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Research Article

Identification of a novel centrosomal protein Crp^{F46} involved in cell cycle progression and mitosis

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ABSTRACT

A novel centrosome-related protein Crp^{F46} was detected using a serum F46 from a patient suffering from progressive systemic sclerosis. We identified the protein by immunoprecipitation and Western blotting followed by tandem mass spectrometry sequencing. The protein Crp^{F46} has an apparent molecular mass of ~60 kDa, is highly homologous to a 527 amino acid sequence of the C-terminal portion of the protein Golgin-245, and appears to be a splice variant of Golgin-245. Immunofluorescence microscopy of synchronized HeLa cells labeled with an anti-Crp^{F46} monoclonal antibody revealed that Crp^{F46} localized exclusively to the centrosome during interphase, although it dispersed throughout the cytoplasm at the onset of mitosis. Domain analysis using *Crp^{F46}* fragments in GFP-expression vectors transformed into HeLa cells revealed that centrosomal targeting is conferred by a C-terminal coiled-coil domain. Antisense *Crp^{F46}* knockdown inhibited cell growth and proliferation and the cell cycle typically stalled at S phase. The knockdown also resulted in the formation of poly-centrosomal and multinucleate cells, which finally became apoptotic. These results suggest that Crp^{F46} is a novel centrosome-related protein that associates with the centrosome in a cell cycle-dependent manner and is involved in the progression of the cell cycle and M phase mechanism.

Introduction

The centrosome is a multifunctional organelle best known for its function as a major microtubule-organizing center in metazoan cells [1–3]. During interphase the centrosome nucleates a radial array of microtubules of uniform polarity, which extend to the cell periphery and drive the cellular distribution of cytoplasmic organelles. The centrosome normally replicates during the S phase, forming two daughter centrosomes that migrate to the opposite poles of the cell where they direct the assembly of a bipolar mitotic spindle and play an important role in the organization and orchestration of mitosis. At cytokinesis, each daughter cell inherits one centrosome. Thus, at any time during interphase, a normal cell contains either one centrosome (G1/G0 phase) or two duplicated centrosomes (late S or G2 phase).

The centrosome consists of a pair of cylindrical centrioles surrounded by a matrix of protein aggregates (pericentriolar

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material, PCM). The two centrioles act as basal bodies for the assembly of cilia and flagella [4]. The PCM harbors hundreds of proteins that perform diverse functions, including the integration of signaling pathways and regulation of cellular processes in addition to its well established role as the primary microtubule-organizing center [3,5,6]. Among the characterized centrosomal proteins are γ-tubulin complexes and other cytoskeletal regulators including microtubule-associated proteins, motor proteins, cell-cycle regulators, checkpoint proteins, kinases and phosphatases, calcium-binding proteins, signaling proteins, structural/scaffold proteins, ubiquitination and protein degradation, and many other proteins whose function remains to be determined [7,8].

The replication of centrosomes is strictly controlled by a molecular mechanism that "licenses" centrosome duplication and "blocks" re-duplication [9]. When a cell loses control of centrosome duplication, centrosome amplification occurs, leading to the formation of aberrant mitotic spindles, multipolarity, and chromosome missegregation during mitosis [10–13]. Aberrant amplification of centrosomes can be induced by mutations in various cell-cycle regulatory proteins [14–17], loss of tumor suppressor proteins and other pathological changes [18–21]. The exact molecular mechanism controlling centrosome replication is unclear.

Antibodies present in the sera of patients with autoimmune diseases, such as systemic lupus erythematosus and scleroderma polymyositis, have become a valuable and important resource for identifying new cellular constituents [22–24]. These include centromere (kinetochore)-related proteins [25,26], SCL-70/topoisomerase I [27], RNA polymerase I [28] and proteins associated with uridine-rich small nuclear RNAs (U RNAs) [29]. Advances in proteomics have dramatically accelerated the process of identifying new proteins. Because the sequencing of some proteins has been complicated by the presence of blocked amino termini, tandem mass spectrometry (MS/MS) has become a powerful tool of direct peptide sequencing for identification and comparison of related proteins [30–33]. In the present study, we used autoimmune serum F46 from a patient suffering from progressive systemic sclerosis to immunoprecipitate the target protein from HeLa cell lysate. MS/MS sequencing [34,35] and deletion fragment analysis showed that it is a novel protein with predicted molecular mass of 62.6 kDa, formed as a splice variant of the C-terminal portion of Golgin-245. Immunofluorescence labeling of synchronized HeLa cells revealed that the protein localizes exclusively to the centrosome during interphase but disappears at the onset of mitosis. We named the protein "centrosome-related protein F46" (Crp^{F46}). Depletion of Crp^{F46} by antisense RNA caused the formation of poly-centrosomal and multinucleate cells, inhibition of cell proliferation, and arrest at the S phase. These results suggest that Crp^{F46} may be a critical factor in cell-cycle progression and M phase regulation.

Materials and methods

Cell culture, reagents, and antibodies

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) containing 10% (v/v) fetal calf serum (FCS) (Sanli Biotechnology, Wuhan, China) at 37 °C in the presence of 5% CO_2. Among the reagents used in this study were the reagent G418 (Merck, USA), rabbit anti-goat and goat anti-mouse IgG conjugates with FITC, and TRITC-conjugated goat anti-rabbit IgG (both from Vector Laboratories, Peterborough, UK). The polyclonal antibody against γ-tubulin was from Santa Cruz Biotechnology, USA, and an anti-PLK1 antibody from Cell Signaling Technology, USA. Oligonucleotides were synthesized by Sangon (Shanghai Sangon Biotechnology, Shanghai, China). Autoantiserum F46 was from a patient with progressive systemic sclerosis (Peking Union Medical Hospital, China).

Immunoprecipitation of Crp^{F46} protein and sequencing

Total protein extracts of HeLa cell were incubated with F46 serum (1:100 dilution) at 4 °C overnight and then incubated with Protein A plus-Sepharose (Dingguo Biotechnology, Beijing, China). The beads were washed three times with phosphate-buffered saline (PBS) containing 0.1% Triton X-100, and bound proteins were eluted by heating at 100 °C for 5 min. The eluted proteins were separated by SDS-PAGE using 12% gels. The polypeptide band of interest was excised and digested *in situ* with trypsin, and the resulting peptides were submitted for protein sequencing by tandem mass spectrometry (MS/MS) [36]. The protein sequence of Crp^{F46} was used to search for homologous proteins in the NCBI/EMBL protein database using BLASTP.

Cloning of Crp^{F46} cDNA

The cDNAs were synthesized by RT-PCR using HeLa total RNA prepared by extraction with Trizol reagent (Invitrogen, USA). The RT-PCR was conducted in single (one-step) reactions by using M-MLV retro-transcriptional enzyme (Promega, USA). Primers for gradient PCR were synthesized according to the Crp^{F46} peptide sequence: 5′-GGAAAGTTCACAGTCAGAAACA-3′ and 5′-AGATCGGAGCCATGACATCA-3′. The PCR product was inserted into TA cloning vector (TaKaRa, Japan) to construct a recombinant plasmid pMD18-T-Crp^{F46}.

Co-immunoprecipitation of Crp^{F46}, γ-tubulin, and Plk1

Cells were washed in PBS and lysed in HEPES buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA) containing protease inhibitors and 0.5% Triton X-100 for 5 min on ice. After centrifugation for 10 min at 16,000 g at 4 °C, cleared lysates were obtained. For immunoprecipitation, extracts were incubated with antibodies against Crp^{F46}, γ-tubulin, PLK1, or Golgin-245 overnight at 4 °C. Subsequently, Protein A plus-Sepharose was added and the mixture incubated for 3–4 h at 4 °C. After centrifuging for 30 s at 4 °C, the pellets were washed five times with 500 μl of cell-lysis buffer while keeping on ice. Washed pellets of protein complexes were resuspended in 20 μl SDS sample buffer, vortexed, boiled, centrifuged for 30 s, and analyzed by SDS-PAGE and Western blotting.

Preparation of monoclonal antibody against Crp^{F46}

The coding sequence of Crp^{F46} was amplified by PCR using the pMD18-T-Crp^{F46} plasmid as a template. The primers were

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5′-ACGCGGATCCATGGAAAGTTCACAGTCAG-3′ and 5′-CCGGAATTCTCAAGATGAAGATCGG-3′. The PCR product was then cleaved with BamHI/EcoRI enzymes and inserted into corresponding restriction sites in pGEX-2Z (constructed by pGEX-2T at the multiple-cloning site). Recombinant protein was expressed in BL21(DE3) cells induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and the purified protein was submitted for the preparation of monoclonal antibody (Welson Biotechnology, Beijing, China).

Construction of recombinant pEGFP expression vectors with Crp^{F46} fragments

Deletion fragments of *Crp*^{F46} sequence corresponding to amino acid residues 1–241, 127–426, and 323–530, each containing one of the three coiled-coil domains, were prepared by PCR amplification of the *Crp*^{F46} cDNA using the following pairs of primer sequences: 5′-GGGAAGCTTGAAAGTTCACAGTCAGAAAC-3′ and 5′-ACGCGGATCCTCCAAGCAGCAG-3′; 5′-CCCAAGCTTGAAAGTTCACAGTC-3′ and 5′-CGCGGATCCGGCTAATTTAA-3′; 5′-CCCAAGCTTAAGCAAAACTTGG-3′ and 5′-CGCGGATCCCTTGCTCTTTC-3′; 5′-CCCAAGCTTCAGGAGGTGGAG-3′ and 5′-CGCGGATCCTCAAGATGAAGAT-3′.

The PCR products were cleaved with HindIII/BamHI enzymes and inserted into corresponding restriction sites in the expression vector pEGFP-C3 (gift from Dr. Wanjie Li, Beijing Normal University, China). The recombinant vectors were transiently transfected into HeLa cells by using high-efficiency transfection reagent Vigofect according to the manufacturer's instruction (Vigoruse, Beijing, China). Forty eight hours after transfection, cells expressing GFP fusion protein were counter-stained with γ-tubulin antibody (diluted 1:30) followed by and TRITC-conjugated goat anti-rabbit IgG antibody (diluted 1:50), and examined with Olympus laser-scanning confocal microscope (Olympus Fluoview FV300, Japan).

Construction of antisense Crp^{F46} RNA and antisense golgin-245 RNA expression vectors and stable transformation of HeLa cells

A pXJ41 antisense-*Crp*^{F46} vector was constructed by PCR amplification of the entire coding sequence of *Crp*^{F46} using pMD18-T-*Crp*^{F46} as a template. The PCR product was ligated in reverse orientation into HindIII/EcoRI sites of pXJ41-neo vector (gift from Dr. Xiaoyu Zhu, Beijing Normal University, China), and the fidelity of ligation was confirmed by sequencing using the primers 5′-GGGAAGCTTGGAAAGTTCACAGTCAG-3′ and 5′-CCGGAATTCGTAGATGAAGATCGG-3′.

A pcDNA3.1+ antisense *golgin-245* vector was constructed by PCR amplification of the N-terminus sequence of *golgin-245*, using pMD18-T-N-terminus of Golgin-245 as a template. Primers for gradient PCR were synthesized according to the 320–1627 bp region of *golgin-245*: 5′-ATGGCACAGGCTAACTCAG-3′ and 5′-TTTATTGGACCAGTCATCTACC-3′. The PCR product was ligated in reverse orientation into BamHI/HindIII sites of pCDNA3.1+ vector (gift from Dr. Wanjie Li, Beijing Normal University, China), and the fidelity of ligation was confirmed by sequencing using the primers 5′-CGGGATCCGGCTAACTCAGC-3′ and 5′-CCCAAGCTTGGACCAGTCATCTA-3′.

The recombinant DNA construct pXJ41 antisense-*Crp*^{F46} and an empty vector pXJ41-neo, and similarly the pCDNA3.1+ antisense *golgin-245* vector and the empty vector, were transfected into HeLa cells by using the transfection reagent Vigofect. Expression of the antisense molecule is driven by the cytomegalovirus promoter of the vector transformed cells were selected by culturing in the presence of G418 reagent (60 ng/ml) for 14 days, and transgenic cell lines that were stably silencing Crp^{F46} were established. These cells were then either fixed for immunostaining or processed for protein extraction and immunoblot analysis.

Cell growth curves

Stably transformed antisense RNA cells and control cells were seeded in 96-well plates at a density of 1×10^4 cells per well in DMEM containing 10% FCS, and grown at 37 °C in the presence of 5% CO_2. Three wells of cells from each cell line were harvested everyday for up to 7 days. A liter stock solution of 5 mg/ml MTT reagent (Sigma, USA) in PBS was then diluted 1:10 in DMEM without FCS, and 100 μl of this solution were added to each well after aspirating the original media. Following 4 h incubation at 37 °C, the cells were lysed with 100 μl DMSO and incubated for a further 10 min at 37 °C with gentle shaking. Absorbance at 540 nm was determined using a microplate analyzer (BIO-RAD Laboratories, USA).

Cell synchronization

HeLa cells in exponential growth phase were cultured in DMEM with 10% FCS and 2 mM TdR for 16 h. The cells were then washed with fresh DMEM containing 10% FCS, and allowed to grow for an additional 9 h. At this time, 2 mM TdR was again added, and the cells were incubated for another 16 h. The cells were then washed again with a fresh medium, and allowed to grow for defined intervals as follows: 0 h in order to collect cells arrested in G1/S phase, 5 h for arrest in S phase, and 9 h for arrest in G2 phase. For M phase, the cells were cultured for 15 h in DMEM containing 10% FCS and 200 ng/ml nocodazole (Sigma, USA).

Flow cytometry

Cells were trypsinized, washed three times with cold PBS, and then fixed with 70% ethanol at 4 °C overnight. Following digestion with RNase for 30 min at 37 °C, propidium iodide (PI) (Sigma, USA) was added to a final concentration of 65 μg/ml. Flow cytometric analysis was performed on a FACS Calibur (BD Biosciences, USA).

Western blot analysis

Total protein extracts of HeLa cells or *E. coli* cells expressing GST-Crp^{F46} fusion protein were loaded onto 12% gels, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane for 2 h at 200 mA. The membrane with transferred polypeptides was immersed in 5% skim milk in PBST (PBS containing 0.1% Tween 20) at room temperature for 1 h. After two rinses with PBST, the proteins were probed with F46 serum (1:2000) or anti-Crp^{F46} monoclonal antibody (1:500) at

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room temperature for 1 h. The membrane was then incubated with alkaline phosphatase-conjugated horse anti-mouse IgG (1:1000) and the immunoreactions detected with nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Promega, USA).

Immunostaining of cells

HeLa cells grown on coverslips were washed with PBS and fixed either with methanol at −20 °C for 8 min or with 3.7% paraformaldehyde at room temperature for 10 min, followed by permeabilization with 0.25% Triton X-100 at room temperature for 10 min. After washing three times with PBS and blocking for 30 min with 5% skim milk in PBS, the coverslips with cells were incubated with primary antibodies for 1 h at room temperature. The primary antibodies were as follows: a mouse anti-Crp^{F46} monoclonal antibody (1:100), rabbit anti-γ-tubulin polyclonal antibody (1:100) (Santa Cruz Biotechnology, USA), and goat anti-α-tubulin polyclonal antibody (1:100) (Santa Cruz Biotechnology, USA).The cells were then washed three times for 10 min each in PBST, and incubated for 1 h at 37 °C with the secondary antibodies. The secondary antibodies used were FITC-conjugated goat anti-mouse IgG (1:100), TRITC-conjugated goat anti-rabbit IgG (1:100), or FITC homo anti-goat IgG (1:100) (all three antibodies were from Vector Laboratories, USA). The cells were then washed three times with PBST, counter-stained with propidium iodide (PI) or diamidino-2-phenylindole (DAPI) (Sigma, USA), and mounted in antifade mounting solution. The preparations were observed with Olympus confocal laser-scanning microscope (Olympus Fluoview FV300, Japan).

Results

Identification and characterization of centrosome-related protein Crp^{F46}

In a preliminary experiment, we used a serum F46 from a patient suffering from progressive systemic sclerosis to immunolabel HeLa cells. The immunoreaction showed that the target protein localized as one or two dots adjacent to the nucleus in interphase cells. This localization was similar to the images shown in the detailed immunofluorescence characterization below. In order to determine the size of the target protein, total proteins were extracted from HeLa cells separated by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with the F46 serum. The immunoreaction revealed a distinct band of apparent molecular mass (M_r) of ~60 kDa (Fig. 1). Thus the F46 serum recognizes a unique protein that appears to localize to the centrosome.

The identity of the protein was determined by immunoprecipitation with the serum F46 followed by SDS-PAGE, isolation of the polypeptide band, digestion with trypsin, and protein sequencing by tandem mass spectrometry (MS/MS) (Supplement 1). Analysis of the peptide sequences using BLASTp showed that the polypeptide is identical to a 527 amino acids sequence of the C-terminal portion of Golgin-245 (Fig. 2). The theoretical p*I* of Crp^{F46} is 5.82, with predicted molecular mass of 62.6 kDa (predicted by PeptideMass) [37]. We named the protein

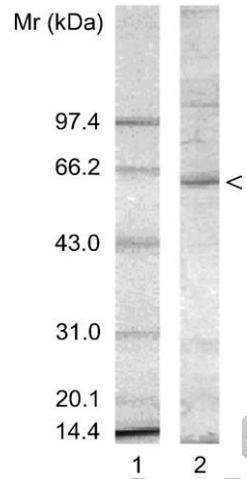

Fig. 1 – Western blot analysis of HeLa proteins using F46 serum. Total HeLa cell proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with F46 serum. The immunoblot (lane 2) reveals a distinct band of M_r ~60 kDa (arrow).

"centrosome-related protein F46" (Crp^{F46}). Analysis of the Crp^{F46} sequence using SMART (simple modular architecture research tool; smart.embl-heidelberg.de) [38,39] revealed three coiled-coil domains at amino acid positions 33–192, 218–399 and 420–454 (Fig. 2). The presence of coiled-coil domains is known to be a characteristic feature of centrosomal proteins [40]. In addition, a search using the PROSITE database [41] revealed twelve potential casein kinase II phosphorylation sites, seven protein kinase C phosphorylation sites, a cAMP-/cGMP-dependent protein kinase phosphorylation site, a tyrosine kinase phosphorylation site, three *N*-glycosylation sites, and an *N*-myristoylation site (Table 1). Multiple phosphorylation sites are also typical for centrosomal proteins [42], while the *N*-myristoylation site indicates interaction with membranes, presumably reflecting a relation to Golgin [43].

The presence of Crp^{F46} in HeLa cells

The polypeptide recognized by the F46 serum on immunoblots is only ~60 kDa, while the homologous Golgin-245 has a molecular mass of 245 kDa. Therefore it was necessary to confirm that a recombinant Crp^{F46}, derived from a cDNA obtained on the basis of the MS/MS sequencing, does correspond to the endogenous Crp^{F46} and is recognized by the F46 serum. We designed primers according to the first and last polypeptides of the MS/MS result (Fig. 2) and used RT-PCR and HeLa total RNA as a template to obtain *Crp^{F46}* cDNA. The cDNA was then used to construct a *GST-Crp^{F46}* chimeric gene, and GST-Crp^{F46} fusion protein was expressed using pGEX-2Z-*Crp^{F46}* expression vector transformed into *Escherichia coli*. The recombinant proteins was purified and submitted for preparation of monoclonal antibodies. Immunoblots with endogenous proteins extracted from HeLa cells showed that both anti-Crp^{F46} monoclonal antibody and the F46 serum gave positive immunoreactions at the expected locations (Fig. 3). Furthermore, both antibodies also gave positive immunoreactions with the recombinant protein. Therefore, the cloned Crp^{F46}

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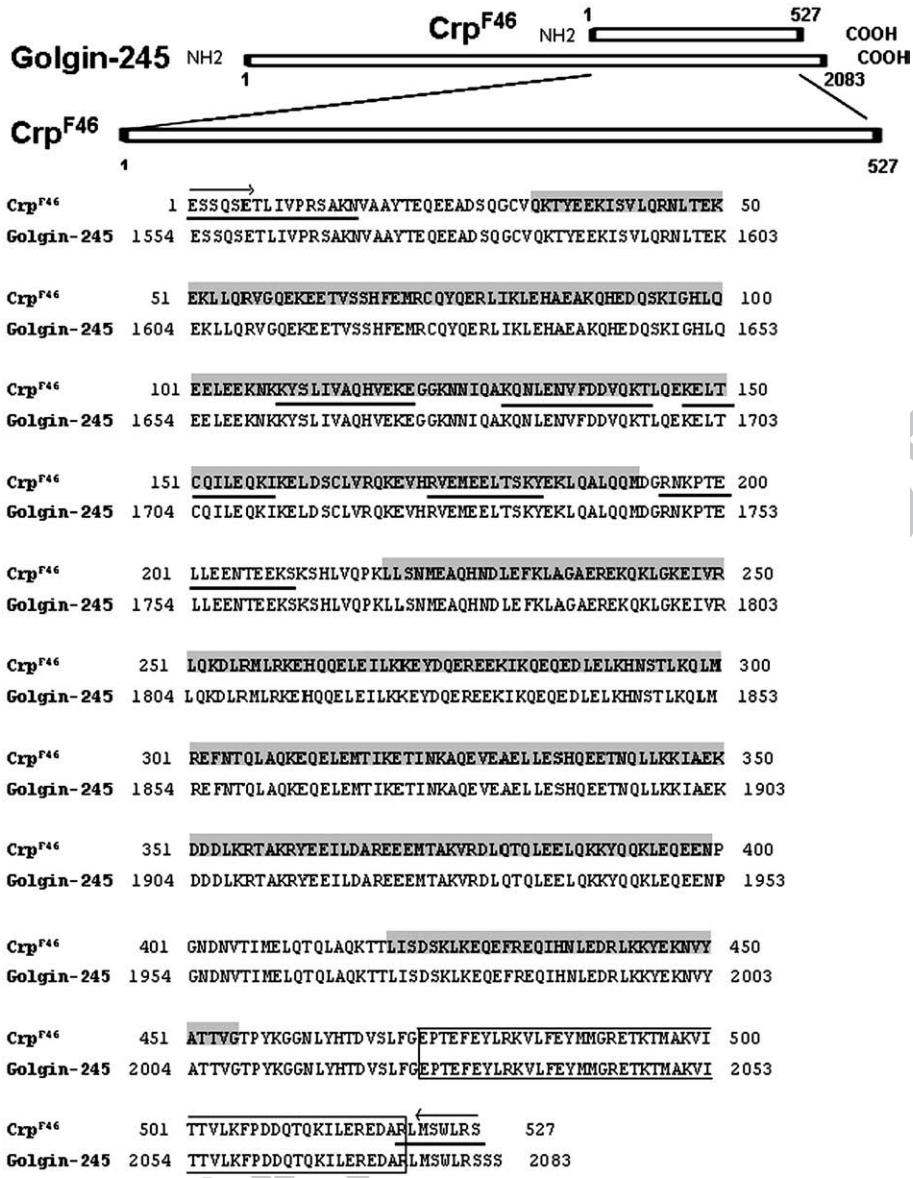

Fig. 2 – Crp^{F46} is highly homologous to a 527 amino acids sequence of the C-terminal domain of Golgin-245. Amino acid sequences were obtained by MS/MS of a protein immunoprecipitated with F46 serum, and aligned with Golgin-245 sequence using BLASTN. SMART analysis identified three coiled-coil domains (shaded) at specified amino acid positions. Amino acid sequences of peptides identified MS/MS are underlined. Amino acids corresponding to GRIP domain are boxed. Arrows indicate the starting positions of segments used for the design of forward and reverse degenerate primers.

does indeed correspond to the endogenous Crp^{F46} protein originally detected by the F46 serum.

Crp^{F46} is a splice variant of Golgin-245

In order to determine if Crp^{F46} is a splice variant of Golgin-245 or encoded by a distinct gene, we constructed a pcDNA3.1+ antisense *golgin-245* vector. This vector targets the nucleotides 320–1627 bp of the N-terminus of *golgin-245*, which is outside the *Crp^{F46}* domain. The antisense vector, or an empty pcDNA3.1+-*neo* vector as a control, were transfected into HeLa cells, and clonal cell lines stably expressing the respective vectors were established using geneticin (G418) selection. Western blot analysis of protein extracts from the two clonal cell lines showed that the antisense *golgin-245* knocked down the expression of Golgin-245 and also the expression of Crp^{F46} (Fig. 4). In contrast, transformation with an antisense *Crp^{F46}* RNA vector showed that only the CrpF46 was depleted but Golgin-245 was still present. We therefore conclude that Crp^{F46} is the product of splicing of *golgin-245*, and not encoded by a distinct gene.

It should be noted that the 527 amino acid sequence at the C-terminus of Golgin-245 that correspond to Crp^{F46} includes a GRIP domain (residues 2026–2070; Fig. 2), which is necessary, though not sufficient, for localization to the Golgi apparatus [44,45]. Unfortunately our MS/MS sequencing did not cover the site of the potential GRIP domain and therefore we have no direct evidence that this domain is either present or absent in the Crp^{F46}. However, our immunostaining assays (Figs. 5, 6, 8,

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Table 1 – Putative protein modification sites identified by searching the PROSITE database

Putative modification site	Amino acid positions	Amino acid motif
Casein kinase II phosphorylation	3–6	SqsE
	21–24	TeqE
	35–38	TyeE
	48–51	TekE
	67–70	ShfE
	143–146	TlqE
	181–184	SkyE
	221–224	SnmE
	317–320	TikE
	335–338	ShqE
	381–384	TqlE
	406–409	TimE
Protein kinase C phosphorylation	13–15	SaK
	48–50	TeK
	180–182	TsK
	295–297	TlK
	317–319	TiK
	357–359	TaK
	373–375	TaK
cAMP- and cGMP-dependent protein kinase phosphorylation	108–111	KKyS
Tyrosine kinase Phosphorylation	443–450	Kky.EknvY
N-glycosylation	46–49	NLTE
	293–296	NSTL
	404–407	NVTI
N-myristoylation	122–127	GGknNI

and 10) consistently showed centrosomal, not Golgi, localization throughout interphase. Further exploration will be required to determine the mechanism of Crp^{F46} targeting to the centrosome.

Cell-cycle-dependent localization of Crp^{F46} to the centrosome in synchronized HeLa cells

To determine the subcellular localization and dynamic distribution of Crp^{F46} during the cell cycle, we synchronized HeLa cells and processed them for immunofluorescence microscopy using the anti-Crp^{F46} monoclonal antibody. During interphase, the immunoreactive material consistently localized to one or two bright dots in the cytoplasm adjacent to the nucleus (Figs. 5A,B). In many cells at G1/S phase, the single bright dot appeared to be dividing into two dots, which were initially interconnected but eventually became completely separated. Once the nucleus entered mitosis, however, the two bright dots of Crp^{F46} were replaced by a weaker, though distinct, punctuate signal throughout the cytoplasm. This indicates that the immunoreactive material dissociates from the centrosomes and becomes fragmented and scattered through the cytoplasm.

The only known cellular structure that behaves similarly to the Crp^{F46} during interphase is the centrosome, which typically shows one- or two-dot pattern in the perinuclear cytoplasm. To test the relation of the Crp^{F46} to the centrosome, we performed double immunolabeling using the anti-Crp^{F46} monoclonal antibody and anti-γ-tubulin polyclonal antibody in order to locate the centrosome. Throughout interphase, the Crp^{F46} was indeed found to co-localize with γ-tubulin and hence the centrosomes (Fig. 5C). Cells at G1 showed a single Crp^{F46} dot, typical of centrosomes during G1 and early S phases. A pair of closely connected dots appeared during S phase, and two separate dots became clearly visible during late S and G2 phases. At the onset of mitosis, when the two centrosomes moved to the opposite poles of the cell, the bright, compact Crp^{F46} dots began to disappear. A weaker but distinct punctuate fluorescent signal again became scattered throughout the cytoplasm during mitosis, without any specific co-localization with the pair of centrosomes marked with the γ-tubulin label. Nevertheless, following cytokinesis the punctate cytoplasmic staining disappeared and a distinct, compact Crp^{F46} dot again re-appeared together with the single centrosome in each of the two daughter cells, suggesting re-association of the punctate signal with the centrosome. Thus the Crp^{F46} accompanies the centrosome during its typical duplication and subcellular localization during interphase, although it disperses during mitosis and re-appears again during the G1 phase of the next cell cycle as a compact dot accompanying the single centrosomes in each daughter cell.

Crp^{F46} deletion fragment (aa 323–530) containing coiled-coil III confers localization to the centrosome

As the first step towards elucidating the mechanism of targeting the Crp^{F46} to the centrosome, we used PCR and specific primers together with *Crp^{F46}* cDNA as a template to prepare three deletion fragments corresponding to the regions of the three coiled-coil domains (Fig. 6A). The fragments were ligated into the expression vector pEGFP-C3 and the recombinant vector transiently transfected into HeLa cells. Cells expressing GFP fusion protein were counter-stained with

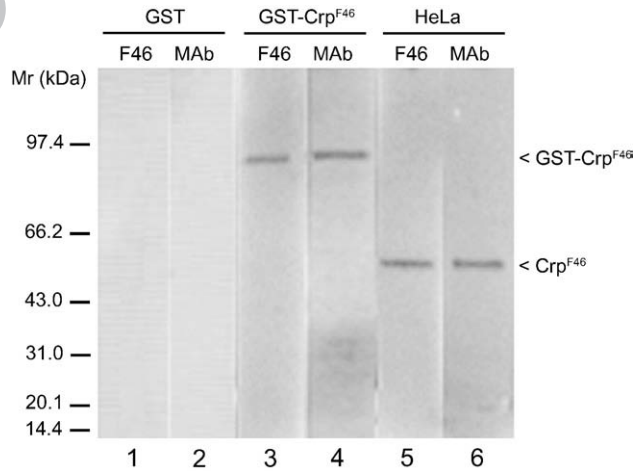

Fig. 3 – Western blot analysis of HeLa proteins and recombinant Crp^{F46} using F46 serum and an anti-Crp^{F46} monoclonal antibody. Total proteins from *E. coli* expressing GST protein (lanes 1 and 2) or Crp^{F46} fusion protein (lanes 3 and 4), and total proteins from HeLa cells (lanes 5 and 6) were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with F46 serum (F46) or anti-Crp^{F46} monoclonal antibody (MAb). Arrows indicate immunoreactive bands at the expected M_r in protein extracts containing either recombinant GST-Crp^{F46} or endogenous Crp^{F46}.

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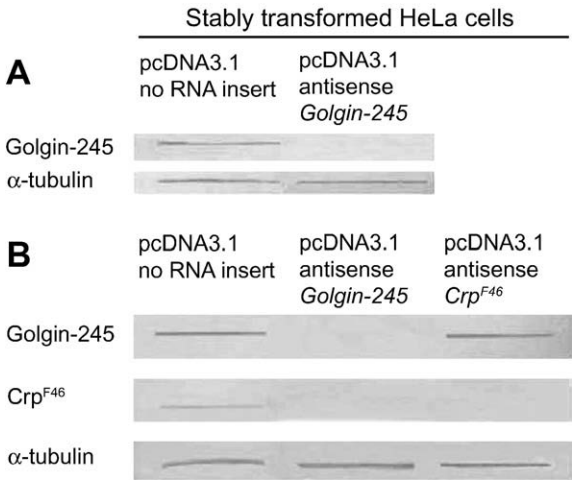

Fig. 4 – Knockdown of Golgin-245 by antisense *golgin-245* also blocks the expression of Crp^{F46}. (A) Protein extracts were prepared from HeLa cell lines transformed with pcDNA3.1+ antisense *golgin-245* RNA or with the empty pcDNA3.1+ vector as a control, separated by SDS-PAGE, and probed by Western blotting using antibody against Golgin-245. While the protein extract from the control cell line showed normal levels of Golgin-245, expression of this protein was effectively knocked down in the cell line stably transformed with the antisense *golgin-245* RNA vector. (B) Stable transformation with antisense *Crp^{F46}* RNA knocked down the expression of Crp^{F46} but did not deplete Golgin-245. Probing with an antibody against γ-tubulin confirms equal protein loading.

antibody against γ-tubulin to identify the centrosome, and examined by confocal microscopy. Only the cells expressing either intact Crp^{F46} or the (aa 323–530) fragment, which contains the C-terminal coiled-coil domain III at residues 420–454, showed typical centrosomal localization in interphase cells (Fig. 6B). By contrast, cells expressing the fragments (aa 1–241) or (aa 127–426), which contain coiled-coil domains I and II, gave only non-specific cytoplasmic GFP signal similar to that in cells expressing the control EGFP. Therefore centrosomal localization is conferred by the Crp^{F46} (aa 323–530) fragment, most likely through the coiled-coil domain III.

Interaction of Crp^{F46} with γ-tubulin and with the centrosomal protein kinase Plk1

To test whether Crp^{F46} interacts with any known centrosomal proteins, we performed co-immunoprecipitation using the anti-Crp^{F46} monoclonal antibody and total protein extract of HeLa cells. Analysis of the immunocomplex by SDS-PAGE and Western immunoblotting using anti-γ-tubulin polyclonal antibody revealed that the complex indeed contains γ-tubulin (Fig. 7). The Crp^{F46} therefore does interact with γ-tubulin, consistent with the co-localization of the two proteins at the centrosome shown by the immunofluorescence microscopy above.

The cell-cycle-dependent association of Crp^{F46} with the centrosome suggests that the mechanism is likely to involve protein phosphorylation, possibly by a known centrosomal kinase such as Plk1 [7,8]. To test this hypothesis, we probed nitrocellulose protein transfers of the Crp^{F46} immunocomplex with an anti-Plk1 antibody. The Western immunoblot revealed a positive reaction, indicating that Crp^{F46} is indeed a target of Plk1. Likewise, reciprocal immunoprecipitation using antibodies against PLK1 or γ-tubulin also gave complexes that consistently contained all three proteins, confirming the three proteins interact. By contrast, immunoprecipitation using anti-Golgin-245 did not form complexes with any of these proteins.

Antisense Crp^{F46} knockdown inhibits the growth and proliferation of HeLa cells

In order to examine the functional role of Crp^{F46}, we inserted an antisense RNA for the whole *Crp^{F46}* coding sequence into pXJ41 expression vector and transfected the resulting construct into HeLa cells. A clonal cell line in which the *Crp^{F46}* was stably transformed was established by using geneticin (G418) selection [46], and silencing was verified by Western blotting and immunofluorescence microscopy using anti-Crp^{F46} monoclonal antibody. Antisense RNA effectively knocked down the expression of Crp^{F46} (Fig. 8A), and it also abolished the typical centrosomal association of the Crp^{F46} (Fig. 8B). A clonal cell line transfected with the control vector pXJ41 alone showed no specific silencing response.

After several passages of the antisense-Crp^{F46} cell line, an increasing number of cells showed dramatic changes in their morphology. The aberrant cells either became abnormally large and multinucleate (Fig. 9A, arrow 2; see also Fig. 10A) or unusually long and narrow (Fig. 9A, arrow 3). The silencing inhibited the growth and proliferation of cells by about 15% (Fig. 9B). The frequency of abnormal cells increased regularly after every passage (Fig. 9C). With continuous subculturing, many cells began to fragment and form conglomerations, indicating that cell growth was severely disrupted (Fig. 9A, arrow 4). Coenocytes containing several mini-nuclei also appeared (Fig. 9A, arrow 1). These results suggest that silencing of Crp^{F46} gradually becomes more effective during continuous subculturing. Eventually, many cells have stopped growing and entered apoptosis, resulting in progressive accumulation of cell debris. The ability of the antisense-*Crp^{F46}* cell line to subculture was also greatly reduced. Flow cytometry analysis revealed that the cell cycle typically stalled at S phase, with marked reduction in the proportion of cells at G2 (Fig. 9D).

The formation of poly-centrosomal and multinucleate cells in antisense-Crp^{F46} cell line

In order to analyze in more detail the subcellular effects of silencing the Crp^{F46}, we processed antisense-*Crp^{F46}* cells and control cells (pXJ41 no RNA insert) for immunofluorescence labeling with anti-Crp^{F46} monoclonal antibody (green) and counter-staining with PI to label DNA (red) (Fig. 10A). Confocal microscopy revealed that silencing of Crp^{F46} caused the formation of coenocytic cells with numerous mini-nuclei and also multinucleate cells, as well as apoptotic cells with aberrant chromatin condensation or fragmented nuclei. To determine if depletion of Crp^{F46} also affected the centrosome, we labeled the cells with anti-γ-tubulin polyclonal antibody. Many cells displayed poly-centrosomal morphology (Fig. 10B).

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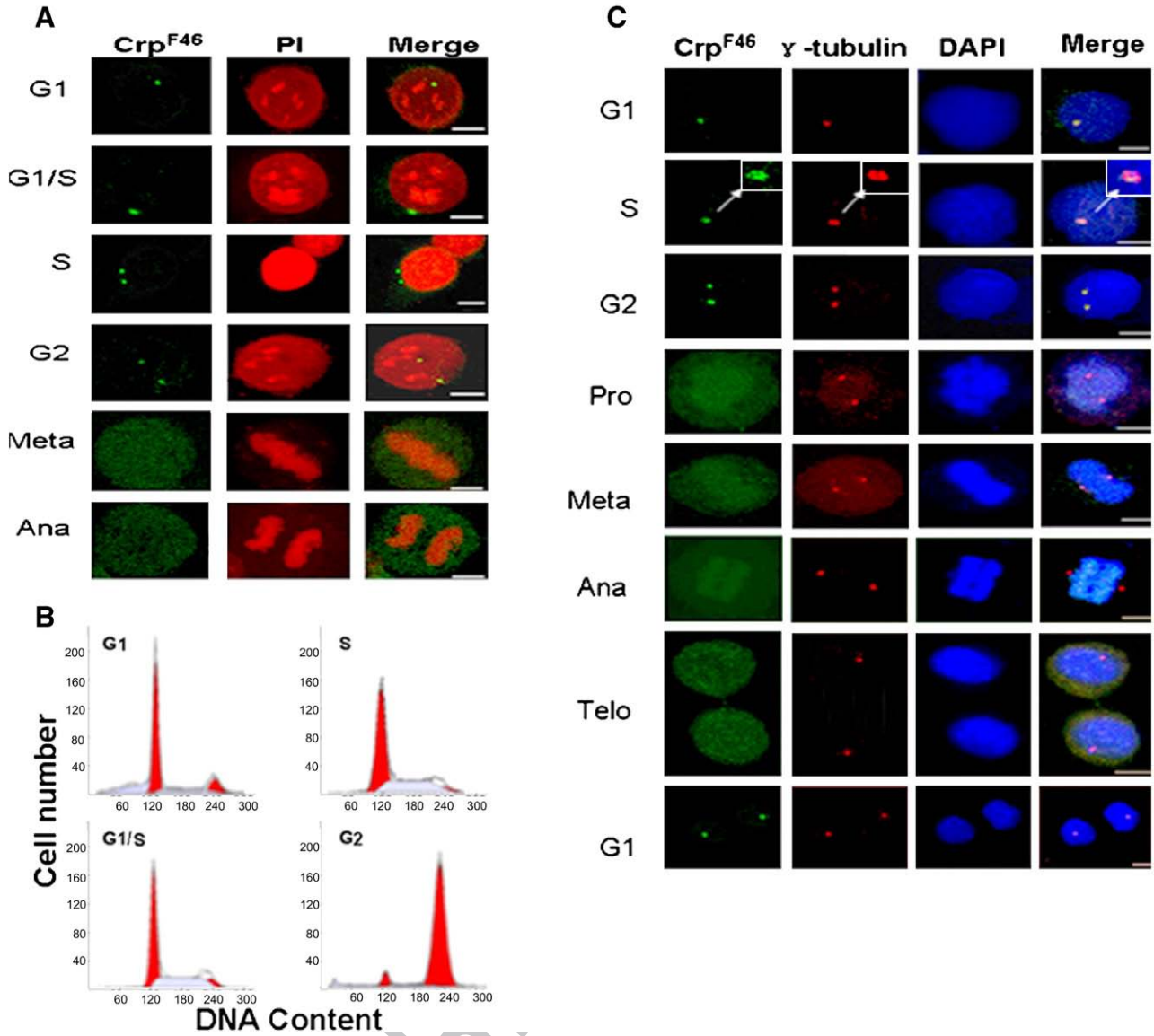

Fig. 5 – Subcellular localization of Crp^{F46} in synchronized HeLa cells, visualized by immunofluorescence labeling and confocal laser-scanning microscopy. HeLa cells were synchronized by culturing in DMEM containing 10% FCS and 2 mM TdR for specific periods (see text). The cells were processed for immunolocalization of Crp^{F46} using anti-Crp^{F46} monoclonal antibody (green) and counter-staining with propidium iodide (PI) to show DNA (red). Individual stages of the cell cycle were determined by FACS analysis. (A) Separate colour images and the merge images show that Crp^{F46} was clearly visible during interphase when it formed one or two bright dots in the cytoplasm next to the nucleus. The bright dots disappeared at the onset of mitosis, although a weaker, punctuate signal became dispersed throughout the cytoplasm. (B) Graphs showing DNA content and cell-cycle phase determined by FACS analysis. (C) Colocalization of Crp^{F46} with γ-tubulin as a centrosomal marker was examined in synchronized cells that were double-labeled with anti-Crp^{F46} monoclonal antibody (green) and anti-γ-tubulin polyclonal antibody (red), and DNA was counter-stained with DAPI (blue). Separate colour images and the merge images show that the Crp^{F46} co-localized with the centrosomes throughout interphase. At the onset of mitosis the bright dots were replaced by a weaker, punctuate signal scattered throughout the cytoplasm. Following cytokinesis, a compact dot of Crp^{F46} appeared again in co-localization with the single centrosome in each daughter cell in G1. Scale bars in A and B, 10 μm.

Furthermore, immunostaining with anti-α-tubulin polyclonal antibody revealed dramatic changes in spindle structure and organization, producing multipolar mitotic spindles (Fig. 10C). The formation of multipolar spindles and asymmetric mitoses are likely to produce coenocytes with mini-nuclei as noted above. In the apoptotic multinucleate cells, the centrosomes generally became smaller and numerous, indicating that apoptosis was the result of a failure to complete the M phase. These results are consistent with the decline in cell growth and proliferation as documented in Fig. 9.

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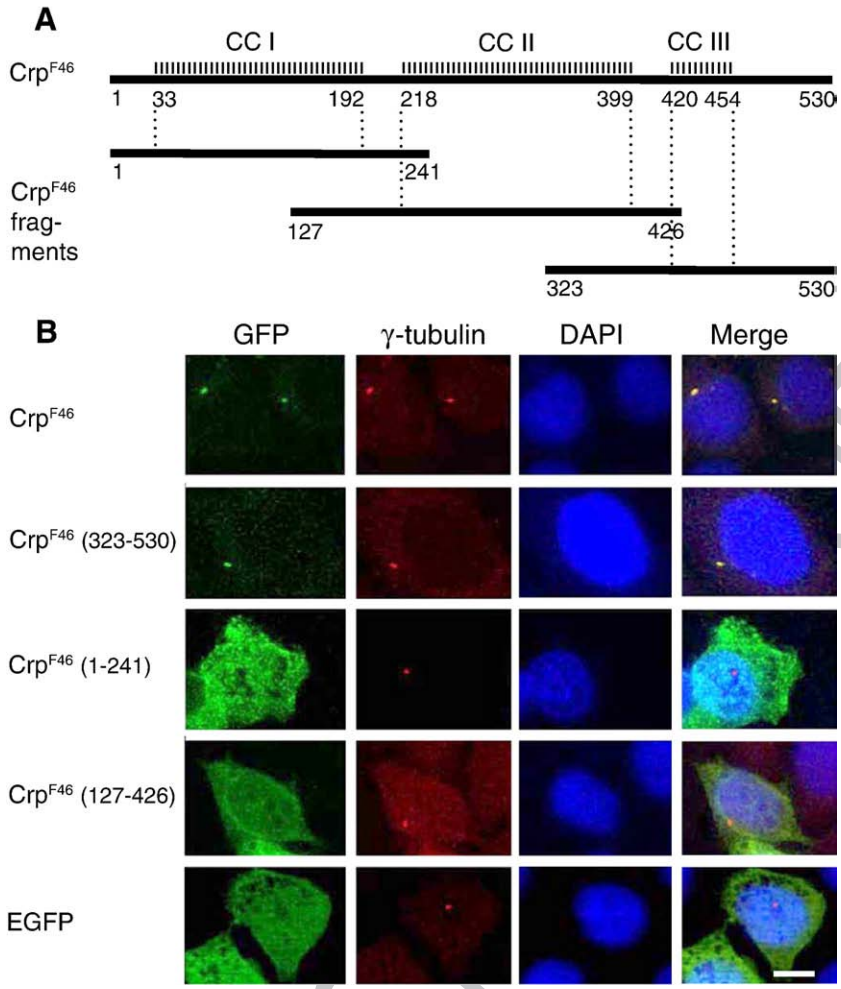

Fig. 6 – Deletion-analysis identification ~~by~~ of a Crp^{F46} domain that confers centrosomal localization. (A) Diagram of amino acid residues defining the positions of Crp^{F46} of coiled-coil domains CC I, CC II, and CC III, and the locations of corresponding deletion fragments. (B) Confocal images of HeLa cells expressing recombinant vectors *pEGFP*-C3 fused with the whole *CrpF46* sequence or its deletion fragments. The HeLa cells were transiently transformed with the recombinant vectors and maintained for 2 days. Cells expressing GFP fusion protein were counter-stained with γ-tubulin antibody and TRITC-conjugated second antibody. Only cells expressing the Crp^{F46} (aa 323–530) deletion fragment, which contains the coiled-coil III domain, showed typical centrosomal localization similar to that seen with the intact Crp^{F46}. Cells expressing the deletion fragments (aa 1–241) or (aa 127–426) showed general cytoplasmic signal. Scale bar, 10 μm.

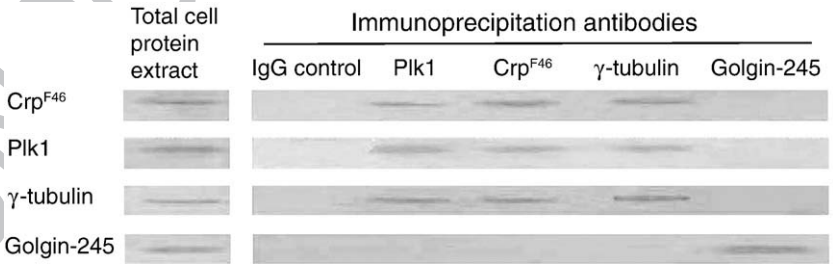

Fig. 7 – Interaction of Crp^{F46} with γ-tubulin and with the protein kinase Plk1. Total protein extracts from HeLa cells were incubated with each of antibodies against Crp^{F46}, Plk1, or γ-tubulin, the individual immunoprecipitated complexes then separated by SDS-PAGE, transferred to nitrocellulose blots and probed with specified antibodies. Immunoprecipitation using anti-Crp^{F46} monoclonal antibody followed by Western immunoblotting gave positive reactions with both anti-Plk1 and anti-γ-tubulin, indicating the three proteins interact as a complex. Reciprocal immunoprecipitation with antibodies against Plk1 or anti-γ-tubulin also showed that the three proteins form a complex. Immunoprecipitation with antibody against Golgin-245 did not form complexes with any of these proteins, and immunoprecipitation with an IgG control was negative.

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Discussion

In this study we identified a novel centrosome-related protein, Crp^{F46}, by using an anti-serum F46 from a patient suffering from progressive systemic sclerosis. The protein attracted our attention because of its unique cell-cycle-dependent localization to the centrosome. We characterized the protein by immunoprecipitation and Western blot using the F46 serum and tandem mass spectrometry (MS/MS) sequencing. Its apparent molecular mass is ~60 kDa, and its predicted amino acid sequence is highly homologous to the C-terminal portion of the protein Golgin-245 using BLAST search of the NCBI/EMBL databases. Western blot of HeLa proteins using a monoclonal antibody raised against a recombinant protein derived from the MS/MS sequencing again showed an immunoreactive band of ~60 kDa, confirming that Crp^{F46} is indeed present in HeLa cells. Immunostaining assay with the anti-Crp^{F46} monoclonal antibody revealed one or two sharp dots near the nuclear envelope at interphase, indicating that Crp^{F46} most likely localizes to the centrosome. Double-labeling experiments using synchronized HeLa cells and anti-γ-tubulin polyclonal antibody showed that Crp^{F46} co-localized with γ-tubulin during interphase but not in mitosis. Therefore, the Crp^{F46} is a novel centrosome-related protein that dissociates from the centrosomes and disperses into the cytoplasm at the onset of mitosis.

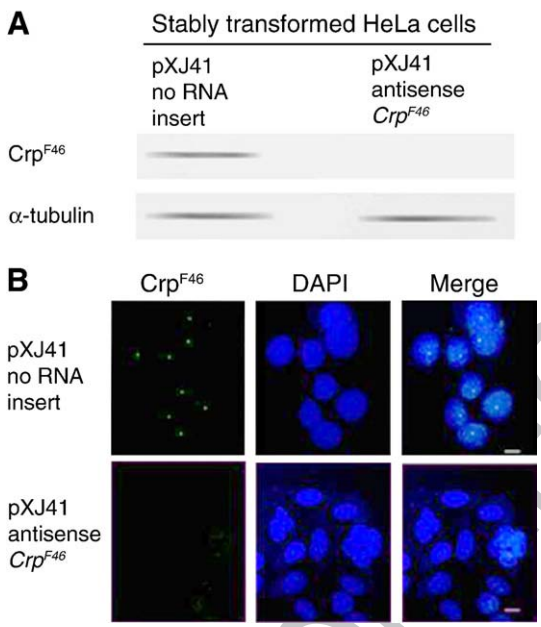

Fig. 8 – Characterization of a stable antisense-*Crp*^{F46} HeLa cell line. (A) Western blot analysis of protein extracts of control cell line (pXJ41no RNA insert) and antisense-*Crp*^{F46} cell line using anti-Crp^{F46} specific monoclonal antibody and α-tubulin antibody to check for equal protein loading. Whereas the control cells showed a normal level of Crp^{F46}, expression of Crp^{F46} was effectively knocked down in the antisense-*Crp*^{F46} cell line. (B) Confocal immunofluorescence images of control cells (pXJ41 no RNA insert) and antisense-*Crp*^{F46} cells labeled with anti-Crp^{F46} specific antibody (green) and counter-stained with DAPI to label DNA (blue). Merge images show that the silencing of *Crp*^{F46} abolished the normal bright staining of centrosomal dots, and a number of cells became multinucleate. Control cells (pXJ41 no RNA insert) appeared normal, without any silencing defects. Scale bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

According to the deletion fragment analysis, the centrosomal localization of Crp^{F46} is conferred by its C-terminal fragment (aa 323–530), most likely through the coiled-coil domain III located at residues 420–454. Many centrosomal proteins, such as TAX1BP2, ninein, rootelin, Cep135, and OFD1, contain coiled-coil domains, and mutations within these domains abolish centrosomal localization [40,47–50]. The coiled-coil domain itself may therefore be sufficient to confer localization to the centrosome. Some centrosomal proteins, such as TAX1BP2, CG-NAP, and pericentrin, can also interact with other centrosomal proteins [40,51,52]. Since our co-immunoprecipitation experiment demonstrated that Crp^{F46} interacts with γ-tubulin, this interaction may serve as a piggyback mechanism for centrosomal localization during interphase. The cell-cycle-dependent association of Crp^{F46} with the centrosome is likely to be regulated by phosphorylation, possibly through interaction with the protein kinase Plk1 as indicated by our co-immunoprecipitation experiment.

Golgin-245 is known to localize specifically to the Golgi apparatus, which performs many unique processes [53]. Despite the distinct localization and function of the Golgin-245 and the Crp^{F46}, the relationship between the Golgi apparatus and the centrosomes is very close as indicated by a list of intimate interactions: (a) The centrosome serves a crucial role in the establishment of the Golgi apparatus, and interaction of the Golgi apparatus with microtubules is important for reconstruction and transport of the Golgi complex following mitosis and cytokinesis; (b) GMAP-210 (Golgi microtubule-associated protein 210) connects cis-Golgi network to the minus ends of centrosome-nucleated microtubules with its respectively N-terminus and C-terminus [54]; (c) CG-NAP (centrosome and Golgi localized PKN-associated protein), locates at centrosome or Golgi apparatus at different phases of the cell cycle [55]; (d) centrosomin's beautiful sister (cbs), a *trans*-Golgi protein, has been found to relate to mature centrosomes [56]; and (e) three members of AKAP350 family (AKAP350A, AKAP350B, and AKAP350C), which are the products of a multiply spliced AKAP, localize to the centrosome and/or the Golgi apparatus after C-terminal splicing events [57]. Thus a protein can contain more than one targeting domain, and splicing events can generate several members that have distinct subcellular localizations and functions. This may be particularly true for proteins containing coiled-coil domains [75].

Our transformation of HeLa cells with antisense *golgin-245* RNA apparently depleted both Golgin-245 and Crp^{F46}. Therefore it is likely that Crp^{F46} is the product of alternative splicing of mRNA transcribed from the entire open reading frame of *golgin-245*, although the process may also involve post-translational modifications in the coiled-coil III domain. Such a splice variant could still include the GRIP domain. Although this domain is necessary for binding to Golgi, it is not sufficient because the binding also requires hierarchical

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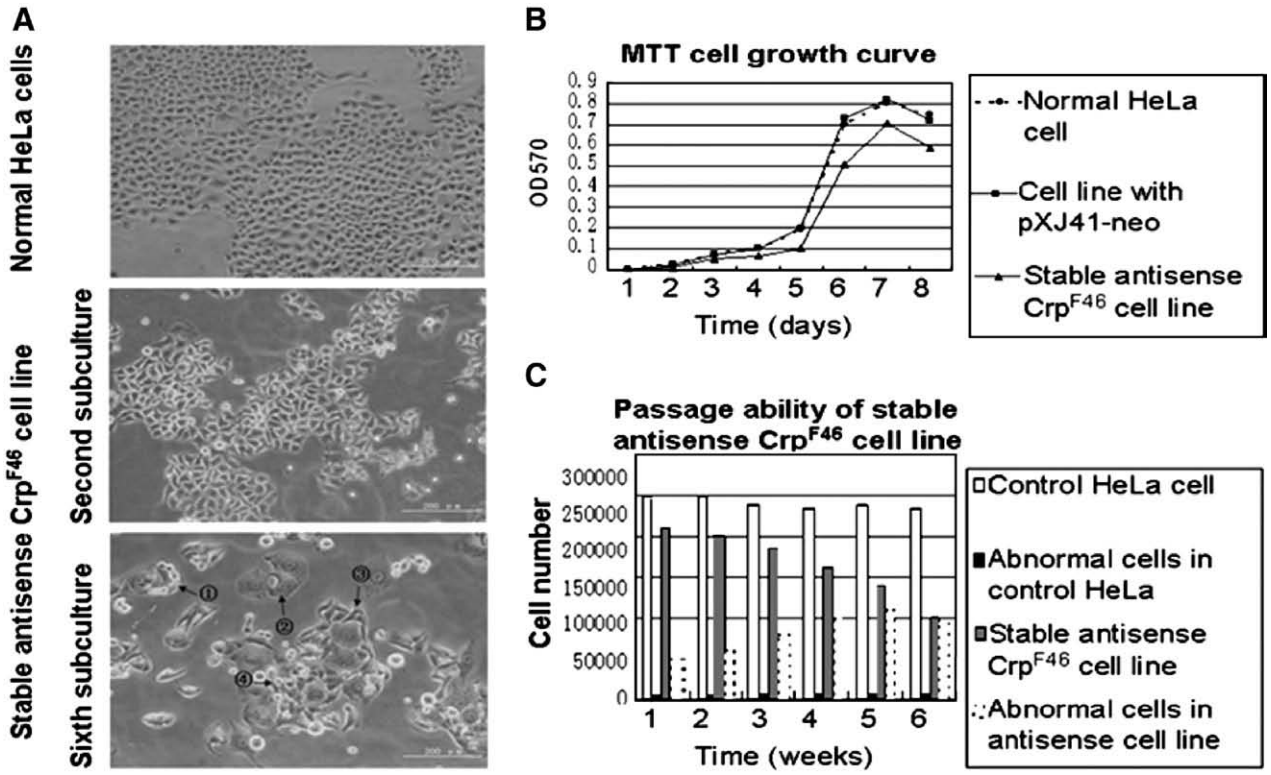

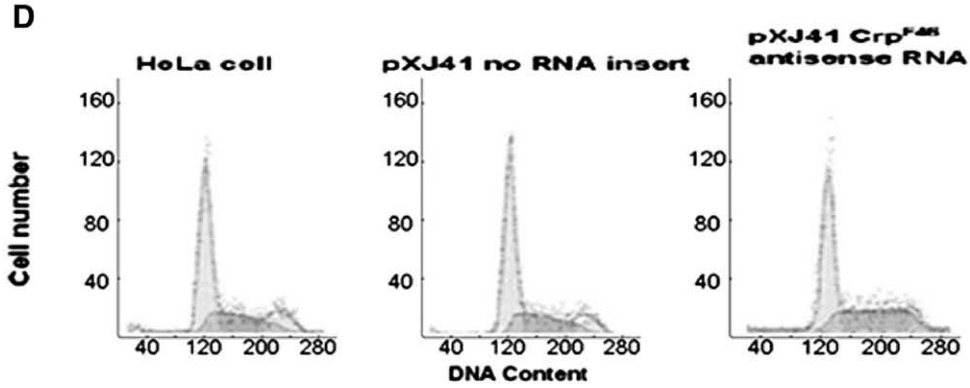

Frequency

Cell lines	Cell cycle phases			
	G1 %	G2 %	S %	G2/G1
HeLa control	57.5	11.9	30.6	1.892
pXJ41 no RNA insert	59.3	8.8	31.9	1.903
pXJ41 antisense *Crp*^{F46}	54.2	3.0	42.8	2.000

Fig. 9 – Mitotic defects and disruption in cell-cycle progression in stable antisense-*Crp*^{F46} HeLa cell line. (A) Accumulation of morphological abnormalities (arrows) with repeated subculturing (passages) of the antisense-*Crp*^{F46} cell line. (B) Cell growth curves, determined by the MTT method, showing that cell growth and proliferation in the antisense-*Crp*^{F46} cell line was inhibited by ~15%. (C) Declining passage vigor and increasing frequency of morphological abnormalities with repeated subculturing of the antisense-*Crp*^{F46} cell line. Wt cell line and antisense-*Crp*^{F46} cell line in exponential phase of growth were repeatedly subcultured at weekly intervals over 6 weeks and grown for 4 days, and the number of normal and abnormal cells determined in each cell line. In the wt cell line, the number of normal cells and the relatively low frequency of abnormal cells remained unchanged over the 6 passages. In contrast, in the antisense-*Crp*^{F46} cell line the total number of normal cells gradually declined and the number of abnormal cells increased with each passage. (D) Flow cytometry analysis of the antisense-*Crp*^{F46} cells showed that the cell cycle was prominently stalled at S phase.

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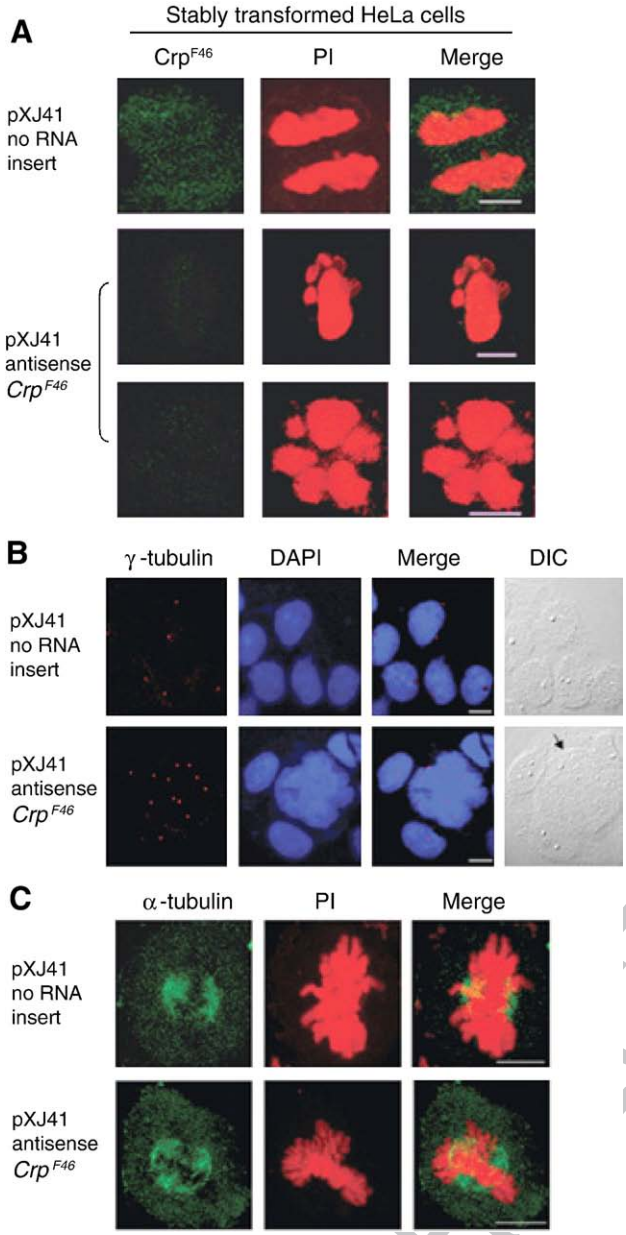

Fig. 10 – Confocal immunofluorescence microscopy of a stable antisense-*Crp*^{F46} cell line. (A) Immunofluorescence images of control cells (pXJ41 no RNA insert) and antisense-*Crp*^{F46} cells probed with anti-Crp^{F46} monoclonal antibody (green) and counter-stained with PI to show DNA (red). Expression of *Crp*^{F46} was effectively knocked down in the antisense-*Crp*^{F46} cells, compared with the normal scattered punctate staining in a control cell at anaphase. The silenced cell line typically contained multinucleate and coenocytic cells, or apoptotic cells with aberrant chromatin condensation and fragmented nuclei. (B) Multiple centrosomes, identified by staining with anti-γ-tubulin polyclonal antibody (red). Counter-staining DNA with DAPI (blue) and DIC reveal a large, multinucleate, poly-centrosomal cell (arrow). (C) Formation of a multipolar mitotic spindle in an antisense-Crp^{F46} cell, revealed by staining with anti-α-tubulin monoclonal antibody (green) and PI (red). Scale bars in A–C, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interactions with other proteins and phospholipids that contribute synergistically to Golgi targeting [45]. Clearly, our results show that Crp^{F46} has its unique localization to the centrosome during interphase and performs a function independent from the Golgi. The exact mechanism of origin of Crp^{F46} remains to be established.

Another unique feature of the Crp^{F46} is its cell-cycle-dependent association with the centrosome. The dissociation of Crp^{F46} from the daughter centrosomes at the onset of mitosis is most likely a part of 'centrosome maturation' in preparation for mitosis [58]. During this process, a number of proteins are recruited to the centrosomes as a result of changes in phosphorylation status, largely involving proteins that increase the microtubule nucleation activity required for the establishment of the microtubule spindle. At the same time, the Golgi apparatus fragments and disperses from the peri-nuclear region into the cytoplasm [59]. It appears that the Crp^{F46} follows a similar behavior, dissociating from the centrosome and scattering through the cytoplasm, a process that most likely involves changes in phosphorylation status. An example of such behavior is Nlp (ninein-like protein), which becomes phosphorylated by Plk1 (polo-like kinase 1) and dissociates from the centrosomes at G2/M transition and then redistributes into the cytoplasm during mitosis [60,61]. Similarly, Chk1 (checkpoint kinase 1) localizes specifically to interphase, but not mitotic, centrosomes [62]. Co-immunoprecipitation of Crp^{F46} with Plk1 in our experiments indicates that the cell-cycle-dependent association of Crp^{F46} with the centrosome likewise involves phosphorylation, consistent with its abundant potential phosphorylation sites.

Phosphorylation on Ser/Thr-Pro sites is a major mechanism regulating many events in cell proliferation and differentiation, including centrosome duplication. Many centrosomal proteins, such as centrin, NPM/B23, Nlp, and Pin1 [13,56,68,69], are known to localize on, or dissociate from, the centrosome and to regulate centrosome duplication with phosphorylation as the prerequisite step.

Our results with stable transformation of HeLa cells with antisense-*Crp*^{F46} RNA show that Crp^{F46} may also play an active role in cell proliferation and influence cell-cycle progression. Thus, Crp^{F46} depletion inhibited cell growth and proliferation, reduced the effectiveness of cell subculturing as the proportion of abnormal cells increased after every passage, and the cell cycle was stalled at S phase. Furthermore, the depletion also caused various morphological changes, including abnormalities in cell volume and the emergence of multinucleate cells containing 2–6 nuclei. Thus the cell-cycle progression was not only inhibited but also seriously disrupted in various ways.

In current view, the centrosome plays an important role not only in microtubule nucleation but also in cell-cycle regulation [63]. Centrosome duplication is closely coupled with other cell-cycle events, especially DNA synthesis [64]. Abnormalities in centrosome-associated proteins like pin1, Rad6, and cyclin D1 can cause centrosome re-duplication and affect the regulation of the cell cycle. Such abnormalities lead to the formation of multinucleate cells, cells with multipolar spindles, mitotic spindle abnormalities, aneuploidy, chromosome instability, oncogenesis, and other defects [13,14,65]. Similarly, the antisense depletion of Crp^{F46} in our experiments included alteration in centrosomal number, formation of multipolar spindles,

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and the formation of coenocytes as a result of failed cytokinesis. Interestingly, the Crp^{F46} association with the centrosome is limited to interphase, suggesting it may be involved in centrosome duplication. Replication of the centrosome is induced by the activity of cyclin E-Cdk2 complex at G1/S phase [66,67], which is regulated by proteins such as p21 or cki-2 [17]. It seems plausible that Crp^{F46} could modulate the activity of these or other proteins that participate in centrosome duplication.

It is unknown exactly how Crp^{F46} may participate in the regulation of the cell cycle. However, its specific localization to the centrosome in interphase is consistent with its proposed role in centrosome duplication. Since the centrosome replicates during the S phase, there is a direct relationship between poly-centrosomes and stalling of the cell cycle at the S phase, both of which can be caused by silencing the Crp^{F46}. Similarly, recent research revealed that the expression of $Cdc25^{string}$, which drives cells into mitosis, is required for the completion of daughter centriole assembly and ensuring that the centrosome replicates only once in the normal cell cycle in *Drosophila* cells [70,71]. Recent research has also shown that components of the large γ-tubulin ring complex participate in the spindle assembly checkpoint [72]. Since Crp^{F46} co-localizes and interacts with γ-tubulin, it is a likely candidate for a role in cell-cycle progression.

The mechanism of cell proliferation remains a hot spot in cancer research. Cancer cells typically have abnormal centrosome number, and the absence of tumor suppressor proteins such as P53 can cause centrosome re-duplication [73]. Silencing of Crp^{F46} has a similar effect. Another potential player in cancer cells is the centromere. Since the centromere/kinetochore complex connects the chromosome to microtubules, and the centrosome acts as a microtubule-organizing center, there is likely to be mutual relationship between centrosome re-duplication, formation of multipolar spindles, centromere abnormalities, and abnormal chromosomal number or structural instabilities [74]. Investigation of centromere proteins in the antisense Crp^{F46} cell line will be required in order to elucidate the role of Crp^{F46} in the mechanism of chromosome separation and morphological abnormalities in the cell.

In conclusion, our research demonstrates a role of a novel centrosomal protein Crp^{F46} in cell-cycle dynamics. Further investigation of the Crp^{F46} is likely to produce new information about the role of the centrosome in cell-cycle regulation, mitosis and cytokinesis, possibly benefiting the treatment of cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2008.02.021.

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