Role of Sec24 isoforms in selective export of membrane proteins from the endoplasmic reticulum

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Sec24 of the COPII (coat protein complex II) vesicle coat mediates the selective export of membrane proteins from the endoplasmic reticulum (ER) in yeast. Human cells express four Sec24 isoforms, but their role is unknown. Here, we report the differential effects of Sec24 isoform-specific silencing on the transport of the membrane reporter protein ERGIC-53 (ER–Golgi intermediate compartment-53) carrying the cytosolic ER export signals di-phenylalanine, di-tyrosine, di-leucine, di-isoleucine, di-valine or terminal valine. Knockdown of single Sec24 isoforms showed dependence of di-leucine-mediated transport on Sec24A, but transport mediated by the other signals was not affected. By contrast, double knockdown of Sec24A with one of the other three Sec24 isoforms impaired all aromatic/hydrophobic signal-dependent transport. Double knockdown of Sec24B/C or Sec24B/D preferentially affected di-leucine-mediated transport, whereas knockdown of Sec24C/D affected di-isoleucine- and valine-mediated transport. The isoform-selective transport correlated with binding preferences of the signals for the corresponding isoforms *in vitro*. Thus, human Sec24 isoforms expand the repertoire of cargo for signal-mediated ER export, but are in part functionally redundant.

Keywords: COPII; ERGIC-53; endoplasmic reticulum export signal; protein transport; Sec24

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INTRODUCTION

Secretory proteins of higher eukaryotic cells are transported from the endoplasmic reticulum (ER) to the Golgi apparatus through the ER–Golgi intermediate compartment (ERGIC; Appenzeller-Herzog & Hauri, 2006). Cargo transport from the ER to the ERGIC is mediated by coat protein complex II (COPII) vesicles that bud from the ER exit sites (Lee *et al*, 2004). Secretory cargo can be

selectively recruited to these vesicles by means of transmembrane receptors that carry an ER export signal in their cytosolic domain recognized by the COPII coat (Barlowe, 2003). Various ER export motifs have been defined, including aromatic, hydrophobic and di-acidic motifs (Kappeler *et al*, 1997; Nishimura & Balch, 1997; Nufer *et al*, 2002).

The mechanism underlying signal-mediated ER export has been described in considerable detail for the type I membrane protein ERGIC-53 that cycles between the ER and the ERGIC (Nufer *et al*, 2003). ERGIC-53 is a mannose-specific lectin that is required for efficient secretion of the blood coagulation factors V and VIII (Nichols *et al*, 1998), and also for the transport of the lysosomal enzymes cathepsin C and cathepsin Z (Vollenweider *et al*, 1998; Appenzeller *et al*, 1999). ER export of ERGIC-53 is directed by a di-phenylalanine (FF) ER export signal that can be functionally substituted by di-tyrosine (YY), di-leucine (LL), di-isoleucine (II), di-valine (VV) or carboxy-terminal valine (Nufer *et al*, 2002, 2003).

ER export signals are thought to be recognized by the Sec24 subunit of the Sec24/Sec23 protein complex that forms the inner layer of the COPII coat. X-ray crystallography and mutagenesis studies suggest at least three cargo recognition sites in yeast Sec24, accounting in part for the spectrum of protein cargo that must be efficiently exported from the ER (Miller *et al*, 2003; Mossessova *et al*, 2003). Human cells express four Sec24 isoforms, termed Sec24A, Sec24B, Sec24C and Sec24D, but it is not known whether all four isoforms are expressed in the same cell or they are functionally different (Pagano *et al*, 1999; Tang *et al*, 1999).

Here, we investigated the role of the four human Sec24 isoforms in signal-mediated ER export by using short interfering RNA (siRNA)-based silencing. Single Sec24 isoform knockdown in HeLa cells failed to produce observable transport phenotypes, with the exception of knockdown of Sec24A that selectively impaired LL-mediated transport. By contrast, double knockdown affected signal-mediated transport in characteristic ways, indicating both selectivity and redundancy of the different Sec24 isoforms.

RESULTS

Di-leucine-mediated ER export depends on Sec24A

To investigate the role of the human Sec24 isoforms in signal-mediated ER export, we took advantage of a previously established reporter transport assay on a variant of the membrane

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protein ERGIC-53 (Kappeler *et al*, 1997; Nufer *et al*, 2002). In this assay, the acquisition of endoglycosidase H (endo H) resistance of Myc-tagged, glycosylated ERGIC-53, lacking its di-lysine retrieval signal (Fig 1A), is measured by [³⁵S]methionine pulse–chase as a read-out for ER export. We first sought to establish the repertoire of Sec24 isoforms expressed in HeLa cells by using immunoblotting. Fig 1B shows that HeLa cells express all four Sec24 isoforms, thus acting as a cell culture model for assessing isoform-dependent processes. By using the reporter transport assay, we examined the effect of siRNA-based knockdown of individual Sec24 isoforms on ER export of the ERGIC-53 reporter carrying the cytosolic transport motifs FF, YY, LL, II, VV or alanine–valine (AV) in positions –1 and –2 from the C terminus (Fig 1C,D). ERGIC-53 possessing a di-alanine (AA) motif, previously shown to promote inefficient ER export, was used as a signal-less reference, although AA might have some weak ER export information, illustrated by the fact that a di-tryptophan motif shows a lower transport rate than AA (Nufer *et al*, 2002; supplementary Fig S1 online).

Single isoform-specific knockdown reduced the protein level of the corresponding Sec24 isoform to less than 10% in 3 days (Fig 1B). Remarkably, the protein levels of the non-targeted isoforms, and also those of Sec23, were not affected suggesting that expression levels of these proteins are not strictly inter-dependent. After single isoform knockdown, signal-mediated ER to Golgi transport was measured by pulse–chase in conjunction with endo H digestion (Fig 1C), and the fraction of endo H-resistant Golgi form of ERGIC-53 was quantified (Fig 1C, arrow; Fig 1D). Interestingly, single knockdown produced minimal effects on signal-mediated export with the exception of Sec24A knockdown that impaired transport of the LL construct (Fig 1D). Of note, transport efficiency mediated by the different motifs in control-siRNA-treated HeLa cells was comparable to that previously established in nontreated COS cells (Nufer *et al*, 2002). Collectively, single-knockdown experiments indicate redundancy and some specificity of the Sec24 isoforms.

Sec24 double knockdowns on ER export

As single knockdown of Sec24 isoforms produced rather minimal effects on signal-dependent transport, we tested the effect of double knockdown combinations of the four Sec24 isoforms. Similar to single knockdown, double knockdown did not affect the protein levels of non-targeted Sec24 isoforms and Sec23 (Fig 2A). Strikingly, however, pairwise knockdown resulted in characteristic signal-dependent effects on ER-to-Golgi transport (Fig 2; Table 1). Double knockdown of Sec24A with any of the three other Sec24 isoforms impaired ER export of all tested signals. For YY and VV, the impairment by Sec24A/D knockdown was not statistically significant, but this is probably due to variation owing to a limited number of experiments. Knockdown combinations excluding Sec24A affected signal-dependent transport more selectively. Double knockdown of Sec24B/C or Sec24B/D reduced LL- and FF-mediated transport, whereas knockdown of Sec24C/D affected II-, VV- and AV-mediated transport. Thus, double knockdown suggested a dominant role of Sec24A and showed cooperative isoform selectivity.

Effect of Sec24 triple and quadruple knockdown

Next, we investigated, by triple knockdown, to what extent a single Sec24 isoform can maintain signal-dependent ER export

Fig 1 | Effects of Sec24 isoform-specific knockdown on signal-mediated transport. (A) A Myc-tagged glycosylated variant of human ERGIC-53 was used as a reporter for endoplasmic reticulum to Golgi transport. The –1 and –2 positions (XX) in the cytoplasmic tail were replaced by the amino acids indicated in (C) and (D). CHO, N-glycosylation site; TMD, transmembrane domain. (B) HeLa cells were transfected with equal amounts of siRNAs specific for the individual Sec24 isoforms or control (Ctr) siRNA. After 3 days, the cells were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblots were probed for the four Sec24 isoforms (Sec24A, Sec24B, Sec24C, Sec24D), Sec23 and tubulin as a loading control. (C) One day after siRNA transfection, the cells were transfected with the ERGIC-53 reporter carrying the indicated amino-acid doublets at the XX position shown in (A); AA, FF, YY, LL, II, VV and AV. After another 2 days, the cells were pulsed for 10 min with [³⁵S]methionine, chased for 1 h and then subjected to immunoprecipitation with Myc antibodies. Precipitates were treated with endo H and separated by SDS-PAGE. Endo H-resistant (arrow) and endo H-sensitive (arrowhead) forms of the ERGIC-53 constructs were visualized by using fluorography, and the relative amount of endo H-resistant form was quantified (D). Values are means $\pm$ s.d.; $n = 3$ –5; statistically significant difference to control siRNA ($P < 0.05$; Student's *t*-test). AA, di-alanine; AV, alanine–valine; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; FF, di-phenylalanine; II, di-isoleucine; LL, di-leucine; siRNA, short interfering RNA; VV, di-valine; YY, di-tyrosine.

(Fig 3). Knockdown of three of the four Sec24 isoforms did not affect the protein levels of non-targeted isoforms and Sec23 (Fig 3A). Remarkably, triple knockdown of Sec24 isoforms strongly reduced transport of all reporter constructs irrespective of the

Fig 2 | Effects of double knockdown of Sec24 isoforms on signal-mediated transport. (A) HeLa cells were transfected with specific siRNAs against two of the four Sec24 isoforms or with control (Ctr) siRNA. After 3 days, the cells were analysed by immunoblotting, as in Fig 1. (B–H) Quantification of the maturation of ERGIC-53 constructs carrying the indicated transport signals after knockdown of Sec24 isoform pairs. Values are means $\pm$ s.d.; $n = 3$ –5; statistically significant difference to control siRNA ($P < 0.05$; Student's t -test). AA, di-alanine; AV, alanine–valine; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; FF, di-phenylalanine; II, di-isoleucine; LL, di-leucine; siRNA, short interfering RNA; VV, di-valine; YY, di-tyrosine.

signal (Fig 3B–H; Table 1). The results indicate that a single isoform of Sec24 is not able to maintain efficient ER export, although export of most of the reporters carrying transport signals was still increased in comparison with the AA motif (Fig 3B).

We next tested whether the transport of our reporter constructs was entirely dependent on COPII. To this end, we knocked down all four Sec24 isoforms (Fig 3A, last lane). Under these conditions, the rate of synthesis of all the reporter constructs, and also an endogenous ER marker B-cell antigen receptor-associated protein 31 (BAP31), was unaffected (not shown), indicating that the residual Sec24 levels were sufficient for cell survival. Moreover, cell growth was not appreciably affected in the 3 days of quadruple knockdown and the localization of BAP31 (ER),

Table 1 | Effects of isoform-selective knockdown of Sec24 on signal-mediated transport of the transmembrane ERGIC-53 reporter from the ER

Sec24 knockdown	Transport signals*					
	FF	YY	LL	II	VV	AV
A	–	–	+	–	–	–
B	–	–	–	–	–	–
C	–	–	–	–	–	–
D	–	–	–	–	–	–
A+B	+	+	+	+	+	+
A+C	+	+	+	+	+	+
A+D	+	–	+	+	–	+
B+C	–	–	+	–	–	–
B+D	+	–	+	–	–	–
C+D	–	–	–	+	+	+
Triple	+	+	+	+	+	+
Quadruple	+	+	+	+	+	+

*The table summarizes the experiments shown in Figs 1–3. Knockdown of Sec24 isoforms either reduced the signal-mediated transport (+) or had no effect (–). AV, alanine–valine; ERGIC-53, endoplasmic reticulum–Golgi intermediate compartment; FF, di-phenylalanine; II, di-isoleucine; LL, di-leucine; VV, di-valine; YY, di-tyrosine.

GM130 (*cis*-Golgi), giantin (*cis*/medial-Golgi) and galactosyltransferase (medial/*trans*-Golgi) was unchanged (supplementary Fig S2A online). However, on Sec24 quadruple knockdown, the fluorescence signal of Sec31—a component of the COPII outer layer—slightly shifted from punctated structures to an additional cytoplasmic staining, probably reflecting a decrease in COPII vesicle formation. Moreover, endogenous ERGIC-53, which cycles between the ER and the ERGIC, showed a more pronounced ER pattern, indicated by the presence of a perinuclear ring (supplementary Fig S2A online). Thus, knockdown of all four Sec24 isoforms results in differences in the steady-state distribution of the COPII component Sec31 and of the cycling membrane protein ERGIC-53, but does not affect Golgi marker localization. This finding confirms our biochemical results and the assumption that our transport assay measures ER export. In the transport assay, the quadruple knockdown led to an almost complete transport block of the ERGIC-53 constructs (Fig 3I), indicating that ER export mediated by aromatic/hydrophobic signals depends exclusively on the COPII pathway. The quadruple knockdown also slightly reduced the protein level of Sec23 (Fig 3A, last lane), suggesting that Sec23 is less stable in the absence of Sec24 proteins.

Sec24D does not rescue the loss of Sec24A and Sec24B

To further investigate the specificity of Sec24 isoforms, we tested whether the effects of single knockdown of Sec24A or double knockdown of Sec24A and Sec24B can be reversed by the seemingly irrelevant isoform Sec24D. As mentioned above, knockdown of Sec24A decreased LL-mediated transport (Fig 1D) and double knockdown of Sec24A and Sec24B decreased the FF-mediated transport of ERGIC-53 (Fig 2C). Fig 4 shows that simultaneous overexpression of Myc–Sec24D and

Fig 3 | Effects of triple and quadruple knockdown of Sec24 isoforms on signal-mediated transport. (A) HeLa cells were transfected with siRNAs against combinations of three or all four Sec24 isoforms and analysed by immunoblotting. (B–H) The maturation of the indicated ERGIC-53 constructs was quantified after triple Sec24 isoform knockdown. (I) Knockdown of all four Sec24 isoforms. A representative fluorogram (left panel) and the quantification of 3–5 fluorograms are shown (right panel). Triple and quadruple Sec24 knockdown combinations result in a significant decrease of endoplasmic reticulum export for all constructs when compared with control (Ctrl) siRNA experiments ($n = 3–5$; values are means $\pm$ s.d.; $P < 0.05$; Student's t -test). AA, di-alanine; AV, alanine-valine; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; FF, di-phenylalanine; II, di-isoleucine; LL, di-leucine; siRNA, short interfering RNA; VV, di-valine; YY, di-tyrosine.

siRNA-mediated knockdown of Sec24A was unable to prevent the defect in LL-mediated transport of ERGIC-53 induced by a Sec24A knockdown (Fig 4A,C). Similarly, the inhibitory effect of Sec24A and Sec24B double knockdown on FF-mediated transport was not compensated by overexpression of Myc–Sec24D (Fig 4B,D). These results further support the specificity of Sec24 isoforms in recognizing subsets of hydrophobic transport signals and argue against the trivial possibility that the observed effects are due to different expression levels of the Sec24 isoforms. Of note, the level

Fig 4 | Sec24D overexpression does not compensate for the loss of Sec24A and Sec24B. (A,B) HeLa cells were transfected with siRNA against Sec24A (A) or Sec24A and Sec24B (B). After 1 day, the cells were co-transfected with Myc–Sec24D and Myc–ERGIC-53 constructs carrying LL (A) or FF (B). After another 2 days, the transport assay was performed (see Fig 1C) and endo H-resistant (arrow) and endo H-sensitive (arrowhead) forms of the ERGIC-53 constructs were visualized by fluorography. Immunoprecipitation of the endogenous marker BAP31 was used as a loading control (Ctrl). (C,D) Quantification of fluorograms shown in (A) and (B). Values are means $\pm$ s.d.; $n = 3$. AA, di-alanine; AV, alanine-valine; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; FF, di-phenylalanine; II, di-isoleucine; LL, di-leucine; siRNA, short interfering RNA; VV, di-valine; YY, di-tyrosine.

of overexpressed Myc–Sec24D was at least ten-fold higher than that of endogenous Sec24D (supplementary Fig S3A online). Moreover, Myc–Sec24 interacted with Sec23 (supplementary Fig S3B online) and substantially colocalized with endogenous Sec31 (supplementary Fig S3C online), suggesting that the Myc–Sec24 protein was functional.

Binding of Sec24 isoforms to transport motifs

Previously, in a peptide-binding assay, we showed that some COPII proteins of HepG2 cells specifically bind to aromatic and hydrophobic ER export signals (Kappeler *et al*, 1997; Nufer *et al*, 2002). We adapted this method to HeLa cells and studied the binding of all four human Sec24 isoforms from HeLa lysates to immobilized peptides carrying the wild-type tail of ERGIC-53 terminating in FF or to peptides in which the FF had been replaced by the transport motifs used in the pulse–chase experiments described above. Sec24A showed strong binding to the YY and FF motifs and weaker binding to the LL, II and V motifs (Fig 5A, upper panel). Sec24B showed a binding pattern similar to Sec24A, although it bound more strongly to the V motifs (Fig 5A, second panel from the top). By contrast, Sec24C preferentially bound to VV and AV, but weakly to FF and YY (Fig 5A, third panel from the top). The V, YY and FF motifs were strongly recognized by Sec24D (Fig 5A, fourth panel from the top). All four Sec24 isoforms bound to II relatively weakly, but still more strongly than to AA. Assuming that Sec23 does not directly bind to the transport motifs and is

indirectly pulled down by its interaction with Sec24, the Sec23 signal indicates the cumulative binding preferences of all Sec24 isoforms for a given motif (Fig 5A, fifth panel from the top). Fig 5B

Fig 5 | Binding of Sec24 isoforms to transport signals *in vitro*. ERGIC-53 tail peptides carrying the indicated motifs were coupled to thiol-activated Sepharose and the beads were incubated with precleared HeLa cell lysate. Bound proteins were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted for the four Sec24 isoforms (Sec24A–Sec24D), Sec23 and tubulin. A representative immunoblot of four independent experiments is shown. (B) Quantification of immunoblots shown in (A). Signal intensities for the Sec24 isoforms were related to those of the respective input signal (A). Values are means $\pm$ s.d.; $n = 4$. (C) The binding preferences of each Sec24 isoform for the different motifs are depicted from high preference (+++) to lowest preference (–). Lowest preference means similar to AA. AA, di-alanine; AV, alanine–valine; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; FF, di-phenylalanine; II, di-isoleucine; LL, di-leucine; VV, di-valine; YY, di-tyrosine.

shows the quantification of Sec24 isoform binding preferences to hydrophobic transport signals. In Fig 5C, these binding preferences are categorized as high (+++) to low (–) depending on the quantification in Fig 5B.

DISCUSSION

Our finding that HeLa cells express all four isoforms of Sec24 allowed us to assess their role in protein transport mediated by various aromatic and hydrophobic ER export signals. This study uncovered a new level of complexity in the regulation of ER export, as was discovered by siRNA-mediated reduction of endogenous Sec24 isoforms in various combinations.

Single knockdown showed a selective requirement of Sec24A for LL-mediated transport and a high degree of redundancy for all Sec24 isoforms in recognizing the tested signals. The Sec24A-mediated transport selectivity is reflected by preferential binding of Sec24A to the LL signal in the peptide-binding assay, although, compared with other signals, Sec24 binding to LL was weak in this assay for unknown reasons. Sec24 isoform redundancy and selectivity observed in HeLa cells are reminiscent of a similar phenomenon in yeast. Overexpression of the non-essential Sec24 isoform Lss1p can partially rescue the knockout of the essential Sec24p isoform, indicating functional redundancy between the two homologous proteins (Kurihara *et al*, 2000). By contrast, the non-essential isoform Lst1p is selectively required for efficient transport of the GPI-anchored protein Gas1p and the plasma membrane proton-ATPase Pma1p (Peng *et al*, 2000; Shimoni *et al*, 2000).

Isoform double knockdown uncovered a dominant role of Sec24A. Most signal-mediated transport was impaired by knockdown combinations that included this isoform, whereby the Sec24A/B knockdown combination had the strongest effect on transport. By contrast, combinations excluding Sec24A affected only few signals. For example, double knockdown of Sec24C/D severely affected V-mediated transport, but had no effect on FF-, YY- or LL-mediated transport. In addition, overexpression of Sec24D did not rescue the effects of single Sec24A knockdown or double Sec24A and Sec24B knockdown on LL- and FF-mediated transport, respectively. Knockdown of three Sec24 isoforms significantly reduced the ER export of all tested constructs. Moreover, decreasing the levels of all four Sec24 isoforms blocked transport entirely, indicating that ER export mediated by these signals is entirely dependent on COPII.

On the basis of sequence homology, the Sec24 isoforms can be divided into two subgroups: A/B and C/D (Pagano *et al*, 1999; Tang *et al*, 1999). This homology-based classification is, to some extent, reflected by the transport phenotypes of double knock-down as well as by the binding patterns observed in the peptide-binding assay. Although the binding assay gives no information on binding affinities, it provides valuable information on which of the transport motifs are favoured by the individual Sec24 isoforms. Isoform preference, however, is not absolute. With the exception of LL, all signals were recognized by all Sec24 isoforms, but clearly with different strength.

Integrating the transport data obtained from the knockdown experiments with the binding patterns of the bead assay, we draw the following conclusions: (1) in HeLa cells, Sec24A is the most important isoform for mediating aromatic/hydrophobic signal-dependent ER export, but another isoform is required for full ER export activity; (2) together the three Sec24 isoforms Sec24B, Sec24C and Sec24D can largely compensate for the lack of Sec24A transport function; and (3) Sec24 isoforms have overlapping selectivity for the different transport signals.

How can a simultaneous redundancy and specificity of Sec24/cargo interaction be explained? Conceivably, the Sec24 isoforms have evolved by gene duplication events that led to redundancy in cargo binding. Given that the yeast Sec24p isoform has at least three cargo binding sites (Miller *et al*, 2003; Mossessova *et al*, 2003), it is possible that mutations in one particular binding site of a duplicated isoform might have changed its cargo preference, whereas other binding sites remained unchanged. These two processes would result in co-evolution of Sec24 isoforms and transport signals, ensuring that cargo is transported independently of the availability of a specific isoform and that the repertoire of cargo for signal-mediated ER export is expanded.

Although our study showed some similarities to the function of Sec24 isoforms in yeast, COPII vesicle budding is more complex in mammalian cells. Purified yeast COPII components are sufficient to generate vesicles and package cargo molecules into vesicles from yeast microsomal membranes in a cell-free system (Barlowe *et al*, 1994), whereas mammalian COPII vesicle formation requires other unknown cytosolic components (Kim *et al*, 2005). The existence of two Sar1 isoforms, two Sec23 isoforms and two Sec31 isoforms with several splice variants adds to the difficulty of functionally reconstituting COPII-dependent ER export *in vitro*.

The knockdown approach used in this study provides a basis for studying COPII-mediated transport in general and for assessing Sec24 isoform dependence of other ER export signals. A particular advantage of our approach is that individual Sec24 isoforms, in various combinations, can be targeted specifically without affecting the expression of the remaining isoforms.

METHODS

Complementary DNAs and short interfering RNA. The construction of Myc-tagged ERGIC-53 was described previously (Kappeler *et al*, 1997); Myc-Sec24D cDNA was kindly provided by W. Hong (Tang *et al*, 1999). siRNA oligonucleotides against the following target sequences were used: hSec24A, 5'-GAGTCAGT GAGCCAAGGAT-3'; hSec24B, 5'-GCCGATCCTGTGCAACGTA TA-3'; hSec24C, 5'-GGCTGCTGTGTAGATCTCT-3'; hSec24D, 5'-CTGTCTTACCCAGGAGGCT-3'; as a control, the nonfunctional siRNA, 5'-CATAAACAAGACCTCACAG-3', was used.

Cell culture, transfections and metabolic labelling. HeLa cells were grown in six-well plates and transfected with siRNA (HiPerFect, Qiagen, Hilden, Germany) at a final concentration of 5 nM. After 24 h, the cells were transfected with ERGIC-53 reporter constructs (Effectene, Qiagen). After 48 h, the cells were pulsed with [³⁵S]methionine for 10 min, chased in the presence of 10 mM unlabelled methionine for 1 h and then subjected to immunoprecipitation with Myc antibodies (Nufer *et al*, 2002). After endo H digestion (Kappeler *et al*, 1997), proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), visualized by fluorography and quantified by phosphor imaging using ImageQuant™ software (Molecular Dynamics, Sunnyvale, CA, USA).

Peptide-binding assay. Peptides of defined sequence were coupled to thiol-activated Sepharose 4B (Nufer *et al*, 2002). Precleared Triton X-100 extracts of HeLa cells were incubated with peptide beads for 2 h at 4 °C under low-salt conditions (50 mM HEPES, 90 mM KCl, 2.5 mM MgOAc, 1% Triton X-100; pH 7.3). Bound proteins were analysed by SDS-PAGE followed by immunoblotting.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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