Quantification of fluorescence levels of basal foot proteins in RPE-1 cells transduced with siRNA targeting NIN. Data are represented as mean ± SD. Statistical analysis was done by unpaired student t-test. (I)Cartoon depicting NIN role in basal foot assembly. See also Figure S2 and Table S1, S2.

**Figure 4. CEP112 is a basal foot protein**

(A)Cartoon depiction showing position of Bir-A\* insertion in CEP128 sequence for BioID experiments. (B)Diagram showing proteins identified via BioID in close proximity to CEP128-BirA\* in ciliated HEK-293 cells. Arrow thickness is proportional to the number of peptides detected. (C)Strategy to identify basal foot region I components.See also Figure S4. (D)Primary sequence of CEP112.(E)3D-SIM volume maximum intensity projection of a primary cilium in RPE-1 cell labelled with anti-CEP112 (green) antibodies labelling C-terminal domain (CTD, top) and middle domain (MD, bottom), and anti-Glut-TUB (red) antibody, showing two distinct subpopulations of the protein at 1) the proximal ends of daughter centriole (DC) and 2) the basal feet (BF) (see arrows). Scale bars represent 1 μm. (F and G)Box plot of radial (F) and axial (G) distances of CEP112 CTD and MD relative to CEP128 CTD in primary cilia of RPE-1 cells (n=40). Glut-TUB and RPGRIP1L were used as basal body wall and transition zone references, respectively. Statistical analysis was done by one-way ANOVA with Tukey's multiple comparison test. (H)Cartoon depicting position of CEP112 in basal foot structure. C: CTD, M: Middle Domain.

**Figure 5. CEP112 KO does not affect basal foot, cilia structure or length**

(A)Linear polypeptide sequence of CEP112 with position of CRISPR/Cas9 indel. (B) Western blot of HEK-293 lysates from CEP112 CRISPR/Cas9 KO cells. Membranes were probed with anti-CEP112 antibodies and anti-actin as control. (C) 2D projection micrograph of 3D-SIM volume of WT or CEP112 KO HEK293 cells by CRISPR-Cas9 (clonally selected), labelled with anti-basal feet markers (green), anti-glutamylated Tubulin (red) antibodies. Scale bar represents 1 μm.(D)Bar graph depicting measurements of ciliation level in CEP112 KO cells. Data are represented as mean ± SD. Statistical analysis was done using unpaired t-test.(E)Bar graph depicting measurements of cilia length in CEP112 KO cells byCRISPR/Cas9 editing. Data are represented as mean ± SD. Statistical analysis was done using unpaired t-test. See also Figure S5.

**Figure 6. The conserved and distinct architectural features of basal foot in motile cilia**

(A) Left: 3D-SIM volume maximum intensity projection of an area of an airway epithelial multiciliated cell (end-on view), labelled with an antibody recognizing a basal body protein (POC1B, red) and a basal foot protein (CNTRL, green). Scale bar represents 200 nm. Right: Cartoon depiction of the strategy used to measure radial distance of basal foot proteins (green) in motile cilia of human airway multiciliated cells using end-on view. Radial distance was measured relative to the basal body center (red). (B)Box plot of radial distributions of basal foot proteins in human airway multiciliated cells (n=80). Region assignment was done based on one-way ANOVA with Tukey's multiple comparison test. Proteins whose distances were not significantly different with at least one other protein were grouped into the same region. See also Figure S5 and Table S4. (C)Representative localization of CEP112 (green) in relative to POC1B (red) in human airway multiciliated cells (end-on view). Note the different patterns of CEP112 staining (white arrows denote 2-dot and complete ring patterns). Scale bar represents 2 μm. Right: High-magnification views of boxed area. Scale bar represents 500 nm.(D)Left: Representative TEM micrograph from a cross section of a human airway multiciliated cell at the basal feet level. Scale bar represents 500 nm. Right: High-magnification view of the boxed region. Note three main electron-dense regions of the basal foot. Red asterisk denotes basal cap region, red arrowhead the spherical symmetrical structures, red empty arrowhead denotes the fibril region and red arrow represents the arch region. Scale bar represents 100 nm. (E)Cartoon depiction of EM-resolved basal foot structure (top) and the distance measurements of its sub-structures in motile cilia (MT triplets, arch tips, spherical structure and basal foot cap) relative to the basal body center based on TEM mapping (bottom).Data are represented as mean ± SD. (F)Left: 2D-STORM micrograph of human airway multiciliated cells labeled with anti-CNTRL antibody (end-on view). Scale bar represents 2 μm. Right: High-magnification views of boxed areas. Scale bar represents 50 nm.

**Figure 7. Proposed model of basal foot molecular architecture in primary (A) and motile (B) cilia.** Basal foot components are organized into spatially distinct regions with different functions (Region I: Basal body anchoring, Region II: Scaffolding, Region III: Microtubule anchoring). Cartoons demonstrate domain localizations of basal foot proteins. C: CTD, N: NTD, M: Middle Domain.

**MAIN TABLES AND LEGENDS**

None in this study

**STAR METHODS**

***RESOURCE AVAILABILITY***

***Lead Contact***

Further information and request for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Vito Mennella (v.mennella@soton.ac.uk)

***Materials Availability***

All unique/stable reagents generated in this study are available from the Lead Contract with a completed Materials Transfer Agreement.

***Data and Code Availability***

The published article includes all datasets generated during this study.

***EXPERIMENTAL MODEL AND SUBJECT DETAILS***

## Immortalized cell lines

hTERT-RPE1 cell line (source: ATC® CRL-4000TM) was culturedin 10% FBS-containing DMEM media. HEK293 Flp-In T-Rex cells were cultured in the Tetracycline-free DMEM media supplemented with 10% FBS. To induce ciliation, RPE-1 and HEK293 cells were serum-starved with DMEM/F-12 media for 48-72 hours.

## Air-Liquid Interphase differentiation of primary human nasal airway cells

Human primary nasal airway cells from healthy volunteers were collected using a cytology brush by a nurse, with a protocol approved by Research Ethics Board at the Hospital for Sick Children, adhering to local and national research and ethical approval. Airway cells were then expanded, seeded on transwells (Corning HTS Transwell-96 and -24 permeable support; 0.4 µm pore size), and differentiated for at least 21 days following Stem Cell Technologies protocols using PneumaCult-Ex and PneumaCult-ALI media (Cao et al., 2020). The media were supplemented with vancomycin, tobramycin, gentamicin and antibiotic-antimycotic antibiotics.

***METHOD DETAILS***

***Immunofluorescence and Antibodies***

RPE-1 cells were grown and fixed on coverslips, while human nasal and mouse tracheal multiciliated cells from ALI cultures were directly fixed on transwell filters with either methanol (20 min at -20 oC) or 4% Paraformaldehyde (PFA; 10 min at RT). For PFA fixation, cells were subsequently reduced with 0.1% Sodium Borohydride (7 min), then permeabilized with 0.2 % Triton X-100 (25 min). Cells were blocked using 5% FBS-containing PBS, incubated with primary antibodies for either 1 hour (RT) or overnight (4 0C), and then secondary antibodies conjugated with Alexafluor -405, -488, -555 and -647 nm (Thermo Fisher Scientific). When appropriate, cells were stained with directly labelled primary antibodies (prepared using APEX Antibody Labelling Kit, Thermo Fisher Scientific). Cells were stained for nuclei with Hoechst 33342. Please refer to key resources table for a list of antibodies used in this study. Trachea labelling was performed as described in previously published protocol (Vladar et al., 2015).

***Super-resolution Imaging***

3D-SIM samples were imaged using ELYRA PS.1 (Carl Zeiss Microscopy) with a Plan-Apochromat 63x or 100x/1.4 Oil immersion objective lens with an additional 1.6x optovar. An Andor iXon 885 EMCCD camera was used to acquire images with 101 nm/pixel z-stack intervals over a 5-10 µm thickness. For each image field, grid excitation patterns were collected for five phases and three rotation angles (-75o; -15o, +45o). The raw data was reconstructed and channel aligned using SIM module of ZEN Black Software (version 8.1). STORM data was collected using PALM mode in ELYRA PS.1 (Carl Zeiss Microscopy) with a Plan-Apochromat 63x or 100x/1.4 Oil immersion objective lens with an additional 1.6x optovar. An Andor iXon 885 EMCCD camera was used to acquire images using TIRF mode. Lasers of wavelength 647 nm and 405 nm were used to activate the fluorophore. Raw data was reconstructed using PALM module of Zen Black Software (version 8.1), with recognition and fitting of overlapping molecules active. Reconstructed data was further processed for drift correction and binning using home-written MATLAB script (can be accessed via the following link: https://drive.google.com/open?id=11fuWn7kmZ-loCn79CKChJI5FeMme0fDU).

***Transmission Electron Microscopy (TEM)***

ALI filters of fully differentiated human nasal multiciliated cells were fixed in 2% glutaraldehyde in 0.1M sodium cacodylate buffer, rinsed in 0.1M sodium cacodylate buffer with 0.2M sucrose, post-fixed in 1% OsO4 in 0.1M sodium cacodylate buffer, dehydrated in a graded ethanol series (70%, 90%, 3X 100%), infiltrated with propylene oxide, and embedded in Quetol-Spurr resin. 90nm-thick serial sections were cut on a Leica Ultracut ultramicrotome, stained with uranyl acetate and lead citrate, and imaged on a FEI Tecnai 20 TEM.

## CRISPR-Cas9 cell line generation

RNA-guided targeting of *CEP112* in HEK-293 cells was performed by SYNTHEGO with Cas9 2NLS nuclease protein from *S. Pyogenes* with chemically modified single guide RNAs (sgRNAs). Briefly, 1.5x105 HEK 293 cells were nucleofected with 20 pmol Cas9 2NLS and 180 pmol of sgRNA according to manufacturer’s instructions with Lonza 4D Nucleofector unit and 16 well nucleocuvette strips. Sequence of *CEP112* gRNA used is as follows: (5′- GCUGUUCUCUUCUUUCAGAG-3′). 72 hours post nucleofection, Sanger sequencing analysis on DNA isolate was performed to determine editing efficiency. For clone selection, single-cell clones were initiated in 10 cm polystyrene dishes starting with cell densities of 5, 10 and 20 cells per dish. Subsequently, single colonies were isolated (Cloning cylinders, Millipore sigma, C1059) and gradually expanded in 24-well, 12-well and 6-well plates. For clones genotyping, the edited region was PCR-amplified from isolated genomic DNA and verified by Sanger sequencing. Western blot analysis was used to further verify the CEP112 KO clones. The clone selected for subsequent analysis bears mutation p.K832EfsX19.

***Cilia length and ciliation level measurements***

After reaching confluency, HEK293 cells were serum starved for 72 hours, fixed and immunolabeled with antibodies. To enhance adherence of HEK293 cells, coverslips were covered with a solution of Poly-L-lysine (0.01%). For measurements of length and ciliation, cilia were identified with anti-ARL13B and GT335 antibodies and measured using ImageJ software. Ciliation percentage was calculated as a ratio of cilia to nuclei (DAPI) in each image.

***BioID Assay***

HEK293 Flp-In T-Rex cells were first co-transfected with the pcDNA5/FRT/TO CEP128-FLAG-BirA\* plasmid and Flp Recombinase Expression plasmid pOG44 (1:20 ratio), and selected for stable expression with Hygromycin B and Blasticidin. Stable CEP128-FLAG-BirA\* HEK293 Flp-In T-Rex cell line was induced for BirA expression and biotinylated for 24 hrs with 1 µg/ml tetracycline and 50 µM biotin. For ciliation experiments, cells were serum-starved to for 72 hrs. Cells were then collected and processed for BioID and FLAG Immunoprecipitation (IP) experiments as described previously (Coyaud et al., 2015). For BioID, 5 x 150 mm diameter subconfluent dishes of cells were pelleted (2000 rpm, 3 min), the pellet was washed twice with PBS, and dried pellets were snap frozen. Pellets were lysed in 10 ml of modified RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 1:500 protease inhibitor mixture (Sigma-Aldrich, Saint-Louis, MO), 250U Turbonuclease (Accelagen, San Diego, CA)) at 4°C for 1 h, then sonicated (30 s at 35% power, Sonic Dismembrator 500; Fisher Scientific) to disrupt visible aggregates. The lysate was centrifuged at 19,000 rpm (45000 × g) for 20 min. Clarified supernatants were incubated with 30 μl packed, pre-equilibrated streptavidin-Sepharose beads (GE) at 4 °C for 3 h. Beads were collected by centrifugation (2,000 rpm, 2 min), washed six times with 50 mm ammonium bicarbonate pH 8.3, and treated with TPCK-trypsin (Promega, Madison, WI, 16 h at 37 °C). The supernatant containing the tryptic peptides was collected and lyophilized. Peptides were resuspended in 0.1% formic acid and 1/6th of the sample was analyzed per MS run.

***Anti-FLAG IP***

For FLAG pulldowns, 5 × 150 mm diameter dishes of cells were scraped into PBS, pooled, washed twice in 25 ml PBS and collected by centrifugation at 300 × g for 5 min at 4 °C. Dried cell pellets were stored at −80 °C until lysis. The cell pellet was weighed and 1:4 pellet weight/lysis buffer (by volume) was added. Lysis buffer consisted of 50 mM HEPES-NaOH (pH 8.0), 100 mM KCl, 2 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 1 mm PMSF, 1 mM DTT and 1:500 protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO). Upon resuspension, cells were incubated on ice for 10 min, subjected to one additional freeze-thaw cycle, then centrifuged at 35,000 × g for 20 min at 4 °C. Supernatant was transferred to a fresh 15 ml conical tube and 250U Turbonuclease (Accelagen) plus 30 μl packed, pre-equilibrated FLAG-M2 agarose beads (Sigma-Aldrich) were added. The mixture was incubated for 2 hrs at 4 °C with end-over-end rotation. Beads were pelleted by centrifugation at 1,000 rpm (100 × g) for 1 min and transferred with 1 ml of lysis buffer to a fresh centrifuge tube. Beads were washed once with 1 ml lysis buffer and twice with 1 ml ammonium bicarbonate (ammbic) rinsing buffer (50 mM ammbic pH 8.0, 75 mM KCl). Elution was performed by incubating the beads with 150 μl of 125 mM ammonium hydroxide (pH >11). The elution step was repeated twice more, and the combined eluate centrifuged at 16,000 × g for 10 min, transferred to a fresh centrifuge tube and lyophilized. Following overnight trypsin digestion (as above), peptides were resuspended in 0.1% formic acid and 1/6th of the sample was analyzed per MS run.

***Mass spectrometry analysis***

Briefly, high performance liquid chromatography was conducted using a 2 cm pre-column (Acclaim PepMap 50 mm x 100 um inner diameter (ID)), and 50 cm analytical column (Acclaim PepMap, 500 mm x 75 um diameter; C18; 2 um; 100 Å, Thermo Fisher Scientific, Waltham, MA), running a 120 min reversed-phase buffer gradient at 225nl/min on a Proxeon EASY-nLC 1000 pump in-line with a Thermo Q-Exactive HF quadrupole-Orbitrap mass spectrometer. A parent ion scan was performed using a resolving power of 60,000, then up to the twenty most intense peaks were selected for MS/MS (minimum ion count of 1,000 for activation) using higher energy collision induced dissociation (HCD) fragmentation. Dynamic exclusion was activated such that MS/MS of the same *m/z* (within a range of 10 ppm; exclusion list size = 500) detected twice within 5 sec were excluded from analysis for 15 sec. For protein identification, Thermo .RAW files were converted to the .mzXML format using Proteowizard (Kessner et al., 2008), then searched using X!Tandem (Craig and Beavis, 2004) and COMET (Eng et al., 2013) against the human Human RefSeq Version 45 database (containing 36,113 entries). Data were analyzed using the trans-proteomic pipeline (TPP) (Pedrioli, 2010), (Deutsch et al., 2010) via the ProHits software suite (v3.3) (Liu et al., 2010). Search parameters specified a parent ion mass tolerance of 10 ppm, and an MS/MS fragment ion tolerance of 0.4 Da, with up to 2 missed cleavages allowed for trypsin. Variable modifications of +16@M and W, +32@M and W, +42@N-terminus, and +1@N and Q were allowed. MS raw files were Proteins identified with an iProphet cut-off of 0.9 (corresponding to ≤1% FDR) and at least two unique peptides were analyzed with SAINT Express v.3.6. Twelve control runs (from cells expressing the FLAG-BirA\* epitope tag, and either processed following the anti-FLAG IP or the BioID protocol) were collapsed to the two highest spectral counts for each prey, and compared to the two technical of each of the two biological replicates of the FlagBirA\*-Cep128. High confidence interactors were defined as those with BFDR≤0.01.

***Plasmids, Transfection and siRNA-mediated knockdown*** Please refer to key resources table for a list of plasmids and primers used in this study. hTERT-RPE1 cells were transfected using Lipofectamine3000 Kit (Invitrogen) according to manufacturer instruction. Cells were analysed for downstream applications at 48-72 hours post transfection (hpt). Smartpool ON-TARGETplus siRNAs for human NIN were purchased from GE Dharmacon. Cells were transfected with siRNAs using Lipofectamine RNAiMAX (Invitrogen). Cells were serum-starved at 24hpt for ciliation and harvested at 90hpt for immunofluorescence or immunoblotting.

## Western Blot

Cells were lysed using RIPA lysis buffer (Pierce, Thermo Fisher Scientific) freshly added with protease inhibitor (Roche, Sigma-Aldrich). Lysates were loaded on 4-12 or 8% Bis-Tris Plus gels. Proteins were transferred to a nitrocellulose membrane and blocked using 5% Skim-milk in TBST. Protein blots were sequentially incubated with primary and HRP-conjugated secondary antibodies diluted in 5% BSA in TBST. Blots were developed using the Novex ECL Chemiluminescent Substrate Kit (Invitrogen).

***QUANTIFICATION AND STATISTICAL ANALYSIS***

Image measurements were done in Zeiss Zen Black and Fiji ImageJ softwares. Data was analysed in Microsoft Excel and Prism software. Statistical tests, sample sizes and number of replicates were specified in figure legends. Differences were regarded as significant if p < 0.05, unless otherwise stated.

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