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# Research Article

# Molecular mechanism underlying the association of Coronin-7 with Golgi membranes

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**Abstract.** Coronin-7 (Crn7) is a ubiquitous mammalian WD40-repeat protein that localizes to the Golgi complex, interacts with AP-1 adaptor complex via binding of a tyrosine-288-based sorting signal to the μ1-subunit of AP-1, and participates in the maintenance of the Golgi structure and function. Here, we define the requirements for the recruitment of Crn7 from the cytosol to the Golgi. We establish that Src activity is indispensable for the interaction of Crn7 with Golgi membranes. Crn7 binds Src *in vivo* and can be phosphorylated by recombinant Src *in vitro*. We demonstrate that tyrosine-758 is the major Src phosphorylation site. Further, to be targeted to membranes Crn7 requires the presence of cargo in the Golgi complex. Finally, downregulation of the μ1-subunit of AP-1 leads to the dispersal of Crn7 from the Golgi membranes. We propose a mechanism whereby sequential events of protein interaction and posttranslational modification result in the membrane targeting of Crn7.

**Keywords.** Coronin family, Golgi complex, Src, phosphorylation, membrane binding.

## Introduction

Coronins are ubiquitous, conserved proteins executing a variety of actin cytoskeleton-related functions in many organisms ranging from yeast to mammals, and most organisms have more than one coronin gene [1, 2]. A canonical structure of a coronin comprises a seven-bladed β-propeller formed by a WD repeat-containing N-terminal domain followed by a C-terminal "extension" with a region unique to each coronin, and a very C-terminally located coiled coil [3]. A well-defined subfamily of coronins consists of proteins possessing a duplicate WD domain and lacking the C-terminal coiled coil region. Such proteins have been studied in *Caenorhabditis elegans* and *Drosophila melanogaster*, and mutant phenotypes suggest that they play a role in vesicular trafficking, embryonic axis formation and axonal guidance [4, 5]. We recently demonstrated that the only human dual WD domain coronin, the Coronin-7 (Crn7), differs from the rest of the family in that it localizes to the

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Golgi complex and is required for the maintenance of Golgi morphology and for protein export from the Golgi [6, 7]. Depletion of Crn7 by RNAi leads to Golgi breakdown and retention of VSVG in the Golgi upon a shift to the permissive temperature. This export block in turn correlated with accumulation of AP-1 clathrin adaptor complexes on intracellular membranes, most probably in connection with arrested cargo. We further showed that the tyrosine-288-based sorting motif of Crn7 is capable of interacting with the μ1-subunit of the AP-1 complex *in vitro*, and that Crn7 binds AP-1, but neither AP-2 nor AP-3 *in vivo*. Based on our data, we hypothesized that Crn7 is required for very late stages of Golgi export, e.g. generation of membrane curvature, interaction with co-adaptors or coat or, less likely, membrane fission. Here, we characterize the requirements for the membrane targeting of Crn7. Lacking a predicted signal sequence or transmembrane domain, Crn7 is likely to be recruited to the outer side of Golgi membranes from the cytosol. Our data establish a network of posttranslational modification and protein-protein interaction leading to such recruitment.

## Materials and methods

**Reagents.** Antibodies to Coronin-7 and Coronin-1C (Coronin-3) were reported previously [6, 8]. Src inhibitor SU6656 and monoclonal antibodies to Src were from Calbiochem. Antibody to mannosidase II was from Serotec, anti GPP130 from Alexis. GFP-Syn5 plasmid and ERGIC53 antibody were kind gifts from Dr. Irina Majoul. GFP-Src, GFP-Src (Y527F) and GFP-Src (K295M) constructs were from Dr. Michael Way. Synthetic peptides, HPLC-purified: "CON" = KVEKIGEGTYGVVYK (from Jena Bioscience, Germany), Y288 = GKGERQLYCYEVVPQ, Y288A = GKGERQLYCAEVAPQ, Y758 = GKGDTRVFLYELLPE, Y758A = GKGDTRVFLAELAPE (from JPT, Germany). Recombinant Src was from Jena Bioscience and Upstate, $\gamma^{32}$P-ATP from Amersham. Fine chemicals were from Sigma unless indicated otherwise. Brefeldin A (BFA) was used at 2 μg/ml, genistein at 25 μg/ml, SU6656 at 0.4–2 μM.

**Cell culture, transfection and immunofluorescence.** HeLa cells were from ATCC. 293TN cells were from Biocat/SBI. Cells were grown and processed for immunofluorescence as described [6]. FuGENE6 reagent (Roche) was used for transfections according to the manufacturer's instructions.

**Immunoprecipitation and western blotting.** Immunoblotting was performed according to standard procedures. Immunoprecipitation using protein A-Sepharose (Amersham) was performed essentially as described [9]. Briefly, cells were lysed in 1 % NP-40 buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 % NP-40) and lysates were precleared with protein A-Sepharose beads for 1 h and incubated with 10 μg of antibody overnight. Sepharose beads were added for 3 h, pelleted, washed five times with 1 % NP-40 buffer and boiled with protein sample buffer. All incubations were performed at 4°C in the presence of protease inhibitors (from 100x stock solution containing 50 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin in ethanol) and phosphatase inhibitors (Calbiochem). In the experiment shown in Figure 5E, chemical crosslinker DSS (Pierce) was used according to the manufacturer's instructions.

**Cell fractionation experiments.** For salt extraction experiments, HeLa cells were grown in 15-cm Petri dishes, scraped into ice-cold PBS, washed with PBS and homogenized by passing through a 21G $1\frac{1}{4}$ needle 12 times in ice-cold HB buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, protease and phosphatase inhibitors). The lysate was precleared at 1000 g for 15 min and centrifuged at 100 000 g for 60 min. Pellets were collected, resuspended either in PBS or in PBS with 1 M KCl, incubated on ice for 30 min and re-centrifuged as above. Alternatively, membrane pellets of 293TN cells resuspended in HB buffer were treated with 100 mM $Na_2CO_3$, pH11.5.
For proteinase K protection assays, 293TN cells grown to confluency in six 15-cm Petri dishes were scraped into ice-cold PBS, washed with PBS, homogenized by five strokes with a loosely fitting Dounce homogenizer in ice-cold PBS without protease inhibitors supplemented with 2 mM EDTA, and precleared at 1000 g for 15 minutes. The resulting supernatant was divided into three equal fractions, one of which was kept untreated, one adjusted to 100 μg/ml Proteinase K, and one to 100 μg/ml Proteinase K and 1 % Triton X-100. After 1 hr incubation at 4°C 5 mM PMSF and 2 mM EGTA were added and the samples were centrifuged at 100 000 g for 45 min at 4°C. The resulting pellets were washed with PBS, solubilized in SDS sample buffer, and analyzed by SDS-PAGE.

***In vitro* kinase assays using Crn7 peptides.** Reactions were carried out in 100 μl volume containing: 50 μl 2x kinase buffer (100 mM HEPES, pH 8.0, 4 mM DTT, 20 mM $MnCl_2$), 10 μl 6 mM substrate peptide, 1 μg GST-Src (>65U/μg), water to 95 μl, started by addition of 5 μl ATP mix (6 μl 2 mM ATP, 2.4 μl $\gamma^{32}$P-ATP, 10 μCi/μl, >6000 Ci/mM, water to 60 μl). The reac-

tions were performed at 30°C and stopped by adding 20 µl 60 mM EDTA, pH 8.0. Ten and 2.5 µl of the resulting solution were spotted onto Protran nitrocellulose membrane, air-dried for 45 min, washed 12 times with 75 mM phosphoric acid and analyzed using a PhosphorImager 445SI system. In parallel, samples were separated from unbound ATP by binding to Chromabond HR-P columns (Macherey-Nagel), and analyzed by scintillation counting using routine methods after repeated washing and elution.

***In vitro* kinase assays of endogenous Crn7.** Crn7 was immunoprecipitated from HeLa cell lysate using anti-Crn7 antibody and Protein A-Sepharose. Aliquots of Sepharose beads were mixed with kinase reaction mix and ATP mix as above and incubated in the presence or absence of 25 µM Src inhibitor SU6656 at 30°C for the indicated periods of time. Reactions were stopped by adding EDTA, boiled with SDS-PAGE sample buffer and resolved by SDS-PAGE. Gels were Coomassie-stained, dried and exposed to Kodak MAX X-Ray film for 72 hours.

***In vitro* kinase assays of recombinant Crn7.** A cDNA coding for both N-terminally GST- and C-terminally His6-tagged human full-length Crn7 was inserted into the backbone of baculoviral expression vector pVL1393, expressed in Sf9 insect cells, and purified according to the manufacturer's manuals (insect cell systems of Pharmingen and Invitrogen). Crn7 was eluted by elution buffer (20 mM glutathione, 200 mM Tris-HCl, pH 8.0, 2 mM EGTA, 0.1 % Triton X-100) and verified by SDS-PAGE, Coomassie staining, western blotting and peptide mass fingerprinting. Kinase reactions were carried out in 100 µl volume as described above for the indicated time periods. Each reaction contained 12 µg recombinant GST-Crn7-His6 and 1.2 µg GST-Src. Reactions were stopped by adding 20 µl 60 mM EDTA, boiled with 30 µl 5x SDS-PAGE sample buffer, and 30 µl of each experiment were resolved by SDS-PAGE. Gels were Coomassie-stained, dried and exposed to Kodak MAX X-Ray film for 72 hours. Controls were without Crn7, ATP, Src, or in the presence of 92.5 µM Src inhibitor SU6656; all experiments contained an appropriate concentration of the solvent DMSO. For quantification, respective densitometry values were normalized to the amount of loaded Crn7 and Src proteins (Coomassie blue stained gel) and corrected for background values using the AlphaEaseFC software (Alpha Innotech).

**Site-directed mutagenesis.** To express the double tyrosine mutant Y288A/Y758A, the Crn7 plasmid GST-hCrn7-His6-VL1393 (see above) was modified by two sequential steps of *in vitro* mutagenesis according to the manufacturer's protocol (QuikChange Site-Directed Mutagenesis; Stratagene). Primer pairs hCrn7Y288AFmut 5′-CGAGAGGCAGCTGTACTGTGCCGAGGTGGTACCGCAGCAGCCGGC-3′ and hCrn7Y288ARevmut 5′-GCCGGCTGCTGCGGTACCACCTCGGCACAGTACAGCTGCCTCTCG-3′ as well as hCrn7Y758AFmut 5′-GCGACACCCGTGTATTCCTGGCCGAGCTCCTCCCCGAGTCCCCTTTCTTC-3′ and hCrn7Y758ARevmut 5′-GAAGAAAGGGGACTCGGGGAGGAGCTCGGCCAGGAATACACGGGTGTCGC-3′ were used to introduce the respective mutations. The resulting plasmid GST-hCrn7-Y288AY758A-His6-VL1393 was verified by restriction enzyme analysis and sequencing.

**HRP assays.** HeLa cells were transiently transfected with a plasmid encoding ssHRP [10, 11] using FuGENE6 reagent (Roche) and plated onto 6-well plates at approx. 60 % confluency. Twelve hours after plating, SU6656 was added to plates at 0.4–2 µM. After 1 hr, medium was replaced with 2 ml fresh, serum-free, SU6656-containing medium. 100 µl aliquots were taken at the indicated periods of time. 600 µl of complete ECL reagent (Amersham) were added to the medium, and three 200 µl aliquots of each reaction were pipetted into white plastic 384-well plates. Chemiluminescence was measured using a microtiter plate reader (Tecan). Cells were trypsinized and counted using a haematocytometer, and luminescence values were normalized using cell count data.

**siRNA experiments.** The following siRNA were used: targeting human µ1-adaptin – validated siRNA cat. # SI00299187 (Qiagen; no sequence information provided by vendor); targeting human Crn7 – $siRNA_{(8)}2454$ [7]; control: siCONTROL Non-Targeting siRNA Pool cat. # D-001206-13 (Dharmacon; no sequence information provided by vendor). After three siRNA transfections and a total of nine days of gene interference, cells were processed for immunofluorescence as described above.

## Results

**Crn7 is a peripheral membrane protein.** At least five lines of evidence suggest that Crn7 is a peripheral membrane protein attached to Golgi membranes at the outer (cytosolic) side rather than a transmembrane or luminal protein. i) Crn7 lacks a predicted signal sequence and transmembrane domains [6]. None of the known coronins is either luminal or transmembrane [1]. ii) The protein is present in both

membrane fraction and cytosol in mammalian cells; in fact, the bulk of the protein is cytosolic rather than membrane-bound as demonstrated by quantitative differential centrifugation at 200 000 g [see Fig. 7 in Ref. 6]. iii) In 100 000 g membrane pellets treated with 1 M KCl in order to dissociate peripheral membrane proteins and re-centrifuged, significant amounts of Crn7 redistribute to the soluble fraction (Fig. 1A). Treatment with 100 mM sodium carbonate, known to have a similar effect [12], also resulted in partial redistribution of Crn7 from the membrane pellet into the supernatant (Fig. 1B). iv) Membrane-bound Crn7 can be digested by treatment with proteinase K in the absence of detergents, which suggests that the protein is not protected from proteolytic cleavage by a compartment membrane (Fig. 1C, upper panel). While membrane-associated proteins localized at the cytosolic side of compartment membranes are cleaved both in the presence and absence of detergent (βCOP, middle panel), luminal proteins such as GPP130 are protected from proteolytic cleavage by compartment membranes and only cleaved in the presence of detergent (lower panel). v) Treatment with brefeldin A (BFA), an agent causing the fusion of Golgi membranes with ER, results in the redistribution of luminal and transmembrane Golgi proteins into the ER [13, 14]. In BFA-treated HeLa cells, Crn7 gradually disperses from Golgi membranes, as do several other peripheral Golgi proteins such as βCOP [Figs. 2 and 3, see also Ref. 6]. Luminal and transmembrane proteins exemplified by the integral membrane protein mannosidase II [15, 16] remain associated with the resulting Golgi-ER compartment (Fig. 2). While not all Golgi/ER-localized proteins demonstrate the characteristic nuclear envelope pattern upon BFA treatment (Fig. 2, compare KDEL-R and mannosidase II patterns), transmembrane proteins co-localize with subdomains of the ER/Golgi chimaeric organelle (Fig. 2D, note the colocalization between KDEL receptor and mannosidase II in subdomains of the ER). Crn7, however, does not show a re-distribution to the nuclear envelope and, unlike KDEL receptor, looses co-localization with mannosidase II (Fig. 2C). Crn7 does not co-localize with the intermediate compartment marker ERGIC53 either. While Crn7- and ERGIC53-positive compartments are closely juxtaposed in control cells, staining patterns of the two proteins are markedly different in BFA-treated cells. In agreement with published data [17], ERGIC53 redistributed into enlarged circular membrane compartments upon BFA treatment, but these compartments were not positive for Crn7 (Fig. 3).

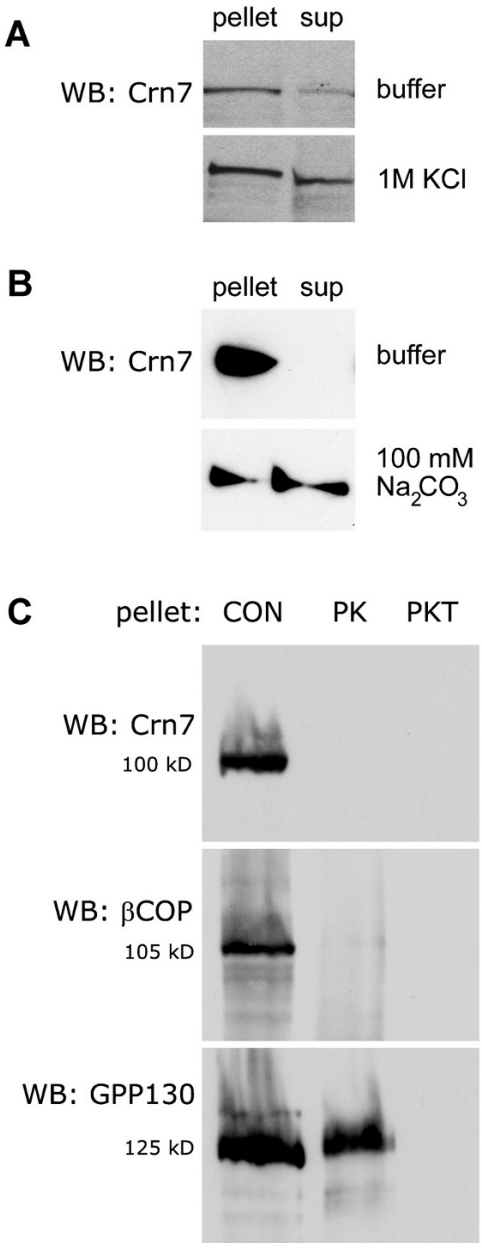


**Figure 1.** Crn7 is a peripheral membrane protein. (*A*) Crn7 (100 kD) is part of a salt-sensitive membrane pellet. Postnuclear supernatants of HeLa cells were processed as described in Materials and methods and probed with Crn7 antibody. Treatment of 100 000 g pellets with 1M KCl resulted in a partial release of Crn7 into the supernatant. (*B*) Treatment of 100 000 g pellets with 0.1 M sodium carbonate, pH 11.5, resulted in a partial release of Crn7 into the supernatant. (*C*) Crn7 is not protected from protease activity by compartment membranes. Treatment of membrane fractions (input – CON, first lanes) with proteinase K (PK, second lanes) resulted in a loss of Crn7 and βCOP signal but not GPP130 signal. Treatment of membrane fractions with proteinase K in the presence of detergent resulted in a loss of all three signals (PKT, third lanes). GPP130 is a type II single-span transmembrane protein. Its cytoplasmic portion consists of only 12 amino acids, while 684 amino acids belong to the transmembrane and luminal domains [56]. The antibody recognizes a part of the luminal domain of GPP130.

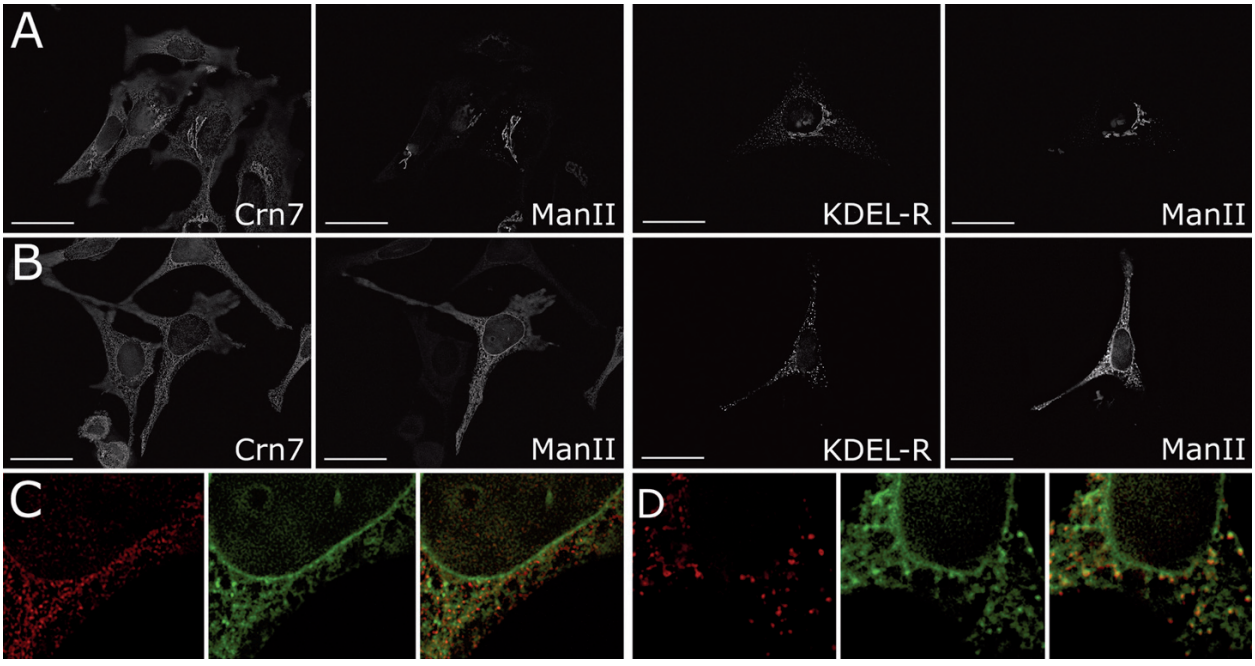


**Figure 2.** Crn7 redistributes to the cytosol in BFA-treated (2 μg/ml, 1 h) HeLa cells. (*A*) in DMSO-treated control cells, Crn7, the resident Golgi enzyme mannosidase II (ManII) and the KDEL receptor (KDEL-R) partially localize to Golgi membranes. (*B*) Upon BFA treatment, Crn7 redistributes to the cytosol, while mannosidase II and KDEL receptor remain associated with the ER. Note the characteristic nuclear envelope pattern for mannosidase II. Bars, 25 μm. (*C*) Higher magnification of a region of a BFA-treated cell demonstrates the absence of Crn7 from the nuclear envelope and absence of co-localization between Crn7 (red) and mannosidase II (green); (*D*) Higher magnification of a region of a BFA-treated cell showing the complete co-localization of KDEL receptor-containing structures (red) with mannosidase II-positive ER membranes (green).

**Crn7 is a target of Src and depends on Src in its membrane targeting.** Our initial experiments, such as 2D-gel electrophoresis and western blotting using anti-phosphotyrosine antibodies, demonstrated that Crn7 is phosphorylated on tyrosine residues in the membrane fraction, but not in the cytosol [6]. Because treatment with the broad-range tyrosine kinase inhibitor genistein significantly decreased the amount of Crn7 in the membrane pellet of HeLa cells, the tyrosine phosphorylation is required for the targeting of Crn7 to membranes (Fig. 4A). More specifically, the application of SU6656, a Src inhibitor [18], markedly redistributed Crn7 to the cytosol upon short-term application (2 h) (Fig. 4B, C). Thus, Src controls the Golgi targeting of Crn7 in either a direct or indirect fashion. To confirm this conclusion, we studied the distribution of Crn7 in HeLa cells overexpressing a GFP-tagged Src mutant unable to bind ATP (Src K295M, Fig. 4D, E). In Src-K295M cells Crn7 was predominantly cytosolic, implying that overproduction of inactive kinase has a dominant-negative effect with regard to Crn7 localization. We did not register any changes in Crn7 distribution in cells overexpressing wild-type or constitutively active Y527F Src (data not shown).

The Crn7 sequence contains two putative Src phosphorylation sites. Tyrosines-288 and 758 located in the C-terminal parts of the two coronin WD domains showed the highest probability values (data not shown), and were analyzed for Src phosphorylation *in vitro*. We used synthetic 15-mer peptides encompassing Y288 (referred to as "peptide Y288") and Y758 ("peptide Y758") and mutated control peptides ("peptides Y288A and Y758A"; see Materials and methods). A known Src substrate peptide $NH_2$-KVEKIGEGTYGVVYK-COOH corresponding to amino acids 6–20 of human cyclin dependent kinases 2 and 3 served as a positive control [19, 20]. Analysis of the incorporation of $\gamma^{32}$P-ATP into the peptides by recombinant GST-fused Src demonstrated that Y758 is a preferred Src target, whereas Y288 is phosphorylated at a significantly lower rate (Fig. 5A). The initial rate of phosphorylation of the Y758-containing peptide was comparable with that of a control substrate peptide, while the rate of phosphorylation of the Y288 peptide was approx. 80 % lower (Fig. 5B). Importantly, the peptide Y288 harbours an additional tyrosine in position 286. This residue is not phosphorylated by Src because the $\gamma^{32}$P-ATP signal from the Y288A control peptide possessing Y286 (but not Y288) is at all time points exactly the same as that of the Y758A peptide not containing any tyrosine residues at all.

Incubation of purified GST-tagged full-length Crn7 protein expressed in Sf9 cells (see Materials and

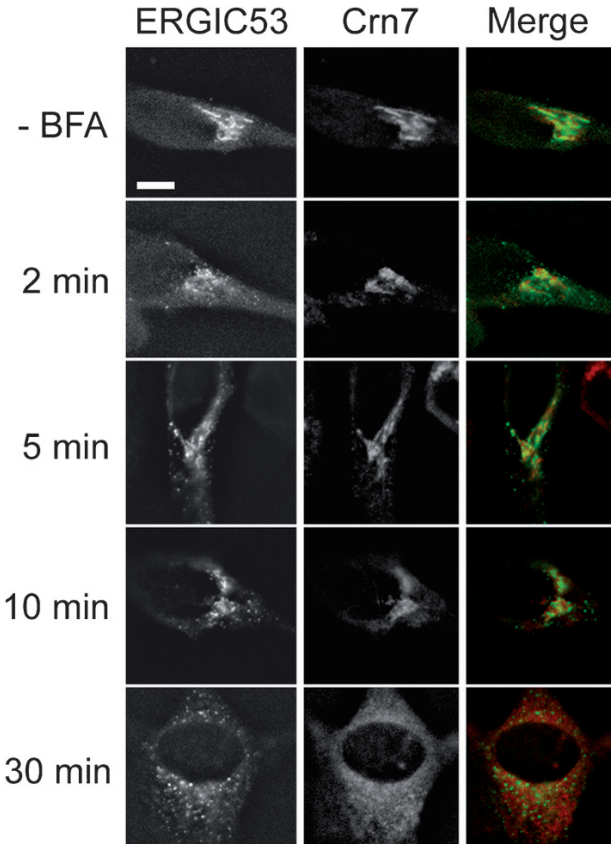


**Figure 3.** Crn7 redistributes to the cytosol in BFA treated (2 μg/ml, 1 h) HeLa cells. Indirect immunofluorescence experiment demonstrating ERGIC53 (left panel) and Crn7 (middle panel) staining and the false color merged image (right panel; ERGIC53 shown in green, Crn7 in red). The dynamics of Crn7 localization upon BFA treatment suggest that the dissociation of the protein from Golgi membranes occurs later than 10 min, but earlier than 30 min after the addition of the compound. Crn7-positive Golgi membranes are localized in the vicinity of the ERGIC53 compartment in control cells (upper panel), but this close juxtaposition is lost as soon as 5 min after the application of BFA. Note that at the latest time point, there is no colocalization between dispersed Crn7 staining and ERGIC53 structures. Bar, 10 μm.

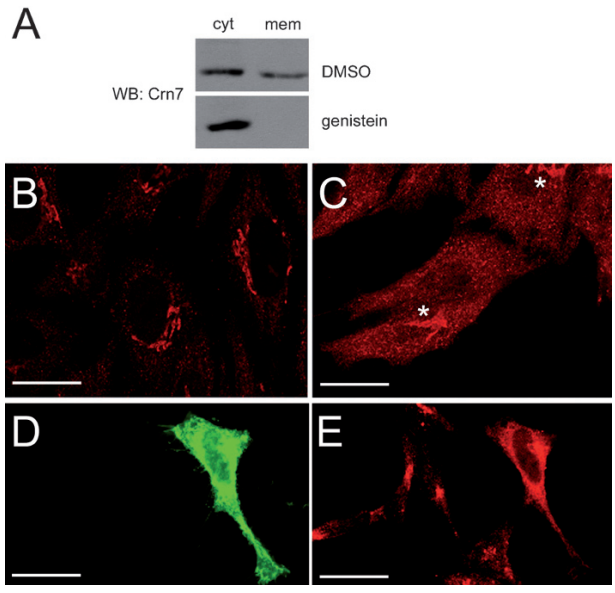


**Figure 4.** Functional Src is required for the targeting of Crn7 to Golgi membranes. (*A*) Inhibition of tyrosine phosphorylation leads to the redistribution of Crn7 from membranes. HeLa cells were treated with either DMSO or genistein (25 μg/ml), fractionated and analysed by western blotting using the Crn7 antibody. Note the complete redistribution of Crn7 into the cytosol. (*B*), (*C*) Inhibition of Src results in the dissociation of Crn7 (red) from the Golgi. (*B*) Control HeLa cells; (*C*) Cells treated with 0.4 μM SU6656 for 2 h. Images were taken at same settings of microscope and software. Asterisks: residual staining in the Golgi zone. (*D*), (*E*) Expression of kinase-dead Src K295M (green) affects the Golgi localization of Crn7 (red). Note that the bulk of Crn7 signal is in the perinuclear Golgi region in GFP-negative cells, while in GFP-SrcK295M cells the protein demonstrates predominantly cytosolic localization (*E*). Bars, 20 μm.

methods) with recombinant GST-tagged full-length Src (Jena Bioscience) resulted in phosphorylation of Crn7 (Fig. 5C), suggesting that our data on phosphorylation of Crn7-derived peptides by Src are relevant to full-length Crn7. The corresponding Coomassie blue stained gel is shown in the lower panel. Mutations in the two predicted Src phosphorylation sites (Y288A/Y758A) resulted in a strong decrease in Crn7 phosphorylation signal. The quantification of this series of experiments indicated that the Y288A/Y758A mutant exhibited a level of phosphorylation indistinguishable from the SU6656 control, while wild type protein was strongly phosphorylated (Fig 5D). While we can not exclude the presence of additional Src target tyrosines in Crn7, our data suggest that tyrosine residues 288 and 758 are major sites of Src-mediated phosphorylation of the full-length protein.

Next, we tested whether the *in vitro* phosphorylation of Crn7 and of peptides harbouring the predicted Src phosphorylation sites has any relevance *in vivo*. Firstly, we performed co-immunoprecipitation experiments in order to reveal the possibility of a physical interaction between Crn7 and Src *in vivo*. Fig. 5E demonstrates that Crn7 can indeed be co-immunoprecipitated with both total Src and activated Src phosphorylated on tyrosine-418 from HeLa cell lysate. Secondly, *in vitro* phosphorylation experiments were further performed with immunoprecipitated endogenous full-length Crn7. Aliquots of immunoprecipitated Crn7 protein on Protein A-Sepharose beads were mixed with the kinase reaction mix (see Materials and methods) and full-length recombinant Src. In this experiment, non-tagged kinase (Upstate) was used instead of the GST-tagged enzyme used in Fig. 5C,D. Although this preparation demonstrated significantly lower activity in both autophosphorylation and phosphorylation of Crn7, it helped achieve better separation of signals from Crn7 and Src. Fig. 5F

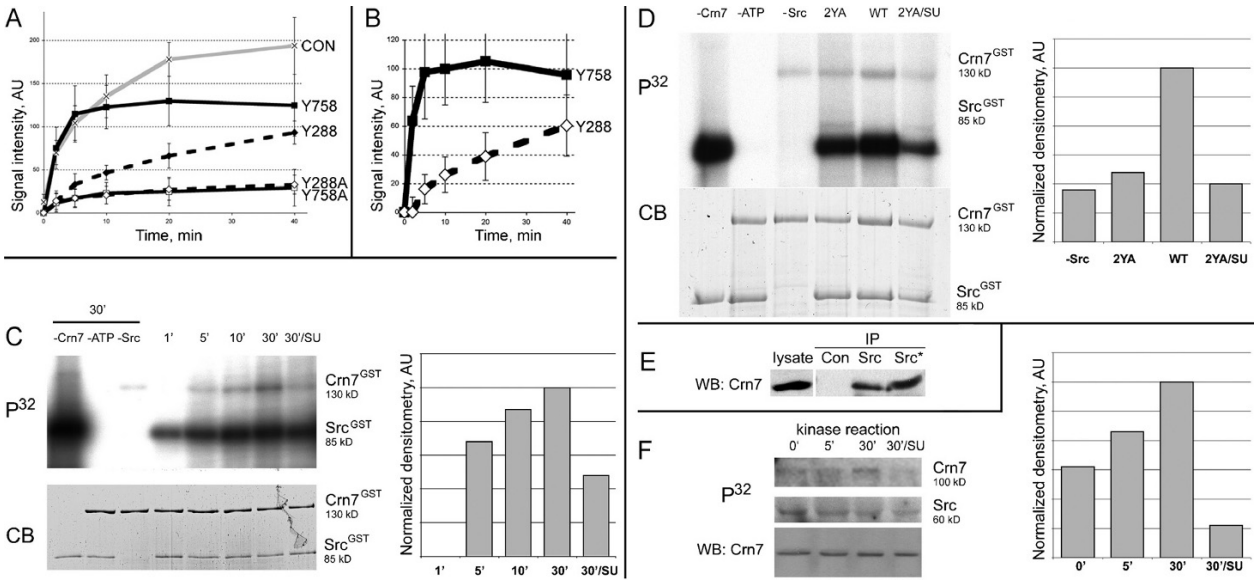


**Figure 5.** Phosphorylation of Crn7 by protein kinase Src *in vivo* and *in vitro*. (*A*) *In vitro* phosphorylation of Crn7-derived synthetic peptides by recombinant Src. Time course of $\gamma^{32}$P-ATP incorporation into a control peptide (CON), peptides enclosing Y288 and Y758, and corresponding mutant peptides (Y288A, Y758A). X-axis, time, minutes; Y-axis, radioisotope signal as measured by the intensity of emitting spots using the PhosphorImager. AU, arbitrary units. See text for details. (*B*) Normalized graph showing the time course of $\gamma^{32}$P-ATP incorporation into the peptides enclosing Y288 and Y758 after the subtraction of mutated peptide values. Axes as in A. (*C*) *In vitro* phosphorylation of full-length recombinant Crn7 protein in the presence of recombinant Src. Upper panel, autoradiogram of time-dependent phosphorylation of Crn7 and autophosphorylation of Src. Lower panel, the corresponding Coomassie brilliant blue stained gel. First three lanes represent control experiments where Crn7 (first lane), ATP (second lane) or Src (third lane) were omitted. Lanes 4–7 represent a time course experiment demonstrating time-dependent phosphorylation of Crn7 by Src after 1–30 minutes of the *in vitro* reaction. The last lane represents the specificity control experiment where Crn7 was incubated with Src for 30 min in the presence of the Src inhibitor SU6656. Right, corresponding $^{32}$P-Crn7 densitometry values were normalized to the amount of Crn7 and Src proteins loaded (Coomassie stained gel) and corrected for background values. (*D*) *In vitro* phosphorylation of full-length recombinant Y288A/Y758A Crn7 mutant. Upper panel, autoradiogram of phosphorylation of Crn7 and autophosphorylation of Src after 30 min of kinase reaction. Lower panel, the corresponding Coomassie brilliant blue stained gel. First three lanes represent control experiments (as in panel C). Lanes 4–6 represent an experiment demonstrating the lack of phosphorylation of the double mutant. Right, corresponding densitometry values calculated as in C. Note that the phosphorylation level of the double mutant after 30 min (lane 4) is comparable with that in the presence of Src inhibitor (lane 6) and is significantly lower than the phosphorylation level of wild type protein (lane 5). (*E*) Physical interaction of endogenous Src and Crn7. Src was immunoprecipitated from HeLa cells as described in Materials and methods using antibodies recognizing either total or activated (phosphorylated on tyrosine-418, star) Src. Crn7 was detected in the precipitate by western blotting using Crn7 antibody. (*F*) *In vitro* phosphorylation of full-length endogenous immunoprecipitated Crn7 protein by recombinant Src. Upper panel, phosphorylation of immunoprecipitated Crn7; middle panel, autophosphorylation of Src in the same reaction. Lower panel: loading control; the western blot was probed with Crn7 antibody (100 kDa). Right, corresponding $^{32}$P-Crn7 densitometry values were normalized to the amount of Crn7 protein loaded (Western blot) and corrected for background values.

demonstrates that immunoprecipitated full-length Crn7 was phosphorylated by recombinant Src *in vitro* in a time-dependent manner. Baseline phosphorylation level was observed in the presence of SU6656. Autophosphorylated Src was present as a band more prominent than that of Crn7, and was utilized as loading control in parallel with control western blotting with Crn7 antibody.

Experiments shown in Figure 4 strongly implied the requirement for Src activity for the targeting of Crn7 to the Golgi. Previously, we reported that Crn7 knockdown leads to the accumulation of cargo in the Golgi complex due to the block of the formation of export intermediates [7]. If we imply that the regulation of Crn7 by Src is important for the function of Crn7 in the biosynthetic pathway, then interfering with Src activity should lead to defects in transport comparable to those of Crn7 knock down cells. To test this hypothesis, we expressed horseradish peroxidase fused to a signal sequence [ssHRP, see 10, 21] in HeLa cells and assayed ssHRP secretion after SU6656 treatment. To this end, ssHRP-expressing cells were pre-treated with SU6656 at 0.4–2 μM for 1 h, then the cell culture medium was replaced and cells were allowed to secrete ssHRP for 2 h. Chemiluminescence of aliquots of cell culture supernatant was measured after mixing with standard ECL reagent as described in Materials and methods. We observed a concentration-dependent (Fig. 6A) and time-dependent (data not shown) decrease in the HRP signal in the medium derived from SU6656-treated cells as compared to cells treated with DMSO alone. Treatment with SU6656 in concentrations of 0.4 μM, 2 μM (Fig. 6) or up to 50 μM (not shown) did not result in major

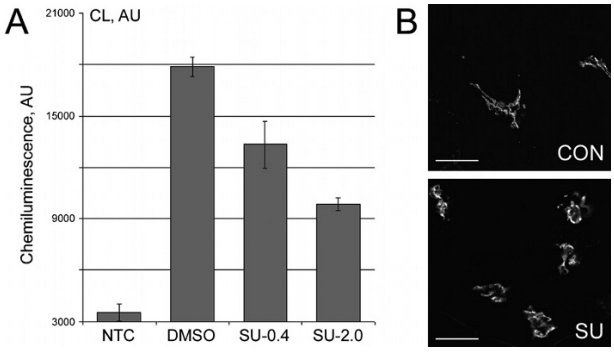


**Figure 6.** (*A*) Effect of SU6656 on secretion of signal sequence-tagged HRP from HeLa cells. Chemiluminescence of secreted ssHRP in medium was measured and plotted vs. control (non-transfected cells) after normalization by cell count as described in Materials and Methods. Y-axis, chemiluminescence, arbitrary units (AU). NTC – non-transfected cells; DMSO – vehicle control; 0.4, 2 – concentrations of SU6656 in μM. (*B*) Effect of SU6656 on Golgi structure in HeLa cells expressing GFP-tagged mannosidase II. Top panel, DMSO-treated control cells; lower panel – cells treated with 0.4 μM SU6656 for 2 h. Although the Golgi structure appears slightly compacted, there is no evidence of Golgi fragmentation or dispersal. Bars, 10 μm.

changes in Golgi morphology similar to those seen in Crn7 siRNA cells [see Ref. 7].

**Crn7 targeting to the Golgi requires the integrity of the AP-1 adaptor complex and presence of cargo.** We previously demonstrated that Crn7 interacts with the cargo adaptor protein complex AP-1 *in vivo* and *in vitro* [7]. To find out whether Crn7 localization on Golgi membranes requires AP-1, we used pre-tested commercial siRNA (Dharmacon) targeting μ1-adaptin, the subunit of AP-1 demonstrated to bind Crn7. In control siRNA-treated cells, Crn7 co-localized with γ-adaptin, another component of the AP-1 complex, on Golgi membranes but not in any other area [Fig. 7A, see also Ref. 7]. Downregulation of the μ1 subunit (AP1M1 RNAi) resulted in gradual dispersal of both γ-adaptin and Crn7 from the Golgi (Fig. 7A). The amount of Crn7 on membranes was directly proportional to that of AP-1. Western blot data indicated that overall levels of Crn7 and γ-adaptin were not affected by μ1 subunit RNAi (not shown). Together with our previous findings these data suggest that the presence of AP-1 on the Golgi is required for targeting of Crn7 to membranes, but not vice versa. That AP-1 complex does not bind to membranes in μ1-adaptin deficient cells has been demonstrated previously by the Schu laboratory [22]. In fact, γ-adaptin staining of cells derived from μ1 knockout mice [see Fig. 3 in Ref. 22] is exactly similar to our γ-adaptin staining of μ1 siRNA cells (Fig. 7A). Importantly, knockdown of μ1-adaptin using RNAi results in a marked fragmentation of Golgi membranes (Fig. 7B). These data are in line with our earlier observation that downregulation of

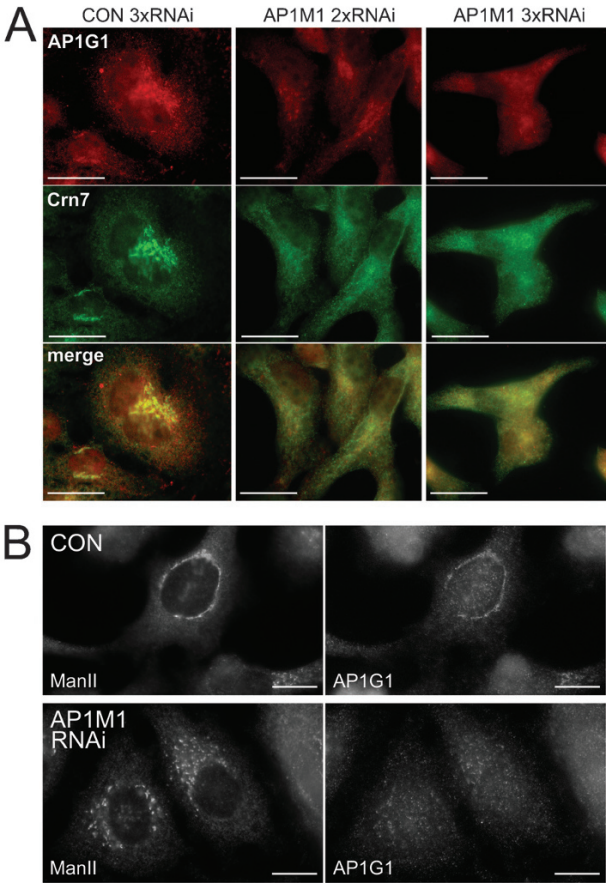


**Figure 7.** Depletion of μ1-adaptin by siRNA leads to the dispersal of Crn7 from the Golgi. (*A*) HeLa cells were transfected with validated siRNA targeting μ1-adaptin (AP1M1) or with control siRNA (CON) for either six (two rounds of siRNA transfection) or nine (three rounds of transfection) days as indicated. Cellular level and distribution of γ-adaptin (AP1G1) and Crn7 were studied by immunofluorescence. Note the gradual dispersal of Crn7 from the Golgi complex correlating with the disappearance of γ-adaptin from Golgi membranes. Bars, 20 μm. (*B*) Cells expressing mannosidase II-GFP were double-transfected with μ1-adaptin (AP1M1 RNAi) or control siRNA (CON) for a total of nine days, and the Golgi morphology (ManII) and localization of γ-adaptin (AP1G1) were examined by fluorescence microscopy. Note the marked Golgi fragmentation in AP1M1, but not in control cells (left). AP1M1 knockdown was verified as in Figure 7A by the dispersal of γ-adaptin (AP1G1) from the Golgi (right). Bars, 10 μm.

Crn7 by siRNA fragments the Golgi [7]. Removal of μ1-adaptin and the resulting disruption of the AP-1 complex lead to dispersal of membrane-attached Crn7 and thus mimic the effect of Crn7 knockdown. One implication hereof is that the depletion of the membrane-attached pool of Crn7 is sufficient for the Golgi fragmentation even in the presence of unchanged cellular levels of Crn7 protein.

The docking of AP-1 on the Golgi correlates with the interaction of its μ1 subunit with characteristic tyrosine-based motifs exposed on cytosolic tails of transmembrane cargo and cargo receptors [reviewed in 23]. We tried to find out whether the depletion of cargo

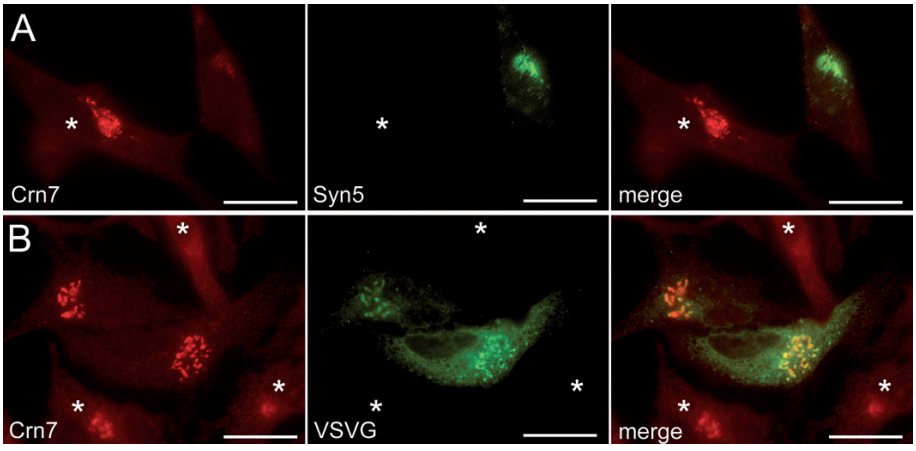


**Figure 8.** The presence of cargo in the Golgi complex is required for the targeting of Crn7. (*A*) Overexpression of GFP-Syn5 (green) leads to the redistribution of Crn7 (red) from the Golgi. HeLa cells were transiently transfected with GFP-Syn5 and analyzed for Crn7 distribution. Note the strong reduction of Crn7 signal in the perinuclear region in the cell overexpressing Syn5 as compared to an untransfected cell (asterisk). (*B*) Redistribution of Crn7 to Golgi membranes in HeLa cells upon the accumulation of cargo in the TGN. Note the strong Golgi Crn7 staining and downregulation of cytosolic Crn7 in VSVG-transfected cell as compared to non-transfected cells (asterisks). Bars, 20 μm.

would result in dissociation of Crn7 from the Golgi and used two alternative methods to test the possible functional link between the presence of cargo in the biosynthetic pathway and localization of Crn7 on Golgi membranes.

A syntaxin 5 containing cis-Golgi t-SNARE complex participates in late stages of ER-to-Golgi transport [24]. Syntaxin 5 (Syn5) specifies docking sites for both COPI and COPII vesicles in the Golgi complex [25 and references therein]. Overexpression of Syn5 inhibits the import of cargo exemplified by VSVG-tsO45 into the Golgi complex, leading to accumulation of cargo in pre-Golgi intermediates [26]. We overexpressed GFP-Syn5 in HeLa cells and examined Crn7 localization by immunofluorescence analysis. Cells expressing GFP-Syn5 exhibited a significant reduction of Crn7 on the Golgi membranes as compared to untransfected cells (Fig. 8A).

Further, we used a 20°C block to accumulate cargo in the TGN [27]. We incubated cells at 40°C overnight to accumulate VSVG in the ER, and then transferred them for 6 h to 20°C to synchronously release the cargo from the ER and arrest it in the TGN. We observed a marked increase of Crn7 staining on TGN membranes, where it co-localized with VSVG (Fig. 8B), and a decrease of cytosolic Crn7 staining. Because we did not block protein synthesis, the VSVG signal is present in both Golgi and ER in Figure 8B. These two sets of experiments infer that an accumulation of cargo in the trans-Golgi network is sufficient to target the bulk of Crn7 from the cytosol to Golgi membranes, while cargo depletion by overexpression of Syn5 leads to Crn7 dispersal.

## Discussion

Our previous data implied that Crn7 is indispensable to Golgi architecture and function, because Crn7 RNAi resulted in Golgi fragmentation and blocked protein export from the TGN. We also showed that Crn7 knockdown does not impair the recruitment of AP-1 complex to TGN membranes [7], suggesting that Crn7 functions downstream of the AP-1 interaction with Golgi cargo or cargo receptors. Here, we demonstrate that the direct and specific phosphorylation of Crn7 by Src is required for the targeting of Crn7 from the cytosol to the Golgi. Crn7 further requires a functional AP-1 complex and cargo for its targeting to the Golgi.

Our data allow us to propose the following model for Crn7 recruitment to the Golgi. Cargo proteins or cargo receptors interact with AP-1, and this complex recruits Crn7 phosphorylated by Src on tyrosine-758 to Golgi membranes. Binding of Crn7 to the Golgi is at least partially mediated by the interaction of a tyrosine-288-based sorting motif of Crn7 with μ1-adaptin [7]. In the absence of cargo, Crn7 remains cytosolic (see Fig. 8A). If the integrity of the AP-1 complex is compromised, Crn7 remains cytosolic as well (Fig. 7A), and the Golgi morphology becomes very similar to that in Crn7 siRNA cells. Redistribution of Crn7 from the Golgi into cytosol can be experimentally achieved by interfering with Src activity (Fig. 4). Importantly, the dispersal of Crn7 due to Src inhibition correlates with a defect in secretion (Fig. 6A), implying that not merely the presence of a physiological amount of Crn7 protein but its localization to Golgi membranes is required for

its function in the secretory pathway. Previously, we demonstrated that Crn7 knockdown by siRNA results in a block of cargo progression as well [7]. We conclude that the effect of Crn7 siRNA reflects the inability of the Golgi complex to maintain its integrity and function as a result of the depletion of the membrane-bound pool of Crn7. Data presented in this manuscript strongly suggests that the cytosolic pool is not relevant to Golgi structure and function. It might merely represent a depot of membrane-unbound protein, as is the case with other membrane traffic regulators like ARNO [28]. Otherwise, cytosolic Crn7 may play some role in Golgi-unrelated cellular process(es), e.g. regulation of the cytoskeleton, a core function of coronin proteins, or other specific functions of Crn7 in the cytosol.

The molecular mechanisms of Crn7 action at the site of formation and detachment of a secretory vesicle for export remain to be elucidated. Current efforts are aimed at the identification of further Crn7 interaction partners and at the analysis of a possible interplay between Crn7 and the Gβγ-PKCη-PKD machinery participating in the membrane fission at the TGN [29–34]. Our previous data suggested that Crn7 here may be functioning directly upstream of the PKD activation [7].

Src is a tyrosine kinase involved in a wide variety of cellular functions [reviewed in 35, 36]. It has been demonstrated to play an important role in secretion in a wide variety of cell types [37–45]. Best known is its role upstream of hormone- and growth factor-induced secretion, where chemical inhibition of Src negatively affects the secretory response [39, 41–43, 45]. Additionally, Src has been shown to mediate lipopolysaccharide-induced secretion of TNFα in macrophages [40] and secretion of gastric mucin regulated by β-adrenergic receptor [44].

While most studies on the molecular mechanism of Src function in the regulation of secretion have been aimed at the characterization of classical secondary messenger cascades downstream of Src [39], this kinase was also suspected to play a more direct trafficking-related role in the Golgi [46, 47]. Src is required for the translocation of activated H-Ras to the Golgi complex where it could activate Erk and Akt signalling [48], although it is not yet clear whether H-Ras is directly phosphorylated by Src. Ectopic expression of Src in SYF cells lacking Src, Yes and Fyn kinases resulted in the relocation of KDEL receptor from the Golgi to the ER and reduction of the rate of retrograde trafficking of *Pseudomonas* toxin [49]. The authors explained these findings by implying that Src decreased the accessibility of the receptor to the toxin because upon Src activation the receptor was recruited back to the ER more rapidly. We demonstrate here a significant effect of Src inhibition on general protein secretion.

What is the mechanism of activation of Src leading to the regulation of Crn7 targeting? An intriguing hypothesis is that the pathway leading to such activation involves growth factor receptor signalling. It has been known for more than a decade that Src is a downstream target of growth factor receptors [50–55]. It is plausible that growth factor-regulated secretion relies on direct or indirect activation of Src by growth factor receptors and subsequent Src-dependent translocation of a major pool of Crn7 to Golgi membranes where it is required for the formation of transport intermediates. Together with growth factor-mediated induction of expression of secretory molecules, such regulation would provide an effective basis for upregulation of both general and specific secretion.

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