Mutations in fission yeast Cut15, an importin α homolog, lead to mitotic progression without chromosome condensation

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Chromosome condensation is a major mitotic event [1]. Fission yeast mutations in topoisomerase II and condensin subunits produce the characteristic 'cut' phenotypes, in which the septum bisects the nuclear material in the absence of normal condensation and sister chromatid separation [2]. We show here that the same condensation defect is produced in *cut15* temperature-sensitive mutants at the restrictive temperature (36°C). The gene product of *cut15*$^+$ is, surprisingly, very similar to importin α [3,4], which binds proteins containing a nuclear localization signal (NLS) and forms the heterodimer with importin β that mediates translocation through the nuclear pore complex [5]. We show that in a nuclear import assay, purified Cut15 protein behaved identically to mammalian importin α but mutant Cut15 did not. Mutant Cut15 failed to bind an NLS-containing protein *in vitro* but could still bind importin β. Unexpectedly, however, NLS proteins were imported into the nucleus in *cut15* mutants. Cut15 is thus essential for mitotic chromosome condensation, but its role in nuclear import might be dispensable. Green fluorescent protein (GFP)-tagged Cut15 was enriched within the nucleus specifically during prometaphase–metaphase, so the interaction of Cut15 with nuclear NLS proteins during mitosis might be important for condensation.

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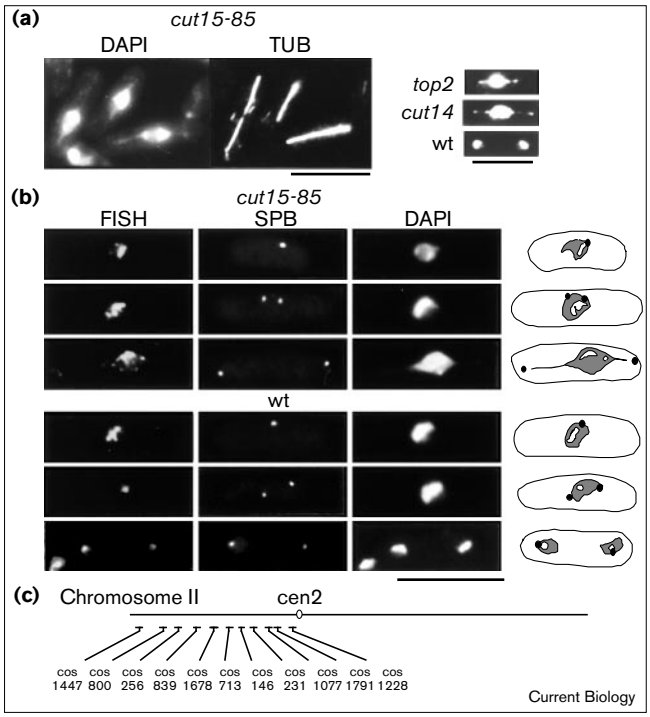
Results and discussion

The cytological phenotypes of the *cut15-85* mutant at 36°C (Figure 1a) were most like those of *top2* and *cut14* mutants that are defective in topoisomerase II and one of the condensin subunits, respectively [2]. Mutant cells could enter and exit mitosis, but nuclear chromatin did not condense during mitosis. Another mutant allele, *cut15-122*, gave identical phenotypes (data not shown). To confirm the condensation defect, the fluorescence *in situ* hybridization (FISH) technique [6] was applied to paint the left arm of chromosome II (Figure 1b) using mixed cosmid probes (Figure 1c). Condensation was completely deficient in *cut15-85* cells, which had diffused signals in mitosis.

The *cut15*$^+$ gene was isolated using a *Schizosaccharomyces pombe* genomic library. Plasmid pCQ100 fully suppressed the temperature-sensitive phenotype of both *cut15-85* and

Figure 1



Loss of chromosome condensation in the *cut15-85* mutant. **(a)** In *cut15-85* cells cultured at 36°C for 2 h, spindle elongation took place (TUB, anti-tubulin stain), whereas nuclear chromatin (diamidinophenylindole, DAPI, stain) did not condense, and tiny portions of chromatin were pulled apart along the spindle. DAPI stains of *top2*, *cut14* and wild-type (wt) cells at 36°C are shown on the right. **(b)** The FISH method was applied to *cut15-85* and wild-type strains at 36°C using **(c)** mixed cosmid probes derived from the left arm of chromosome II [6]. (b) Nuclear chromatin and spindle pole bodies (SPBs) were stained by DAPI and anti-SPB antibodies, respectively [12]; the cartoons show the merged images. Cells containing one and two SPBs were in interphase and mitosis, respectively. The rod-like FISH signal in interphase became a compact dot in mitotic wild-type cells, but a diffused form in *cut15-85* mutant mitotic cells. The bars represent 10 μm.

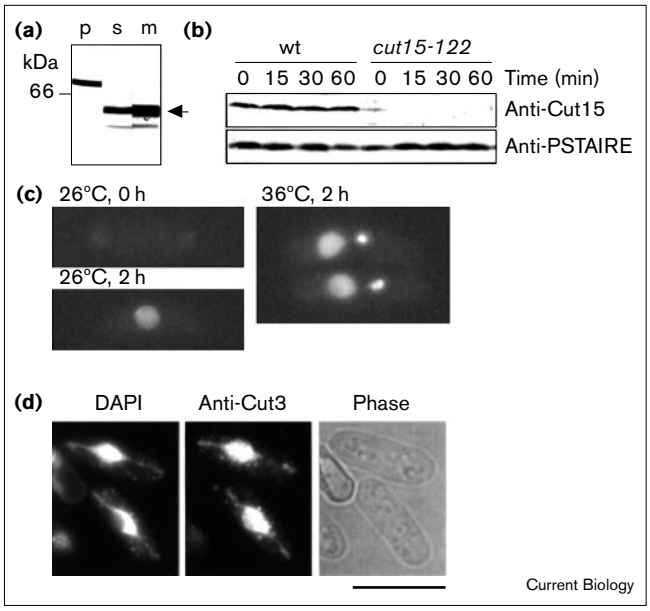
cut15-122 strains. The 2.3 kb insert containing one open-reading frame was verified to be derived from the *cut15*$^+$ gene by integrating it into the chromosome by homologous recombination, followed by tetrad analysis (the sequence is deposited in the DNA database of Japan, accession number AB010574). A database search indicated that Cut15, which has 542 predicted amino acids, is very similar to importin α (karyopherin α) and budding yeast Srp1 (see the Supplementary material published with this paper on the internet). Its overall identity to Srp1, mouse importin 1 and human Rch1 [7,8] is 56.6%, 43.6% and 44.0%, respectively. Nuclear division is also defective in a budding yeast *srp1* mutant [4,9], but chromosome condensation in the mutant has not been examined.

This unexpected identification of the *cut15*$^+$ gene prompted us to determine the position of the mutations, as importin α contains 'arm repeats' implicated in protein–protein interactions, and its basic amino terminus is sufficient for binding importin β [10,11]. Substitution mutations determined by sequencing the mutant gene were found at amino acid residues 419 and 450 (both mutated from Gly to Ser) for *cut15-85*, and at position 395 (Cys to Arg) for *cut15-122*. These mutated residues are in the arm repeats, suggesting that the Cut15 mutant protein might be defective in protein–protein interactions, for example with NLS proteins, but not in its interaction with importin β.

Polyclonal anti-Cut15 antibodies were raised against recombinant Cut15 protein. Affinity-purified antibodies detected the 60 kDa polypeptide in wild-type strains (Figure 2a, indicated by the arrow). Mutant Cut15-122 protein was unstable at 36°C (Figure 2b). Its staining intensity on a gel was low even at 26°C, the permissive temperature, and barely detected after 15 minutes at 36°C. The other mutant protein Cut15-85 was also unstable (data not shown). The temperature-sensitive mutations thus caused instability. Gene disruption showed that the *cut15*$^+$ gene was absolutely required for cell viability. The *cut15* null spores germinated and produced the same cut phenotype with spindle elongation but no chromosome condensation (data not shown).

Unexpectedly, the nuclear import of a protein with an NLS was apparently normal in *cut15-85* mutant cells (Figure 2c). NLS-tagged GFP (GFP–NLS) accumulated in the nuclei when it was expressed in mutant cells from the inducible nmt1 promoter. The GFP–NLS signal was initially not seen at 26°C, but became abundant in the nuclei after 2 hours at both 26°C and 36°C (Figure 2c shows two cells with the cut phenotype at 36°C). Moreover, in *cut15-85* mutant cells at 36°C, Cut3 (a condensin subunit) was localized exclusively in the nucleus (Figure 2d). Note that Cut3 colocalized with the chromatin region. Therefore, the condensation defect in the *cut15* mutant occurred in spite of the nuclear accumulation of Cut3 and GFP–NLS.

Figure 2



Mutations in Cut15 sustain the nuclear import of NLS proteins and a condensin subunit. **(a)** An immunoblot of wild-type extracts. Pre-immune serum (p) gave a 70 kDa band that disappeared when using the immunized serum. Affinity-purified anti-Cut15 antibodies (s) gave a 60 kDa band (indicated by the arrow), the lower band might be a proteolytic product. Introduction of plasmid pCUT15 carrying the wild-type gene resulted in an increased intensity of the 60 kDa band (m). **(b)** Wild-type (wt) and *cut15-122* cells grown at 26°C were shifted to 36°C for the indicated times. Cdc2 detected by the anti-PSTAIRE antibody is the loading control. **(c)** Mutant *cut15-85* cells which express GFP–NLS under the inducible nmt1 promoter (REP1) were cultured at 26°C under the repressed condition (0 h time point). The culture was either maintained at 26°C or shifted to 36°C to inactivate Cut15 and 2 h later, GFP–NLS was expressed. Abundant GFP–NLS was found in the mutant nuclei which had the cut phenotype. **(d)** The *cut15-85* cells cultured at 36°C for 2 h were stained with DAPI and anti-Cut3 antibodies. Mutant cells with a chromosome condensation defect contained normal levels of Cut3 staining. The bar represents 10 μm.

To obtain clues about the nuclear function of Cut15 protein, GFP-tagged *cut15*$^+$ (Cut15–GFP), which was functional because the plasmid carrying it could complement temperature sensitive *cut15*, was integrated into the chromosome with the native *cut15* promoter. Cells expressing Cut15–GFP were observed by a confocal microscope. Two different optical sections of the same cells are shown in Figure 3a. A mitotic cell was distinguished by the presence of two spindle pole bodies (SPBs). The nuclear envelope signal given by Cut15–GFP was intense in both interphase and mitosis, but the intranuclear signal was enhanced in mitotic cells only.

The time course of Cut15–GFP localization was then followed in living wild-type cells at 26°C [12]. The GFP signals are shown in Figure 3b (another cell is shown in the Supplementary material). The nuclear envelope signal was relatively strong throughout the cell cycle. The additional

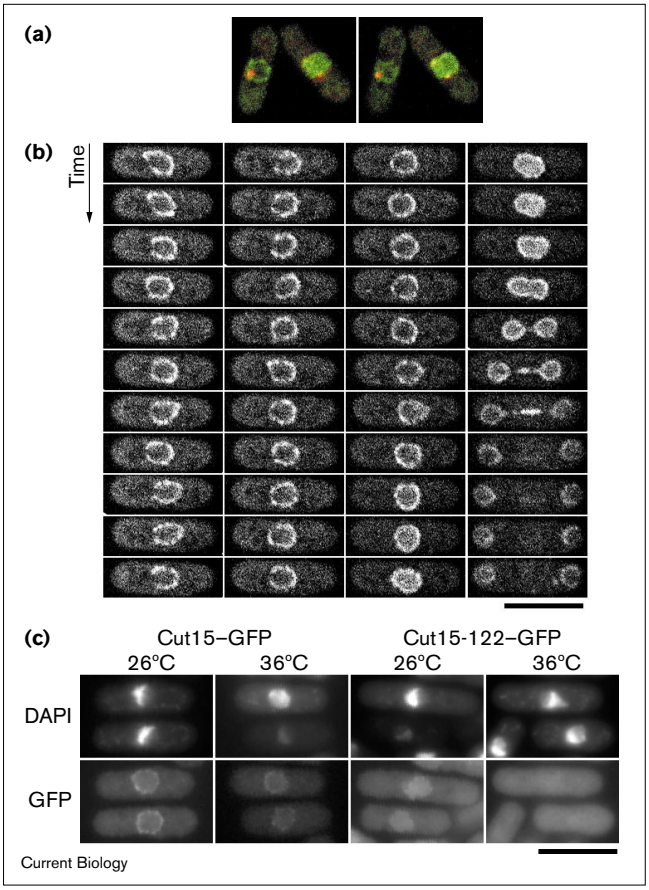
intranuclear signal increased specifically during mid-mitosis, and the intensity was maximal 4–6 minutes before nuclear division, coincident with the prometaphase–metaphase stage. The mutant Cut15 protein was highly abundant in the cytoplasm at 36°C, however (Figure 3c), indicating that the carboxy-terminal mutation impaired its ability to enter the nucleus.

Although the sequences of Cut15 and importin α are very similar, Cut15 might not act like importin α in the nuclear transport assay [13], as evidence *in vivo* did not give any indication that the Cut15 mutants had a defect in nuclear import. To examine this point, Cut15 with a tag consisting of the hemagglutinin (HA) epitope and six copies of the His epitope was overproduced in wild-type cells from the inducible promoter REP1 and purified by affinity chromatography followed by a MonoQ chromatography step (see Supplementary material). SDS–PAGE showed that the MonoQ preparations largely consisted of Cut15 polypeptide, and a Coomassie-blue-stained 60 kDa band reacted with anti-Cut15 antibodies (see Supplementary material). Cut15-122 mutant protein was also purified from cells at 26°C overproducing the mutant protein from plasmid REP1Cut15-122HA6His. Fresh preparations of these proteins were used for the assays that follow.

Wild-type Cut15 protein behaved exactly like mammalian importin α in the standard transport assay. Madin-Darby bovine kidney (MDBK) cells treated with digitonin were incubated with the NLS protein allophycocyanin (T-APC) which displays red fluorescence, Cut15, importin β (p97), the Ran GTPase and NTF2 (p10) in the presence of ATP and GTP at 26°C for 30 minutes. T-APC was found to enter the nuclei under these standard conditions (Figure 4a). In the absence of Cut15, however, T-APC was not transported into the nuclei. The results in Figure 4a establish that Cut15 could substitute for mammalian importin α in the nuclear transport assay.

Cut15-122 mutant protein was then used in the transport assay (Figure 4b) with or without heat pretreatment (37°C for 30 minutes). Heat-treated Cut15-122 did not transport T-APC into the nuclei whereas non-treated Cut15-122 did. Heat-treated Cut15 was not degraded (data not shown). Control wild-type Cut15 showed normal transport after the heat treatment. The temperature-sensitive nature of mutant Cut15 was thus in parallel with the heat-sensitive defect in the nuclear transport assay. To understand the defect of the mutant Cut15-122 protein, we examined whether it was capable of binding importin β and/or an NLS protein. Cut15-122 coprecipitated with importin β but not with glutathione-S-transferase (GST)–NLS after heat treatment (Figure 4c). The inability of Cut15-122 to bind GST–NLS was consistent with the site of the protein known to be mutated, and could well explain the failure in the nuclear transport assay *in vitro*. We conclude that Cut15

Figure 3

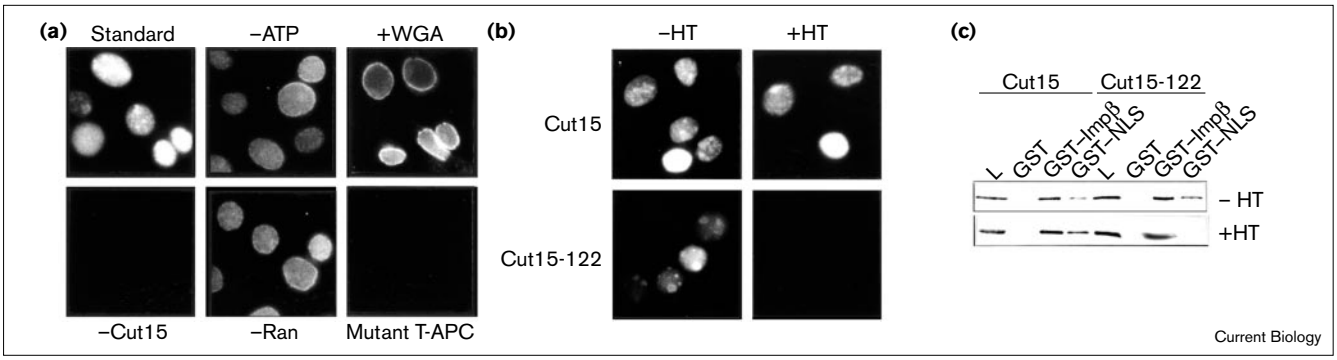


Transient nuclear localization of Cut15–GFP in living mitotic cells. **(a)** Wild-type cells expressing Cut15–GFP (green) and Sad1 (red, the SPB marker) were fixed and observed after anti-Sad1 staining. These are confocal optical sections and the mitotic cell is on the right of each panel. **(b)** The time course of the Cut15–GFP signal in living wild-type cells at 26°C by confocal optical sections. Images of one example cell taken at 2 min intervals are shown in order down the columns starting on the left. Strong intranuclear signals transiently accumulated at prometaphase–metaphase. The GFP signal was also intense in the middle of the late anaphase spindle. **(c)** Wild-type Cut15 and mutant Cut15-122 tagged with GFP were expressed at 26°C or 36°C and observed by conventional fluorescence microscopy after DAPI staining. Localization of mutant Cut15 was largely cytoplasmic at 36°C. The bars represent 10 μm.

is an importin α homolog, as defined by its sequence, its ability to bind NLS proteins and its import activity *in vitro*. The mutations in Cut15 abolished its ability to bind NLS proteins but not importin β. A possible explanation for the difference between transport *in vitro* and *in vivo* is that unidentified protein(s) may substitute for Cut15 function in the nuclear import of NLS proteins *in vivo*.

We propose that the binding defect between mutant Cut15 and NLS proteins within the nucleus results in condensation defects. Accumulation of Cut15 in the chromosomal region at prometaphase–metaphase may thus be

Figure 4



Cut15 substitutes for the activity of importin α in the nuclear import assay but the Cut15-122 mutant does not. **(a)** MDBK cells were permeabilized in transport buffer (TB) containing digitonin for 5 min to remove cytoplasm, and then washed twice in TB. Digitonin-treated cells were incubated with the transport substrate T-APC, Cut15 (6 pmol), importin β, Ran and NTF2 in the presence of ATP, GTP and ATP-regenerating reagents at 26°C for 30 min. The nuclear import took place only under standard conditions and not when either ATP, Cut15 or Ran were missing, nor when wheat germ agglutinin (WGA; a nuclear transport inhibitor) was added to the reaction nor when T-APC with a mutant NLS was used. **(b)** Cut15 and Cut15-122 (6 pmol each) were heat-treated (+HT) or not heat-treated (–HT) at 37°C for 30 min, and the nuclear transport assay was carried out as in (a). **(c)** To examine whether Cut15-122 could interact with GST-tagged mouse importin β (GST–Impβ) and GST–NLS proteins, Cut15 and Cut15-122 were treated at 37°C for 30 min, and then incubated with GST–Impβ or GST–NLS, followed by a pull-down using glutathione beads. Cut15 was able to bind both GST–Impβ and GST–NLS with (+) or without (–) the heat treatment. Cut15-122 lost the ability to bind GST–NLS when it was heat-treated, however. The lane labeled L shows samples before the GST pull-down; GST indicates a pull-down with GST alone.

functionally relevant. The *Drosophila* importin-α-like protein pendulin also binds to the surface of condensed metaphase chromosomes [14]. Cut15 possibly interacts with a crucial regulator required for condensation inside the nucleus during mitosis. In *cut15* mutants, the interaction is lost, so that chromosome condensation fails but other cell cycle events proceed. Alternatively, Cut15 might interact directly with topoisomerase II or condensin inside the nucleus during mitosis and aid their correct positioning on the mitotic chromosomes. There is a genetic interaction between *cut15* and *cut3*: *cut15-85* is synthetically lethal when combined with any of three *cut3* alleles. Cut15 and Cut3 might share an essential function in assembling chromosome loops and coils during condensation.

Supplementary material

Figures showing a sequence alignment of Cut15, further time-lapse images of Cut15–GFP and the purification of Cut15 proteins, and additional methodological details, are published with this paper on the internet.

Acknowledgements

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Supplementary material

Mutations in fission yeast Cut15, an importin α homolog, lead to mitotic progression without chromosome condensation

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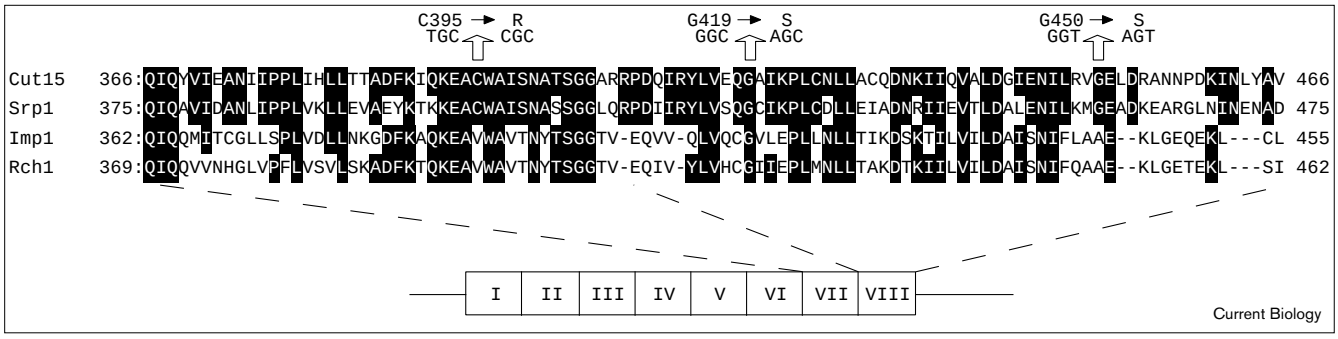
Figure S1



Partial sequences of Cut15, Srp1, importin 1 (Imp1) and Rch1. Identical amino acids are shaded. Mutation sites are indicated. The full sequence of Cut15 is deposited in the DNA database of Japan (DDBJ; accession number AB010574).

Materials and methods

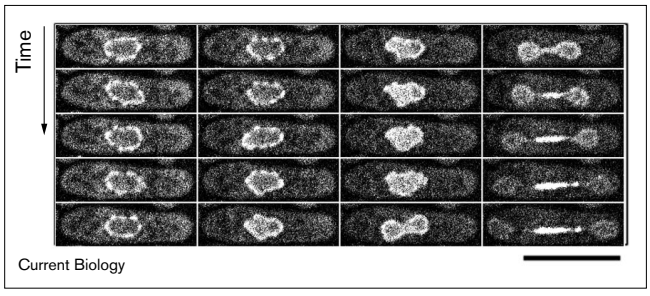
Strains, plasmids and extracts

S. pombe strains used were previously described [S1]. Plasmid pCQ100 carrying the *cut15*$^+$ gene was isolated from an *S. pombe* genomic library by transformation of the *cut15-85* mutant. The cloned sequence integrated onto the chromosome by homologous recombination was tightly linked to the *cut15* locus (less than 1.7 cM as PD:NPD:TT = 28:0:0). The GFP plasmid adapted for *S. pombe* was described previously [12].

Determination of mutation sites

The *cut15*$^+$ gene sequence was digested with exonuclease III from the 3′ end, and resulting fragments were used for integration into the chromosome of *cut15-85* and *cut15-122*. Fragments containing the carboxyl terminus could suppress them. The genomic DNA of this region was then isolated from both alleles by the PCR cloning method, followed by nucleotide sequencing.

Figure S2



The time course of the Cut15–GFP signal in living wild-type cells at 26°C by confocal microscopy. See Figure 3 legend for details. This is a second example cell with images taken at 2 min intervals. The bar represents 10 μm.

Light microscopy and FISH

Confocal microscopy for *S. pombe* cells carrying the plasmid encoding Cut15–GFP was previously described [S2,S3]. The FISH method was used to paint the left arm of chromosome II [6].

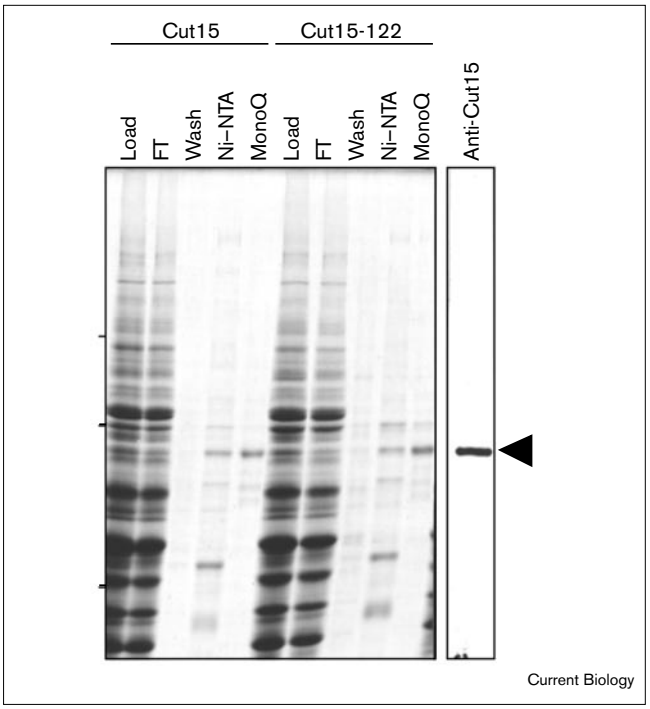
Purification of Cut15 protein

Wild-type strain HM123 carrying plasmid REP1Cut15HA6His was cultured in the synthetic medium EMM2 at 26°C for 22 h. Cells were collected by centrifugation and washed once by TE buffer (10 mM Tris HCl, 0.1 mM EDTA). Cells (6×10^{10}) were suspended in 20 ml buffer D (20 mM Tris HCl at pH 7.5 containing 150 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol and 1 mM phenyl methyl sulfonyl fluoride, PMSF) and disrupted with glass beads, followed by ultracentrifugation at 100,000 × *g* for 1 h. Imidazole (final concentration 5 mM) was added to the supernatant, which was then mixed with 5 ml Ni^{2+}–NTA agarose previously equilibrated with buffer D plus 5 mM imidazole and incubated at 4°C for 2 h. Agarose beads were then washed twice with buffer D plus 20 mM imidazole for 5 min each time, and eluted with buffer D containing 200 mM imidazole for 15 min. The eluant was dialyzed against 20 mM HEPES pH 7.3, 110 mM potassium acetate, 2 mM DTT and pepstatin A (1 μg/ml) overnight, and applied to a MonoQ column using a 100 mM to 1 M NaCl gradient. Eluted Cut15 protein was dialyzed against the transport buffer TB (20 mM HEPES pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 2 mM DTT), and proteinase inhibitors (1 μg/ml each of aprotinin, pepstatin and leupeptin). Mutant Cut15-122 protein was purified by the same procedures using plasmid REP1Cut15-122HA6His. This purification method has been described previously [S4].

In vitro *nuclear transport assay*

MDBK cells were treated with digitonin (40 μg/ml) in TB for 5 min. Cells were then washed twice in TB without digitonin. Cut15 (6 pmol), importin β (3 pmol), Ran (40 pmol), p10 (16 pmol), 1 mM ATP, 5 mM creatine phosphate, creatine phosphokinase (20 units/ml), 0.5 mM

Figure S3



Purification of wild-type Cut15 and mutant Cut15-122 proteins by affinity chromatography (Ni–NTA) followed by a MonoQ chromatography step. A Coomassie-blue-stained gel is on the left. The MonoQ preparations largely consisted of Cut15 polypeptide (60 kDa). An immunoblot of the purified preparation using anti-Cut15 antibodies is shown on the right. The lane labeled load contains cell extracts; the flow-through (FT) lane, fractions of the Ni–NTA column; the wash lane, washes of the Ni–NTA column with buffer D.

GTP and the transport substrate T-APC (100 ng; Calbiochem-Noalbiochem) were added, and cells were incubated at 26°C for 30 min. Cells were washed twice, fixed with formaldehyde (3.7%) and observed by fluorescence microscopy (the substrate APC produced red fluorescence by green excitation). In the absence of ATP, Apyrase (1 unit/ml) was added. WGA (0.2 mg/ml) was added to block the nuclear import. This assay has been described previously [13].

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