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ER-associated protein degradation is a common mechanism underpinning numerous monogenic diseases including Robinow syndrome

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Correct folding of nascent polypeptide chains within the ER is critical for function, assembly into multi-subunit complexes and trafficking through the exocytic pathway for secretory and cell surface proteins. This process is rather inefficient, and a substantial proportion of nascent polypeptides is rejected by an ER quality control system and targeted for degradation. In some cases, only a minor fraction of nascent chains is correctly folded, and the smallest alteration to polypeptide primary structure (i.e. point mutation) can result in the complete loss of function with inherent pathological consequences; cystic fibrosis and emphysema result from such mutations. We have taken a bioinformatic approach to parse a large database of known disease susceptibility genes for candidates whose disease-associated alleles are likely prone to misfolding in the ER. Surprisingly, we find that proteins with ER-targeting signals are over represented in this database when compared with all predicted proteins in the human genome (45 versus 30%). We selected a subgroup of proteins that were positive for both an ER-targeting signal and a membrane-anchoring domain and thereby identified several ER-associated degradation diseases candidates. To determine whether our analysis had identified new ER-degradation substrates, we established that ER retention is indeed the mechanism underlying Robinow syndrome (RRS), one of the identified candidates. Specifically, mutant alleles of *ROR2* that are associated with RRS are retained within the ER, whereas wild-type and non-pathogenic alleles are exported to the plasma membrane. These data both uncover a major pathogenic factor for RRS and indicate that misfolding of secretory proteins is likely to significantly contribute to human disease and morbidity.

INTRODUCTION

Rapid and efficient polypeptide folding is an essential cellular process required for functionality of the native protein. Recently, it has emerged that a significant number of pathological states arise via protein misfolding, including responses to environmental stress and infectious and hereditary diseases. The stress response, for example, to xenobiotics, oxidants and elevated temperatures, can involve promotion of protein misfolding, which is partly compensated for by activation of the unfolded protein response, an ER-based mechanism for increasing the folding efficiency of proteins within the secretory system (1). For infectious diseases, potent examples include the prion-related diseases, specifically the spongiform encephalopathies, whereby a malfolded prion protein acts as a template to catalyze misfolding of normal conformers (2). Prion diseases can also be inherited, e.g. Creutzfeldt–Jacob's disease, and appear to result primarily from point mutations within the prion gene, which increase misfolding potential (2). Further examples of hereditary disease include alleles associated with cystic fibrosis, emphysema and hypercholesterolemia, where again point mutations in disease-associated genes have a profound impact on health through loss of function (3). These latter examples are all

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secretory proteins and associated with misfolding at an early stage of trafficking and specifically retention within the ER. A number of diseases associated with misfolding have unknown etiology, for example, Alzheimer's disease: accumulation of amyloid fibrils following abnormal processing of the amyloid precursor protein in the late secretory system leads to central nervous system pathology and neurological damage; the trigger for promotion of abnormal processing of the precursor protein remains poorly understood (4).

Defective protein trafficking, as a result of defects either within the secretory protein itself or the trafficking machinery, has been implicated in the pathogenesis of many human disorders (5,6). From a survey of trafficking defects, ER-associated degradation (ERAD) was implicated as contributing to the mechanism of at least 30 human diseases (3,5,6). ERAD is responsible for the quality control (QC) of secretory protein folding and requires the coordinated action of at least three groups of ER lumenal proteins (7,8): the calnexin system, a chaperone system and a redox system. Rejection by the QC system results in diversion into a degradation pathway, involving re-export to the cytosol and proteosome-mediated proteolysis (9). However, the diseases surveyed were restricted to those where the molecular mechanism was known. As ~30% of all cellular proteins contain a predicted ER-targeting signal, we reasoned that many more ERAD diseases remain undiscovered. Knowledge of the molecular mechanisms underpinning a disease is clearly vital and serves to facilitate understanding both pathology and potentially the selection and development of appropriate therapeutic approaches. Here, a bioinformatic strategy was used to search for ERAD and secretory pathway disease candidates, which identified several new ERAD disease candidates.

Robinow syndrome (RRS) is an autosomal recessive disease characterized by severe skeletal dysplasia, dysmorphic facial appearance, segmental defects of the spine, brachydactyly (shortening of digits) and genital hypoplasia (10,11). The incidence of RRS is about one in 500 000 (12), with an increased prevalence in populations in Turkey, Oman and the former Czechoslovakia, where consanguineous marriages are frequent (13). The *ROR2* gene present on chromosome 9 encodes an orphan receptor tyrosine kinase and mutant alleles are responsible for RRS: both homozygous missense and nonsense mutations in the extracellular and intracellular domains of *ROR2* have been reported, suggesting that RRS is associated with loss of *ROR2* function, although the precise mechanism remains unclear (11,14). Homozygous mutations in the mouse *Ror2* gene cause mesomelic dwarfing (15,16), and Ror2 knockout mouse showed similar phenotypes as found in RRS patients (17). In addition, mutations in *ROR2* cause the autosomal dominant gain of function condition brachydactyly type B (18). The *ROR2* gene, which was identified by informatics as a strong ERAD disease candidate, was selected for further investigation and to validate the predictions. Remarkably, mutant alleles of mouse Ror2 are indeed ER retained, providing a mechanism for loss of function and the first insight into the molecular basis for RRS. These data therefore support ER retention as a major pathological factor among monogenic diseases of unknown etiology.

RESULTS

ERAD candidates in the GeneCards database

All ERAD substrates must, by definition, contain an ER-targeting signal. To identify new ERAD substrates among human disease genes, we parsed the GeneCards (http://bioinfo.weizmann.ac.il/cards/index.shtml) data set using SignalP to detect sequences predicted to contain an ER-signal sequence. Of a total of 2656 entries, we retrieved sequences corresponding to 1730 unique genes (Supplementary Material, Table S2). Of these, SignalP detected an N-terminal ER-signal sequence in 668 entries and a signal anchor in a further 103. Significantly, ER-targeted proteins contribute 45% (771/1730) of the disease genes retrieved, somewhat higher than the predicted proportion within the human genome (~30%). Importantly, ~85% of known disease-associated genes that are authentic ERAD substrates (5,6) were present in this cohort (Supplementary Material, Table S3). This gave us confidence that the retrieved data set contained novel ERAD diseases. Six ERAD substrates were not found because of failure of SignalP to predict a signal sequence that is not located at the N-terminus (Supplementary Material, Table S4). An implication of this is that ERAD diseases might be contributing >45% of the total human disease genes.

The cohort of sequences positive for ER-targeting signal was further selected for the presence of additional subcellular targeting signals (Supplementary Material, Table S5). We mainly concentrated on proteins with a transmembrane domain (TMD), i.e. membrane proteins. Using TMHMM v2.0, 295 proteins with signal sequences and 96 proteins with signal anchors were returned. We further rejected proteins where the disease-associated allele was known to be due to premature termination and positively selected for proteins where disease alleles contained missense mutations and mutations likely to affect folding. The criteria for the latter include introduction of charged amino acids into a TMD, the presence of multiple extracellular domains, non-conservative substitutions and mutations to cysteine codons. Further, we also looked for experimental evidence for mistargeting, stability or trafficking defects associated with the mutated gene product. Using these criteria, we arrived at a final set of 15 strong candidates (Table 1) and about 40 weak candidates (data not shown).

Selection of ROR2 for validation and generation of mutant alleles

With a level of accuracy in prediction exceeding 80%, we selected a disease gene for validation of the data set and hence the informatics strategy. This was particularly important as the latter part of the selection was essentially non-quantitative and statistical significance could not be applied to the entries in the final cohort. We selected the *ROR2* gene and RRS-associated alleles for several reasons (Fig. 1): (i) mutations were in cysteine codons or resulted in substitution of charged amino acids with strongly hydrophobic residues, (ii) the majority of mutations associated with RRS were in the extracellular domain, (iii) mutations in kringle

Table 1 Strong ERAD candidate disease genes

Gene	Disease	System affected	Potential effect of pathogenic mutation(s)
ROR2	Robinow syndrome	Skeletal, heart	Disruption to protein folding
POMT1	Walker–Warburg syndrome	Musculoskeletal	Disruption to TMD
GUCY2D	Leber congenital amaurosis, type I	Ocular	Possible retention in the ER reported[a]
COLQ	Endplate acetyl-cholinesterase deficiency	Muscular	Disruption to protein folding
MPZ	Charcot–Marie–Tooth neuropathy-IB	Neurological	Possible retention in the ER reported[b]
SLC2A1	Glucose transport defect	Blood–brain barrier	Disruption to TMD
CSF2RB	Pulmonary alveolar proteinosis	Lung	Disruption to tertiary structure
ACVRL1	Hereditary hemorrhagic telangiectasia	Vascular/pulmonary	Possible retention in the ER reported[c]
BMPR2	Familial primary pulmonary hypertension	Vascular/pulmonary	Disruption to protein folding
GJB3	Erythrokeratodermia variabilis	Skin	Possible retention in the ER reported[d]
GJB4	Erythrokeratodermia variabilis	Skin	Disruption to TMD
ABCA1	Tangier disease and HDL deficiency	Cardiovascular	Disruption to protein folding[e]
DHCR7	Smith–Lemli–Opitz syndrome	Cardiovascular	Disruption to protein folding
DHCR24	Desmosterolosis	Cardiovascular	Disruption to protein folding
ELN	Supravalvular aortic stenosis	Cardiovascular	Disruption to protein folding

The genes listed here are those identified as strong ER-retention candidates. They include diseases where the location of the mutation or experimental data suggest a defect in folding or trafficking, in addition to conforming to the informatics criteria, i.e. possessing an ER-targeting signal.

[a]Cremers, F.P., van den Hurk, J.A. and den Hollander, A.I. (2002) Molecular genetics of Leber congenital amaurosis. *Hum. Mol. Genet.*, **11**, 1169–1176.

[b]Matsuyama, W., Nakagawa, M., Takashima, H. and Osame, M. (2002) Altered trafficking and adhesion function of MPZ mutations and phenotypes of Charcot–Marie–Tooth disease 1B. *Acta Neuropathol. (Berl.)*, **103**, 501–508.

[c]Harrison, R.E., Flanagan, J.A., Sankelo, M., Abdalla, S.A., Rowell, J., Machado, R.D., Elliott, C.G., Robbins, I.M., Olschewski, H., McLaughlin, V. *et al.* (2003) Molecular and functional analysis identifies ALK-1 as the predominant cause of pulmonary hypertension related to hereditary haemorrhagic telangiectasia. *J. Med. Genet.*, **40**, 865–871.

[d]Gottfried, I., Landau, M., Glaser, F., Di, W.L., Ophir, J., Mevorah, B., Ben-Tal, N., Kelsell, D.P. and Avraham, K.B. (2002) A mutation in GJB3 is associated with recessive erythrokeratodermia variabilis (EKV) and leads to defective trafficking of the connexin 31 protein. *Hum. Mol. Genet.*, **11**, 1311–1316.

[e]Retention in the ER and engagement of ERAD has been recently reported for this disease. (Albrecht, C., Baynes, K., Sardini, A., Schepelmann, S., Eden, E.R., Davies, S.W., Higgins, C.F., Feher, M.D., Owen, J.S. and Soutar, A.K. (2004) Two novel missense mutations in ABCA1 result in altered trafficking and cause severe autosomal recessive HDL deficiency. *Biochim. Biophys. Acta*, **1689**, 47–57.)

domains are difficult to accommodate without disrupting folding because the domain is small and folded in a complex manner, (iv) the protein has multiple ectodomains, suggesting a significant burden on the ER-folding environment, (v) the mechanism underlying RRS is unknown and (vi) RRS is recessive and likely to result from loss of function, which would be predicted if the ROR2 protein failed to reach its site of action, the plasma membrane.

To investigate and compare the trafficking of ROR2 wild-type and mutant proteins, we introduced single amino acid substitutions by PCR-based site-directed mutagenesis to generate HA-tagged mouse Ror2 mutants: C182Y, R184C, R189W, R366W and N620K (Fig. 1). These encompass all known missense mutations that are associated with RRS and represent a wide range of missense mutations in *ROR2* gene: C182Y, R184C and R189W locating within the cysteine-rich domain, R366W in the kringle domain and N620K in the tyrosine kinase domain. We also generated a Ror2 double mutant, pcDNA3-Ror2RRWW-HA, which contains both R189W and R366W mutations to simulate the case of an affected individual who carries both R189W and R366W mutations on the same mutant allele (19).

Ror2 mutant proteins are hypersensitive to EndoH digestion

We used sensitivity to EndoH as screen for an ER exit defect (20). Immunoprecipitation and immunoblotting results showed that for the Ror2 wild-type protein, a single protein band was detected at ~130 kDa, but following EndoH digestion, this single band resolved into two species (Fig. 2, lanes 1 and 2). The upper band (M) likely represents mature Ror2 protein with complex *N*-glycans resistant to EndoH digestion, and the lower band (P) retaining EndoH-sensitive oligomannosyl *N*-glycans, suggestive of a precursor form that is yet to enter the Golgi complex or has not been modified by Golgi glucosidases and glycosyltransferases. The approximate equal proportions of the mature (M) and putative precursor (P) ER forms suggest that wild-type Ror2 potentially has a significant immature population and therefore may itself be a slow folding protein.

For the Ror2 mutants (C182Y, R184C, R189W, R366W, N620K and RRWW), a band was also detected in immunoprecipitates from transiently transfected cells, which migrated similar to the M form of the Ror2 protein (Fig. 2). However,

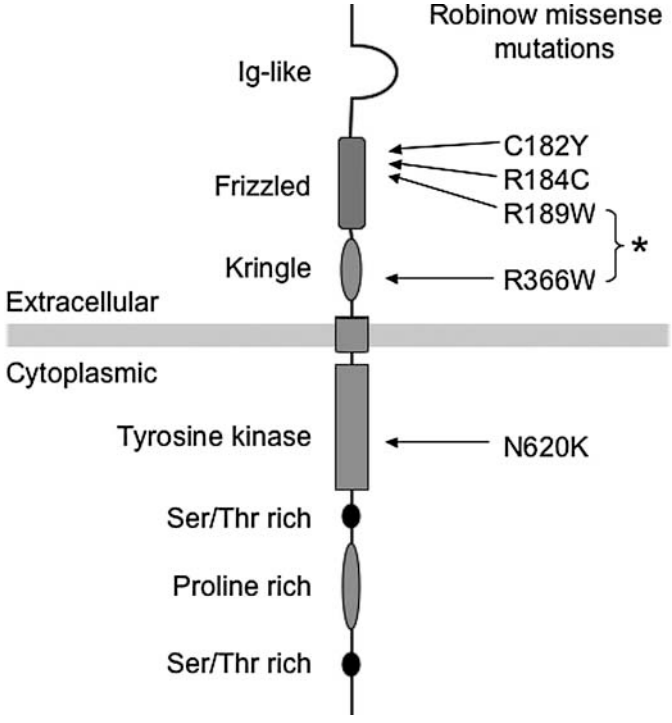

Figure 1. Schematic representation of the structure of the ROR2 protein and locations of mutations analyzed. The ROR2 protein contains immunoglobulin-like cysteine-rich (CRD) and kringle domains in the extracellular region and tyrosine kinase, serine/threonine-rich and proline-rich domains in the intracellular region. Mutations causing BDB1 are truncating mutations located in the intracellular part of the ROR2 protein (data not shown). In contrast, mutations causing RRS are scattered throughout the ROR2 protein and can be divided into two groups: missense mutations and premature stop mutations. An allele with two missense mutations, R189W in the CRD and R366W in the kringle domain, is indicated by an asterisk. Adapted from Afzal and Jeffery (19).

in contrast to the wild-type protein, these forms of Ror2 were fully sensitive to EndoH (Fig. 2). These data are highly suggestive that the Ror2 mutants are quantitatively retained in a pre-Golgi compartment.

Ror2 wild-type protein localized predominantly to plasma membrane, whereas the Ror2 mutant proteins are retained intracellularly

We co-expressed the Ror2 wild-type and mutant proteins with EGFP-hRas protein in HeLa cells, and their subcellular localization was examined by immunofluorescence analysis under confocal laser scanning microscopy. Examination of intrinsic GFP fluorescence revealed that EGFP-hRas protein is expressed exclusively at the plasma membrane (Fig. 3), consistent with earlier work (21). By labeling cells with mouse anti-HA-Tag monoclonal antibody, immunofluorescence of transfected HeLa cells showed that Ror2 wild-type protein co-localized very well with the EGFP-hRas protein predominantly to the plasma membrane, with additional staining throughout the *trans*-Golgi network and a small pool present in the ER region (Fig. 3C), confirming that Ror2 wild-type proteins are transported out of the ER system and expressed in the plasma membrane. The ER resident population likely represents a newly synthesized pool of Ror2 wild-type proteins at the time of fixation of the cells and corresponds to the EndoH-sensitive fraction.

In contrast, none of the Ror2 mutant proteins, including the RRWW double mutant, co-localized with the EGFP-hRas protein to the plasma membrane (Fig. 3D–U). Rather, these forms of Ror2 were observed to be distributed intracellularly in a perinuclear and reticular pattern typical of the ER. These data indicate that the Ror2 mutants fail to be exported to the cell surface, and taken together with the immunoblotting analysis, are most likely to be retained within the ER.

Ror2 mutant proteins are localized in the ER region of the cells

HeLa cells expressing each of the HA-tagged Ror2 isoforms were double labeled with the mouse anti-HA-Tag monoclonal antibody and a rabbit anti-calnexin polyclonal antibody. Double labeling revealed that there was no significant co-localization of the Ror2 wild-type protein with calnexin (Fig. 4). In contrast, all of the mutant proteins co-localized with the ER marker calnexin and were observed to be associated with individual ER tubules as well as the nuclear envelope, which is an extension of the ER membrane (Fig. 4). Taken together with the previous results of a lack of co-localization with EGFP-hRas, these data strongly suggest that the Ror2 mutant proteins are expressed and most likely retained in the ER. Significantly, no difference in the locations between the Ror2 double mutant RRWW and the single mutants R189W and R366W was seen (Fig. 4), and it indicates that a single amino acid substitution is sufficient to impair the trafficking of Ror2. This is in contrast to the earlier suggestion that the R366W mutation may exert the predominant pathogenic effect in this patient (14). It is suspected that R189W could cause a defect in a potential glycosylation site of the cysteine-rich domain and thus affects the modification of the ROR2 protein by the N-linked glycans (22,23).

Non-pathogenic alleles of ROR2 are transported to the cell surface

To confirm the specificity of the ER retention phenotype of the ROR2 RRS-associated mutant alleles, we expressed two further naturally occurring alleles of the Ror2 protein, but which are not associated with disease. There are two non-synonymous mutations reported in human ROR2 (19): 733 G to A results in an Ala to Thr substitution at position 245 (24) and 2455 G to A results in Val to Ile substitution in position 819 (18). In mouse, Ile is the common amino acid at position 819 (25), and therefore, we mutated this residue to Val. When Ror2 A245T and I819V were expressed in HeLa cells, we observed the proteins present in the plasma membrane, as well as having a population in the ER, similar to the wild-type allele (Fig. 5), confirming that only RRS-associated alleles of Ror2 are ER retained.

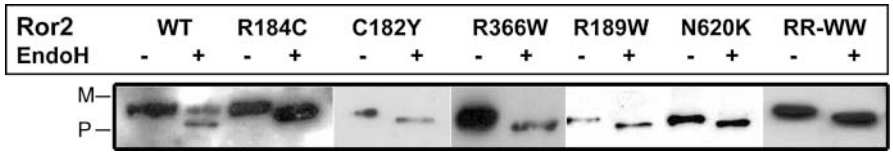

Figure 2. Immunoprecipitation and EndoH digestion of transiently transfected HEK293 cell lysates. HEK293 cells transiently expressing mouse Ror2 wild-type or mutant proteins were harvested and cell lysates were prepared as described earlier. The cell lysates were immunoprecipitated with mouse anti-HA monoclonal antibody and digested with EndoH. Treated and untreated immune complexes were separated by SDS–PAGE and immunoblotted with mouse anti-HA monoclonal antibody. After EndoH treatment, two bands are present for the Ror2 wild-type protein, with the upper band representing mature (M) protein resistant to EndoH digestion and the lower band representing immature precursor (P) protein sensitive to EndoH. For the Ror2 mutants, the proteins are completely sensitive to EndoH digestion, with only the lower band corresponding to precursor observed after EndoH treatment being observed.

DISCUSSION

The ER is the cellular site where secretory proteins are synthesized and where a variety of QC mechanisms operate to ensure that only correctly folded proteins are transported to their target compartments (9). Any proteins that are misfolded, incompletely assembled or lack the correct post-translational modifications are retained by the ER and eventually retranslocated to the cytosol and degraded by the proteasome, a mechanism known as ERAD (3). ERAD has been implicated for loss of function of many disease-associated proteins (5,6,26–29). Using a combination of informatics and literature resources, we parsed 1730 protein sequences for the identification of genes, whose disease-associated alleles may be ERAD substrates. The correct identification of 85% of established ERAD disease-associated substrates suggests that for a complex and partially qualitative analysis, the data set is of high reliability. The elevated proportion (45%) of ER-targeted proteins in the disease gene data set indicates that ERAD is likely to contribute to a large number of diseases. By inspecting the disease genes that have both ER-targeting signal and a TMD, we detected 15 strong ERAD disease candidates and an additional 40 genes with lower support.

ROR2 was implicated with high probability as an ERAD disease gene (Table 1). Additional considerations strengthened this proposal. In humans, mutations in ROR2 cause two distinct pathologies: an autosomal dominant condition of brachydactyly type B, characterized by terminal deficiencies in fingers and toes, and autosomal recessive RRS. Mutations associated with brachydactyly B are localized to the intracellular region of ROR2, causing truncation of the protein and predicted to be associated with gain of function (24). RRS is caused by distinct homozygous missense, nonsense and frameshift mutations scattered throughout the intracellular and extracellular regions of the protein and likely to cause loss of function (Fig. 1) (19). Additionally, the similarity in phenotype between autosomal RRS and a homozygous mouse model (14,18) and consistency of phenotype of RRS mutations located in different domains of ROR2 suggest that these mutations have similar consequences for protein function (14,19). Furthermore, several of these mutations involve structurally important cysteine residues participating in disulfide bridges or are non-conservative amino acid changes, i.e. charged to hydrophobic residues.

Our analysis indicates that wild-type and neutral polymorphisms of the ROR2 protein are efficiently targeted to the plasma membrane consistent with previous work (30), albeit with a significant population within the ER at steady state. In contrast, all of the RRS Ror2 mutant proteins were completely localized in the ER, with no evidence for Golgi processing or a population in the plasma membrane. This behavior extends to a rare double mutant (RRWW), where R189W and R366W occur in the same individual (14). Significantly, either of single mutations resulted in ER retention, in contrast to an earlier proposal that the R366W mutation may exert the predominant pathogenic effect in this patient (14). The most likely interpretation of these findings is that ROR2 RRS mutants are retained in the ER because of inefficient folding and that explains why severity of RRS is independent of the position or nature of the mutation. This is further supported by the finding that at steady state, a significant proportion of Ror2 carries oligomannose *N*-glycans, suggesting that the protein requires a considerable period to exit the ER and progress through the Golgi complex. Hence, even wild-type Ror2 protein may fold inefficiently, consistent with previous work demonstrating that Ror2 associates with the MAGE family protein, Dlxin-1, in the plasma membrane and ER (30).

Probably, the best-known example of protein misfolding responsible for disease is the ΔF508 mutation in the gene encoding cystic fibrosis transmembrane domain (CFTR), the molecular basis for cystic fibrosis. Mutant versions of CFTR are recognized as abnormal and degraded by the ERAD pathway (26,31). Significantly, the wild-type CFTR is poorly exported such that ∼90% of the protein never exits the ER. It is probable that Ror2 is also poorly transported, as our data suggest that 50% of the protein is in the immature ER form at steady state. Hence, our second conclusion is that the molecular mechanism underpinning RRS is ER retention of the affected gene product, the ROR2 protein, and provides further evidence for loss of function in RRS.

The evidence provided in this report suggests that ERAD is an important factor in many diseases. The set of over 50 disease-associated genes identified here (including both the strong and less well supported candidates), together with the accuracy of the analysis suggests that the set contains a considerable number of novel ERAD substrates. Evidence already exists to partly support a number of these candidates, although direct analysis of the locations of both the wild-type and disease-associated alleles is required to rigorously validate this proposal. Indeed two missense mutations in ABCA1 causing Tangier disease and HDL deficiency, an ERAD disease candidate identified in this study, has recently been shown to be retained in the ER and degraded by ERAD

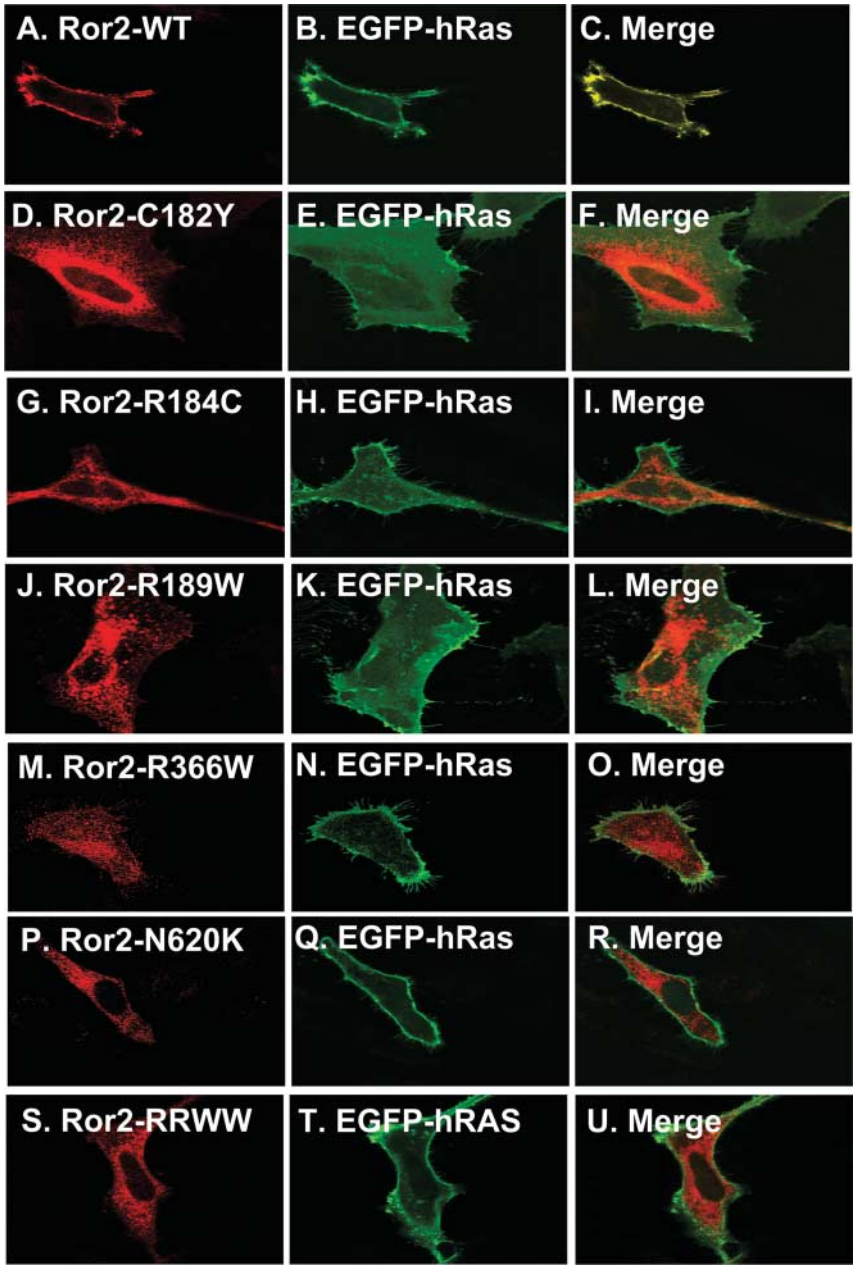

Figure 3. Co-expression of Ror2 wild-type and mutant proteins with EGFP-hRas protein indicates intracellular retention of the mutant allele proteins. HA-tagged Ror2 wild-type and mutant DNAs were co-transfected with EGFP-hRas DNA into HeLa cells. Cells were fixed and stained using mouse anti-HA monoclonal antibody, which was detected using Alexa 568-conjugated goat anti-mouse secondary antibodies (red) (**A**, **D**, **G**, **J**, **M**, **P** and **S**). Expression of EGFP-hRas protein was detected by intrinsic GFP fluorescence (**B**, **E**, **H**, **K**, **N**, **Q** and **T**). Merged fluorescence shows that Ror2 wild-type protein co-localizes with the EGFP-hRas protein to the plasma membrane of the cells (**C**), whereas Ror2 mutant proteins do not but are expressed intracellularly in a reticular pattern typical of the ER region of the cell (**F**, **I**, **L**, **O**, **R** and **U**). Data also show that the Ror2RRWW mutant protein is localized intracellularly in a similar pattern as the Ror2 mutants R189W and R366W (L, O and U).

(32). Clearly, identifying the molecular mechanism of a disease is crucial for management and therapy, and significantly, loss of function underpinning a particular disease might be due to several mechanisms. For example, cystic fibrosis is caused by over 800 mutations within the CFTR gene, some of which result in loss of function because of lack of CFTR protein synthesis, whereas others (such as ΔF508) arise from ER. One emerging and promising approach for the possible treatment of ERAD diseases is to overcome their ER retention because some pathological mutant alleles are functional if they exit the ER and reach their normal cellular location (33–35). With a potentially large number of ERAD diseases, such approaches could significantly contribute to relief of the burden of genetic diseases.

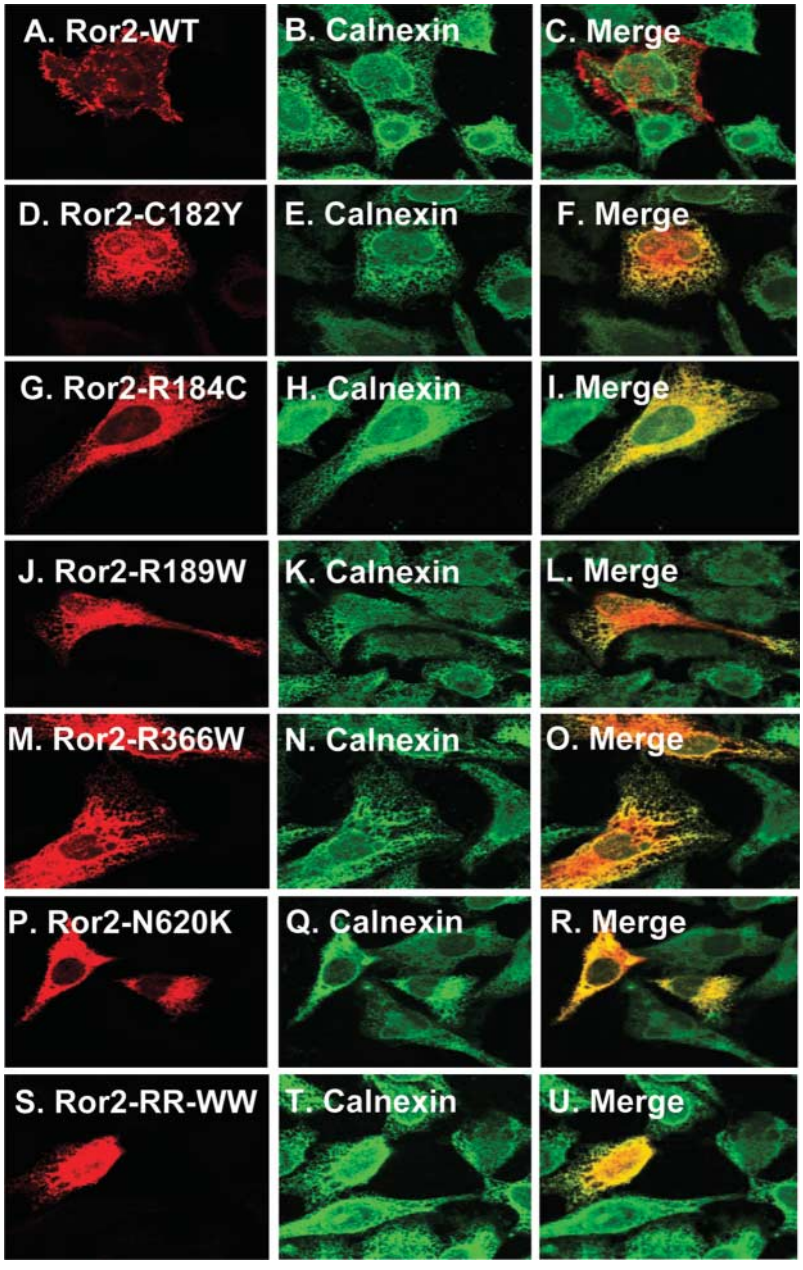

Figure 4. Ror2 mutant proteins are localized in the ER. HeLa cells were transfected with HA-tagged Ror2 wild-type and mutant DNAs and fixed. Cells were co-stained using mouse anti-HA monoclonal antibody (**A**, **D**, **G**, **J**, **M**, **P** and **S**) and rabbit polyclonal antibody reactive to the ER marker protein calnexin (**B**, **E**, **H**, **K**, **N**, **Q** and **T**). Monoclonal antibody was detected using Alexa 568-conjugated goat anti-mouse secondary antibody (red) and polyclonal antibody was detected using Alexa 488-conjugated goat anti-rabbit secondary antibody (green). The merged images show that all of the Ror2 mutant proteins co-localized with the ER marker (**F**, **I**, **L**, **O**, **R** and **U**). In contrast, no significant co-localization was observed between the Ror2 wild-type protein and calnexin (**C**). The Ror2RRWW mutant protein was also completely co-localized with calnexin, consistent with the Ror2 single mutants R189W and R366W.

METHODS AND MATERIALS

Bioinformatics

As of July 2003, 2656 disease genes were listed on the GeneCards database (http://bioinfo.weizmann.ac.il/cards/index.shtml). Using a custom PERL-script, 2365 sequences that comprised 1730 unique disease genes and 635 alternative transcripts were extracted. Four signal peptide prediction programs were tested using a test set of 19 protein sequences (10 proteins with known N-terminal ER-signal sequences and nine without them): SignalP HMM 2.0, SignalP NN 2.0 (http://www.cbs.dtu.dk/services/signalp-2.0/), PSORT (http://psort.nibb.ac.jp/) and SigCleave (minweight 3.5) (http://www.hgmp.mrc.ac.uk/software/emboss/apps/sigcleave.html (Supplementary Material, Table S1). SignalP HMM 2.0 was chosen as it performed similar to SignalP NN 2.0 but allowed discrimination between signal peptides and signal anchors, which SignalP NN 2.0 did not (36). Hypothetical

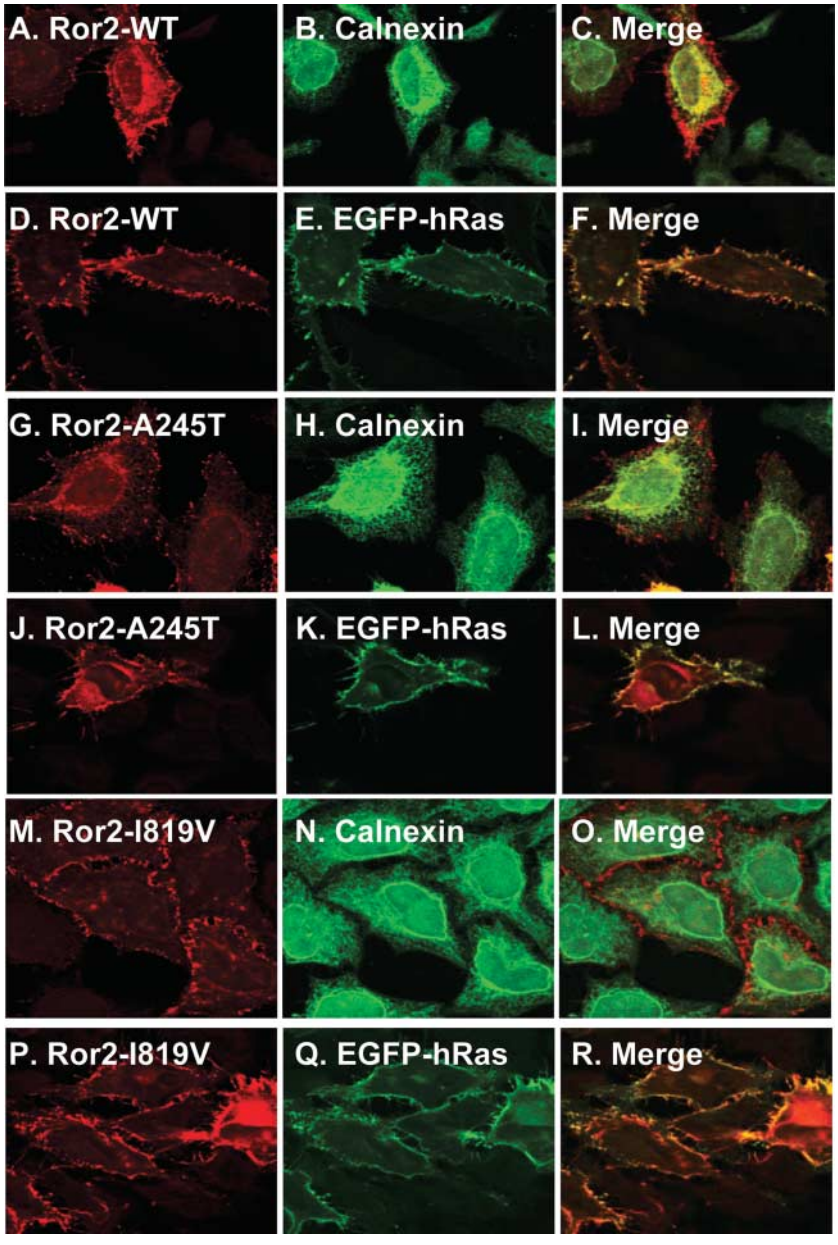

Figure 5. Ror2 neutral polymorphism mutant proteins are localized to the cell surface. HeLa cells were transfected with HA-tagged Ror2 wild-type and mutant DNAs and fixed. Cells were then co-stained using the mouse anti-HA monoclonal antibody (**A**, **D**, **G**, **J**, **M** and **P**) and rabbit polyclonal antibody reactive to the ER marker protein calnexin (**B**, **H** and **N**) or imaged for EGFP-hRas protein using the intrinsic GFP fluorescence signal (**E**, **K** and **Q**). Monoclonal antibody was detected using Alexa 568-conjugated goat anti-mouse secondary antibody (red) and polyclonal antibody was detected using Alexa 488-conjugated goat anti-rabbit secondary antibody (green). The merged images (**I**, **L**, **O** and **R**) show that both of the Ror2 neutral polymorphism mutant proteins (A245T and I819V) are expressed on the cell surface and co-localize with hRas, similar to the wild-type Ror2 protein (**C** and **F**).

translations of all 2365 transcripts were submitted to SignalP HMM 2.0 and the results were parsed (Supplementary Material, Table S2). One hundred and twenty-eight signal anchor predicted sequences were also found, with 103 of these from unique genes. All predicted signal peptide/signal anchor positive proteins were then submitted to TMHMM 2.0 to predict the presence of transmembrane helices, to big-PI to predict the presence of a GPI-anchor and to PSORT II to predict the presence of ER-retrieval signals (XXRR, KKXX and K/HDEL) and the YQRL motif required for internalization of proteins via clathrin-coated pits (Supplementary Material, Table S5).

Cell culture and transfection

HeLa cells and human epithelial kidney cells (HEK293) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM L-glutamine

and 100 U/ml penicillin/streptomycin at 37°C with 10% CO_2. For immunofluorescence, HeLa cells were grown on cover slips in a 24-well plate for 24 h and transiently transfected using the liposomal transfection reagent FuGENE 6 (Roche Biochemicals), according to manufacture's instructions. In co-transfection, a mixture of 0.5 μg of EGFP-hRas, 1 μg of mRor2 wild-type or mutant DNA and 5 μl of FuGENE 6 in 94 μl of OPTIMEM I medium (Invitrogen) was applied to each well of the HeLa cells at ~80% confluence. In single transfection, only a mixture of 1 μg of mRor2 wild-type or mutant DNA and 4 μl of FuGENE 6 in 94 μl of OPTIMEM I medium was applied to each well of the HeLa cells. The cells were then fixed and processed for microscopy 24 h later. For immunoblotting, HEK293 cells were seeded in a six-well plate for 24 h, and at ~80% confluence of the cells, a mixture of 3 μg of mRor2 DNA and 10 μl of FuGENE 6 in 250 μl of OPTIMEM I medium was applied to each well of the HEK293 cells. Cells were incubated with the transfection mixture for 24 h before being processed for western blot analysis.

Construction of mouse Ror2 mutants

The plasmid pcDNA3-ROR2WT-HA (gift of Drs Y. Minami and S. Kani, Kobe University, Japan) served as a template to generate Ror2 mutants (pcDNA3-Ror2C182Y-HA, pcDNA3-Ror2R184C-HA, pcDNA3-Ror2R189W-HA, pcDNA3-Ror2R-366W-HA, pcDNA3-Ror2N620K-HA, pcDNA3-Ror2A245T-HA and pcDNA3-Ror2I819V-HA) using QuickChange™ Mutagenesis (Stratagene), according to the manufacturer's instructions using the following primers (mutagenic bases in bold, codons underlined): C182Y: 5′-CCGTACCGAGGGATCGCTT**A**TGCGCGCTTCATTGGG-3′ and 5′-CCCAATGAAGCGCGCA**T**AAGCGATCCCTCGGTACGG-3′; R184C: 5′-GGGATCGCTTGTGCG**T**GCTTCATTGGGAACCGG-3′ and 5′-CCGGTTCCCAATGAAG**CA**CGCACAAGCGATCCC-3′; R189W:-CGCTTCATTGGGAAC**T**GGACTATTTATGTGGACTCC-3′ and 5′-GGAGTCCACATAAATAGTCC**A**GTTCCCAATGAAGCG-3′; R366W: 5′-GGCCATGCCTACTGC**T**GGAACCCCGGGGGC-3′ and 5′-GCCCCCGGGGTTCC**A**GCAGTAGGCATGGCC-3′; N620K: 5′-GACCTGGCCACACGCAA**G**GTGCTGGTGTACGAC-3′ and 5′-GTCGTACACCAGCAC**CT**TGCGTGTGGCCAGGTC-3′; A245T: 5′-GACGCATGCTCCCGG**A**CGCCCAAGCCTCGCGAAC-3′ and 5′-GTTCGCGAGGCTTGGGCG**T**CCGGGAGCATGCGTC-3′; and I819V: 5′-CCCGCACAGCTGTAC**G**TCCCGGTGAACGGC-3′ and 5′-GCCGTTCACCGGGA**C**GTACAGCTGTGCGGG-3′.

To simulate the ROR2 double mutant R189W–R366W (pcDNA3-Ror2RR-WW), pcDNA3-Ror2R366W-HA was mutated further with the R189W primers. All constructs were confirmed by DNA sequencing (Advanced Biotechnology Centre, Imperial College London, UK).

Immunochemistry

Antibodies were purchased from the following sources: mouse anti-HA-Tag monoclonal antibody (dilution 1:200 for immunofluorescence and 1:1000 for western blotting; Cell Signaling Technology), rabbit anti-calnexin polyclonal antibody (dilution 1:500; StressGen Biotechnologies), Alexa Fluor 568-goat anti-mouse IgG (dilution 1:200; Molecular Probes), Alexa Fluor 488-goat anti-rabbit IgG (dilution 1:200; Molecular Probes) and HRP-conjugated to goat anti-mouse IgG (dilution 1:5000; Dako).

For immunofluorescence, cover slip-grown HeLa cells were washed with phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde in PBS for 15 min at room temperature, washed in PBS three times, quenched with 50 mM NH_4Cl in PBS for 10 min and incubated in permeabilization/blocking solution (0.05% saponin, 0.5% BSA and 1× PBS) for 15 min. An alternative fixation method was used for visualizing the ER network and involved adding cold methanol (−20°C) to the cover slips and incubating at that temperature for 4 min and then in blocking solution (0.5% BSA and 1× PBS) for 30 min. The fixed cells were then incubated at room temperature for 1 h with either mouse monoclonal anti-HA antibody alone or co-stained with both mouse monoclonal anti-HA antibody and rabbit polyclonal anti-calnexin antibody. After washing with PBS, the cells were incubated with the appropriate secondary antibodies for 1 h at room temperature, washed several times with PBS and mounted in immuno fluor medium (ICN Biomedicals) and visualized under a Leica DM-IRBE confocal microscope. Images were acquired using Leica TCS-NT software associated with the microscope and processed with Adobe Photoshop® (Adobe Inc.). For immunoblotting, HEK293 cells transiently expressing mouse Ror2 wild-type or mutant proteins were detached with a cell scraper, washed with PBS on ice and harvested by centrifugation at 1200 r.p.m. (100 *g*) for 5 min at 4°C. Cell pellets were then lysed in buffer containing 50 mM HEPES pH 7.4, 10 mM NaCl, 1 mM DTT, 1× protease inhibitor cocktail (Roche Biochemicals) and 1% Triton X-100 and benzonase (1/100 μl lysis buffer). Lysates were centrifuged at 10 000 r.p.m. for 10 min and 4× SDS sample buffer was added to the supernatants. The samples were boiled for 3 min, separated on 8% SDS–polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA), according to the manufacturers' instructions. Membranes were blocked in PBSTM (PBS, 0.25% Tween-20 and 5% non-fat milk) for 1 h and sequentially incubated with the mouse anti-HA-Tag monoclonal antibody diluted in PBSTM for 1 h, washed in PBST (PBS and 0.25% Tween-20), incubated in HRP-conjugated goat anti-mouse antibody diluted in PBSTM for 1 h and washed as earlier. Bound antibodies were detected using a supersignal West Pico chemiluminescent substrate detection kit (Perbio Science UK Ltd) and the ECL system (Amersham Pharamcia Biotech).

For immunoprecipitation, transiently transfected HEK293 cells were harvested and cell lysates were prepared as mentioned previously. The supernatants of the cell lysates were incubated with mouse monoclonal anti-HA antibody overnight at 4°C with end-over-end rotation and followed by addition of a 50% slurry of protein G-Sepharose fast flow beads (Amersham Pharmacia Biotech), with a further incubation for 1–2 h at 4°C with end-over-end rotation. The immunocomplexes were precipitated by low speed centrifugation, and the pellets were washed twice with lysis buffer. Precipitated proteins were separated on 8% SDS–polyacrylamide gels, transferred to Immobilon-P membranes and immunoblotted.

Endoglycosidase H digestion

Immunoprecipitated samples were denatured at 100°C for 10 min in 25 μl of denaturing buffer (5% SDS and 10% β-mercaptoethanol). After adding 5 μl of 0.5 M sodium citrate, pH 5.5, samples were incubated with $EndoH_f$ for 1 h at 37°C. Samples were then subjected to SDS–PAGE and immunoblotted.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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