Hypomorphic variants of SEL1L-HRD1 ER-associated degradation are associated with neurodevelopmental disorders

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**Graphical abstract**

ERAD-associated neurodevelopmental disorders with onset in infancy (ENDI)

Developmental delay, intellectual disability and locomotor dysfunction
Hypomorphic variants of SEL1L-HRD1 ER-associated degradation are associated with neurodevelopmental disorders

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**Running title:** Disease-causing *SEL1L-HRD1* ERAD variants in humans

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**Summary:** ERAD-associated neurodevelopmental disorder with onset in infancy (ENDI) is caused by hypomorphic SEL1L-HRD1 ERAD variants and characterized by infantile-onset developmental delay, intellectual disability, microcephaly, facial dysmorphisms, hypotonia and/or ataxia.
Recent studies using cell type-specific knockout mouse models have improved our understanding of the pathophysiological relevance of SEL1L-HRD1 endoplasmic reticulum (ER)-associated degradation (ERAD); however, its importance in humans remains unclear as no disease variant has been identified. Here we report the identification of three bi-allelic missense variants of \textit{SEL1L} and \textit{HRD1} (or \textit{SYVN1}) in six children from three independent families presenting with developmental delay, intellectual disability, microcephaly, facial dysmorphisms, hypotonia and/or ataxia. These \textit{SEL1L} (p.Gly585Asp, p.Met528Arg) and \textit{HRD1} (p.Pro398Leu) variants were hypomorphic and impaired ERAD function at distinct steps of ERAD including substrate recruitment (\textit{SEL1L} p.Gly585Asp), \textit{SEL1L-HRD1} complex formation (\textit{SEL1L} p.Met528Arg), and \textit{HRD1} activity (\textit{HRD1} p.Pro398Leu). Our study not only provide new insights into the structure-function relationship of SEL1L-HRD1 ERAD, but also establish the importance of SEL1L-HRD1 ERAD in humans.
INTRODUCTION

Nascent membrane or secretory proteins are synthesized and folded in the endoplasmic reticulum (ER), which is prone to misfolding. It may have pathogenic consequences if not cleared effectively (1-5). The suppressor of lin-12-like (SEL1L) - HMG-CoA reductase degradation 1 (HRD1) complex represents one such quality-control mechanism in the cell, known as ER-associated degradation (ERAD) (6-8). In SEL1L-HRD1 ERAD, misfolded proteins are recognized and recruited to the SEL1L-HRD1 protein complex via ER chaperones such as ostesarcoma amplified 9 (OS9) and ER lectin 1 (ERLEC1, also known as XTP3B) (9-14) followed by retrotranslocation and poly-ubiquitination by the E3 ligase HRD1 (12, 15, 16) and proteasome degradation in the cytosol (17, 18). In this complex, SEL1L is an obligatory cofactor for the E3 ligase HRD1 (19, 20), not only controlling the protein stability of HRD1 (6, 19, 20), but functioning as a scaffold for other ERAD components such as OS9, ERLEC1, and degradation in ER (DERLIN) proteins (10, 19, 21-27). In yeast, SEL1L homolog Hrd3p may regulate Hrd1p autoubiquitination and self-degradation (28, 29).

Global or acute deletion of Sel1L or Hrd1 in germline and adult mice cause embryonic or premature lethality, respectively (20, 30-32), pointing to the requirement of SEL1L-HRD1 ERAD function at both embryonic developmental and adult stages. Subsequent studies using cell type-specific gene knockout mouse models have established its vital importance in many physiological processes, including food intake, water balance, thermogenesis, energy homeostasis, gut homeostasis, β cell identity/function, immune cell development/function, and hematopoietic cell quiescence (2, 3, 5, 33-51). Although these advances in mouse models have provided critical insights into the physiological importance of this complex, its relevance in humans remain unknown as no disease variant has been identified in humans.

Using whole exome sequencing, here we report the identification of three autosomal recessive variants, SEL1L p.Gly585Asp, p.Met528Arg and HRD1 p.Pro398Leu, in six children from three unrelated families with similar neurodevelopmental disorders - termed ERAD-associated neurodevelopmental disorder with onset in infancy (ENDI). These variants are hypomorphic and attenuate ERAD function, likely via distinct mechanisms including substrate recruitment, SEL1L-HRD1 complex formation, and HRD1 activity. Hence, this study establishes the pathophysiological importance of SEL1L-HRD1 ERAD in humans.
RESULTS

Identification of bi-allelic SEL1L and HRD1 variants in humans

Six patients from 3 unrelated families in Saudi Arabia (Patient 1), Morocco (Patient 2-5) and Italy (Patient 6) were suspected of inherited genetic disease during clinical visits (Figure 1, A-C). Patient 1, 2-3 and 6 were subjected to whole exome sequencing (WES) of DNA samples (Figure 1, D-F). WES results were stringently filtered for novel variants by excluding variants with low sequencing quality, in the non-coding region, with high frequency in the population, or likely to be benign in silico (Figure 1, D-F). We failed to identify any known variants linked to inherited neurological or metabolic disorders, but rather noted three variants linked to the same protein complex/pathway, namely SEL1L-HRD1 ERAD: SEL1L p.Gly585Asp (NM_005065: exon 17: c.1754G>A) in Saudi Arabian patient (Patient 1), SEL1L p.Met528Arg (exon 16: c.1583T>G) in Moroccan patients (Patient 2-3), and HRD1 p.Pro398Leu (NM_172230: exon 12: c.1193C>T) in Italian patient (Patient 6) (Figure 1, D-F and Table 1). Indeed, homozygous SEL1L p.Met528Arg was the only variant shared between Patient 2 and 3, but not found in the healthy parents. Although additional variants including homozygous, heterozygous, compound heterozygous and de novo mutations were identified in the Saudi Arabian (Patient 1) and Italian (Patient 6) patients (Figure 1, D and F, Supplemental Table 1), SEL1L p.Gly585Asp and HRD1 p.Pro398Leu variants were considered as potential candidates based on their biological relevance as reported in mice (2, 3, 5), and also according to the American College of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP) 2015 guideline for clinical interpretation of genetic variants (52). Indeed, neither of the SEL1L variants was found in public genetic variants databases such as 1000gp3, ESP6500, ExAC and gnomAD, and both variants were consistently predicted to be damaging in various prediction tools such as CADD, PolyPhen2-HVAR, SIFT, LIST-S2, M-CAP, BayesDel addAF, DEOGEN2, FATHMM-MKL, MutationAssessor, MutationTaster, and PrimateAI (Table 1). Similarly, allele frequency of HRD1 p.Pro398Leu was low in the population and was predicted to be benign to damaging in various prediction databases (Table 1). SEL1L and HRD1 genes are located on chromosomes 14 and 11, respectively (Figure 1, G and H). Using Sanger sequencing, we confirmed that the three variants were found to be homozygous in all patients, but heterozygous in all parents (Figure 1, G and H). Three siblings for Patient 1 were either heterozygous or wildtype (WT) for the SEL1L allele (Figure 1, G and H).

Clinical features of the patients
Patient 1, a 14-year-old boy born to healthy consanguineous Saudi Arabian parents (with 3 healthy siblings), showed hypotonia (poor sucking and floppiness) and microcephaly at 4 months of age. Three siblings, two WT and one heterozygous at the SEL1L G585 locus, were healthy. The patient presented with global developmental delay (sat at 2 years of age, walked at 5, uttered mama/dada at 6 and is not yet toilet trained), moderate-severe intellectual disability (limited two-word sentences, and cannot count to 3 with an IQ of 35), and central hypotonia (with brisk deep tendon reflexes, wide-based gait with hands held on the side to support his balance) (Figure 2A, Table 1, Supplemental Table 2/Video 1). Physical examination performed at the age of 13 years revealed short stature (-3.9 Standard Deviation (SD)), underweight (-3.9 SD), microcephaly (-4.2 SD), subtle facial dysmorphism (downslanting palpebral fissures and overbite), pectus excavatum (arrows, Figure 2B), a moderate degree of joint hyperlaxity, and shawl scrotum. The patient had a total of 3 seizures at 8 years of age with largely normal electroencephalogram (EEG). His medical history was notable for frequent airway infections although his workup did not suggest immunodeficiency. Other than hypotonia, neurological examination was largely normal.

Patient 2-5, four Moroccan siblings, 1 female (the proband, born 2005) and three males (born 2007, 2011 and 2017), born to healthy consanguineous parents (Figure 1B), presented since a few months of age with developmental delay, intellectual disability, speech delay, short stature, seizures, and ataxic gait (progressive with age). The two older siblings (Patient 2 and 3) had single seizure history and the two younger siblings (Patient 4 and 5) showed microcephaly. The proband showed severe spastic and ataxic gait, falls, wide-based gait, pes cavus and equinus, mild dystonia, paraparesis with pyramidal signs of lower limbs, with brisk, diffused tendon reflexes and clonus, pyramidal extension of the first toe, and bilateral positive Babinski sign (Figure 2C, Table 1, Supplemental Table 2/Video 2). The proband showed varus equus, scoliosis, and arched palate. The four patients also shared facial dysmorphism including downslanting palpebral fissures and overbite (Figure 2D), and were diagnosed with unilateral maculopathy, pallor of temporal poles, and severe corneal dystrophy. Magnetic resonance imaging (MRI) of the proband showed small cavities in the frontal periventricular area with non-specific ventricular dilatation (arrows, Supplemental Figure 1). EEG of the proband showed generalized discharges of polyspikes and slow waves.

Patient 6, an Italian girl born to healthy non-consanguineous parents in 2001, presented since her first months of age with hypotonia and severe drug-resistant seizures that were resolved by
the age of 14 years (Figure 1C). She exhibited intellectual disability, speech delay, stereotypic movements, a clumsy gait (Figure 2E, Table 1, Supplemental Table 2/Video 3) and dysmorphic facial features (Figure 2F). Physical examination at 16 years of age revealed underweight (37.5 kg body weight, < 5th percentile, z-score=-3.45), short stature (height 139.5 cm, < 5th percentile, z-score=-3.9), and microcephaly (head circumference 51.8 cm, < 5th percentile, z-score=-2.6). Brain MRI performed in the first year of life revealed a cerebellar cyst without other notable findings. When repeated at the age of 21 years, an abnormal signal was detected in the globus pallidum and substantia nigra (not shown). Cardiac and abdomen ultrasounds were both normal. The patient has been on risperidone (Table 1) since COVID-19 outbreak because of worsening of her behavior with agitation and aggression.

In summary, all 6 patients were symptomatic at infancy and presented with neurodevelopmental disorder, developmental delay, intellectual disability and facial dysmorphisms (Table 1 and Supplemental Table 2). Four out of six patients had microcephaly. Two patients showed hypotonia with floppiness, unsteady and clumsy gait, and difficulty in walking, but no frank ataxia, while the other four patients from the Moroccan family exhibited severe ataxia that progressed with age. Interestingly, hypotonia in the two patients did not progress with age, and in fact the Italian patient was no longer hypotonic in the most recent evaluation in June 2022. No notable abnormalities were observed in routine blood chemistry tests (glucose, electrolytes, blood urea nitrogen, creatinine, aspartate aminotransaminase, alanine aminotransaminase and albumin) and complete blood counts in all 6 patients.

**SEL1L and HRD1 variants are hypomorphic with impaired ERAD function**

To investigate whether and how these disease variants affect SEL1L-HRD1 ERAD function, we generated knock-in (KI) HEK293T cells carrying the biallelic variants using the CRISPR/Cas9 system and verified by Sanger sequencing (Supplemental Figure 2, A-E). We then tested whether these disease variants affect ERAD function by measuring protein stability and levels of known endogenous ERAD substrates such as inositol-requiring enzyme 1α (IRE1α) (41), OS9 (53) and cluster of differentiation 147 (CD147) (54), as well as the disease mutant of pro-arginine vasopressin (proAVP) Gly57Ser (Gly-to-Ser at residue 57) (47). Indeed, endogenous substrates became accumulated in all three KI HEK293T cell lines (Figure 3, A and B) due to protein stabilization, similar to SEL1L- or HRD1- knock-out (KO) HEK293T cells (Figure 3, C and D and Supplemental Figure 3). Similarly, proAVP(Gly57Ser) accumulated in transfected KI
cells, forming much more extensive high-molecular-weight (HMW) aggregates than those in WT cells (Figure 3, E and F).

Couple points are worth noting here: first, although all variants caused substrate accumulation, the extent of substrate accumulation differed among the variants, the highest being the SEL1L M528R variant, and the lowest being either SEL1L G585D or HRD1 P398L variant, which are consistent for both endogenous and model substrates (Figure 3, B and F). Second, the extent of substrate accumulation and HMW aggregation in KI cells was modest compared to those in KO cells (Figure 3, B and F), pointing to the hypomorphic nature, rather than loss-of-function, of these variants. Taken together, these data suggest that three variants are hypomorphic with moderate to severe ERAD dysfunction.

Lack of an overt unfolded protein response (UPR) in KI cells

Intriguingly, we did not observe an overt UPR in these KI cells, as demonstrated by the lack of IRE1α phosphorylation, X-box-binding protein 1 (XBP1) mRNA splicing, as well as phosphorylation of protein kinase R-like endoplasmic reticulum kinase (PERK) and eukaryotic initiation factor-2α (eIF2α) (Supplemental Figure 4, A-D). ER chaperones such as immunoglobulin heavy chain-binding protein (BiP) and protein disulfide isomerase (PDI) were accumulated in KI HEK293T cells (Supplemental Figure 4, E and F). These data point to a cellular adaptive response in cells expressing these disease variants.

Sequence and structural analyses of SEL1L and HRD1 variants

We next asked how these variants affect ERAD function. We first performed the in silico conservation and structural analyses. These variants affect conserved residues from yeast or drosophila to humans, with the exception of HRD1 Pro398, which is absent in yeast (Figure 4, A-C). Position-specific scoring matrix (PSSM) analysis (55) showed that all three variants replaced evolutionarily selected amino acids and that the mutations may be detrimental to SEL1L and HRD1 function (Figure 4, D-F). The SEL1L variants, p.Met528Arg and p.Gly585Asp, affect residues in the Sel1-like repeat (SLR)-M and the linker region between SLR-M and -C, respectively (Figure 4G). To visualize these variants in ERAD complex, we performed the Al-based AlphaFold2 prediction network analysis (56) to model the structure of the human SEL1L (107-723 aa)-HRD1 (1-334 aa)-OS9 (33-655 aa)-DERLIN1 (1-213 aa) protein complex (Figure 4H). Structural modeling of human SEL1L-HRD1 complex showed a great similarity to the cryogenic electron microscopy (Cryo-EM) structure of the yeast Hrd1p-Hrd3p complex (PDB ID...
6VJZ) (12) (Supplemental Figure 5A). ConSurf conservation analysis (57) of SEL1L confirmed that both Met528 and Gly585 residues were in highly conserved patches (Supplemental Figure 5B).

SEL1L Met528 is predicted to be a part of α-helix facing outward away from the putative substrate binding groove (red line) and OS9 (gold, Figure 4I). Mutation of Met528 to Arg is expected to seriously disrupt α-helical structure and destabilize the protein. On the other hand, Gly585 is located on a loop between the two helices in the putative substrate binding groove (Figure 4J). While mutation of Gly585 to Asp is not predicted to disrupt the α-helical structure, it is located in the substrate binding groove in close proximity to the substrate(s) and lectins (OS9 and ERLEC1). Moreover, Pro398 of HRD1 is located in the proline-rich region (~ 50 Pro in a stretch of 140 amino acids) of its cytosolic domain, C-terminal to the Really Interesting New Gene (RING)-finger domain (Figure 4K). This proline-rich region is disordered based on IUPred2 prediction (58) (Figure 4L), with no predictable structure.

**SEL1L and HRD1 variants impair ERAD function via distinct mechanisms**

We next explored how these variants cause ERAD defects using KI HEK293T cells. We first measured protein levels of SEL1L-HRD1 ERAD complex. Noticeably, SEL1L<sup>M528R</sup> KI HEK293T exhibited reduced SEL1L and HRD1 protein levels by ~80 and 60%, respectively (Figure 5, A and B), uncoupled from their gene transcription (Figure 5, C and D). Indeed, both SEL1L and HRD1 proteins were unstable in SEL1L<sup>M528R</sup> KI cells (Figure 5, E and F). By contrast, SEL1L<sup>G585D</sup> exhibited a modest reduction of SEL1L and HRD1 protein levels by ~20-30% (Figure 5, A and B), without changes in mRNA levels (Figure 5, C and D). SEL1L<sup>G585D</sup> had a subtle effect on the stability of SEL1L protein, but not HRD1 protein, in KI HEK293T cells (Figure 5, E and F). This reduction in ERAD protein levels was unlikely to explain the ERAD defects associated with SEL1L<sup>G585D</sup>-expressing cells as heterozygosity of SEL1L or HRD1 is sufficient for ERAD function (20, 34, 43, 44). On the other hand, HRD1<sup>P398L</sup> had no effect on either protein levels or stability of SEL1L and HRD1 (Figure 5, A-B and E-F). Hence, SEL1L<sup>M528R</sup> variant causes ERAD dysfunction by reducing the protein stability and levels of the SEL1L-HRD1 complex, but not SEL1L<sup>G585D</sup> and HRD1<sup>P398L</sup>.

Given the location of SEL1L G585 residue, we next asked whether SEL1L<sup>G585D</sup> affect substrate recruitment. During ERAD, substrates are recruited by lectins such as OS9 and ERLEC1 to the SEL1L-HRD1 complex, which also includes ubiquitin-conjugating E2 enzyme J1 (UBE2J1) and
DERLIN proteins (18). To circumvent the confounding issue of reduced SEL1L and HRD1 protein levels in SEL1L KI cells, we used an overexpression system in SEL1L-/HEK293T cells. Surprisingly, SEL1L<sup>G585D</sup> variant significantly reduced its interactions with two lectin proteins (ERLEC1 and OS9) by ~70-80%, and with HRD1 by ~50% compared to that of WT SEL1L (Figure 6, A and B). By contrast, SEL1L interaction with UBE2J1 and DERLIN2 was not affected in SEL1L<sup>G585D</sup>-expressing cells (Figure 6, A and B). By contrast, SEL1L<sup>M528R</sup> did not affect the interaction between SEL1L and HRD1 or other ERAD components in transfected SEL1L<sup>-/-</sup> HEK293T cells (Figure 6, A and B). Hence, unlike SEL1L<sup>M528R</sup>, SEL1L<sup>G585D</sup> variant impairs ERAD function by attenuating substrate recruitment. This conclusion is in line with the prediction that SEL1L G585 faces substrate binding groove and is in close proximity to OS9 (Figure 4J).

In HRD1<sup>P398L</sup> KI HEK293T cells, the interactions of HRD1 with other ERAD components such as SEL1L, UBE2J1, DER2, valosin-containing protein (p97/VCP), and Family With Sequence Similarity 8 Member A1 (FAM8A1) were comparable to those in WT HEK293T cells (Figure 7, A and B). However, substrate ubiquitination was significantly attenuated in HRD1<sup>P398L</sup> KI HEK293T cells, similar to the other two SEL1L variants (Figure 7, C and D), providing further support for the ERAD dysfunction. Given that HRD1 P398L is close to the RING domain (Figure 4K), we next asked whether HRD1 P398L may affect HRD1 activity by modulating its ubiquitination using denaturing immunoprecipitation followed by Western blot. For the unknown reason, we failed to detect ubiquitination of endogenous HRD1 proteins in WT and KI cells even with the treatment of MG132 (Supplemental Figure 6). Upon transfection in HRD1<sup>-/-</sup> HEK293T cells, P398L mutation attenuated HRD1 ubiquitination compared to those in WT cells (lanes 3 and 9 vs. 2 and 8, Figure 7, E-F). The effect of P398L on HRD1 ubiquitination was similar to that of HRD1 C2A mutation (lane 4 and 10), a mutation in the RING domain known to abolish HRD1 E3 activity (8). Interestingly, mutation of the neighboring HRD1 Pro to Leu (P397L or P396L) had much milder effect on HRD1 ubiquitination (lanes 5-6 and 11-12, Figure 7, E-F). MG132 treatment enhanced HRD1 ubiquitination in all samples (lanes 8-12 vs. 2-6, Figure 7, E-F), suggesting that HRD1 ubiquitination may contribute to its turnover. These data suggest that HRD1 P398L affects HRD1 ubiquitination, which may contribute to its dysfunction. Taken together, we conclude that these three variants cause ERAD dysfunction at distinct steps of ERAD, including substrate recruitment (SEL1L<sup>G585D</sup>), SEL1L-HRD1 protein stability and complex formation (SEL1L<sup>M528R</sup>), and HRD1 activity (HRD1<sup>P398L</sup>).
DISCUSSION

This study reports three novel variants in SEL1L and HRD1 genes in six patients from three unrelated families. These patients manifest similar clinical features including developmental delay, microcephaly, intellectual disability, facial dysmorphism, hypotonia, and ataxia. Using KI HEK293T cells expressing individual variants, we further show that these variants impair ERAD function at distinct steps of ERAD, including substrate recruitment, SEL1L-HRD1 protein stability and complex formation, and HRD1 activity (Figure 8). We speculate that the phenotypic variations among these patients may reflect different levels of ERAD dysfunction associated with the variants, and/or less likely, possible effects of other non-ERAD variants.

A few additional variants were identified from the Saudi Arabian (Patient 1) and Italian (Patient 6) patients (Supplemental Table 1). Most of the heterozygous and compound heterozygous variants were predicted to be benign by the pLI score (the intolerance of the gene to loss-of-function) and variant effect prediction tools (CADD, PolyPhen-2 HVAR, SIFT), except for the heterozygous Furry (FRY) - like transcription coactivator (FRYL) variant (FRLY c.7490C>G, p.T2497R) identified in Patient 1; however, Fryl heterozygous mutant mice were found normal compared to WT littermates, while homozygous mutant mice showed lower birth rate and renal defects (hydronephrosis) if survived (59), suggesting that the FRYL variant may not be disease relevant in Patient 1. Similarly, although several additional homozygous variants were identified, the reported functions of these protein are not biologically relevant to the symptoms observed in our patients, e.g. Ras-associated protein rab17 (RAB17) which encodes a GTPase to enable GDP binding activity (60), von Willebrand factor A containing 5B2 domain (VWA5BA) which belongs to the family of von Willebrand factors crucial for primary platelet and collagen adhesion function (61), and Solute carrier family 25, member 53 (SLC25A53) which is predicted to be an integral component of mitochondrial inner membrane (Alliance of Genome Resources) with unknown function. Among them, although mutations of other RAB family proteins have been linked to neurological disorders (62), RAB17 is an epithelial cell-specific GTPase (63) and is expressed at a very low level in the central nervous system (gtexportal.org). Similarly, two other variants identified in patient 6, Membrane-spanning 4-domains, subfamily a, member 12 (MS4A12) and Protein phosphatase 1 regulatory subunit 32 (PPP1R32), are associated with colon cancer (64, 65) and ciliary movements (66), respectively. Given that the (patho-)physiological importance of SEL1L-HRD1 ERAD (2, 3, 5, 67) and given that M528R
variant is the only variant shared among the affected siblings from the Moroccan family, we believe that SEL1L-HRD1 ERAD variants are most likely to be disease causing in these patients.

Comparing disease variant KI HEK293T cells to ERAD KO HEK293T cells, our biochemical analyses showed that these variants attenuated ERAD function. Since all the parents and some healthy siblings are heterozygous for the variant, we propose that all these variants cause more than 50% reduction in ERAD function. Further comparisons among the three variants showed that SEL1L M528R may be the most severe one. This may account for the differences in clinical features between the Patient 2-5 with SEL1L M528R variant (ataxia and microcephaly) vs. the other two Patient 1 and 6 with SEL1L G585D and HRD1 P398L variants (hypotonia). While the underlying molecular mechanisms are distinct for these variants in causing ERAD dysfunction, they all invariably cause ERAD dysfunction, leading to the stabilization and accumulation of endogenous ERAD substrates. Hence, these studies suggest that there is a threshold requirement for SEL1L-HRD1 ERAD function essential for normal neuronal function in humans.

We reported that these human SEL1L-HRD1 variants compromised ERAD via distinct mechanisms. Specifically, in HRD1 P398L KI HEK293T cells, HRD1 ERAD function was impaired. Following overexpression in HRD1-deficient HEK293T cells, we found that HRD1 P398L variant impaired HRD1 ubiquitination. While this finding is potentially interesting as it may reflect HRD1 autoubiquitination as reported by Baldridge et al. (28), we are aware that overexpression of HRD1 likely alters the stoichiometric ratios of the ERAD components that do not accurately reflect those of endogenous HRD1 complex. The “ubiquitinated HRD1” may be resulted from (partially) unassembled and misfolded HRD1 that are targeted for proteasome-dependent degradation. Studies are under way to explore whether HRD1 P398L affects autoubiquitination of the RING domain specifically related to channel gating, or other lysine residues.

In the accompanying paper, we reported additional 5 patients carrying another SEL1L variant (SEL1L p.Cys141Tyr) identified from a Slovakian Romani family. This group of patients exhibited not only similar neurological disorders, but severe agammaglobulinemia resulting from the lack of B cells. This difference in clinical manifestation is likely due to the fact that SEL1L p.Cys141Tyr is the most severe variant among the four. Moreover, a SEL1L mutation (p.Ser658Pro) was previously identified in Finnish Hounds with cerebellar ataxia (a.k.a.
Cerebellar Ataxia Finnish Hound Type, CAFH) (68), further suggesting that SEL1L may play an important role in maintaining normal neurological function or neurodevelopment. These findings provide strong experimental support for the notion that hypomorphic SEL1L-HRD1 variants are pathogenic in humans. With that said, how these variants are linked to neurological defects in these patients remains to be investigated and is of great interest going forward. Although lacking substantial evidence in humans, we speculate that SEL1L -HRD1 ERAD variants cause disease via substrate-dependent and cell -type specific manner, as none is associated with an overt UPR. Other mechanisms such as organellar dysfunction may also contribute to this pathological process.

This study reports the first set of human patients carrying variants in the core components of a key protein degradative machinery, providing key evidence for its pathophysiological importance in humans. It's worth noting that several variants have been identified in p97/VCP, another key component of the ERAD machinery involved in protein retrotranslocation from the ER. However, unlike SEL1L-HRD1 ERAD variants, these p97/VCP variants cause multisystem disorders (69-71). Differences in clinical features between these two sets of patients are likely due to the fact that, in addition to ERAD, p97/VCP is involved in a wide variety of other cellular functions, including genomic stability, translational stress response, and RNA biology (70). Moreover, a number of variants in genes involved in protein glycosylation have been reported to cause congenital disorders of glycosylation (CDG), of which manifestations also largely include neurodevelopmental delay and variable facial dysmorphism (72-77) - similar clinical consequences as our ENDI patients described in this study. This similarity is not surprising as glycosylation is intimately associated with ER protein folding, maturation, and degradation (78). However, CDG patients also exhibit multi-systemic symptoms, including hypoglycemia, liver, skin, gastrointestinal, coagulation abnormalities (74), which were not observed in ENDI patients. Hence, identifying Mendelian disorders caused by mutations in core ERAD components is essential in delineating the importance of ERAD in humans.

ENDI is a rare neurodevelopmental disorder associated with SEL1L-HRD1 ERAD and characterized by infantile-onset developmental delay, intellectual disability, microcephaly, facial dysmorphisms, hypotonia and/or ataxia. Intellectual disability affects about 1-3% of the population (79, 80), while ataxia has an estimated overall prevalence of 26/100,000 in children (81). Our data suggest that evaluating SEL1L-HRD1 ERAD has diagnostic values for those with intellectual disability, developmental delay, and ataxia. While it is currently rare, we expect that
more SEL1L-HRD1 ERAD variants will surface as evidence grows for its importance in humans. Options for treating patients with ENDI are currently very limited, but this study provides a framework for our future effort to target this important ERAD complex.

METHODS

*Human subjects.* Six patients from three families were identified and included in the study. The patient cases were gathered through the web-based tool GeneMatcher (82) ([https://genematcher.org/statistics/](https://genematcher.org/statistics/)). The Saudi Arabian boy was born on 10/2009 to a Gravida 2 Para 1 Abortion 0 29-year-old healthy mother and a 29-year-old father following an uneventful full-term pregnancy and spontaneous vaginal delivery. He presented with global developmental delay, intellectual disability, and hypotonia. MRI at 4 months of age suggested nonspecific periventricular white matter signal. The patient was officially diagnosed with short stature at 5 years of age and has been showing limited response to growth hormone therapy. The patient was diagnosed with cataract at age of 6 and was treated with lens extraction and intraocular lens implant placement. The patient was diagnosed with hypothyroidism and has been on 25mcg of l-thyroxine because of elevated TSH. Other than hypotonia, neurological examination was largely normal. The last doctor visit was in May 2022.

Four Moroccan siblings, 1 female (born on 06/2005) and three males (born on 12/2007, 02/2011 and 10/2017) displayed developmental delay, intellectual disability, speech delay, short stature, seizures, and ataxic gait (progressive with age). Brain MRIs of the patient 2 (Moroccan family proband) aged 14 years showed small cavities in the frontal periventricular area with nonspecific ventricular dilatation on coronal T2, coronal FLAIR and axial T2 weighted images and thin corpus callosum with no anomalies of basal ganglia or at the infra tentorial level of sagittal plane. Blood test suggested vitamin D deficiency and an infection at the time of tests on 06/22. Family history is notable for parents being first cousins. No similarly affected relatives were found in the family. The last doctor visit was in June 2022.

The Italian girl (born on 11/2001) presented with intellectual disability, speech delay, hypotonia, severe drug-resistant seizures, stereotypies, and dysmorphic features. The patient showed no autism spectrum disorder traits. The last doctor visit date was in June 2022.

**CRISPR/Cas9-based knockout (KO) and KI HEK293T cells.** HEK293T cells, obtained from the American Type Culture Collection (ATCC), were cultured at 37°C with 5% CO2 in DMEM with
10% fetal bovine serum (Fisher Scientific). To generate SEL1L- or HRD1-KO HEK293T cells, sgRNA oligonucleotides designed for human SEL1L (5’-GGCTGAACAGGGCTATGAAG-3’) and human HRD1 (5’-GGACAAAGGCCTGGATGTAC-3’) were inserted into lentiCRISPR v2 (Addgene 52961). Cells grown in 10 cm petri dishes were transfected with indicated plasmids using 5 μl 1 mg/ml polyethylenimine (PEI, Sigma) per 1μg of plasmids for HEK293T cells. The cells were cultured 24 hours after transfection in medium containing 2 μg/ml puromycin for 24 hours and then in normal growth media.

SEL1LM528R, SEL1LG585D, and HRD1P398L Ki HEK293T cells were generated using the CRISPR-Cas9 Homology-Directed Repair (HDR) system (Integrated DNA Technologies, IDT). 5 μL 100 μM Alt-R crRNA (IDT) with gRNA sequence was mixed with 5 μL 100 μM Alt-R tracrRNA (IDT) containing Cas9 interacting sequence. To anneal the oligos, the duplex mixture was heated at 95°C for 5 min and then cooled at room temperature for 20 min. 9 μL of the guide complex was incubated with 6 μL 62 μM Alt-R Cas9 enzyme (IDT) at room temperature for 20 min. 5 μL of the ribonucleoprotein (RNP) complex, together with 1.2 μL 100 μM HDR Donor Oligo (IDT), 1.2 μL 100 μM Alt-R Cas9 Electroporation Enhancer (IDT), was added into 100 μL HEK293T cell suspension (about 5x10^5 cells) in Electroporation Solution (Ingenio). The mixture was transferred into a 0.2 cm cuvette and electroporation was performed using Lonza Nucleofector IIb (Lonza). To prepare cell culture media, 3.4 μL 0.69 mM Alt-R HDR Enhancer V2 (IDT) was added to 2000 μL DMEM with 10% fetal bovine serum (Fisher Scientific). After electroporation, cell suspension was added to the cell culture media, and the mixture was incubated in 4 wells of a 24-well plate (500 μL per well). The cells were cultured at 37°C with 5% CO2. After 5 days of incubation, the genomic DNA of the cell culture was extracted with 50 mM NaOH. DNA fragments covering the target sites were amplified by PCR, using HotStart Taq 2X PCR Master (ABclonal), and analyzed by Sangar Sequencing (Eurofins Genomics US) to estimate the percentage of mutant allele in the cell pool. In parallel, cells were diluted into 8 cells per mL and cultured in 96-well plates (100 μL per well) for single cell isolation. After 10 days, 100 single cell colonies were transferred into 24-well plates. The SEL1LM528 region of each colony was amplified by a 50 μL PCR reaction, and 25 μL of the PCR product was treated with endonuclease NsiI (NEB) in rCutSmart Buffer (NEB), incubated at 37°C overnight. PCR products that were resistant to NsiI digestion were further analyzed by Sangar Sequencing. The SEL1LG585D, and the HRD1P398L regions were amplified using a 25 μL PCR reaction and sequenced. Cell colonies with homozygous SEL1LM528R, SEL1LG585D, or HRD1P398L alleles were transferred into a 6-well plate for further experiments.
crRNA (guide sequence):

```
SEL1^{M528R}: 5'-CTAGCTCAGATGCATGCCAG-3'
SEL1^{G585D}: 5'-TACCTCCTCGTGCTGAACA-3'
HRD1^{P398L}: 5'-CACACGCTCCTCGAGCTGG-3'
```

HDR Donor Oligo: (mutation sites are underlined)

```
SEL1^{M528R}: 5'-
AATTTAGCTTCTCAGGGAGGCAATCTTGGCTTTCTATAACCTAGGCTCAGAGGCATGCCA
GTGCGACCCGCGTATGCGATCATGTCACACTGCAGTGGAG-3'
```

```
SEL1^{G585D}: 5'-
GGCGATTACAATGCTGCAGTGATCCAGTACCTCCTCGTGCTGAACAGGACTATGAG-3'
GCACAAAGCAATGCACTCTCCCTTATTTCTGTCACTCAGATGTAAGG-3'
```

```
HRD1^{P398L}: 5'-
TGGCCCCCCATGGGCCCCTTTCCACTGTCCCGGCCTGCCCTCAGTCAGAGGAGGGTCG
GCTCCTCCATCCACCAGTGCGAG-3'
```

Amplification PCR primers:

```
SEL1^{M528R}: 
F: 5'-AATCTGTATCAGTGTGTTAGCTTGTATTA-3'
R: 5'-AGACTTTCCTGCTGGGCAA-3'
```

```
SEL1^{G585D}: 
F: 5'-AAACCTGTTGACTTCTAAAGAGTAAGTGAAAACTT-3'
R: 5'-AATGTCAAATCCATTTCTACAGTCAACTCG-3'
```

```
HRD1^{P398L}: 
F: 5'-CAGTCAGTGTGACCAGTGCT-3',
R: 5'-CTCACCCCCAAGAAGAACCC-3'
```

Sequencing primers:

```
SEL1^{M528R}: 5'-CTTGACAGATGGCATTGGAGTCAAGAGA-3'
```

```
SEL1^{G585D}: 5'-CCCACCTCACACAGTTGTTTAAGAATGT-3'
```

```
HRD1^{P398L}: 5'-CCTCCGTCTTCTCTGCAG-3'
```
Plasmids. The following plasmids were used in the study (h denotes human genes; m denotes mouse genes): pcDNA3-h-proAVP(G57S)-HA were described previously (47); mSel1L cDNA was cloned from mouse liver cDNA and inserted into the pcDNA3 to generate pcDNA3-mSel1L(WT)-FLAG. Point mutations of Sel1L in this study were generated using site-directed mutagenesis. The SEL1L-FLAG mutants G585D and M528R were generated using the plasmid pcDNA3-mSel1L(WT)-FLAG as the template. All plasmids were validated by DNA sequencing. The mutagenesis primers are: mSel1L-FLAG-F: 5’- CGCGGATCCACCATGCAGGTCCGCGTCAGGCTGTCG-3’, R: 5’- CGCTCTAGACTATTATATCATCATCATCTTTTATAATCTCCGCCCCTGTGTTGGCTGCTGCTGCTCTG G-3’. G585D-F: 5’-TGGCTGAGCAGGACTACGAGGTGGC-3’, R: 5’- GCCACCTCGTAGTCCTGCTCAGCCA-3’. M528R-F: 5’ - CCTCGCACAGAGGCACGCCAGCGGC-3’, R: 5’-GCGCCTGGCGTGCCTCTGTGCGAGG-3’. hHRD1 cDNA was cloned from pcDNA3-hHRD1(WT)-Myc-His (a kind gift from Y. Ye, NIDDK, USA) and inserted into the pcDNA3 to generate pcDNA3-hHRD1(WT)-FLAG. Point mutations of HRD1 in this study were also generated using site-directed mutagenesis. The HRD1-FLAG mutants P398L, C2A(C291A/C294A), P397L, P396L were generated using the plasmid pcDNA3-hHRD1(WT)-FLAG as the template.

Western blot and antibodies. Cells were harvested and snap-frozen in liquid nitrogen. The proteins were extracted by sonication in NP-40 lysis buffer (50 mM Tris-HCl at pH7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA) with protease inhibitor (Sigma), DTT (Sigma, 1 mM) and phosphatase inhibitor cocktail (Sigma). Lysates were incubated on ice for 30 min and centrifuged at 16,000 g for 10 min. Supernatants were collected and analyzed for protein concentration using the Bio-Rad Protein Assay Dye (Bio-Rad). 20-50 μg of protein were denatured at 95°C for 5 min in 5x SDS sample buffer (250 mM Tris-HCl pH 6.8, 10% sodium
dodecyl sulfate, 0.05% Bromophenol blue, 50% glycerol, and 1.44 M β-mercaptoethanol).

Protein was separated on SDS-PAGE or Phos-tag gel (as described previously (83, 84))
followed by electrophoretic transfer to PVDF (Fisher Scientific) membrane. The blots were
incubated in 2% BSA/Tri-buffered saline tween-20 (TBST) with primary antibodies overnight at
4°C: anti-HSP90 (Santa Cruz, sc-13119, 1:5,000), anti-SEL1L (home-made, ref. (33),
1:10,000), anti-HRD1 (Proteintech, 13473-1, 1:2,000), anti-OS9 (Abcam, ab109510, 1:5,000),
anti-CD147 (Proteintech, 11989-1, 1:3,000), anti-IRE1α (Cell Signaling, 3294, 1:2,000), anti-
ERLEC1 (Abcam, ab181166, 1:5,000), anti-UBE2J1 (Santa Cruz, sc-377002, 1:3,000), anti-
DER2 (gift from Chih-Chi Andrew Hu, ref (85). 1:1,000), anti-VCP (Proteintech, 10736-1,
1:3000), anti-FAM8A1 (Proteintech, 24746-1, 1:3000), anti-FLAG (Sigma, F1804, 1:1,000), anti-
HA (Sigma, H3663, 1:5,000), anti-PERK (Cell Signaling, #3192, 1:5000), anti-eIF2α (Cell
Signaling, #9722, 1:5000), anti-p-eIF2α (Cell Signaling, #9721, 1:1000), anti-GRP78 BiP
(Abcam, #ab21685, 1:5000), anti-PDI (Enzo Life Sciences, #ADI-SPA-890-D, 1:5000).
Membranes were washed with TBST and incubated with secondary antibodies, either HRP
conjugated (Bio-Rad, 1:10,000), anti-Rabbit IgG TrueBlot HRP (Rockland, 18-8816-33, 1:500)
or anti-Mouse IgG TrueBlot-HRP (Rockland 18-8817-31, 1:500) at room temperature for 1h for
ECL chemiluminescence detection system (Bio-Rad) development. Band intensity was
determined using Image lab (Bio-Rad) software.

**Immunoprecipitation (IP).** For SEL1L-FLAG and HRD1 IP, HEK293T cells transfected with the
indicated plasmids or KI HEK293T cells were snap-frozen in liquid nitrogen and whole cell
lysate was prepared in the IP lysis buffer [150 mM NaCl, 0.2% Nonidet P-40 (NP40), 0.1%
Triton X-100, 25 mM Tris-HCl pH 7.5] at 4°C, supplemented with protease inhibitors, protein
phosphatase inhibitors, and 10 mM N-ethylmaleimide. A total of ~5 mg protein lysates were
incubated with 15 μl anti-FLAG agarose (Sigma, A2220) or 2 μl anti-HRD1 antibody
(Proteintech, 13473-1) overnight at 4°C with gentle rocking. HRD1 IP lysates were incubated
with 10 μl Protein A agarose (Invitrogen, 20333) at 4°C for 2 hours after the incubation. The
incubated agaroses were washed three times with the IP lysis buffer and eluted in the SDS
sample buffer at 95°C for 5 min followed by SDS-PAGE and Immunoblot.

**Denaturing IP for ubiquitination assay.** HEK293T cells were transfected with proAVP(G57S)-HA
plasmids for 24 hours and then treated with 10 μM MG132 for 2 hours. The cells were snap-
frozen in liquid nitrogen and whole cell lysate was prepared in the NP-40 lysis buffer [50 mM
Tris-HCl at pH7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA] with 1% SDS and 5 mM DTT, and
denatured at 95°C for 10 min and centrifuged at 16,000×g for 10 min. Subsequently, supernatants were diluted 1:10 with NP-40 lysis buffer and incubated with 15 μl anti-HA agarose (ThermoFisher, 26182) overnight at 4°C with gentle rocking. The incubated agaroses were washed three times with the NP-40 lysis buffer and eluted in the SDS sample buffer at 95°C for 5 min followed by SDS-PAGE and Immunoblot.

Chemical treatment. The cells were treated with 50 μg/ml cycloheximide for the indicated times followed by Western blot analysis or treated with 10 μM MG132 followed by denaturing IP. WT HEK293 cells treated with 100 nM thapsigargin for 4 hours were included as positive controls for UPR.

Statistical analysis. Statistics tests were performed in GraphPad Prism version 8.0 (GraphPad Software). Unless indicated otherwise, values are presented as mean ± standard error of the mean (SEM). All experiments have been repeated at least two to three times and/or performed with multiple independent biological samples from which representative data are shown. All datasets passed normality and equal variance tests. Statistical differences between the groups were compared using the unpaired two-tailed Student’s t-test for two groups or one-way ANOVA or two-way ANOVA for multiple groups. P < 0.05 was considered statistically significant.

The intensity of Western-blot band between different samples in some experiments were also statistically compared using one-way ANOVA with post hoc Tukey-Kramer Test in the R environment. The input data were first examined for homoscedasticity using the Breusch-Pagan Test implemented in the ncvTest function in the R package car. In our experience, the data that did not satisfy a constant variance usually display log-normal distribution. Therefore, the log-transformed data were used as input in that case.

Study approval. Study protocols and written informed consents have been approved by the local institutional review boards at the Research Advisory Council (RAC, King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia, KFSHRC RAC# 2080006), APHP-Délégation Interrégionale à la Recherche Clinique (DIRC, Assistance Publique-Hôpitaux de Paris, Paris, France, #2015-03-03/DC 2014–2272), the Ethical Committee of University of Naples Federico II (Federico II University Hospital, Naples, Italy, #48/16), the Institutional Review Boards of the University of Michigan Medical School (IRBMED, University of Michigan, Ann Arbor, USA, HUM00227482), and the Institutional Review Board for Health Sciences
Research (IRB-HSR, University of Virginia, Charlottesville, USA, HSR230351). The patients and the parents have been provided with written informed consent prior to participation in the study. The written informed consent was received for the use of the photographs and the record of informed consent has been retained.

Data and materials availability. The materials and reagents used are either commercially available or available upon the request. All data and materials for the manuscript are included in the Methods and Supporting Data Value.

AUTHOR CONTRIBUTIONS
H.H.W., L.L.L. designed and performed most experiments; Z.J.L. generated all the KI cells; X.W. performed the structural analysis; O.A., G.C., M.O.H., L.H., A.M., M.A., F.S.A., C.B., K.P. and N.B.P. performed the exome sequencing analysis, identified variants and acquired clinical data; Q.P., S.S. and M.B. provided insightful discussions; Y.L. provided assistance on statistical analysis; S.S. and L.Q. directed the study, designed experiments, and wrote the manuscript with help from H.H.W., L.L.L., and Z.J.L.; H.H.W., L.L.L., and Z.J.L. wrote the methods and figure legends; all authors commented on and approved the manuscript.

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REFERENCES


FIGURE LEGENDS

Figure 1. Genetic analysis pipeline and identification of bi-allelic SEL1L and HRD1 variants in patients.

(A-C) Family pedigrees for the kindreds from Saudi Arabia (A, Patient 1, consanguineous), Morocco (B, Patient 2-5, consanguineous), and Italy (C, patient 6), showing autosomal recessive inheritance. Shaded shapes, individuals with symptoms; arrows, the probands. The age indicated is as of 2022.

(D-F) Genetic analysis pipeline of whole exome sequencing data for

 SEL1L c.1583T>G p.Met528Arg
 RAB17 splice site mutation
 VWA5B2 c. 1475C>G; p.S492C
 SLC25A53 c.347T>A; p.H116L

 HRD1 c.1193C>T p.Pro398Leu
 MS4A12 c.277G>A; p.V93M
 PPP1R32 c.473A>G; p.Q158R
(D) Patient 1 (SEL1L p.G585D), (E) Patient 2-3 (SEL1L p.M528R) and (F) Patient 6 (HRD1 p.P398L). (G-H) Exonic and chromosomal location of the SEL1L (G) and HRD1 (H) variants as well as Sanger sequencing confirmation of the patients and healthy family members from Figure 1. Red arrowheads, the nucleotide changes; K, heterozygosity.
Figure 2. Clinical features of patients carrying SEL1L and HRD1 variants.

(A, C, E) Photos of Patient 1 (A), Patient 2 (C) and Patient 6 (E) showing clumsy and wide gait, with need of external supports in (C and E). (B, D, F) Photos of the patients showing dysmorphisms (red arrows): (B) Patient 1 at 13 years (left, middle) and 11 years (right) of age, showing overbite (left, middle), downslanting palpebral fissures and pectus excavatum (right);

(D) Patient 2-5 at age of 17 (Patient 2), 15 (Patient 3), 11 (Patient 3), and 5 years (Patient 4), showing overbite (Patient 2 and 3) and down slanting palpebral fissures (Patient 3 and 4); and

(F) Patient 6 at 16 years old, showing hypertelorism and flat nasal bridge (right).
Reducing and non-reducing SDS-PAGE and Western blot analysis of high molecular-weight ERAD KO samples shown in Supplemental Figure 3. (A-B) Western blot analysis of known ERAD endogenous substrates IRE1α, OS9 and CD147 in KI HEK293T cells with quantitation shown in (B) (n=4-16 for IRE1α, n=4-12 for OS9 and n=3-12 for CD147 per group). OS9.1 and OS9.2 were quantified together as OS9. SEL1L⁻/⁻ and HRD1⁻/⁻ HEK293T cells were included as controls. (C-D) Cycloheximide (CHX) chase analysis of known ERAD endogenous substrates IRE1α, OS9 and CD147 in KI HEK293T cells with quantitation shown in (D) (n=3-8 for IRE1α, n=3-6 for OS9 and n=3-8 for CD147 per group). SEL1L and HRD1 variants were analyzed separately with their own WT controls. Quantitation normalized to WT controls. Western blot data for ERAD KO samples shown in Supplemental Figure 3. (E-F) Cycloheximide (CHX) chase analysis of known ERAD endogenous substrates IRE1α, OS9 and CD147 in KI HEK293T cells with quantitation shown in (E) (n=4-16 for IRE1α, n=4-12 for OS9 and n=3-12 for CD147 per group). In (F) ERAD KO samples shown in Supplemental Figure 3. (A-B) Western blot analysis of known ERAD endogenous substrates IRE1α, OS9 and CD147 in KI HEK293T cells with quantitation shown in (B) (n=4-16 for IRE1α, n=4-12 for OS9 and n=3-12 for CD147 per group). OS9.1 and OS9.2 were quantified together as OS9. SEL1L⁻/⁻ and HRD1⁻/⁻ HEK293T cells were included as controls. (C-D) Cycloheximide (CHX) chase analysis of known ERAD endogenous substrates IRE1α, OS9 and CD147 in KI HEK293T cells with quantitation shown in (D) (n=3-8 for IRE1α, n=3-6 for OS9 and n=3-8 for CD147 per group). SEL1L and HRD1 variants were analyzed separately with their own WT controls. Quantitation normalized to WT controls. Western blot data for ERAD KO samples shown in Supplemental Figure 3. (E-F) Cycloheximide (CHX) chase analysis of known ERAD endogenous substrates IRE1α, OS9 and CD147 in KI HEK293T cells with quantitation shown in (E) (n=4-16 for IRE1α, n=4-12 for OS9 and n=3-12 for CD147 per group). In (F) ERAD KO samples shown in Supplemental Figure 3.
(HMW) aggregates of proAVP(G57S) in KI HEK293T cells with quantitation shown in (F) (n = 3-6 for group). “n” indicates individual cell samples. In (A, C and E), SEL1L and HRD1 KO HEK293T cells were included as controls and quantitated as “ERAD KO”. The replicates in Western blot are technical replicates. Data are presented as mean ± SEM. Quantitation of the band intensities was compared using one-way ANOVA with post hoc Tukey-Kramer Test (B and F), or two-way ANOVA followed by multiple comparisons test (D). In (B), comparisons between different letters (a,b,c,d) represents p<0.05; n.s., not significant. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.
Figure 4. Sequence and structural analyses of SEL1L and HRD1 variants.

(A-C) Amino acid sequence alignment of SEL1L (A-B) and HRD1 (C) showing the conservation of residues across species. (D-F) Position-specific scoring matrix (PSSM) scores for amino acids position in SEL1L (D-E) and HRD1 proteins (F), with WT in green and variants in red.

(G-K) Schematic diagrams of human SEL1L (G) and HRD1 (K) with the location of the variants indicated. SP, signal peptide; FNII, fibronectin type II domain; SLR-N/M/C, Sel1-like repeats at N-terminal, Middle-, and C-terminal; TM, transmembrane; CYTO, cytosol; RING, really interesting new gene domain; Pro-rich, Proline-rich domain. (H-J) Structural prediction of human...
SEL1L/OS9/HRD1/DERLIN ERAD complex using AlphaFold2 with close-up views of SEL1L-M528 (blue) and G585 (green) areas shown in (I) and (J). Red (dotted) line marks the putative substrate binding groove. (L) Comparison of disordered region of HRD1 across species, highlighting the disordered nature of the proline-rich domain.
Figure 5. Reduced SEL1L-HRD1 protein level and stability for SEL1L M528R variant, not the other two variants.

(A-B) Western blot analysis of SEL1L and HRD1 in knock-in (KI) HEK293T cells expressing indicated variants with quantitation shown in (B) (n = 8-10, 4-6, 7-9, 5 for WT, G585D, M528R and P398L).

(C-D) RT-PCR analysis of SEL1L and HRD1 transcript levels in KI HEK293T cells with quantitation shown in (D) (n = 4-7 per group). L32, a loading control.

(E-F) Cycloheximide (CHX) chase analysis of SEL1L and HRD1 in KI HEK293T cells, with quantitation shown in (F) (n = 10, 3-4, 4-5, 3-5 for WT, G585D, M528R and P398L). SEL1L and HRD1 variants were analyzed separately with their own WT controls. Quantitation normalized to WT controls. “n” indicates individual cell samples. Data are presented as mean ± SEM. n.s., not significant.

*p<0.05, **p<0.01 and ****p<0.0001 by one-way ANOVA followed by Dunnett’s multiple comparisons test (B and D), and two-way ANOVA followed by multiple comparisons test (F).
Figure 6. SEL1L G585D variant, not M528R, impairs substrate recruitment.

(A-B) Immunoprecipitation of FLAG-agarose in SEL1L-/HEK293T cells transfected with G585D or M528R SEL1L-FLAG to test their interactions with components of the ERAD complex with quantitation shown in (B) (n=3-4 per group). “n” indicates individual cell samples. Data are presented as mean ± SEM. n.s., not significant. ***p<0.001 and ****p<0.0001 by one-way ANOVA followed by Dunnett’s multiple comparisons test (B).
**Figure 7. HRD1 P398L variant impairs HRD1 ubiquitination.**

(A-B) Immunoprecipitation of HRD1 in HRD1<sup>P398L</sup> KI HEK293T cells to test its interaction with components of the ERAD complex with quantitation shown in (B) (n = 3 per group). (C-D) Denaturing immunoprecipitation of HA-agarose in KI HEK293T cells expressing indicated variants transfected with a model substrate proAVP (G57S)-HA to measure substrate ubiquitination with quantitation shown in (D) (n = 3 per group). (E-F) Denaturing immunoprecipitation of FLAG-agarose in HRD1<sup>-/-</sup> HEK293T cells transfected with indicated HRD1 variants, with or without 10 μM MG132 for 2hr, to measure HRD1 ubiquitination with quantitation was shown in (F) (n = 3 per group). “n” indicates individual cell samples. Data are presented as mean ± SEM. n.s., not significant. **p<0.01, ***p<0.001 and ****p<0.0001 by two-tailed Student’s t-test (B), one-way ANOVA followed by Dunnett’s multiple comparisons test (D and F).
Figure 8. Our model for disease-causing SEL1L-HRD1 hypomorphic variants in ENDI.

Human ENDI variants identified in patients through whole exome sequencing are hypomorphic and cause a partial loss of function of SEL1L-HRD1 ERAD via distinct mechanisms such as SEL1L protein and ERAD complex stability ($\text{SEL1L}^{M528R}$), substrate recruitment ($\text{SEL1L}^{G585D}$), and HRD1 activity ($\text{HRD1}^{P398L}$).
Table 1. Genetic and clinical characteristics of patients with ENDI.

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<td>DEOGEN2_pred</td>
<td>Damaging</td>
<td>Benign</td>
<td>Benign</td>
</tr>
<tr>
<td>FATHMM-MKL_pred</td>
<td>Damaging</td>
<td>Benign</td>
<td>Benign</td>
</tr>
<tr>
<td>MutationAssessor_pred</td>
<td>Medium</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>MutationTaster_pred</td>
<td>Damaging</td>
<td>Damaging</td>
<td>Benign</td>
</tr>
<tr>
<td>PrimateAI_pred</td>
<td>0.874478221</td>
<td>0.928470612</td>
<td>0.586824059</td>
</tr>
</tbody>
</table>

Clinical presentation

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2-5</th>
<th>Patient 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (as of 2023)</td>
<td>14yrs</td>
<td>6-18yrs</td>
<td>22yrs</td>
</tr>
<tr>
<td>Developmental delay</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Intellectual disability</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Short stature</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Underweight</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Microcephaly</td>
<td>+</td>
<td>(2/4)</td>
<td>+</td>
</tr>
<tr>
<td>Seizures</td>
<td>+</td>
<td>(2/4)</td>
<td>+</td>
</tr>
<tr>
<td>Hypotonia</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Progressive ataxia</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Early death</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vomiting after eating</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Frequent infections</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Immune deficiency</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dysmorphisms</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Brain MRI abnormality</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Eye symptoms</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Therapy (in chronological order first used)

<table>
<thead>
<tr>
<th></th>
<th>GH</th>
<th>Depakine</th>
<th>Risperidone</th>
</tr>
</thead>
<tbody>
<tr>
<td>IOL placement</td>
<td></td>
<td>Vitamin D</td>
<td></td>
</tr>
<tr>
<td>l-thyroxine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- no significant findings; +, showed evidence; GH, growth hormone; IOL, intraocular lens implant