SHORT REPORT

Cep78 is a new centriolar protein involved in Plk4-induced centriole overduplication

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ABSTRACT

Centrioles are core components of centrosomes, the major microtubule-organizing centers of animal cells, and act as basal bodies for cilia formation. Control of centriole number is therefore crucial for genome stability and embryogenesis. Centriole duplication requires the serine/threonine protein kinase Plk4. Here, we identify Cep78 as a human centrosomal protein and a new interaction partner of Plk4. Cep78 is mainly a centriolar protein that localizes to the centriolar wall. Furthermore, we find that Plk4 binds to Cep78 through its N-terminal domain but that Cep78 is not an *in vitro* Plk4 substrate. Cep78 colocalizes with Plk4 at centrioles and is required for Plk4-induced centriole overduplication. Interestingly, upon depletion of Cep78, newly synthesized Plk4 is not localized to centrosomes. Our results suggest that the interaction between Cep78 and the N-terminal catalytic domain of Plk4 is a new and important element in the centrosome overduplication process.

KEY WORDS: Cep78, Plk4, Centriole duplication, Centrosome

INTRODUCTION

In animal cells, centrioles are the major microtubule-organizing centers and are surrounded by a proteinaceous matrix denoted the pericentriolar material (PCM). Centrioles exist as pairs and are composed of an older (mother) centriole and a younger (daughter) centriole. Centrioles duplicate once per cell cycle in a tightly controlled manner to ensure that an interphase centrosome is duplicated exactly once per cell cycle (Firat-Karalar and Stearns, 2014; Brito et al., 2012; Gonczy, 2012). Abnormalities in centriole number and structure can lead to the formation of extra centrosomes that can perturb spindle organization and chromosome segregation giving rise to aneuploidy (Gonczy, 2015). A number of diseases including cancer and developmental disorders, such as microcephaly, dwarfism and ciliopathies, arise from the malfunction of centriolar and centrosomal proteins (Gonczy, 2015; Valente et al., 2014; Bettencourt-Dias et al., 2011).

An evolutionary conserved pathway of centriole formation has been described from *Caenorhabditis elegans* and *Drosophila melanogaster* to humans (Pelletier et al., 2006; Delattre et al.,

2006; Brito et al., 2012). Major components of the pathway in human cells are the serine/threonine polo-like kinase 4 (Plk4), Cep192, Cep152, Sas-6, STIL and CPAP (also known as CENPJ) (Brito et al., 2012). In human cells, Plk4, Sas-6 and STIL localize to the sites of procentriole formation and collaborate to induce cartwheel assembly during daughter centriole formation. Plk4 is a structurally divergent polo-like kinase family member as it harbors three polo-boxes (PB1–PB3) whereas Plk1, Plk2 and Plk3 only have two polo-boxes, PB1 and PB2 (Slevin et al., 2012).

Few substrates of Plk4 have been described to date, including STIL (Ohta et al., 2014; Dzhindzhev et al., 2014; Kratz et al., 2015; Arquint et al., 2015; Moyer et al., 2015), GCP6 (also known as TUBGCP6) (Bahtz et al., 2012; Martin et al., 2014) and the F-box protein Fbxw5, a component of the SCF ubiquitin ligase (Puklowski et al., 2011). Cep152 and Cep192 collaborate to recruit Plk4 to centrioles (Cizmecioglu et al., 2010; Hatch et al., 2010; Dzhindzhev et al., 2010; Sonnen et al., 2013; Kim et al., 2013) in order to concentrate Plk4 at the centrioles and facilitate subsequent activation. The identification of additional binding partners of Plk4 will help to unravel the function and regulation of this key kinase in centriole duplication.

Here, we identify the centriolar protein Cep78 as a new binding partner of Plk4. Cep78 binds to the N-terminal catalytic domain of Plk4. We further find that Cep78 is required for Plk4-induced centriole overduplication and for re-duplication in aphidicolin-arrested U2OS cells. Upon depletion of Cep78 newly synthesized Cep78 does not localize to centrioles. Thus, Cep78 is a new player in the regulation of Plk4-induced centriole overduplication.

RESULTS AND DISCUSSION

Cep78 is a Plk4-interacting protein

To identify new proteins involved in centriole duplication we searched for Plk4-interacting proteins. A GST-tagged Plk4 C-terminal fragment (amino acids 581–971) was used as bait in pulldown experiments with U2OS cell lysates, followed by mass spectrometrical analysis of binding partners (Fig. S1A). Among already known Plk4-interacting proteins, we identified Cep78, a so far poorly described protein (Fig. S1B). Cep78 is conserved among vertebrates (Fig. S1C). To analyze the function of Cep78, we used a rabbit polyclonal anti-Cep78 antibody (targeting amino acid residues 639–689) of human Cep78 or an anti-Cep78 antibody generated against amino acid residues 709–722. In western blots the antibodies recognized a Cep78-specific band migrating at 78 kDa, which was absent upon Cep78 depletion with small interfering RNA (siRNA) (Fig. S2A,B). Cep78 has also been previously identified in a proteomic screen for centrosomal proteins in human cells (Andersen et al., 2003). In this study, we verified the specific centrosomal localization of Cep78 (Fig. S2C). To confirm the binding between full-length (FL) Cep78 and Plk4, we expressed GFP-tagged Cep78 together with Flag-tagged Plk4

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or N-terminal (1–580) and C-terminal (581–971) fragments of Plk4 in 293T cells and performed co-immunoprecipitation (Fig. 1A). In contrast to previously identified binding partners or substrates of Plk4, which predominantly interact with the polo-box domains PB1 and PB2 (Cizmecioglu et al., 2010; Hatch et al., 2010), we found that Cep78 binds with higher affinity to the N-terminal catalytic domain of Plk4 (Fig. 1A). The recently identified Plk4 substrate STIL also binds to regions of Plk4 that are situated outside of the Plk4 polo-box domain (Kratz et al., 2015; Arquint et al., 2015), suggesting different interaction platforms within Plk4. The observed interaction between Cep78 and Plk4 could further be confirmed in GST pulldown experiments using bacterially expressed GST–Cep78 to pull down Flag–Plk4 transfected in 293T cells (Fig. 1B). We could verify that recombinant GST–Cep78 binds to the full-length and the N-terminal fragments of Plk4 (1–580) (Fig. 1B). In our initial pulldown assay, Cep78 was identified by mass spectrometry with a C-terminal Plk4 fragment as bait. Our biochemical assays reveal that Cep78 interacts predominantly with the N-terminal domain of Plk4 and only weakly with the C-terminus. Given that the C-terminal Plk4 fragment heterodimerizes with full-length Plk4 (Klebba et al., 2015), it is conceivable that Cep78 was pulled out bound to the N-terminal part of full-length Plk4. We further

established the interaction by immunoprecipitation of endogenous Cep78 and Plk4 (Fig. 1C). In addition, the interaction between Cep78 and Plk4 was confirmed *in vitro* in GST pulldown experiments using recombinant GST-tagged Cep78 and MBP–Plk4, and a Zz pulldown using Zz–Plk4 and GST–Cep78 (Fig. 1D).

To explore the possibility that Cep78 is directly phosphorylated by Plk4, we carried out *in vitro* kinase assays. We used either GFP-tagged kinase-active and inactive Plk4 (KD, D154R) or active recombinant Zz-tagged and MBP-tagged Plk4 and incubated these with GST-tagged Cep78. Interestingly, we found that Cep78 is not phosphorylated by Plk4 (Fig. S3). Although Cep78 clearly bound to Plk4 it does not seem to act as an *in vitro* substrate. In summary, these results show that Cep78 is a new Plk4-interacting protein which predominantly binds to the N-terminal part of Plk4.

Cep78 is a centriolar protein and colocalizes with Plk4
We further asked whether Cep78 protein levels are regulated throughout the cell cycle. We found that Cep78 protein levels were low in mitosis but started to increase during late G1, coinciding with the initiation of centriole duplication. Cep78 levels then gradually increased until the S/G2 transition (Fig. 2A). We then analyzed the centrosomal localization of Cep78 co-staining with centrin-2, a
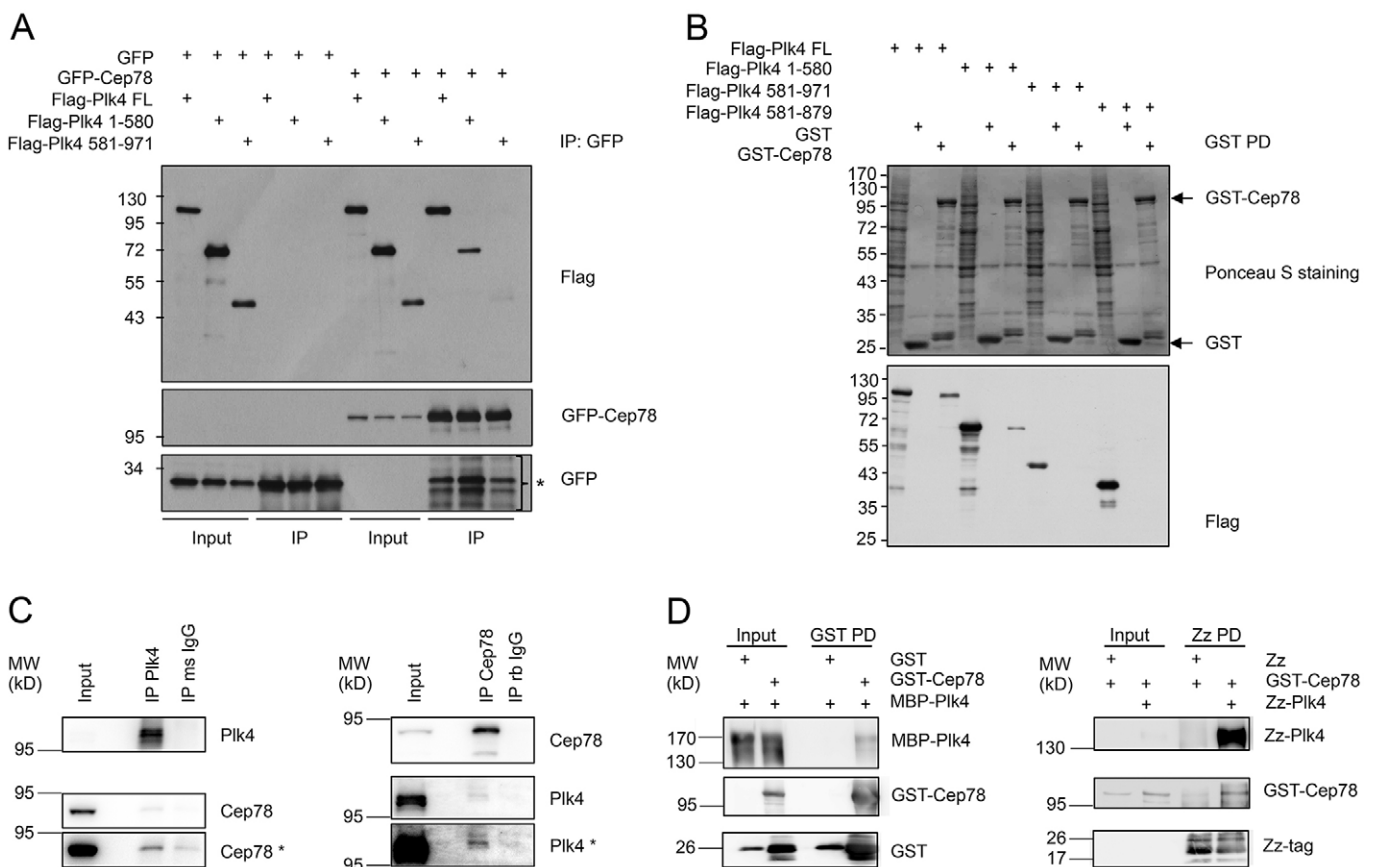

Fig. 1. Cep78 is a conserved protein and interacts with Plk4. (A) GFP–Cep78 and Flag–Plk4 or its fragments were co-transfected into 293T cells and immunoprecipitated with antibodies against the GFP tag. Co-immunoprecipitated (IP) proteins were detected by western blotting. The asterisk (*) marks degradation bands from GFP–Cep78. (B) Flag–Plk4 full-length (FL) or Flag-tagged truncated versions were transfected in 293T cells. Following GST pulldown (PD), the interaction was detected by western blotting using anti-Flag antibodies. (C) Co-immunoprecipitation of endogenous Plk4 and Cep78 from 293T cell lysates was detected by western blotting with rabbit anti-Cep78 (amino acids 709–722) or anti-Plk4 antibody, respectively. *, contrast-adjusted image. ms, mouse antibody; rb, rabbit anitibody. (D) *In vitro* interaction of Cep78 and Plk4 was determined in GST and Zz pulldown assays. MBP–Plk4 co-precipitating in the GST–Cep78 pulldown was detected using anti-Plk4 antibodies. GST–Cep78 was detected in the Zz–Plk4 pulldown using anti-GST antibodies. The Zz tag was detected using HRP-conjugated anti-rabbit-IgG antibodies.
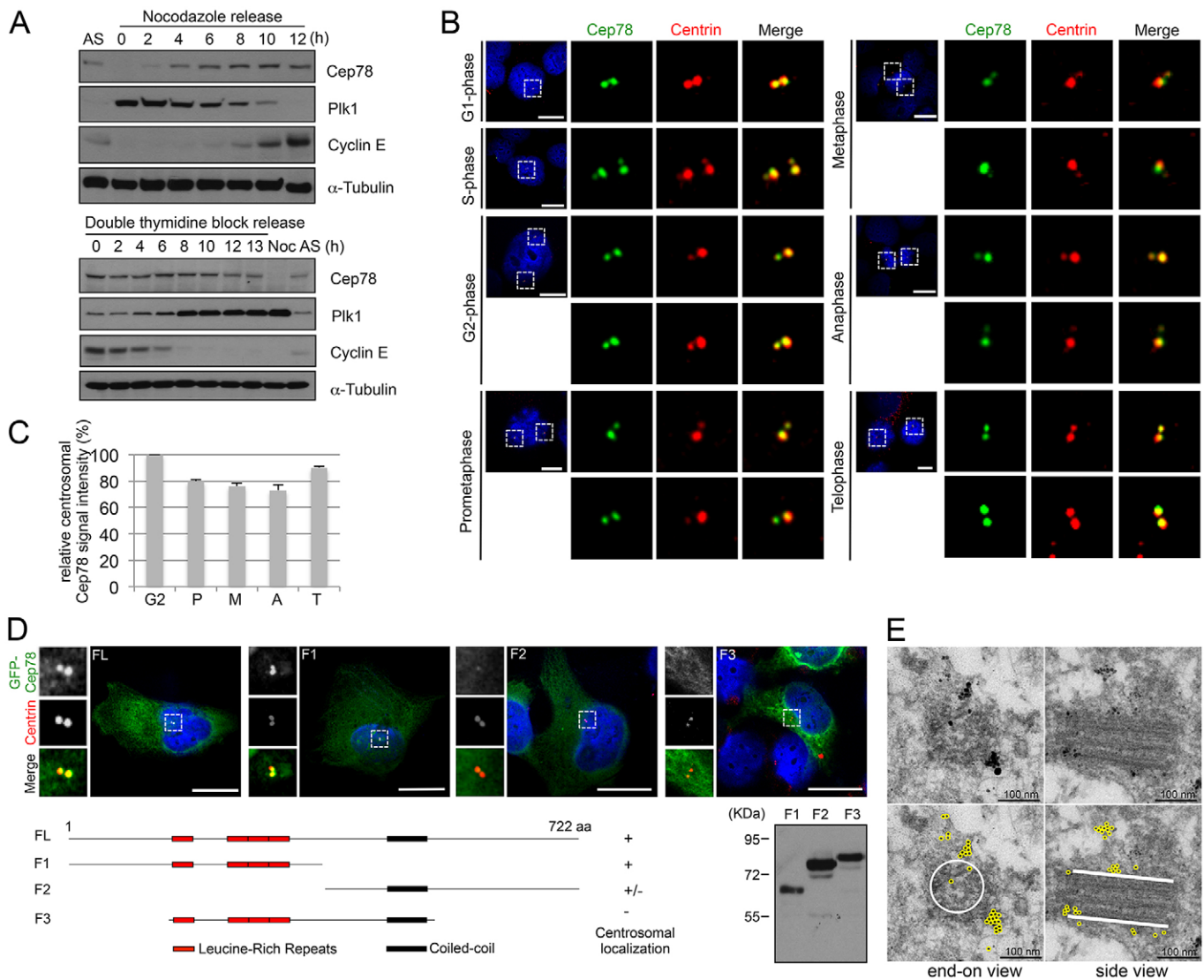
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Fig. 2. Cep78 is a component of the centriole and mainly expressed during interphase. (A) Upper panel, HeLa cells were released from nocodazole block. Lower panel, HeLa cells were released from double-thymidine block. Noc, nocodazole; AS, asynchronous. At the indicated time points, cells were collected and cell extracts were subjected to western blot analysis using the indicated antibodies. (B) Immunofluorescence images of HeLa cells in interphase and different phases of mitosis. Scale bars: 10 μm. (C) Quantification of the experiment shown in B. Results are mean±s.e.m. (n=100). (D) GFP–Cep78 FL and three different fragments (F1–F3) were transfected into U2OS cells and their centrosomal localization was analyzed by immunofluorescence. Scale bars: 10 μm. Below right: western blot showing the expression levels of Cep78 fragments. (E) Immunogold electron microscopy images of HeLa cells labeled with anti-Cep78 antibodies. Scale bars: 100 nm. Yellow circles highlight gold bead positions. White lines highlight the putative position of the centriole wall.

centriolar marker. We found that during all cell cycle phases, Cep78 localized to centrioles (Fig. 2B). However, the intensity of the Cep78 signal at centrioles was slightly reduced during prometaphase until anaphase (Fig. 2C). The reduced Cep78 signal at centrioles during early phases of mitosis was confirmed by the lower expression of Cep78 in extracts from metaphase-blocked cells in the western blot analysis (Fig. 2A). To determine which domain of Cep78 is required for its centrosomal localization, we generated truncated versions of Cep78 (F1–F3, Fig. 2D) and analyzed their centriolar localization. Whereas fragment F1, which includes the complete N-terminus and the two leucine-rich repeats, localized to the centrioles, we found that the centrosomal localization of the F2 fragment, which contains the coiled-coil domain and the C-terminus, was reduced. Interestingly, the F3 fragment, which lacks the N-terminal domain of Cep78, failed to localize to centrioles. These results suggest that the N-terminal domain of

Cep78 is required for its localization to centrioles. To obtain more detailed insights into the localization of Cep78, immunoelectron microscopy was applied. We found that Cep78 was mainly localized at the centriolar wall (Fig. 2E), whereas a subfraction was also found in the PCM cloud surrounding the centrioles.

Cep78 is involved in Plk4-induced centriole overduplication

We have established that Cep78 and Plk4 interact, and now analyzed whether Cep78 colocalized with Plk4. As shown in Fig. 3A, Cep78 partially associated with Plk4 on both centrioles. Furthermore, using a cold-treatment assay, we found that the localization of Cep78 to centrosomes appears to be independent of microtubules (Fig. S4A). As Plk4 is the key kinase regulating the centriole duplication process, we asked whether Cep78 has a role in centriole duplication. siRNA-mediated down-regulation of Cep78 did not lead to a loss of centrioles or the formation of
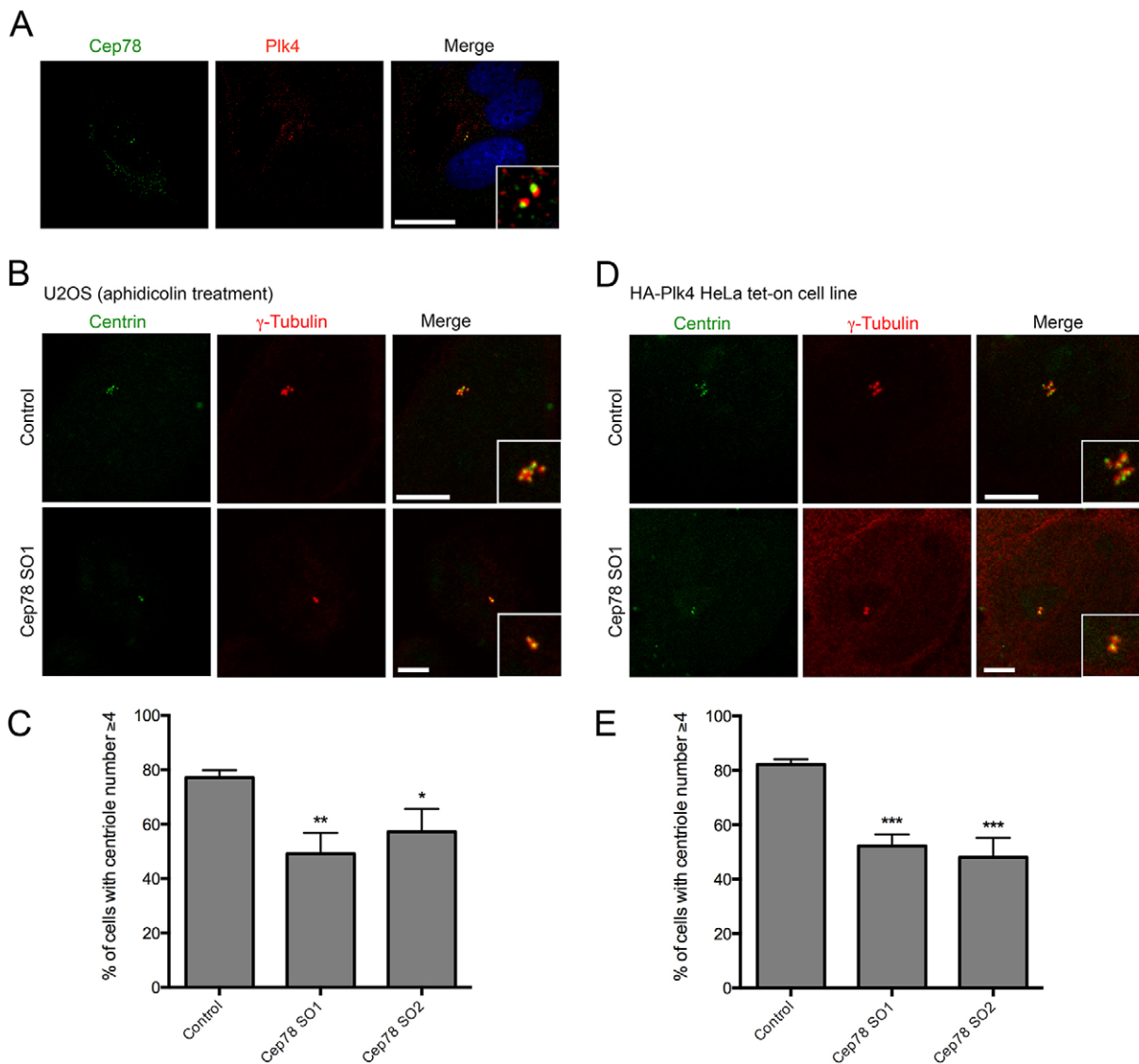
Fig. 3. Cep78 is required for Plk4-induced centriole overduplication. (A) Co-staining of U2OS cells with antibodies against Cep78 (green) and Plk4 (red). Scale bar: 10 µm. (B) U2OS cells were transfected with siRNAs targeting Cep78 (SO1, SO2), or control siRNA and arrested in S phase by aphidicolin treatment. After 70 h, cells were fixed and immunostained with the indicated antibodies. Scale bars: 10 µm. (C) Quantification of the experiments shown in B. (D) HeLa Tet on cells expressing HA–Plk4 were transfected with siRNAs against Cep78 or control siRNA for 72 h and Plk4 expression was induced in the last 24 h. Fixed cells were stained with the indicated antibodies. Scale bar, 10 µm. (E) Quantification of phenotype of the experiment shown in (D). Results in C and E are mean± s.e.m. (three individual experiments, $n{\geq}150$; error bars denote s.d.). *$P<0.05$, **$P<0.005$, ***$P<0.0003$ [one-way ANOVA test using GraphPad PRISM (GraphPad)].

monopolar spindles (data not shown) and Cep78 is therefore not involved in the canonical centriole duplication pathway. To assess whether Cep78 might play a role in centriole overduplication, a previously described centriole reduplication assay was performed (Balczon et al., 1995; Bobinnec et al., 1998; Meraldi et al., 1999). Cep78 expression was silenced in GFP–centrin-expressing U2OS cells by treatment with two different siRNA oligonucleotides. After siRNA transfections, U2OS cells were incubated with aphidicolin to induce centriole reduplication. As shown in Fig. 3B, nearly 80% of control siRNA-treated cells contained more than four centrioles. This phenotype was reduced from 78% in control cells down to a mean of 55% when Cep78 expression was inhibited (Fig. 3C).

Overexpression of Plk4 triggers the formation of multiple centrioles around one maternal template (Kleylein-Sohn et al., 2007). To analyze whether Cep78 is required for Plk4-induced

centriole overduplication, Cep78 was depleted in HeLa Tet-on cells that stably express HA-tagged Plk4. Fig. 3D shows that doxycycline-induced Plk4 expression stimulated the formation of multiple centrioles in cells treated with control siRNA. The quantification in Fig. 3E shows that 82% of the control cells exhibit $\geq$4 centrioles. Interfering with Cep78 expression resulted in a reduction of this overduplication phenotype to ∼50% of cells, indicating that Cep78 plays a role in the Plk4-induced centriole amplification process.

Localization of newly synthesized Plk4 to centrosomes depends on Cep78

We then analyzed the localization of Cep78 on overduplicated centrosomes following Flag–Plk4 overexpression in U2OS cells and found that Cep78 colocalized with centrin-2 and Plk4 on duplicated centrioles (Fig. 4A), suggesting that Cep78 is recruited to
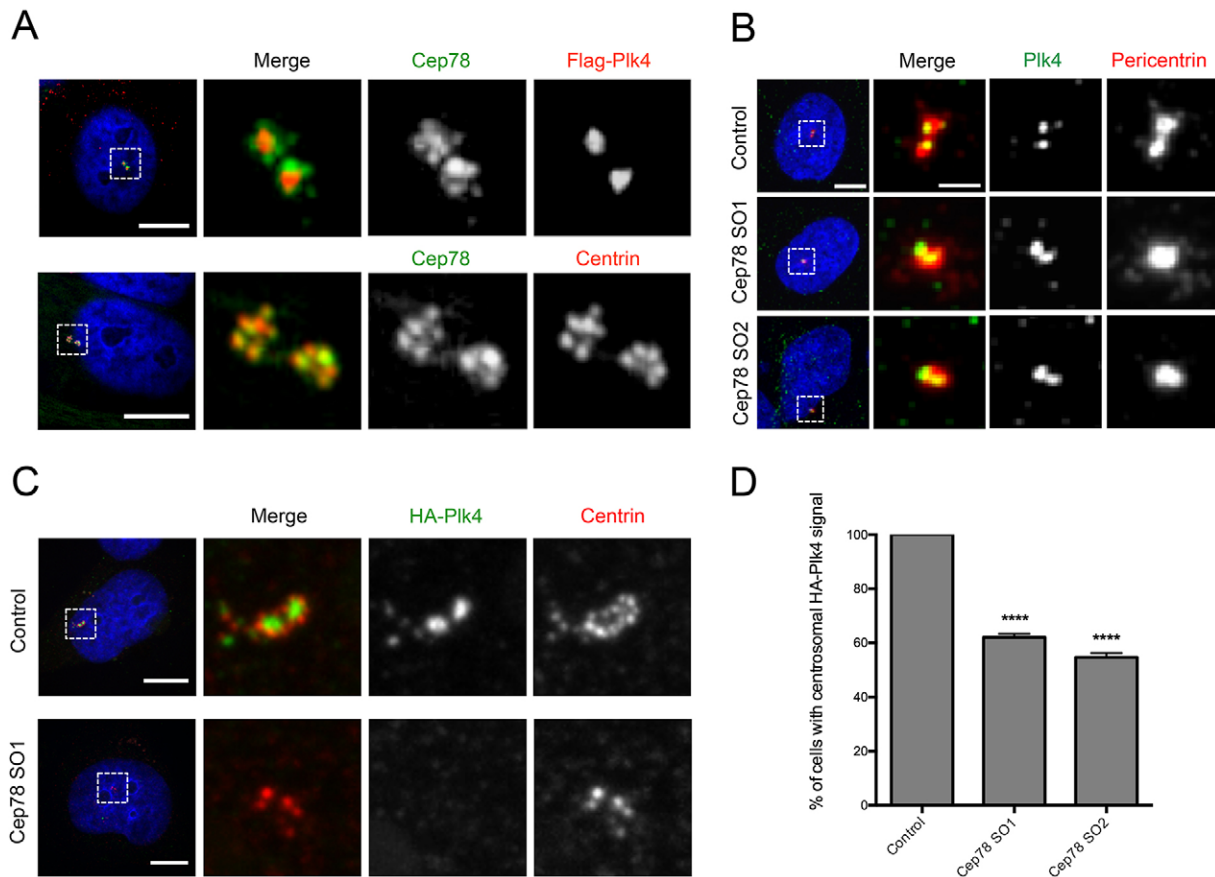
Fig. 4. Localization of newly synthesized Plk4 to the centrosome depends on Cep78. (A) Flag–Plk4 was overexpressed in asynchronous U2OS cells for 48 h and cells were stained with the indicated antibodies. (B) Immunofluorescence analysis of U2OS cells after depletion of Cep78 with two different siRNA oligonucleotides for 48 h. Data are representative of three individual experiments. Scale bars: 2 μm. (C) HeLa Tet-on cells expressing HA–Plk4 were transfected with siRNAs against Cep78 or control siRNA for 48 h and Plk4 was induced as described in Cizmecioglu et al., 2010. Fixed cells were arrested in S-phase by supplementing with 1.6 nM aphidicolin for the final 20 h of the experiment. Cells were immunostained for the indicated antibodies. Scale bars: 10 μm. (D) Quantification of centriolar HA–Plk4 localization from C (mean±s.e.m. from three individual experiments, $n{\geq}150$ each). ****$P<0.0001$ (two-tailed unpaired Student's t-test).

procentrioles in Plk4-overexpressing cells. To identify the role of Cep78 in Plk4-induced centriole overduplication, we first analyzed whether the centrosomal localization of Plk4 was dependent on Cep78 and vice versa. We found that the centrosomal localization of endogenous Plk4 was independent of Cep78 (Fig. 4B). Similarly, depletion of Plk4 did not alter the centrosomal localization of Cep78 (Fig. S4B). Given that Cep78 is involved in Plk4-induced centriole overduplication, we aimed to analyze whether Cep78 would regulate excessive centrosomal levels of Plk4 caused by ectopic expression of HA–Plk4. Upon analysis of the centrosomal HA signal, we found that, in the absence of Cep78, recruitment of HA–Plk4 to the centrosome was markedly impaired (Fig. 4C); ~40–50% of the cells could not localize HA–Plk4 to centrosomes (Fig. 4D). Interestingly, it has been shown that Cep78-siRNA-treated planaria exhibits cilia assembly defects in multicentriolar, multiciliated cells. Cep78 depletion in these cells, where Plk4 is strongly upregulated, results in a reduced number of centriole-based cillia basal bodies per cell (Azimzadeh et al., 2012), indicating that in this model organism Cep78 also appears to participate in the regulation of centriole amplification.

In conclusion, our work identifies Cep78 as a new Plk4-interacting protein that is involved in Plk4-induced centriole overduplication. The identification of Cep78 as a Plk4-associated factor provides a new tool for understanding the function of Plk4 in centriole overduplication.

MATERIALS AND METHODS

Antibodies

Anti-Cep78 rabbit polyclonal antibody against amino acids 639–689 was from Bethyl Laboratories (A301-800A) and was used at 1:500. Rabbit polyclonal anti-Cep78 antibodies were also raised against a Cep78 peptide sequence and was used at 1 μg/ml (amino acids 709-722, this antibody was only used for experiments described in Fig. 1C). Mouse anti-Plk4 antibody (used at 1 μg/ml) was as previously described (Cizmecioglu et al., 2010). Rabbit anti-centrin-2 (N-17-R, 1:100) was from Santa Cruz Biotechnology. Mouse anti-α-tubulin (T5168, 1:5000), rabbit anti-γ-tubulin (T3559, 1:2000) and mouse anti-FLAG M2 (F3165, 1:1000) antibodies were from Sigma-Aldrich. Rabbit anti-GFP (NB600-303, 1:5000) antibody was from Novus Biologicals.

Cell synchronization and transfection

To arrest cells in mitosis, they were treated with 100 ng/ml nocodazole for 16 h and released by subsequent replacement with medium. For the double-thymidine block, cells were treated with 4 mM thymidine for 20 h, then released for 12 h and again blocked for 16 h. HeLa Tet-on cells stably expressing HA–Plk4 under a doxycycline-inducible promoter and centriole reduplication assays in U2OS cells were used as described previously (Cizmecioglu et al., 2010).

For siRNA and plasmid DNA transfections, Lipofectamine 2000 (Invitrogen) was used. The following siRNA sequences were used: Cep78 SO1, 636–654, 5′-GGCUGAGAGUCUUCGCUAUtt-3′; Cep78 SO2, 858–876, 5′-GGUCGUUCUGGAUAUAAGAtt-3′; firefly luciferase (GL2, control siRNA), 5′-CGUACGCGGAAUACUUCGAtt-3′. Plk4 siRNA

sequences were as described in Cizmecioglu et al., 2010. siRNA oligonucleotides were purchased from Ambion.

Immunofluorescence microscopy

For immunofluorescence, cells grown on coverslips were fixed with −20°C cold methanol for 10 min at −20°C. Cells were washed once with PBS and blocked for 30 min with 3% BSA in PBS. Cells were incubated with primary antibodies for 1 h. Alexa-Fluor-488-, -594- or -647-conjugated secondary antibodies (Invitrogen) were incubated with the fixed cells for 30 min. DNA was stained with Hoechst 33258 (Molecular Probes) for 10 min.

Images were acquired using a PerkinElmer Ultra-View spinning disc confocal on a Nikon Ti inverted microscope equipped with a 1006 NA 1.0 oil immersion objective and an electron multiplying charge-coupled device camera (Hamamatsu Photonics) or a Zeiss motorized inverted Observer.Z1 microscope, containing mercury arc burner HXP 120 C and LED module Colibri. Images were processed with NIH ImageJ and FIJI.

Mass spectrometry analysis

For identification of Plk4-interacting proteins, GST–Plk4 C-terminal elution fractions were resolved by SDS-PAGE and stained with Colloidal Coomassie. Mass spectrometry analysis was performed at the ZMBH Protein Analysis Facility (ZMBH, University of Heidelberg).

Database searches were performed against the SwissProt database with taxonomy 'human' using the MASCOT search engine (Matrix Science, version 2.2.2).

Supplementary information
Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.184093.supplemental

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