

Cargo Selectivity of the ERGIC-53/MCFD2 Transport Receptor Complex

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Exit of soluble secretory proteins from the endoplasmic reticulum (ER) can occur by receptor-mediated export as exemplified by blood coagulation factors V and VIII. Their efficient secretion requires the membrane lectin ER Golgi intermediate compartment protein-53 (ERGIC-53) and its soluble luminal interaction partner multiple coagulation factor deficiency protein 2 (MCFD2), which form a cargo receptor complex in the early secretory pathway. ERGIC-53 also interacts with the two lysosomal glycoproteins cathepsin Z and cathepsin C. Here, we tested the subunit interdependence and cargo selectivity of ERGIC-53 and MCFD2 by short interference RNA-based knockdown. In the absence of ERGIC-53, MCFD2 was secreted, whereas knocking down MCFD2 had no effect on the localization of ERGIC-53. Cargo binding properties of the ERGIC-53/MCFD2 complex were analyzed *in vivo* using yellow fluorescent protein fragment complementation. We found that MCFD2 is dispensable for the binding of cathepsin Z and cathepsin C to ERGIC-53. The results indicate that ERGIC-53 can bind cargo glycoproteins in an MCFD2-independent fashion and suggest that MCFD2 is a recruitment factor for blood coagulation factors V and VIII.

Key words: cargo receptor, endoplasmic reticulum, ER-Golgi intermediate compartment, lectin, protein fragment complementation, protein retention, protein secretion

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After folding, N-glycosylation and oligomerization, newly synthesized secretory proteins leave the endoplasmic reticulum (ER) in coat protein II (COP II)-coated vesicles (1,2). Membrane proteins can be recruited into COP II-coated vesicles by interaction of their cytosolic tails with the Sec23/24 complex of the COP II coat (3–6). In contrast, soluble secretory proteins cannot rely on a direct interaction with the COP II coat for topological reasons. Their export is believed to occur by either bulk flow or receptor-mediated export (7). According to the bulk flow model, proteins enter COP II-coated vesicles by default due to their high concen-

tration in the ER (8). In the receptor-mediated export model, membrane receptors bind to soluble cargo proteins, thereby recruiting them into COP II-coated vesicles (9).

Interactions of cargo receptors with ER export signals of soluble secretory proteins have been characterized only recently. Two such cargo receptors have been studied in detail. The yeast membrane protein Erv29p binds a hydrophobic ER export signal in its cargo protein glycosylated pro- α -factor (10,11). The mammalian membrane protein ER Golgi intermediate compartment protein-53 (ERGIC-53) recognizes an ER export signal in the cargo protein cathepsin Z that is composed of a combined oligosaccharide/peptide structure (12,13). ERGIC-53 is a 53-kDa type 1 membrane protein that operates as a mannose lectin cycling between the ER and the ERGIC (14–17). The cytosolic diphenylalanine motif in ERGIC-53 interacts with the COP II coat, thereby recruiting ERGIC-53 and its bound cargo to anterograde vesicles (6). A dilysine motif in the cytosolic tail of ERGIC-53 mediates retrieval back to the ER by interacting with the coat protein I (COP I) coat (18). ERGIC-53 acts as a cargo receptor for two lysosomal glycoproteins cathepsin Z and cathepsin C (12,19,20). Mutations in ERGIC-53 can lead to combined factor V and factor VIII deficiency in humans (OMIM #227300). Patients with loss of function mutations in ERGIC-53 show reduced levels of blood coagulation factors V and VIII in their plasma (21). Biochemical studies established a role of ERGIC-53 as a cargo receptor required for efficient transport of factors V and VIII in cultured mammalian cells (22). Recently, the multiple coagulation factor deficiency 2 gene (MCFD2) was identified as a second locus responsible for blood coagulation factor V and VIII deficiency (23). The MCFD2 gene encodes a soluble 16-kDa protein in the lumen of the ER. The protein possesses two EF-hand domains and interacts with ERGIC-53 in a calcium-dependent manner. Chemical cross-linking showed an interaction of factor VIII with both MCFD2 and ERGIC-53, suggesting that ERGIC-53 and MCFD2 operate as a cargo receptor complex (23,24).

Here, we have investigated the interdependence and cargo selectivity of the two subunits of the ERGIC-53/MCFD2 receptor complex by a knockdown approach using short interference RNA (siRNA). We show that ERGIC-53 is strictly required for the retention of MCFD2 in the early secretory pathway. Depletion of MCFD2 by siRNA did not affect binding of cathepsin Z and cathepsin C to ERGIC-53. The results suggest that MCFD2 is a protein specifically required for factors V and VIII but not for the lectin function of ERGIC-53.

Results

Localization of endogenous MCFD2

Overexpressed MCFD2 was previously shown to co-localize with ERGIC-53 to the ERGIC (23). To study the localization of endogenous MCFD2, immunofluorescence microscopy experiments were performed in HeLa cells. Figure 1 shows that endogenous MCFD2 co-localized with ERGIC-53 but only minimally with the two ER markers B-cell receptor associated protein 31 (BAP31) and cytoskeleton-linking membrane protein 63 (CLIMP-63). Next, we examined if MCFD2 cycles in the early secretory pathway. Cycling proteins are known to accumulate in ERGIC clusters in response to brefeldin A (BFA) (25). Brefeldin A treatment indeed led to the accumulation of MCFD2 in ERGIC-53 clusters, while the localization of the two ER resident proteins BAP31 and CLIMP-63 remained unchanged (Figure 1). These data suggest that endogenous MCFD2 localizes to the ERGIC and cycles in the early secretory pathway.

ERGIC-53 retains MCFD2 in the early secretory pathway

Do MCFD2 and ERGIC-53 form a stable complex during their entire cycling journey? There are indications that ER and ERGIC may differ in their luminal pH and ionic properties (26). It is possible, therefore, that the calcium-dependent interaction of MCFD2 and ERGIC-53 does not persist throughout the entire cycling process. Co-immunoprecipitation of MCFD2 and ERGIC-53 from

BFA-treated cells (not shown) suggests that MCFD2 and ERGIC-53 interact in ERGIC clusters. Moreover, ERGIC-53-deficient lymphoblasts contain only trace amounts of intracellular MCFD2, indicating that MCFD2 requires ERGIC-53 either for stabilization or for intracellular retention (23). Experimental demonstration of a requirement for intracellular retention of MCFD2 by ERGIC-53 would directly prove a physiologically relevant post-ER interaction of the two cycling proteins. To test this, we studied the effect of siRNA-based ERGIC-53 silencing on the localization of MCFD2. Three siRNA duplexes designed against different ERGIC-53 target sequences were tested for knockdown efficiencies. The most efficient siRNA duplex reduced ERGIC-53 levels in HeLa cells to less than 10% within 3 days. In ERGIC-53-depleted cells, only trace amounts of intracellular MCFD2 could be detected by immunoblotting. Immunofluorescence microscopy confirmed that MCFD2 was no longer detectable in these cells. The effect on MCFD2 is specific because the localization of BAP31 and giantin was not affected (Figure 2A) and total secretion of ³⁵S-methionine-labeled proteins was unchanged (not shown). Although ERGIC-53 is a major protein of the ERGIC, its depletion does not seem to impair the morphology of the early secretory pathway as indicated by the normal localization of organelle markers of the early secretory pathway including numerous ER, ERGIC and Golgi proteins (not shown). An intact early secretory pathway after ERGIC-53 depletion is in line with previous observations showing that mistargeting of ERGIC-53 to the ER does not result in morphological changes of the early secretory pathway (19).

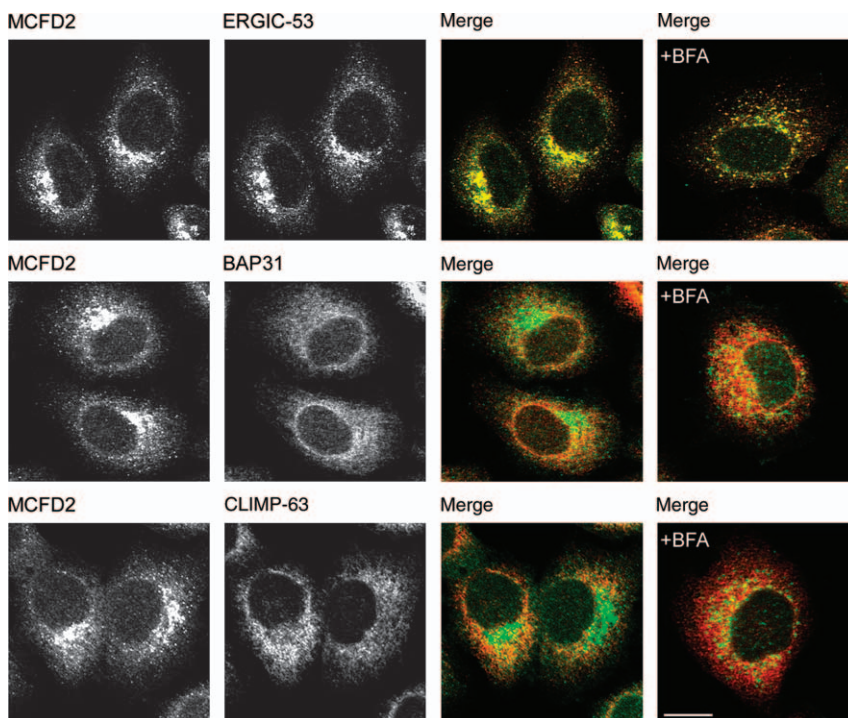


Figure 1: MCFD2 co-localizes with ERGIC-53. Localization of MCFD2 in HeLa cells visualized by double immunofluorescence microscopy using organelle marker antibodies for ERGIC (ERGIC-53, affinity-purified polyclonal antibody) and ER (BAP31 and CLIMP-63; mAbs). The cells were left untreated or incubated with brefeldin A (+BFA) for 90 min at a concentration of 10 μ g/mL. The MCFD2 is shown in green, while BAP31, CLIMP-63 and ERGIC-53 are shown in red. Bar = 10 μ m.

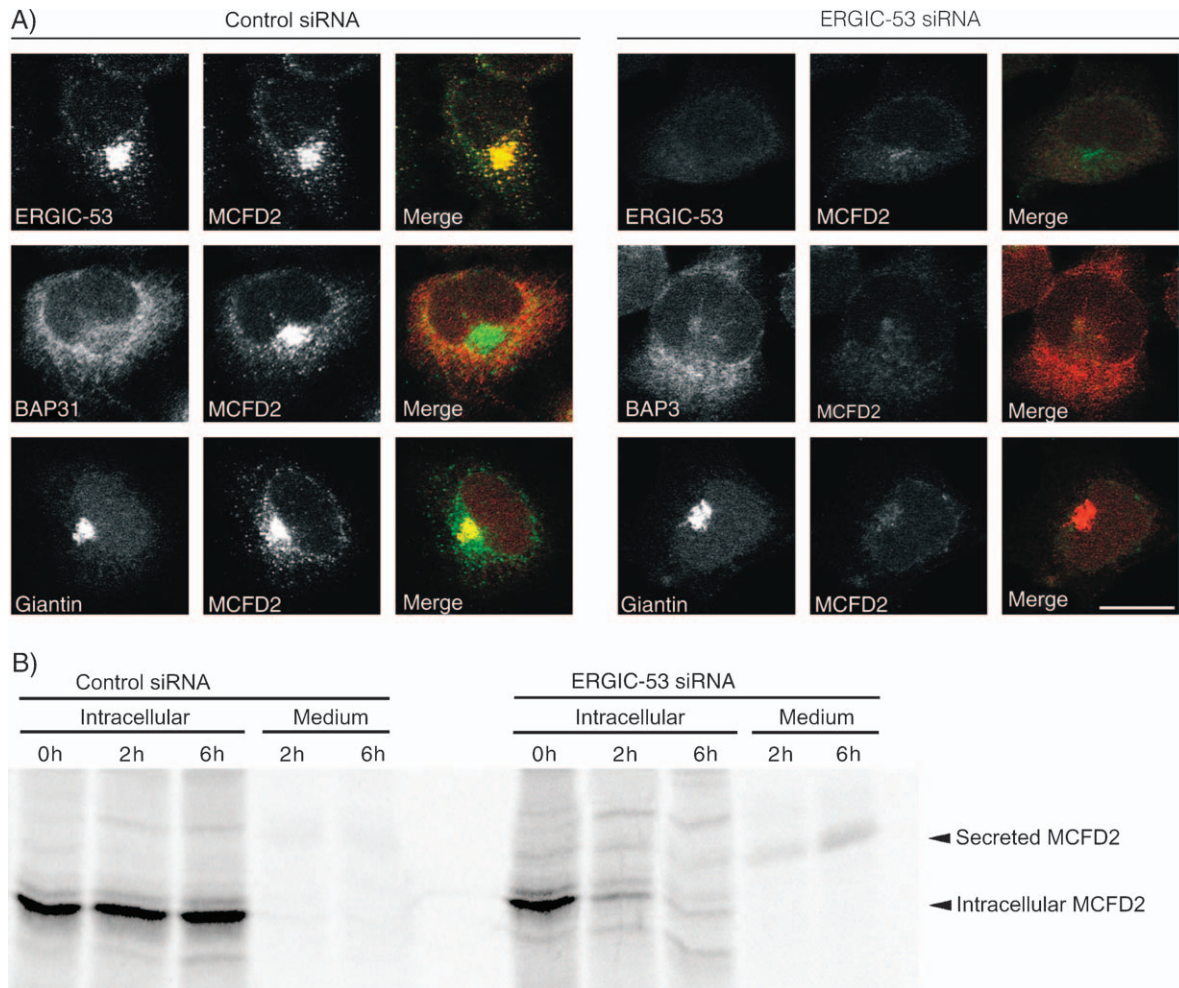


Figure 2: ERGIC-53 is required for the intracellular retention of MCFD2. A) HeLa cells were transiently transfected with control or ERGIC-53 siRNA duplexes. Seventy-two hours after transfection, MCFD2 was visualized by double immunofluorescence microscopy together with ERGIC-53, BAP31 or giantin (Golgi). In ERGIC-53-depleted HeLa cells, only trace amounts of MCFD2 are detectable. The effect on MCFD2 is specific because BAP31 and giantin are unaffected. Bar = 10 μ m. B) Control and ERGIC-53 siRNA-transfected HeLa cells were pulsed with 35 S-methionine for 1 h and chased for the indicated times. The MCFD2 was immunoprecipitated from cell lysates and from conditioned medium. Note that depletion of ERGIC-53 leads to secretion of MCFD2.

To investigate the disappearance of MCFD2 after ERGIC-53 knockdown, pulse-chase experiments with 35 S-methionine were performed. Cell lysate and conditioned medium of control and ERGIC-53 siRNA-transfected HeLa cells were probed for 35 S-methionine-labeled MCFD2 after 2 and 6-h chase periods. In ERGIC-53-depleted cells, intracellular MCFD2 disappeared and was detected as secreted protein in the conditioned medium (Figure 2B). Secreted MCFD2 showed a higher apparent M_r than the initially synthesized protein due to *O*-glycosylation (24). *O*-glycosylation may render MCFD2 less accessible to the antibody, accounting for the only partial recovery of MCFD2 in the conditioned medium. Alternatively, a fraction of MCFD2 may be degraded rather than secreted. Secreted, *O*-glycosylated MCFD2 can also be recovered from conditioned medium on overexpression of the protein (Figure 3). The results shown in Figure 2 show that ERGIC-53 is strictly required

for intracellular retention of MCFD2, indicating that MCFD2 and ERGIC-53 interact in post-ER compartments of unperturbed cells. In HeLa, COS, HepG2 and several glioblastoma cell lines, endogenous ERGIC-53 retains all MCFD2. No secreted MCFD2 could be detected in the conditioned medium (data not shown).

MCFD2 interacts with ERGIC-53 in the ER

Next, we examined if MCFD2 and ERGIC-53 can also interact in the ER. To this end, MCFD2 was localized in HeLa KKAA cells that express a dominant-negative form of ERGIC-53 that is retained in the ER by a C-terminal KKAA retention signal (19,27). In the presence of tetracycline, the expression of ERGIC-53-KKAA is repressed. Under these conditions, ERGIC-53 and MCFD2 co-localized to the ERGIC and were both BFA sensitive (Figure 4A) like in

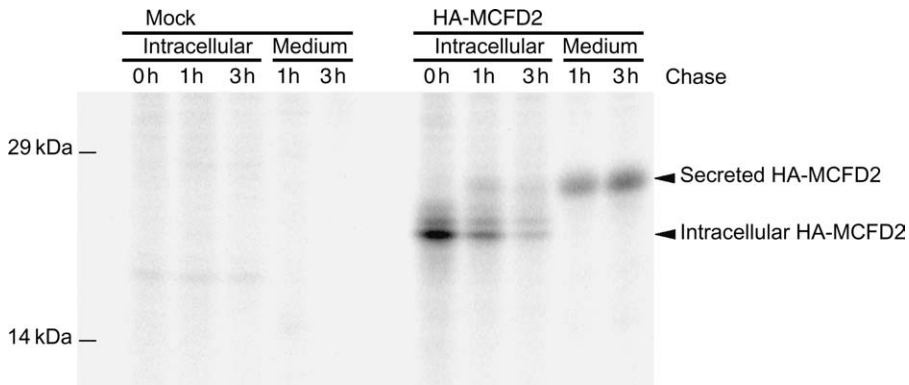


Figure 3: Overexpressed MCFD2 is secreted. HA-MCFD2-transfected and mock-transfected HeLa cells were labeled with ³⁵S-methionine for 30 min and chased for the indicated times. MCFD2 was immunoprecipitated from cell lysates and conditioned medium using polyclonal anti-MCFD2. Intracellular HA-MCFD2 disappears during the 3-h chase period and can be recovered from conditioned medium. Secreted HA-MCFD2 shows a shift in *M_r* due to *O*-glycosylation (24).

wild-type HeLa cells. Removal of tetracycline, which induces the expression of ERGIC-53-KKAA, mislocalizes MCFD2 to the ER (Figure 4B). ER localization of MCFD2 is indicated by co-localization with BAP31 as well as resistance to BFA. The mislocalization of MCFD2 by ER-retained ERGIC-53 shows that the two proteins can also interact in the ER.

ERGIC-53 binds certain cargo glycoproteins in an MCFD2-independent fashion

Previous genetic and biochemical data showed that ERGIC-53 and MCFD2 form a cargo receptor complex, recognizing factors V and VIII (23,24). Factor VIII can be cross-linked to both ERGIC-53 and MCFD2 with similar efficiency, indicating the existence of a triple complex.

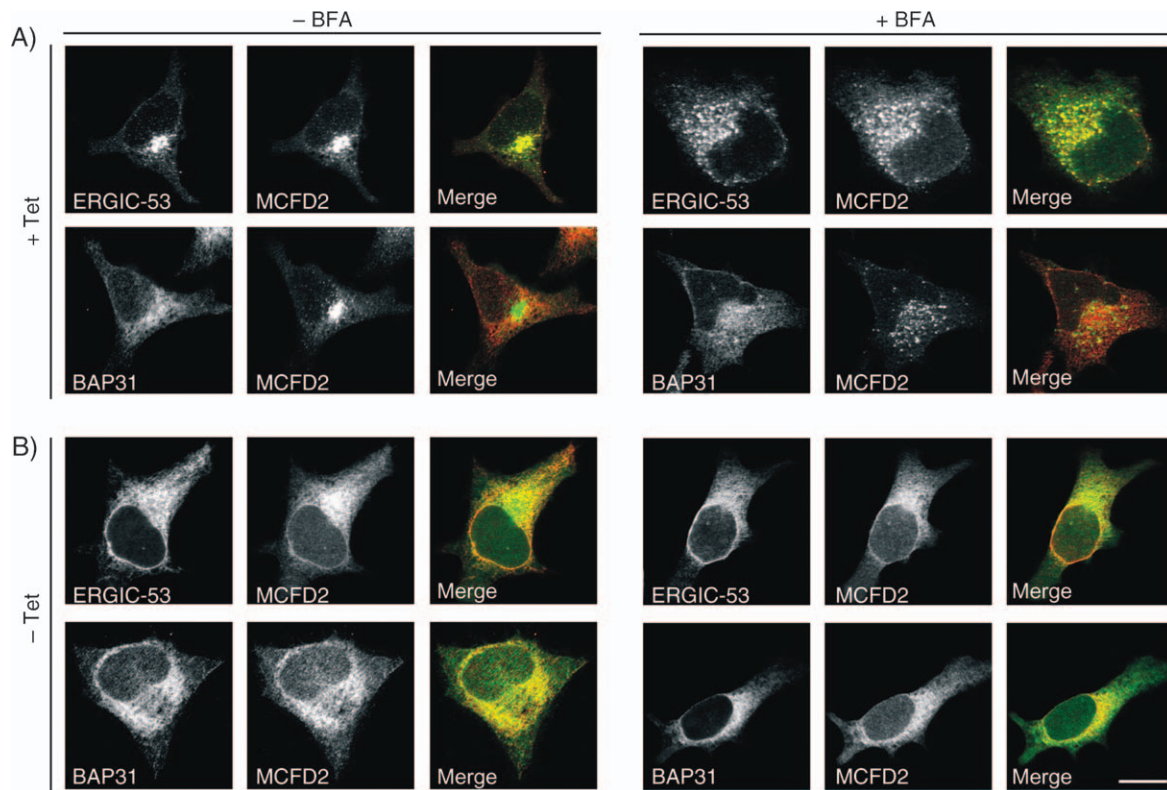


Figure 4: ERGIC-53-KKAA retains MCFD2 in the ER. MCFD2, BAP31 and ERGIC-53 were visualized by immunofluorescence microscopy in HeLa cells expressing ER-retained ERGIC-53-KKAA in a tetracycline-dependent inducible manner. Prior to fixation, the cells were treated with BFA (+BFA) (10 μg/mL for 90 min) or left untreated (–BFA). A) In the presence of tetracycline (+Tet), the expression of ERGIC-53-KKAA is repressed and the cells express only endogenous ERGIC-53. MCFD2 co-localizes with ERGIC-53 and is BFA sensitive. B) Withdrawal of tetracycline (–Tet) for 48 h induces ERGIC-53-KKAA that retains all ERGIC-53 in the ER due to a dominant-negative effect. Expression of ERGIC-53-KKAA relocalizes MCFD2 to the ER. Co-localization with BAP31 and BFA resistance show the ER retention of MCFD2. Bar = 10 μm.

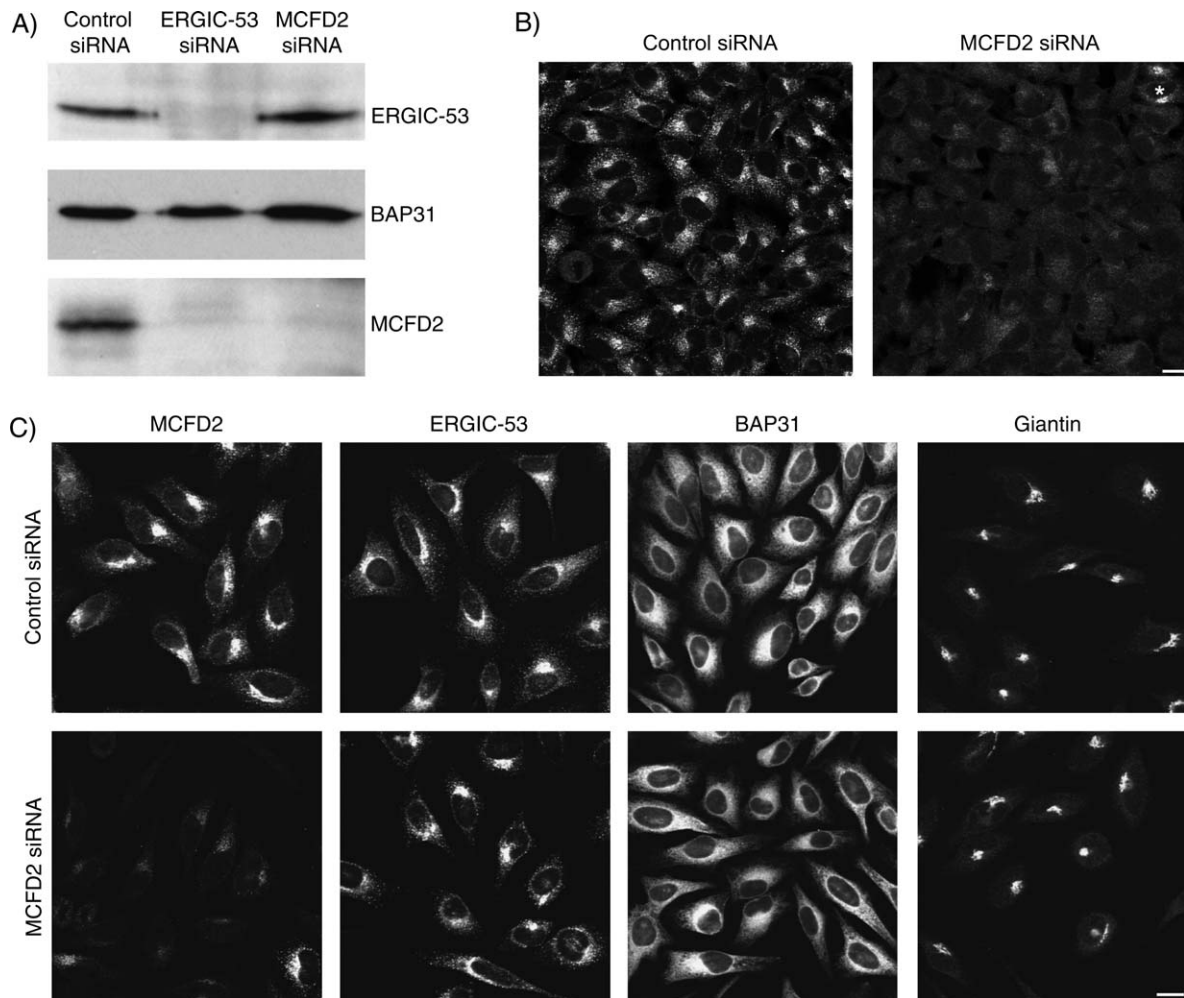


Figure 5: Depletion of MCFD2 affects neither localization nor expression of ERGIC-53. A) HeLa cells were transiently transfected with control, ERGIC-53 and MCFD2 siRNA duplexes. Seventy-two hours after transfection, ERGIC-53, BAP31 and MCFD2 were visualized by immunoblotting. Transfection of ERGIC-53 siRNA depletes both ERGIC-53 and MCFD2, whereas transfection of MCFD2 siRNA only depletes MCFD2. BAP31 levels are unaffected by ERGIC-53- and MCFD2-specific siRNA transfection. B) The very high (>95%) transfection efficiency of MCFD2 siRNA duplexes was visualized by immunofluorescence microscopy in control and MCFD2 siRNA-transfected HeLa cells 72 h after transfection. A nontransfected cell is marked (*). C) MCFD2, ERGIC-53, BAP31 and giantin were visualized by immunofluorescence microscopy in control and MCFD2 siRNA-transfected cells. Depletion of MCFD2 from HeLa cells does not change the localization pattern of ERGIC-53, BAP31 or giantin. Bar = 10 μ m.

ERGIC-53 also interacts with the two lysosomal glycoproteins cathepsin Z and cathepsin C (12,19,20). To test if the MCFD2 subunit of the cargo receptor complex is required more generally for cargo binding, an siRNA-based knock-down approach was taken. Six siRNA duplexes, designed against different MCFD2 target sequences, were probed for knockdown efficiencies. The most efficient siRNA duplex reduced MCFD2 in HeLa cells to less than 10% within 3 days. Transfection efficiency of siRNA was very high as only very few cells stained positive for MCFD2 72 h after transfection (Figure 5B). Depletion of MCFD2 in HeLa cells affected neither the localization nor the protein level of ERGIC-53 (Figure 5A,C), which is consistent with the data from MCFD2-deficient lymphoblasts (23). Likewise, the localization of BAP31 and giantin was unaffected (Figure 5C).

To analyze whether cargo binding of ERGIC-53 requires MCFD2, we combined siRNA-mediated MCFD2 depletion with a recently established yellow fluorescent protein (YFP)-based protein fragment complementation assay (PCA) (20). The basic concept of the YFP PCA relies on splitting YFP into two fragments (YFP1 and YFP2) that exhibit no fluorescence by themselves. When fused to two interacting proteins, the two YFP fragments can be brought into close proximity where they can complement to functional, fluorescent YFP by folding into an active 3D structure (28). Using YFP PCA, we have previously visualized the oligomerization of ERGIC-53, its interaction with MCFD2 and its lectin-mediated interactions with cathepsin Z and cathepsin C (20). Mutagenesis of the lectin domain of ERGIC-53 selectively abolished YFP complementation

with cathepsin Z and cathepsin C (20), demonstrating that YFP PCA is a powerful technique to study specific, carbohydrate-mediated interactions between ERGIC-53 and cargo proteins *in vivo*.

Here, we applied the YFP PCA in MCFD2-depleted HeLa cells to investigate the role of MCFD2 on the oligomerization and glycoprotein-binding properties of ERGIC-53. Figure 6B shows that ERGIC-53 oligomerization and its interaction with cathepsin Z and cathepsin C are independent on the presence of MCFD2. Immunoblotting revealed equal expression of the YFP PCA constructs in control and MCFD2 siRNA-transfected cells, and silencing of MCFD2 was efficient (Figure 6C). As an internal control, the

interaction between ERGIC-53 and MCFD2 was analyzed. After transfection of MCFD2-specific siRNA, no YFP complementation of ERGIC-53 and MCFD2 was observed because the expression of MCFD2-YFP2 was silenced. The results shown in Figure 6 clearly show that ERGIC-53 does not require MCFD2 to bind cathepsin Z and cathepsin C *in vivo*. Furthermore, MCFD2 is not required for ERGIC-53 oligomerization, which is consistent with data derived from MCFD2-deficient lymphoblasts (24). MCFD2-independent binding of cathepsin Z to ERGIC-53 is further supported by the fact that neither chemical cross-linking nor YFP PCA-based experiments showed a direct interaction of MCFD2 and cathepsin Z (not shown).

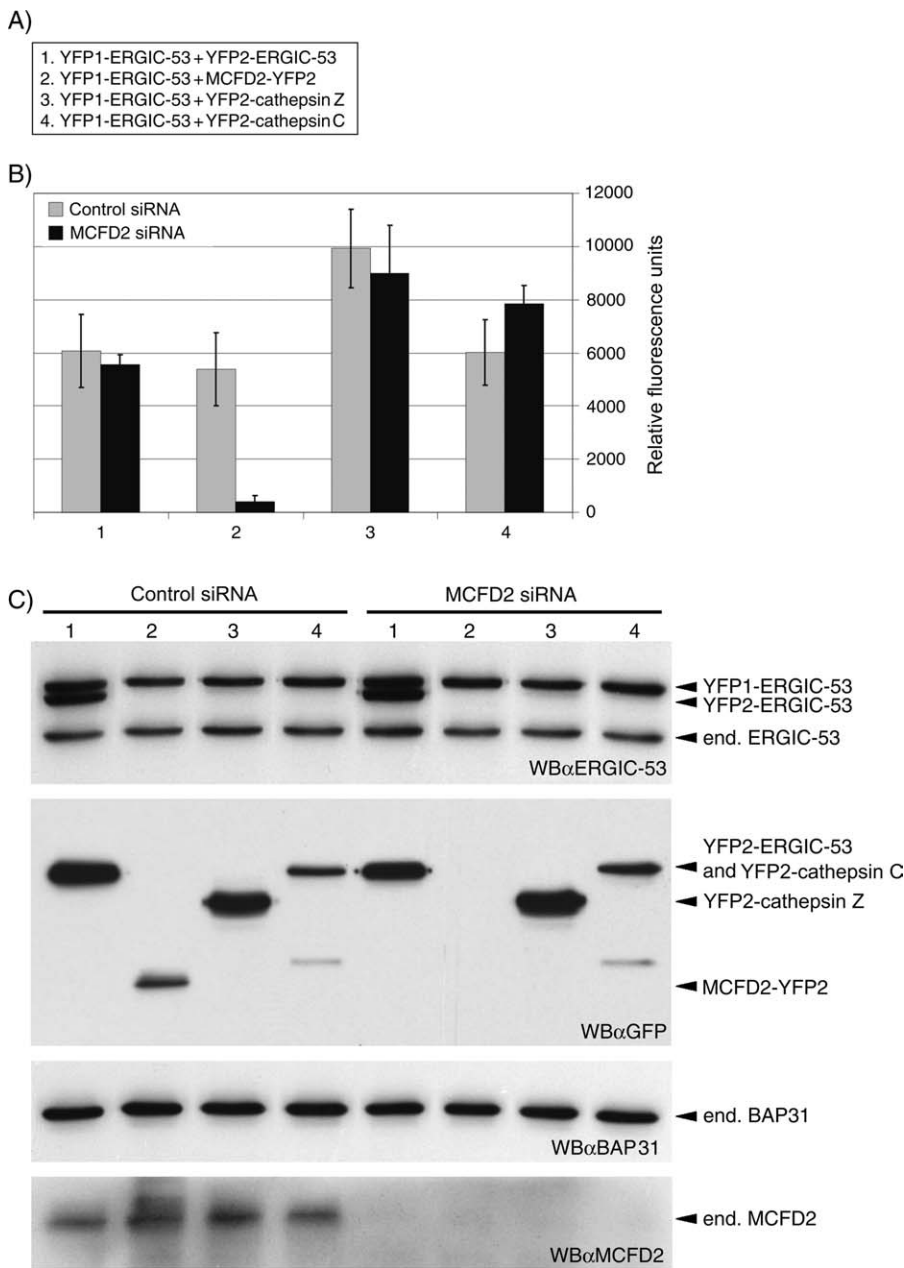


Figure 6: Yellow fluorescent protein PCA-based analysis of ERGIC-53-mediated protein interactions. A) The indicated pairs of YFP PCA constructs were expressed in control and MCFD2 siRNA-transfected HeLa cells. B) YFP fragment complementation was detected by fluorometric analysis using microtiter plates with cell suspensions of transfected HeLa cells (20). Background fluorescence of mock-transfected cells was subtracted, and the relative fluorescence units are shown. Data from three independent experiments were averaged and the error bars represent the standard deviation. MCFD2 siRNA affects neither the ERGIC-53 oligomerization nor the interactions between ERGIC-53 and cathepsin Z or cathepsin C. C) Lysates of fluorometrically analyzed cells were probed by Western blotting (WB) using mAbs against ERGIC-53, GFP, BAP31 and MCFD2. Equal expression of the YFP PCA constructs in control and MCFD2 siRNA-transfected cells is visualized by immunoblotting using anti-ERGIC-53 and anti-GFP antibodies. Equal protein amounts are present in all lanes as revealed by similar levels of endogenous ERGIC-53 and BAP31. MCFD2 siRNA depletes endogenous MCFD2 and silences the expression of the MCFD2-YFP2 construct.

Discussion

Receptor-mediated export of secretory proteins from the ER is a universal feature of eukaryotic cells; yet, the diversity of this process is still largely unknown. Conceivably, receptor-mediated ER export is required to increase transport efficiency of less abundant secretory proteins or for secretory cargo undergoing a second quality control step (2). Receptor-mediated ER export can also account for temporal and spatial co-ordination of the secretion of specific signaling molecules (29). The molecular basis underlying cargo receptor interactions is beginning to emerge although only few cases have been studied (11,13). ERGIC-53 and MCFD2 are particularly interesting because they are both required for the efficient secretion of blood coagulation factors V and VIII and constitute a cargo receptor complex composed of two subunits (23,24,30).

Here, we have described a detailed characterization of the ERGIC-53/MCFD2 receptor complex using a combination of immunofluorescence, gene silencing and PCA approaches to provide insight into the interdependence and cargo selectivity of the two subunits. Endogenous MCFD2 co-localized with ERGIC-53 in untreated and BFA-treated HeLa cells and thus shows characteristic features of a protein cycling between the ERGIC and the ER. Remarkably, both cycling and intracellular retention of MCFD2 are strictly dependent on ERGIC-53. ER-retained ERGIC-53-KKAA mislocalized MCFD2 to the ER, indicating that MCFD2 can bind to ERGIC-53 in the ER and requires ERGIC-53 for anterograde transport. Depletion of ERGIC-53 by siRNA resulted in the secretion of MCFD2; hence, MCFD2 requires ERGIC-53 also for retrograde transport back to the ER. We conclude that ERGIC-53 and MCFD2 form a stable complex and cycle together in the early secretory pathway due to the cytosolic diphenylalanine and dilysine motifs in ERGIC-53. In COS cells, endogenous ERGIC-53 and MCFD2 have similarly long half-lives (>26 h) (24), which is in line with a stable complex of the two proteins.

ERGIC-53-deficient patients have only trace amounts of intracellular MCFD2 (23). Our results provide now an explanation for this lack of MCFD2 by showing MCFD2 secretion on ERGIC-53 depletion in cell culture. Most soluble secretory proteins that localize to the early secretory pathway of mammalian cells carry a C-terminal KDEL tetrapeptide motif and are retained by binding to the KDEL receptor (31). This receptor captures the KDEL proteins in ERGIC and *cis* Golgi and recycles them back to the ER, providing a general mechanism for protein retention early in the secretory pathway (32). In contrast, MCFD2 is retained by a specific interaction with ERGIC-53, exemplifying a KDEL-receptor-independent retention mechanism in the early secretory pathway.

Overexpression of MCFD2 also results in its secretion, suggesting that the protein is secreted as soon as the

retention capacity of ERGIC-53 is saturated. This finding may have physiological implications in view of a recent report proposing that rat MCFD2 (formerly termed stem cell-derived neuronal stem cell supporting factor (SDNSF)) can act as an autocrine or paracrine factor in maintaining stem cell potential and neurogenesis in the adult central nervous system (33). In HeLa, COS, HepG2 and several glioblastoma cell lines, however, we could not detect any secreted endogenous MCFD2, suggesting that endogenous ERGIC-53 is capable of retaining all MCFD2 in these cells, although secretion of minute amounts below the detection limit cannot be excluded. ERGIC-53 and MCFD2 levels are induced in response to cellular stress (34,35). This raises the intriguing possibility that abundant MCFD2 can be secreted and act in some signaling events under certain conditions.

Factor VIII can be cross-linked to both MCFD2 and ERGIC-53, arguing for a triple complex early in the secretory pathway (24). Interestingly, factor VIII can also be cross-linked to the MCFD2 D129E mutant that is unable to interact with ERGIC-53 (24). Thus, MCFD2 interacts with factor VIII in an ERGIC-53-independent manner. To determine whether MCFD2 is a general cargo recruitment factor for ERGIC-53, we tested a putative interaction of MCFD2 with cathepsin Z or cathepsin C. Although serving as a cargo for ERGIC-53, cathepsin Z could not be directly cross-linked to MCFD2. Likewise, protein interaction studies using YFP PCA could not reveal an interaction between MCFD2 and cathepsin Z or cathepsin C, arguing against a role of MCFD2 in the recruitment of these cargo proteins. To confirm these findings, the interaction between ERGIC-53 and cathepsin Z or cathepsin C was studied after MCFD2 knockdown. YFP PCA showed that the interaction between ERGIC-53 and cathepsin Z or cathepsin C in living cells is not affected by the depletion of MCFD2. Binding of cathepsin Z and cathepsin C to ERGIC-53 is unlikely to be mediated by residual MCFD2. First, siRNA-mediated knockdown is very efficient, and hardly any residual MCFD2 is detectable by immunoblot (Figures 5A and 6C). Second, immunofluorescence-based analysis of siRNA transfection efficiency (Figure 5B) shows that residual MCFD2 derives from only very few apparently nontransfected cells, while the big majority of cells is entirely depleted of MCFD2. Third, in the YFP PCA-based protein interaction analysis, endogenous ERGIC-53 and YFP1-ERGIC-53 compete for residual MCFD2, which amplifies the MCFD2 depletion effect. In support of these *in vivo* data, ERGIC-53 can bind to immobilized mannose *in vitro* when purified without MCFD2 in the absence of calcium (26).

The results of our study indicate that MCFD2 is not required for the lectin-mediated binding of certain glycoproteins to ERGIC-53 in intact cells. We propose that the MCFD2/ERGIC-53 receptor complex possesses dual-binding properties. ERGIC-53 binds glycoproteins, such as

cathepsin Z or cathepsin C, in an MCFD2-independent fashion. Conversely, MCFD2 interacts with factors V and VIII in an ERGIC-53-independent manner. Binding of MCFD2 to ERGIC-53 recruits factors V and VIII to ERGIC-53, thereby ensuring efficient ER export. In support of this model, unglycosylated factor VIII but not unglycosylated cathepsin Z can be cross-linked to the MCFD2/ERGIC-53 complex (12,24). Recruitment of specific cargo molecules by a luminal subunit of a receptor complex adds another layer of complexity to receptor-mediated ER export. It will be interesting in the future to elucidate how MCFD2 recruits factors V and VIII to ERGIC-53. This will require new methodology as we were unable to visualize this process by YFP PCA. Mechanistic insight into this recruitment process will be important to understand how soluble secretory proteins can be captured for ER export.

Materials and Methods

Antibodies

The following antibodies were used: mouse monoclonal antibody (mAb) G1/93 against human ERGIC-53 (14) (ALX-804-602; Alexis, Lausen, Switzerland), mouse mAb against human MCFD2 (23), goat polyclonal antibody (pAb) against human MCFD2 (R&D Systems, Minneapolis, MN, USA), rabbit pAb against human MCFD2 (23), mouse mAb A1/182 against BAP31 (16) (ALX-804-601; Alexis), mouse mAb G1/133 against giantin (36) (ALX-804-600; Alexis), mouse mAb against hemagglutinin (HA; Covance, Princeton, NJ, USA) and mouse mAb against green fluorescent protein (GFP; Roche Applied Science, Basel, Switzerland).

Cell culture

HeLa cells (CCL-2; ATCC, Manassas, VA, USA) were grown in DMEM, supplemented with 10% fetal bovine serum, 1× nonessential amino acids and antibiotics. HeLa KKA cells were cultured as described previously (19). For fluorometric analysis and metabolic labeling, cells were grown in six-well plates. For fluorescence microscopy cells, were grown on poly-L-lysine-coated glass slides.

siRNA transfection

siRNA oligos were purchased from Qiagen (Venlo, The Netherlands) and Eurogentec (Seraing, Belgium). Three and six siRNA oligos were designed against ERGIC-53 and MCFD2, respectively. The most efficient siRNA oligo was determined by immunoblotting and chosen for all further experiments. ERGIC-53 was knocked down using 5'-GGACAGAAUCGUAUUAUCdTdT-3' as sense and 5'-GAUGAAUACGAUUCUGUCCdTdT-3' as antisense oligo. The MCFD2 was knocked down using 5'-AGAAGGUGUCAUCAA-CAAAdTdT-3' as sense and 5'-UUUGUUGAUGACACCUUCdAdG-3' as antisense oligo. Nonsilencing control siRNA was purchased from Qiagen. A final siRNA concentration of 5 nM was used for transfection directly after cell plating using HiPerFect (Qiagen) according to the manufacturer's instructions.

DNA transfection

Cloning of pcDNA3[YFP1-p53], pcDNA3[YFP2-p53], pcDNA3[MCFD2-YFP2], pcDNA3[YFP2-catZ] and pcDNA3[YFP2-catC] was described previously (13,20). pcDNA3[HA-MCFD2] was generated by inserting HA-MCFD2 without its signal sequence (generated by polymerase chain reaction amplification) into the pcDNA3 vector containing the artificial signal sequence of calreticulin (20). DNA constructs were transfected with FuGENE6 (Roche Applied Science) according to the manufacturer's instructions. For siRNA and DNA co-transfection, DNA constructs were trans-

ected 24 h after cell plating and siRNA transfection. Cells were analyzed 48 h after DNA transfection, which corresponds to 72 h after siRNA transfection.

Immunofluorescence microscopy

Brefeldin A (10 µg/mL; Epicentre, Madison, WI, USA) was added to the cells 90 min prior to fixation. Cells were fixed in 3% para-formaldehyde and permeabilized for 5 min in PBS containing 3% BSA and 0.2% Triton-X-100. Primary antibodies were added for 30 min in PBS containing 3% BSA. After rinsing, the secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 568 (Molecular Probes, Leiden, The Netherlands) were added for 30 min. Cells were washed in PBS, embedded and analyzed by laser scanning confocal microscopy (TCS NT; Leica, Wetzlar, Germany). For indirect immunofluorescence using mouse mAb against human MCFD2, 0.5% SDS and 5% β-mercaptoethanol was added during permeabilization step (37). The use of mouse immunoglobulin G (IgG) 1 (for MCFD2) and mouse IgG2a (for BAP31 and CLIMP-63) specific secondary antibodies allowed double staining of MCFD2 with BAP31 or CLIMP-63.

Immunoblotting

Protein samples were prepared as described previously (20), separated by SDS-PAGE; transferred to nitrocellulose membranes; immunoblotted with anti-ERGIC-53, anti-BAP31, anti-MCFD2 and anti-GFP and visualized by enhanced chemiluminescence (Amersham Bioscience, Uppsala, Sweden).

Metabolic labeling

Cells were deprived of L-methionine for 20 min, pulsed for 60 min with 100 µCi ³⁵S-methionine (Perkin Elmer, Wellesley, MA, USA) and chased for the indicated times in HeLa culture medium containing 10 mM L-methionine. Cells were lysed in 1% Triton-X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM CaCl₂ and phenylmethylsulfonylfluoride (PMSF), and the lysate was cleared by centrifugation at 100 000 × g for 1 h. The chase medium was cleared from cell debris by centrifugation at 20 000 × g for 5 min. Cleared samples were immunoprecipitated with anti-MCFD2. Immunoprecipitates were separated by SDS-PAGE, and radiolabeled bands were imaged using a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

Yellow fluorescent protein fluorometric analysis

Fluorometric analysis was performed as described previously (20). Data from three independent experiments were averaged.

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