

INTERACTION OF TYROSINASE AND ITS SOLUBLE MUTANT WITH BIOLOGICAL PARTNERS IN MELANOMA CELLS

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In the present study we aim to identify and differentiate between interactors of wild-type tyrosinase (WT-TYR) and its soluble mutant (ST-TYR) overexpressed in A375 amelanotic melanoma cells. Protein complexes of the two tyrosinases were isolated by co-immunoprecipitation and identified by mass spectrometry (MS). By this protocol a large number of proteins involved in various cellular processes were identified as potential interactors of WT-TYR and ST-TYR, and the two sets of interactors only partially overlap. A discussion on this is provided. The results presented herein will form the basis of further investigations intended to confirm from the lists the real interactors of these two tyrosinase form by complementary methods.

Keywords: co-immunoprecipitation, mass spectrometry, A375 cells, tyrosinase, interactors.

INTRODUCTION

Melanoma is the most aggressive form of skin cancer and although many studies were aimed in identifying new molecules for melanoma therapy, only few proved to be effective for this disease. Peptides derived from tyrosinase are presented by the MHC complex on the surface of tumoral cells indicating a role of this antigen in the immune response to melanoma (1). Tyrosinase is a key enzyme in skin pigmentation, responsible for melanin synthesis in melanocytes. Melanin synthesis is a complex process catalysed by tyrosinase and tyrosinase related proteins in melanosomes. These organelles acquire tyrosinase after its maturation in the lumen of endoplasmic reticulum (ER) and Golgi.

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Monophenol monooxygenase is a type I membrane protein of 529 amino acids, with seven potential N-glycosylation sites, 17 cysteine residues grouped in two Cys rich domains and two copper binding domains (2). N-glycans exposed on tyrosinase mediate the interaction with the lectin chaperones calnexin/calreticulin that ensures efficient folding. The protein must pass the endoplasmic reticulum quality control cycle, that releases only mature proteins from ER, in order to reach melanosomes. A small fraction of the molecules that are misfolded proteins is targeted for degradation (3-5).

The interaction with calnexin increases folding efficiency, however not only the glycans, but also the transmembrane domain was shown to be involved in tyrosinase maturation (2). When the C-terminal end of tyrosinase, corresponding to its transmembrane domain, is deleted, it results a soluble mutant (ST-TYR) that fails to fold properly and is retained in the ER. This is further retro-translocated in the cytoplasm and degraded mainly through the proteasomal pathway. It was found that WT-TYR associates with calnexin during the folding process, while ST-TYR mainly interacts with the molecular chaperones calreticulin and BiP. Additionally, soluble tyrosinase is a typical ERAD (ER-Associated protein Degradation) substrate, subjected to ER quality control and does not undergo Golgi transport and subsequent retrieval (2).

Maturation and processing of tyrosinase depends heavily on N-glycan trimming as most ER glycoproteins. Monoglucosylated glycans are signals for calnexin and calreticulin and recognition further demannosylated glycans are signals either for transport to Golgi or for degradation. In recent years several papers showed that EDEM1, 2 and 3 are active players of ERAD and accelerate protein degradation (6). Tyrosinase degradation was shown to be partly performed by the ubiquitin proteasomal system and ERAD. Recent studies showed that the degradation of wild type as well as misfolded tyrosinase mutants is accelerated by EDEM1 (ER degradation-enhancing alpha-mannosidase-like protein 1) (7).

This study aims to optimise the detection of possible interactors of wild type (WT) and soluble (ST) tyrosinases and by doing this to compare the interactomes of the two tyrosinase forms. Along with complementary investigations this is intended to help elucidating the molecular mechanism driving the transport from the ER either to the secretory pathway or to degradation.

MATERIALS AND METHODS

EXPRESSION OF WILD TYPE AND SOLUBLE TYROSINASE IN A375 CELLS

A375, amelanotic melanoma cells, were stably transfected with wild type and soluble tyrosinase using the Retroviral Gene Transfer and Expression System (Clontech). For this, the genes encoding for WT and ST tyrosinase were cloned into pLNCX2 that allows tyrosinase expression under the CMV promoter.

Plasmids pLNCX2-WT, pLNCX2-ST and empty vector (pLNCX2) were transfected into pt67 retroviral packaging cell line grown in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 4 mM Glutamax, for 24 h. Medium containing retrovirus was collected from pt67 cells centrifuged at 500 xg for 10 minutes and filtered through a 45 µm cellulose acetate membrane.

A375 cells, cultivated in the same conditions at confluency of 30-50%, were infected with medium collected from pt67 in the presence of 8µg/mL polybrene for 24 h. Selection of infected cells was made with geneticin 600 µg/mL for 24-72 h and further sub-cultivated in 400 µg/mL. The heterogeneous cell population was cloned by limiting dilution; one selected clone, for each construct, was used for further experiments.

For mass spectrometry analysis we performed a single experiment. The cells were grown in T75 cm² dish to a confluence of 80-90%, harvested and centrifuged at 4°C. The pelleted cells were lysed with 1% PBS-T (1X PBS + 1% Triton X-100) supplemented with protease inhibitors (Roche), for 20 minutes at 4°C. The lysate were centrifuged at 25,000 × g, for 40 min to clarify the supernatant, that was further used to determine the total amount of protein by BCA assay.

ELECTROPHORESIS AND WESTERN BLOTTING

Equal amounts of protein were denatured and separated by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS-PAGE). For Western blotting, the proteins were transferred from the gel to a nitrocellulose membrane that was blocked in a solution of 10% skimmed milk powder in PBS at 4°C, overnight (ON). The membrane was incubated with mouse monoclonal α-tyrosinase (T311) (Santa Cruz Biotechnology, Inc.) primary antibody (1/400) and rabbit α -mouse IgG-HRP (Santa Cruz Biotechnology, Inc.) secondary antibody (1/10000) both diluted in 5% milk in PBS-0.1% Tween 20 for 1 hour at room temperature (RT). The proteins were detected using a chemiluminescence kit ECL (Pierce).

SAMPLE PREPARATION AND MASS SPECTROMETRY ANALYSIS

Immune complexes were captured with protein A or protein G beads, 1/50 ratio for 2 hours with swirling. The non-specifically bound proteins were removed by washing as follows: 3 times with PBS-T 0.2% and 1 time with PBS. The isolated complexes were eluted with SEB (soft elution buffer) (0.2% SDS, 0.1% Tween-20, 50 mM Tris-HCl, pH 8.0) (8) (SEB-beads 4/1 ratio), and shaken for 7 min at 1000 rpm and 25°C. A second elution was performed with 100 mM glycine-HCl, pH 2.8, in 4/1 ratio, and whirled for 20 minutes at 8 rpm and 4 °C.

After centrifugation, the two elutions were merged and used for subsequent protocols.

Gel staining, excision and dehydration. The gel stained with Coomassie Brilliant Blue R-250 was excised into pieces and every lane was divided into three parts that were separately injected for MS analysis. Excised parts were a) below 35 kDa, b) between 35-70 kDa and c) above 70 kDa. Gel pieces were destained with 50 mM ammonium bicarbonate (ABC) in 50% acetonitrile (ACN) and dehydrated with ACN, for 15 minutes at room temperature.

Reduction and alkylation of proteins. Disulfide bonds were reduced with 10 mM dithiothreitol (DTT) in 100 mM ABC, by incubation for 45 minutes in the dark at 56°C with occasional vortexing. The alkylation of the free cysteines was achieved in the presence of 55 mM iodoacetamide (IAA) in 100 mM ABC, by incubation for 45 minutes in the dark with occasional vortexing. Gel pieces were washed with 50 mM ABC in 50% ACN, and ACN by incubation for 15 minutes at room temperature, followed by the drying stage (30 minutes) in the Speed-Vac concentrator.

Enzymatic digestion. For proteins trypsinization, gel pieces were hydrated with 10 ng/μL trypsin solution and incubated 30 minutes on ice, followed by a supplementary trypsin solution addition, depending on the volume of solution absorbed, and incubated for 1 hour on ice. The samples were further incubated overnight at 37°C with gentle shaking.

Peptide extraction. The peptides resulted after trypsinization were extracted with 5% formic acid (FA) and 50% ACN (in 1:2 ratio digestion volume/extraction volume), by incubation for 15 minutes at 37°C (2 times) and 5% FA in ACN, for 30 minutes at 37°C (1 time) with moderate agitation. All solutions containing mixtures of peptides were combined and dried in Speed-Vac concentrator, and used further for LC-MS/MS analysis (9-11).

LC-MS/MS analysis. The dried peptides were solubilized in 20 μL mobile phase A (0.1% FA + 2% ACN) and 5 μL from each sample were injected into a nano-liquid chromatograph (nano LC) Easy nano LC II (Proxeon Biosystems) coupled online to a LTQ Orbitrap Velos Pro instrument (Thermo Fisher Scientific). For peptide separation a 2-30% gradient of mobile phase B (0.1% FA + 98% ACN) was applied to a dual system composed of a C18 trap column 2 cm × 100 μm (Proxeon Biosystems) and a C18 analytical column, 10 cm × 75 μm (Proxeon Biosystems) connected to a Stainless Steel emitter (Thermo Scientific) for electrospray ionization. The mass spectrometer was operated in a data-dependent mode, from which the method involved the acquisition of a full MS scan between 300 and 1800 m/z in the Orbitrap at a resolution of 30000 at m/z 400, followed by 10 consecutive MS/MS scans in the ion trap using Collision Induced Dissociation (CID) for peptide fragmentation (12). Only charges of +2, +3 or higher were selected for fragmentation into the ion trap.

Peptide and protein identification. The spectrum files were searched using the SEQUEST HT algorithm, integrated into Proteome Discoverer v1.4 against a human version of Swiss prot database (20238 sequences as of 08/2013) with the following settings: precursor mass tolerance of 20 ppm, ion fragment mass tolerance of 0.6 Da, carbamidomethylation of Cys residues as a static modification, and oxidation of methionine and deamidated of asparagine and glutamine as the dynamic modifications. For peptide confidence assignment a decoy database was searched in parallel containing the reversed sequences from the initial database. Only peptides with a precursor mass tolerance of maximum 10 ppm and a 99% estimated confidence were kept in the final results. At least 1 unique peptide was required to validate the identification of a protein group.

RESULTS AND DISCUSSION

Herein are presented some preliminary tests aimed to optimise the detection of possible interactors of soluble (ST-TYR) and wild-type tyrosinase (WT-TYR) based on co-immunoprecipitation followed by nano-liquid chromatography peptide separation coupled with tandem mass spectrometry detection (nano LC-MS/MS).

To this end we used A375 cell lines transduced with WT-TYR, ST-TYR and empty pLNCX2 vector as a control. Lysates were immunoprecipitated with rabbit polyclonal α -tyrosinase (produced in our laboratory) and mouse monoclonal α -tyrosinase (T311) antibodies in a 1/100 dilution overnight. The immune complexes were isolated by immobilization on Protein A and Protein G Sepharose beads, respectively, and eluted as described in Materials and methods.

The immune complexes were separated by SDS-PAGE; one gel was stained with Coomassie Brilliant Blue R-250 and used for MS analysis (Fig. 2), whilst the other two gels were used for Western blotting (Fig. 1).

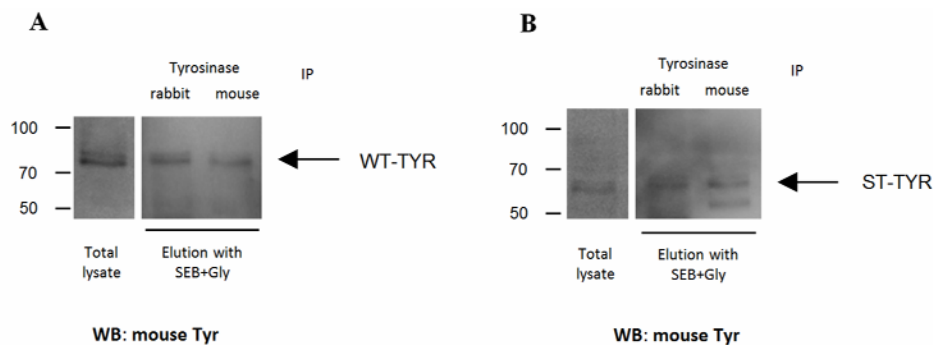


Fig. 1 – Identification of tyrosinases in the immunoprecipitates by Western blotting. Stable cell lines transduced with WT-TYR, ST-TYR and pLNCX2 retroviral vector were lysed, immunoprecipitated with rabbit polyclonal α -tyrosinase and mouse monoclonal α -tyrosinase (T311) antibodies. Eluted samples were separated by SDS-PAGE and further used for Western blotting experiments to detect WT-TYR and ST-TYR using mouse monoclonal α -tyrosinase (T311) antibody.

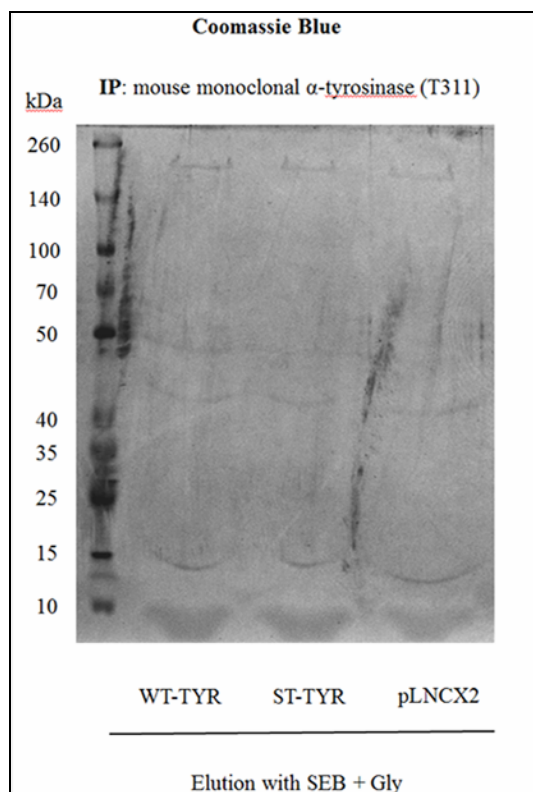


Fig. 2 – SDS PAGE of tyrosinases immunoprecipitated with specific antibodies and Coomassie Blue staining. A375 cells expressing WT-TYR and ST-TYR were lysed and used for immunoprecipitation with mouse monoclonal α -tyrosinase (T311) antibody. The eluted fractions were separated in acrylamide gels and the whole lane was separated into fractions and, subsequently, peptides were extracted as mentioned above and injected into the MS.

As observed in Fig. 1, Western blotting experiments indicate that both WT-TYR and ST-TYR were expressed in A375 cells and efficiently purified by affinity chromatography.

The Coomassie gel was processed for MS analysis as described in Materials and methods and MS results were analyzed with specific softwares. By grouping proteins based on at least one unique peptide with medium scoring confidence, and 10 ppm in mass deviation, we identified in all samples an overall number of 203 proteins excepting the well documented contaminants. These were distributed as follows: 118 in WT-TYR sample, 98 in ST-TYR and 120 in pLNCX2.

Tyrosinase derived peptides were identified in WT and ST samples only with 5 and 8 unique peptides, respectively.

The overall 203 proteins fall in seven classes as follows: specific for WT-TYR [43], ST-TYR [26] and pLNCX₂ [53], common to WT-TYR and ST-TYR

[14], WT-TYR and pLNCX2 [9], and ST-TYR and pLNCX₂ [6], common to all three [52] (Fig. 3). This relatively low number of interactors is probably mainly due to the fact that tyrosinase, even if very active, is expressed in very low quantities in the cell and has an enzymatic rather than receptor or hub type of function.

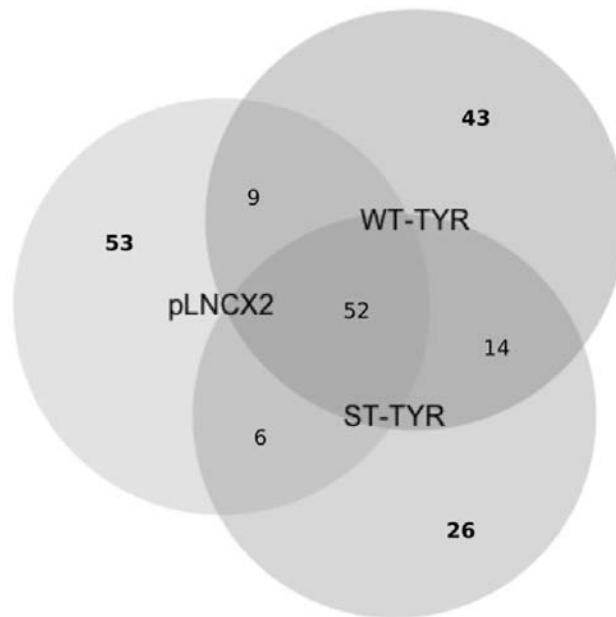


Fig. 3 – Venn diagram distribution of the protein groups identified using nano LC-MS/MS analysis in the WT tyrosinase (WT-TYR), the soluble form (ST-TYR) and in the control vector (pLNCX2). The numbers represent the protein groups unique/common between each condition.

The distributions on cellular compartment and molecular function of the proteins interacting specifically with WT-TYR and ST-TYR, are shown in Fig. 4.

In the wild type case the most interesting interactors that will be followed up in further studies are the ADP/ATP translocase 2 which is involved in catalyzing exchange of mitochondrial ATP with cytosolic ADP (13), calmodulin-like protein 5 which is involved in the regulation of keratinocyte differentiation (14), and putative tripartite motif-containing protein 64C (a fusion protein) (15).

For the misfolded ST-TYR form destined to degradation, highly interesting are the interactors related to the secretory pathway and its derivatives: SEC23-interacting protein which is involved in ER-Golgi transport mediated by COPII (16), kinesin-like protein KIF1C, involved in the retrograde transport of Golgi vesicles to the endoplasmic reticulum (17), and Golgin subfamily B member 1, a cis/medial Golgi protein, involved in intra-Golgi trafficking (18).

It is also interesting to mention here the identification of annexin A2 and inactive caspase-12 as interactors for both WT and ST tyrosinase. Annexin A2 is involved in endocytosis, exocytosis, and cell adhesion (19), and inactive caspase-12 is activated by ER stress leading to accumulation of excess proteins in ER (20).

Other interesting proteins such as filaggrin-2, protein S100-A8, protein S100-A9 and stress-70 protein mitochondrial were also identified in the control sample and thus were considered nonspecific interactors.

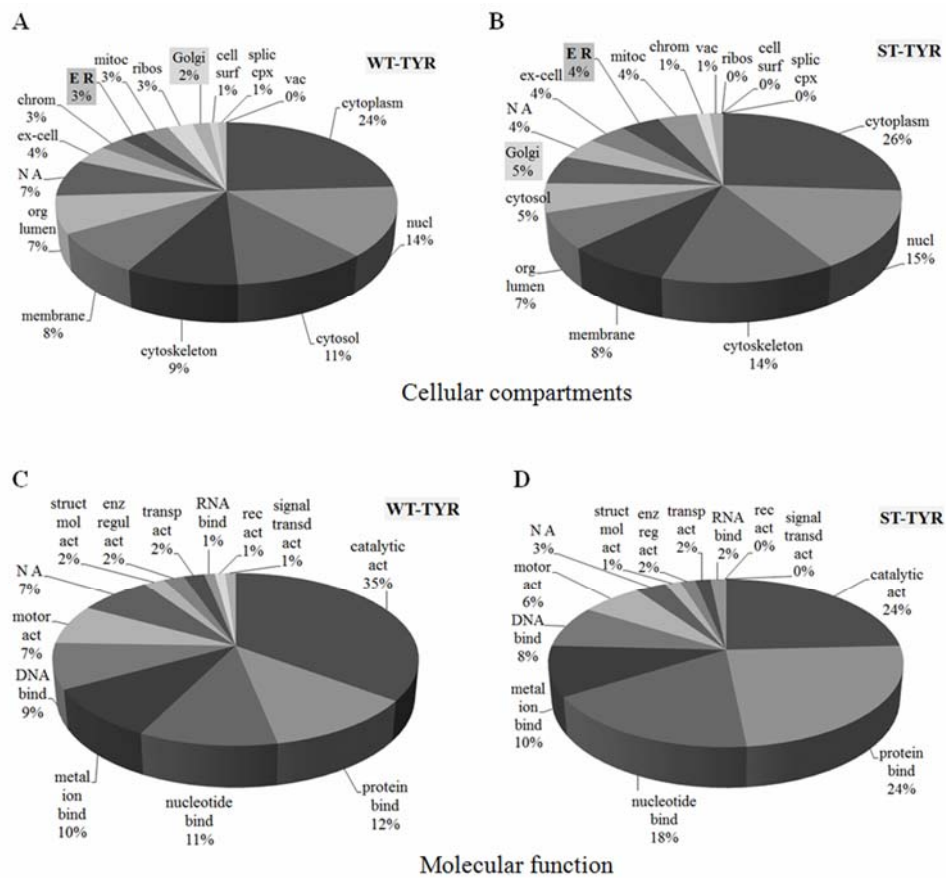


Fig. 4 – Gene ontology annotation of the cellular compartment (GOCC) and the molecular function (GOMF) for the proteins identified in each condition.

SEC23 which is critical in the protein traffic might mediate the transport of unfolded tyrosinase forms into the cytosol for proteasomal degradation. Future studies to confirm these results by complementary methods will be employed. The results presented herein suggest a different traffic for the wild type and the soluble form of tyrosinase in A375 cells.

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REFERENCES

1. Petrescu SM, Popescu CI, Petrescu AJ, Dwek RA, *The glycosylation of tyrosinase in melanoma cells and the effect on antigen presentation*, *Adv. Exp. Med. Biol.*, **535**, 257–269 (2003).
2. Popescu CI, Paduraru C, Dwek RA, Petrescu SM, *Soluble tyrosinase is an endoplasmic reticulum (ER)-associated degradation substrate retained in the ER by calreticulin and BiP/GRP78 and not calnexin*, *J. Biol. Chem.*, **280**, 13833–13840 (2005).
3. Petrescu SM, Branza-Nichita N, Negroiu G, Petrescu AJ, Dwek RA, *Tyrosinase and glycoprotein folding: Roles of chaperones that recognize glycans*, *Biochemistry*, **39**, 5229–5237 (2000).
4. Branza-Nichita N, Petrescu AJ, Negroiu G, Dwek RA, Petrescu SM, *N-glycosylation processing and glycoprotein folding-lessons from the tyrosinase-related proteins*, *Chem. Rev.*, **100**, 4697–4712 (2000).
5. Cioaca D, Ghenea S, Spiridon LN, Marin M, Petrescu AJ, *et al.*, *C-terminus glycans with critical functional role in the maturation of secretory glycoproteins*, *PLoS One*, **6**, e19979 (2011).
6. Olivari S, Molinari M, *Glycoprotein folding and the role of EDEM1, EDEM2 and EDEM3 in degradation of folding-defective glycoproteins*, *FEBS Lett.*, **581**, 3658–3664 (2007).
7. Marin MB, Ghenea S, Spiridon LN, Chiritoiu GN, Petrescu AJ, *et al.*, *Tyrosinase degradation is prevented when EDEM1 lacks the intrinsically disordered region*, *PLoS One*, **7**, e42998 (2012).
8. Antrobus R, Borner GH, *Improved elution conditions for native co-immunoprecipitation*, *PLoS One*, **6**, e18218 (2011).
9. Gundry RL, White MY, Murray CI, Kane LA, Fu Q, *et al.*, *Preparation of proteins and peptides for mass spectrometry analysis in a bottom-up proteomics workflow*, *Curr. Protoc. Mol. Biol.*, **Chapter 10**, Unit10 25 (2009).
10. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M, *In-gel digestion for mass spectrometric characterization of proteins and proteomes*, *Nat. Protoc.*, **1**, 2856–2860 (2006).
11. Sokolowska I, Dorobantu C, Woods AG, Macovei A, Branza-Nichita N, *et al.*, *Proteomic analysis of plasma membranes isolated from undifferentiated and differentiated HepaRG cells*, *Proteome Sci.*, **10**, 47 (2012).
12. Wells JM, McLuckey SA, *Collision-induced dissociation (CID) of peptides and proteins*, *Methods Enzymol.*, **402**, 148–185 (2005).
13. Ito S, Tan LJ, Andoh D, Narita T, Seki M, *et al.*, *MMXD, a TFIIF-independent XPD-MMS19 protein complex involved in chromosome segregation*, *Mol. Cell*, **39**, 632–640 (2010).
14. Donovan M, Ambach A, Thomas-Collignon A, Prado C, Bernard D, *et al.*, *Calmodulin-like skin protein level increases in the differentiated epidermal layers in atopic dermatitis*, *Exp. Dermatol.*, **22**, 836–837 (2013).
15. Taylor TD, Noguchi H, Totoki Y, Toyoda A, Kuroki Y, *et al.*, *Human chromosome 11 DNA sequence and analysis including novel gene identification*, *Nature*, **440**, 497–500 (2006).
16. Amodio G, Venditti R, De Matteis MA, Moltedo O, Pignataro P, *et al.*, *Endoplasmic reticulum stress reduces COPII vesicle formation and modifies Sec23a cycling at ERESs*, *FEBS Lett.*, **587**, 3261–3266 (2013).
17. Dorner C, Ciossek T, Muller S, Moller PH, Ullrich A, *et al.*, *Characterization of KIF1C, a new kinesin-like protein involved in vesicle transport from the Golgi apparatus to the endoplasmic reticulum*, *J. Biol. Chem.*, **273**, 20267–20275 (1998).

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18. Shorter J, Beard MB, Seemann J, Dirac-Svejstrup AB, Warren G, *Sequential tethering of Golgins and catalysis of SNAREpin assembly by the vesicle-tethering protein p115*, **J. Cell Biol.**, **157**, 45–62 (2002).
 19. Mussunoor S, Murray GI, *The role of annexins in tumour development and progression*, **J. Pathol.**, **216**, 131–140 (2008).
 20. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, *et al.*, *Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta*, **Nature**, **403**, 98–103 (2000).